

Programme and Abstracts

**International Symposium on Environmental
and Molecular Toxicology of Chemicals**

ToxChem2023

7 December 2023
Institute for Medical Research and Occupational Health, Zagreb, Croatia

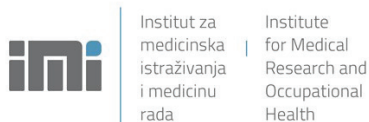
Organized by
Zrinka Kovarik, Chair of ToxChem2023

Scientific Committee:

Jasna Jurasović (Croatia)
Maja Katalinić (Croatia)
Zoran Radić (USA)
Heidi Qunhui Xie (China)

Organizing Committee:

Tena Čadež (Croatia)
Dora Kolić (Croatia)
Antonio Zandona (Croatia)



Programme

8:30-9:00	Registration
9:00-9:05	Opening
Session I – Pesticides and cholinesterases (Chairs: Zoran Radić and Zrinka Kovarik)	
9:05-9:20	Zrinka Kovarik (IMROH, HR): Environment and chemicals in the focus of 75 years of research at the Institute for Medical Research and Occupational Health
9:20-9:40	Heidi Qunhui Xie (RCEES, CN): New perspective on the regulation of acetylcholinesterase by dioxins
9:40-10:00	Zoran Radić (UCSD, USA): Structural basis of organophosphate pesticide toxicity inferred from atomic level studies of acetylcholinesterase structure
10:00-10:20	Goran Šinko (IMROH, HR): <i>In silico</i> examination of pesticide cholinesterase interactions via molecular modelling
10:20-10:35	Dora Kolić (IMROH, HR): Neurotoxic impact of organophosphate pesticides via inhibition of cholinesterase activity
10:35-11:00	Coffee break
Session II – New approaches in chemical toxicology (Chairs: Heidi Qunhui Xie and Maja Katalinić)	
11:00-11:20	Višnja Stepanić (IRB, HR): Use of chemoinformatics in toxicology
11:20-11:40	Maja Katalinić (IMROH, HR): Xenobiotics' effects on the living organism: a cell-based research
11:40-11:55	Yangsheng Chen (RCEES, CN): Aryl hydrocarbon receptor (AhR) pathway mediated activation of primary cultured astrocytes after 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure
11:55-12:10	Ruihong Zhu (RCEES, CN): Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on human renal tubulointerstitial fibrosis
12:10-12:30	Davor Želježić (IMROH, HR): Increasing resolution of pesticide genotoxicity evaluation by implementing fluorescent <i>in situ</i> hybridization
12:30-13:30	Lunch break
Session III – Environmental pollutants and biomonitoring (Chairs: Jasna Jurasović and Gordana Pehnec)	
13:30-13:50	Jasna Jurasović (IMROH, HR): Human biomonitoring as a tool in assessment of prenatal environmental exposure to metal(loid)s
13:50-14:10	Antonija Sulimanec (IMROH, HR): Metals in fish and rice available on the Croatian market: Is there a risk for consumers health?
14:10-14:30	Darija Klinčić (IMROH, HR): Research on polybrominated diphenyl ethers in Croatia
14:30-14:50	Goran Gajski (IMROH, HR): Impact of air pollutants on genome stability and health-related biomarkers
14:50-15:10	Gordana Pehnec (IMROH, HR): Air quality in Croatia – levels, legislation and recent research
15:10-15:30	Coffee Break
Session IV – Exposure to chemicals and adverse effects (Chairs: Irena Brčić Karačonji, Veda Marija Varnai)	
15:30-15:50	Irena Brčić Karačonji (IMROH, HR): Hair analysis as a toxicological tool for the detection of psychoactive substances
15:50-16:10	Veda Marija Varnai (IMROH, HR): PyrOPECh project – risk assessment for adverse effects of dietary pesticide exposure on neuropsychological and sexual development in pubertal boys
16:10-16:25	Marija Macan (IMROH, HR): Insecticide residues in food samples from the Total Diet Study – Zagreb region: preliminary results
16:25-16:45	Nikolina Maček Hrvat (IMROH, HR): Oxime therapy diminishes the neurotoxic effect of organophosphorus compounds in mice
16:45-17:00	Closing

Environment and chemicals in the focus of 75 years of research at the Institute for Medical Research and Occupational Health

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From its early beginnings in 1948, the mission of the Institute for Industrial Hygiene, a newly founded institution by the Academy of Sciences and Arts, was based on the study of harmful impacts of chemicals on health, primarily due to occupational exposure in Yugoslavia's developing industry. Being a true visionary, Andrija Štampar, an internationally recognised pioneer in public health, who also served as the president of the first World Health Organisation (WHO) assembly (Geneva, 1948), recruited leading chemists and medical doctors of the time to work at the Institute. He successfully secured funding from the WHO and The Rockefeller Foundation for the refurbishment of the existing buildings of the Institute and the education of the first class of scientists. A number of remarkable Croatian scientists initially led by professor Božo Težak, Velimir Vouk, and Zdenko Topolnik contributed immensely to the development of chemical toxicology, whose footsteps among others were followed by Milutin Vandekar, Mirka Fugaš, Katja Wilhelm, and many others. Noteworthy figures included Krista Kostial, who worked in the field of physiology of mineral metabolism, including interaction of toxic and essential elements in humans and animal models. In addition to research on calcium metabolism and osteoporosis, she and her collaborators made an important contribution by studying the effects of aging on toxic metal metabolism and toxicology, influencing international recommendations on acceptable levels of exposure to toxic metals, especially in sensitive population groups such as children and, later, also in women of reproductive age. Danica Prpić Majić, an expert in human biomonitoring of exposure to metals and solvents, established a clinical toxicology laboratory at the Institute and initiated the postgraduate study of Toxicology at the University of Zagreb. Interactions of organophosphorus compounds and other xenobiotics with cholinesterases were in the focus of research of Elsa Reiner, a world-leading scientist in enzymology. The Aldridge & Reiner book "Enzyme Inhibitors as Substrates: Interaction of Esterases with Esters of Organophosphorus and Carbamic Acids" (North Holland Pub. Co. Amsterdam, 1972) is considered as a fundamental textbook for enzyme kinetics. From its early years, occupational health has been a significant research topic at the Institute, led by academics Marko Šarić and Tihomir Beritić, founder of the Poison Control Centre. It is worth mentioning that the Institute has been a long-standing publisher of the journal *Archives of Industrial Hygiene and Toxicology* (nowadays with an impact factor of 2.1 and an increasing trend) with the 1st volume appearing in 1950. Already at first glance into the contents of those early issues, one gets the impression that the Institute has always been an interdisciplinary hub with studies on topics that remain highly relevant to general toxicology and specific toxicological and other correlated disciplines to this day. In other words, the *Archives* serves as witness to the 75-year long tradition of research on human health and environmental topics such as quality of air, soil, and water systems, including radioecology and dosimetry. Today, with the realisation of the infrastructural project *ReC-IMI*, the research activity of the Institute for Medical Research and Occupational Health is gaining new momentum and a great opportunity to increase its capacities both scientifically and in terms of its recognition in Croatia and abroad. A bright future in terms of new international, European, and national research projects seems not far away, especially if we combine the footsteps of our legendary scientists with new generations of researchers – skilled in interdisciplinary biomedical and environmental research topics to promptly respond to today's environmental and health challenges.

Zrinka Kovarik is a permanent research adviser at the Institute for Medical Research and Occupational Health, Zagreb and an associate professor of biochemistry and medicinal chemistry at the University of Zagreb, Faculty of Science, Croatia. She graduated at the Faculty of Food and Biotechnology and received MSc and PhD degrees at the Faculty of Science, University of Zagreb. For doctoral and later on for postdoctoral study she was trained at the University of California at San Diego, USA. Her research is focused on cholinergic mechanisms and modulation of neurotransmission in poisonings and cholinesterases – enzymes with key roles in organophosphorus poisoning and in the treatment of neurodegenerative diseases. She was the principal investigator of more than 10 research projects, supervisor of 9 PhD theses, co-author of >100 papers with >1700 citations and an *h*-index of 30. She has been involved in the organisation of more than 30 different scientific conferences and the main organiser of the 17th International Symposium on Cholinergic Mechanisms, held in Dubrovnik, Croatia in 2022. She was a member of several international expert committees (FEBS and OPCW) and president of the Croatian Society of Natural Sciences (2017-2022) and the Croatian Society for Biochemistry and Molecular Biology (2014-2017). She serves as member of the executive board of the Croatian Chemical Society, a section editor of *Periodicum Biologorum*, and a member of executive editorial board of *Archives of Industrial Hygiene and Toxicology*. Since 2023 she serves as Associate Editor of *Biofactors*, an IUBMB journal.

New perspective on the regulation of acetylcholinesterase by dioxins

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Acetylcholinesterase (AChE, EC3.1.1.7) plays important roles in cholinergic neurotransmission, which has been widely recognized as a biomarker for monitoring pollution by organophosphate and carbamate pesticides. Emerging evidence suggests a broad spectrum of environmental toxic substances is able to decrease AChE activity in various species. Dioxins are amongst these emerging environmental AChE disruptors, which are persistent organic pollutants with multiple toxic effects on the nervous system. By using enzyme-based and cell-based methods, we have demonstrated that dioxin decreased AChE activity by suppressing gene expression via the aryl hydrocarbon receptor (AhR) in human derived neuroblastoma cells. Both transcriptional and posttranscriptional regulations were involved in the AhR-mediated mechanisms. On the other hand, AChE activity was also decreased by dioxin treatment during myogenic differentiation of mouse C2C12 cells. The gene expression and enzymatic activity were both significantly suppressed; however, via non-AhR-dependent mechanisms. The dioxin-induced inhibition of AChE expression has different mechanisms of action among different species. This difference may be due to differences in the position and number of consensus sequences of dioxin response elements on the promoters of human and mouse AChE genes.

Heidi Qunhui Xie is an associate professor at the Research Center for Eco-Environmental Sciences (RCEES), Chinese Academy of Sciences, in Beijing, China. In 2002, she graduated at the Health Science Center of Peking University and earned her Bachelor and Master degrees of Clinical Medicine, while 4 years later, she graduated from the Hong Kong University of Science and Technology and received a PhD degree from the Department of Biology. For postdoctoral study, she was trained at the Department of Biology, École normale supérieure, Paris, France supervised by Prof Jean Massoulié. Her research is focused on the regulation of the protein assembly and transcriptional regulation of acetylcholinesterase (AChE), which is a very important enzyme in the nervous system. In the past decade, she has been working on the effects and action mechanisms of dioxins, typical organic persistent pollutants. She and her colleagues have done systematic work on the regulation of AChE by dioxins and published papers in *Environ Health Perspect*, *ES&T*, etc.

Structural basis of organophosphate pesticide toxicity inferred from atomic level studies of acetylcholinesterase structure

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Inhibition of catalytic activity of acetylcholinesterase (AChE; EC 3.1.1.7) by organophosphates (OPs) can fatally compromise cholinergic neurotransmission in vertebrates. The primary mode of inhibition is the formation of a stable covalent bond between the phosphorus of an OP and the O_γ of the active Ser 203 of AChE that disables its nucleophilicity and blocks catalysis. After the first X-ray-derived 3D structure of an AChE was solved in 1991, hundreds of 3D structures of this enzyme have been deposited to the Worldwide Protein Data Bank (PDB). A vast majority of those structures reveals a single main conformation of the protein, for both the backbone and most of the sidechains. Careful analysis, however, reveals small, yet systematic changes in backbone conformations of some of the AChEs inhibited by OP pesticides. We have demonstrated by X-ray crystallography, small angle X-ray scattering (SAXS), mutagenesis, and inelastic neutron scattering (INS) that those small conformational changes can cause larger disturbances of AChE structure with physiologically relevant consequences.

Acknowledgement: This research was supported by the CounterACT Program, National Institutes of Health Office of the Director (NIH OD), and the National Institute of Neurological Disorders and Stroke (NINDS), [Grant Numbers U01 NS083451, 1R21NS120839-01A1, 1R21NS120884-01A1] and by the UCSD Academic Senate grant RG103477.

Zoran Radić, PhD, Adjunct Professor at the Skaggs School of Pharmacy and Pharmaceutical Sciences, and Research Advisor at the University of Zagreb, Zagreb, Croatia is a graduate of the University of Zagreb in Croatia where he started his research under the mentorship of the late Dr Elsa Reiner in 1978 and continued as a graduate student and post-doctoral fellow in the Palmer Taylor laboratory at UC San Diego in 1986 and 1990. His primary interest is in the structure and activity of acetylcholinesterase and cholinesterase family of enzymes. To date, he has closely collaborated with numerous scientists in the field including two-time Nobel laureate Dr Barry Sharpless of The Scripps Research Institute in La Jolla, Dr Andrii Kovalevskyi of the Oak Ridge National Laboratory, Dr Donald Blumenthal of the University of Utah at Salt Lake City, Dr Xiaolin Cheng of the Ohio State University, Dr Carlo Ballatore of UC San Diego, Dr Jonah Cheung of the New York Structural Biology Center, Dr Zrinka Kovarik of the Institute for Medical Research and Occupational Health in Zagreb, Croatia, Dr Kamil Kuča of the University of Hradec Kralovy, Czech Republic, Dr Ondrej Soukup and Dr Jan Korabecny of the University Hospital Hradec Kralovy. His current research leads towards the development of novel, improved oxime antidote reactivators for therapy of organophosphate intoxication.

***In silico* examination of pesticide cholinesterase interactions via molecular modelling**

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Pesticides are a group of widely used chemicals in modern agronomy. Besides the clear benefit of their use, more concerns are being raised about their negative effects on the environment and the general population. Organophosphorus pesticides are known for inhibition of cholinesterases where AChE is a key enzyme in the cholinergic neurotransmission, forming covalently inhibited enzymes and causing cholinergic syndrome due to overstimulation of nicotinic and muscarinic receptors by acetylcholine. Knowledge about specific interactions between OP compounds and AChE and BChE can improve the safety of their use and more importantly species specificity, thus reducing harm to non-targeted entities, e.g. bees. The *in silico* study of organophosphorus pesticides interactions with ChE rationalizes the design of a novel class of pesticides; not only organophosphorus ones but also carbamates and other anti-cholinesterase compounds. Covalently inhibited ChEs can be reactivated by oximes by displacing the phosphoester conjugate through a process called reactivation leading to acetylcholine hydrolysis by ChEs. A novel *in silico* approach has been developed where oxime reactivation efficacy is evaluated by modelling a near-attack conformation in phosphorylated AChE via a phosphorylated oxime.

Goran Šinko was born in Zagreb Croatia in 1974 where he received his BSc in chemistry in 1998 and PhD in biochemistry in 2007, both at the Faculty of Science University of Zagreb. In his scientific area, he studied the kinetics of cholinesterases including enantioselectivity of inhibition, *in silico* methods of molecular modelling, and analysing the interaction of ligands with active site amino acids of both AChE and BChE by molecular docking.

Neurotoxic impact of organophosphate pesticides via inhibition of cholinesterase activity

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Public interest in pesticides and pesticide-free farming has increased in recent years, reinforced by frequent media reports about contaminations of food or aquatic ecosystems with pesticide residues. Pesticides are often available on the global market for an extended period of time before delayed adverse health effects in humans are recognized. Some of the most widely employed insecticides and herbicides are organophosphate (OP) compounds, known for their potential neurotoxicity in humans either as a result of chronic exposure to lower doses or acute poisoning. OPs increase the concentration of the neurotransmitter acetylcholine within the synapse by inhibiting the hydrolysing enzymes acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), resulting in cholinergic crisis, respiratory failure and long-term neurological damage. Our aim was to investigate the neurotoxic potency of several OP herbicides and insecticides by determining the inhibition of human AChE and BChE activity in their presence. Overall, OP insecticides progressively inhibited cholinesterases over time and were more potent inhibitors than the tested herbicides. The docking study identified important interactions within the active site, and cytotoxicity tested in a concentration- and time-dependent manner on neural and liver cells, as well as erythroblasts, corroborated the neurotoxic impact of tested pesticides. Since medical therapy for restoring enzyme activity includes oxime reactivators that dephosphylate the cholinesterase active centre, we also evaluated the potential of several oximes to reactivate pesticide-inhibited AChE. Our findings could assist in the further development of chemicals safer for use and a better response in case of poisonings.

Acknowledgement: This study was supported by the Croatian-Chinese Scientific and Technological Cooperation (2020-2023).

Dora Kolić finished her graduate studies in 2019 and acquired a degree in molecular biology at the Faculty of Science (University of Zagreb, Croatia). She started her PhD research at the Institute for Medical Research and Occupational Health, Zagreb in 2020 and enrolled in the doctoral study of Chemistry at the Faculty of Science (University of Zagreb). Her research is mainly focused on kinetic characterization of novel oxime antidotes which recover activity of cholinesterases inhibited by organophosphates (OPs), that would be used in the treatment of OP nerve warfare agent and pesticide poisoning. Another area of research involves investigating *in vivo* neuroprotective role of antidotes in the brain of mice exposed to OPs by monitoring neuroinflammation markers. So far, she has been an associate researcher on two international scientific projects and a Croatian-Slovenian bilateral project. She is a co-author or equal first author of original scientific papers in journals indexed in the WoSCC database (2 papers) and of several poster and oral presentations at international and domestic scientific congresses.

Use of chemoinformatics in toxicology

Višnja Stepanić

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Toxicity is one of the most important problems of modern society. There is an enormous use of chemicals, including several millions of tonnes of pesticides, as well as extensive development of innovative cosmetics and foods products containing mixtures of various biologically active components, which requires serious screening for possible adverse effects. *In silico* chemoinformatics methods such as chemical similarity assessment and use of models for prediction of toxicity of chemicals are an integral part of such screening protocols. The predictive models for toxicity endpoints should be appropriately developed, validated, and presented to end users so that they can be appropriately and correctly interpreted. In research studies, chemoinformatics methods are used to gain insight into structure-activity/toxicity relationships and to filter out compounds for *in vitro* testing from a set of chemicals. This will be presented by means of case studies on herbicides and ferroptosis modulators.

Dr **Višnja Stepanić** is a computational chemist devoted to finding and designing novel compounds with biological activities. Dr Stepanić applies molecular modelling methods (virtual screening, molecular docking, molecular dynamics), quantum-chemical calculations (DFT), and machine learning approaches to find structural-activity relationships that are practical for end users. She obtained her PhD in 2001, after which she worked in the modelling groups of the pharmaceutical industry PLIVA and GSK, where she received the GlaxoSmithKline Exceptional Science Award for a recognised international contribution. She has been a scientist at the Ruđer Bošković Institute since 2009. Dr Stepanić has participated in more than 15 research projects and published more than 50 articles and several book chapters. Dr Stepanić has given 15 oral presentations at international conferences and participated in the organisation of international events.

Xenobiotics' effects on the living organism: a cell-based research air

Maja Katalinić¹, Antonio Zandona¹, Karla Jagić², Marija Dvorščak², Josip Madunić¹, and Darija Klinčić²

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Knowing the mechanism of action of diverse xenobiotics of natural and/or synthetic origin, to which we are exposed in the short or long term in our daily life, is extremely important for assessing possible negative effects on our health. According to legislation, each substance that is in any type of use has to have a characterized biological activity regarding its purpose. However, for most there are no data on the specific or long-term health effects. Therefore, risk assessments are of a great importance as is the constant monitoring and re-evaluation of the known facts. In the aspect of ever demanding animal studies reduction and refinement, cell-based *in vitro* toxicology has emerged as the most promising method for overcoming this issue. By the simple addition of a cell-based battery of assays, identification of a specific tested compounds' potential adverse or undesirable effects is highly probable. The cell culture techniques available on the market today allow for almost all cell types, of both animal and human origin, to be effectively grown using both 2D and 3D techniques. Moreover, the possible utilization of human cells in research could benefit risk assessments for human situations and overcome potential cross-species differences. Though such tests could not replace animal/human studies fully, they could be the first step towards chemical risk assessment based on mechanistic reasoning and raise red flags for further detailed analysis. For example, studies performed in our lab on the effects of polybrominated diphenyl ethers (PBDEs) on cells indicated that exposure to these chemicals increased membrane disruption and a release of lactate dehydrogenase, accompanied by oxidative stress in cells through the formation of reactive oxygen species (ROS) and a decrease in mitochondrial membrane potential. This could be indicative as risk for human health with regard to the constant exposure to these chemicals through different pathways. As such, our findings point to the importance of further studies on the negative effects of PBDEs to understand their mechanism of action in detail.

Acknowledgement: This research was supported by the Croatian Science Foundation grants UIP-2017-05-7260 and UIP-2017-05-6713.

Maja Katalinić obtained her PhD in Biochemistry and Medicinal Chemistry at the Faculty of Science of the University of Zagreb in 2011, and currently works as a Research Advisor at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. She is an expert in the area of drug discovery and evaluation of potential drugs' effects on cell level in the early phase of studies. Her main focus is on conducting and optimising experiments in order to minimise consumption of reagents/chemicals/enzymes and obtaining reliable data on compound profiling for *in vivo* tests. A biotechnologist in the background and extensively trained in biochemistry and medicinal chemistry, she has strong organisation skills and is engaged through professional societies in the popularisation of science and presentation of scientific results to the public. She is also engaged in the training of students and young scientists. Principal investigator or a researcher on project financed by the Croatian agencies and EU HORIZON programme.

Aryl hydrocarbon receptor (AhR) pathway mediated activation of primary cultured astrocytes after 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) exposure

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Astrocytes play a pivotal role in responding to external factors in the nervous system. However, the understanding of mechanism for the response of astrocytes to environmental pollutant, like dioxins, is still insufficient. In this study, we found that 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) induced the expression of protective phenotype (termed as A2 type) marker genes in astrocytes after TCDD treatment at concentrations ranging from 0.01 to 0.1 nM. After activation, enhanced autonomous motility, and increased expression of certain chemokines (Cxcl10, Cxcl2, and Ccl7), were observed in astrocytes. Above responses were mediated by TCDD-induced activation of aryl hydrocarbon receptor (AhR) pathway. We also found different astrocytic activation mechanism between TCDD and an endogenous AhR ligand 6-formylindolo[3,2-b] carbazole (FICZ). Our study provides new evidence for understanding the specific response of astrocytes to dioxins.

Yangsheng Chen is a postdoctorate researcher in the environmental molecular toxicology group at the Research Center for Eco-Environmental Sciences (RCEES), Chinese Academy of Sciences, in Beijing, China. He also graduated and received PhD degree from the RCEES. His research is focused on the neurotoxic effects and mechanisms of dioxin and other aryl hydrocarbon receptor (AhR) ligands. His work includes identifying neural molecular targets for pollutants and elucidating signalling pathway regulation mechanisms in primary cultured cortical neurons, astrocytes and other neurotumour cell lines.

Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) on human renal tubulointerstitial fibrosis

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Exposure to dioxins can cause various toxic reactions. Nephrotoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and its adverse effects on renal fibrosis in experimental animals has been reported. Although the aryl hydrocarbon receptor (AhR) is a classic signalling molecule mediating TCDD toxicities, the specific mechanism by which TCDD causes renal fibrosis remains elusive. Renal tubulointerstitial fibrosis is the main pathological manifestation of renal fibrosis. In this study, the effects of TCDD on the expression of fibrosis marker genes and the role of an activated AhR signalling pathway in the effects were investigated in human renal proximal tubular epithelial cells (HKC). We found that the migration ability was enhanced in TCDD-treated HKC cells, indicating that the transition of cells from epithelioid to fibrotic phenotype occurred upon TCDD treatment. Furthermore, based on the crucial function of the TGF- β /Smad signalling pathway in cell fibrosis, we further revealed the regulation of TGF- β /Smad downstream genes by the AhR signalling pathway under TCDD stress. We hypothesize that the cross-talking effects of the AhR and TGF- β /Smad signalling pathways might be the underlining mechanisms for the TCDD-induced epithelioid to fibrotic phenotype transition of HKC cells.

Diana Ruihong Zhu is a PhD student at the Research Center for Eco-Environmental Sciences (RCEES), Chinese Academy of Sciences, in Beijing, China. In 2019, she graduated at the China University of Mining & Technology and got her Bachelor degree in Environmental Engineering. She is currently studying at the Research Center for Eco-Environmental Sciences (RCEES) for her PhD degree. Her research is focused on the effects of dioxins and natural nephrotoxins on renal tubulointerstitial fibrosis and the cross-talk of the AhR pathway with the TGF- β /Smad pathway in renal tubular epithelial cells.

Increasing resolution of pesticide genotoxicity evaluation by implementing fluorescent *in situ* hybridization

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Genotoxicity represents the ability of an agent to adversely affect the structure and physiology of the genome whether by getting into direct contact with it or by hindering processes crucial for DNA physiology and gene expression. Genotoxicity represents both the trigger and the driving force of carcinogenicity, thus being of the highest significance in evaluating the population safety of chemicals with which a person may get into contact. The two most relevant methods in evaluating the potential genotoxicity of chemicals, used either for experimental or regulatory purposes, are: alkaline comet assay and micronucleus assay. Alkaline comet assay detects primary damage to DNA, the one that occurs first in direct contact between the substance and DNA. These are allylations, binding of bulky chemical groups to nitrogen bases (DNA-adducts), covalent binding of proteins, covalent binding of complementary DNA chains (cross-linking), dimers, apurinic and apyrimidinic sites, etc. Micronucleus assay detects fixed damage in the structure or number of chromosomes in cells and directly corresponds to the risk from carcinogenesis. Although both methods are relevant and provide relevant results on genotoxicity testing, the information they provide is quantitative and at the general genome level, without specifying which part of it has been affected by genotoxic activity, which is crucial in assessing carcinogenicity. Fluorescent *in situ* hybridization (FISH) enables to visualize and localize specific genes of interest, concerning carcinogenesis these would be tumour suppressor genes and proto-oncogenes. Thus, combining FISH with alkaline comet or micronucleus assay provides information whether genes crucial for a cell cycle and, consequently, carcinogenesis were affected. We will present such a coupling of methods on human lymphocytes treated by carbofuran, terbuthylazine, or α -cypermethrin and the results on genotoxicity it may provide.

Prof Davor Želježić obtained basic education in molecular biology, while his PhD was obtained in the field of toxicology; more precisely, genetic toxicology. He is an experienced researcher who has worked in the field of genetic toxicology for more than 25 years with more than 90 scientific and 20 professional papers in journals cited in Web of Science, 4 book chapters, and 1 book. His scientific work has been cited more than 2,100 times. He has been appointed by the Croatian Ministry of Health to the European Chemicals Agency (ECHA) as an expert on the methodologies of testing genotoxicity, reproductive toxicity, and endocrine disruption. He is a member of relevant groups appointed to improve the EC regulation REACH in evaluating substances prior to the approval of their use in industry. As an expert of the European Food Safety Authority (EFSA), he contributes in the evaluation of substances being used in food and feed, as well in their packaging. The Ministry of Health appointed him as a second-tier expert in negotiations regarding the accession of the Republic Croatia to the Organisation for Economic Co-operation and Development (OECD).

Human biomonitoring as a tool in the assessment of prenatal environmental exposure to metal(loid)s

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Beginning with blood and urine Pb testing in the 1950s, methods of human biomonitoring (HBM) have been used for the assessment of occupational, and, a few decades later, environmental exposure to metal(loid)s and their health effects in numerous epidemiological studies conducted at the Institute for Medical Research and Occupational Health. The use of new sensitive analytical techniques, such as inductively coupled plasma-mass spectrometry (ICP-MS), has significantly advanced trace element analysis, laying the foundation for a better understanding of elemental distribution in the body (metallome), and included a wide range of biological samples (e.g., blood, serum/plasma, hair, breast milk, cerebrospinal fluid, placental tissue, seminal plasma, biopsic material, etc.) in these analyses. The presentation will give highlights of the results from our most recent study in postpartum women and their newborns (n = 156 mother-infants pairs), encompassing the assessment of exposure, status, and interaction of toxic and essential metal(oid)s, along with an assessment of the risk of their effects on antioxidant capacity and metallothionein levels, epigenetic markers (selected micro-RNAs' expression and DNA methylation), and sex steroids' levels in the maternal-placental-foetal unit.

Acknowledgement: The study was funded by the Croatian Science Foundation project "Assessment of Daily Exposure to Metals and Maternal Individual Susceptibility as Factors of Developmental Origins of Health and Disease – METALORIGINS" (HRZZ-IP-2016-06-1998).

Jasna Jurasović is a permanent scientific advisor at the Division of Occupational and Environmental Health of the Institute for Medical Research and Occupational Health, Zagreb. She served as the head of the Analytical Toxicology and Mineral Metabolism Unit (2007–2023). As an analytical chemist, she has been dedicated to the development and implementation of state-of-the art analytical techniques and methods for qualitative and quantitative determination of environmental contaminants, primarily metal(oid)s, in various environmental and biological matrices. For years, her research was focused on the adverse effects of toxic metals, primarily lead and cadmium, and their interactions with essential elements on male reproductive function and blood pressure in both occupationally and environmentally exposed populations. Most recently, her research has been in the area of exposure and risk assessments of toxic metals, mainly as constituents of food and tobacco smoke in high-risk population groups such as women of reproductive age and their progeny.

Metals in rice and fish available on the Croatian market: Is there risk to consumers' health?

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There is growing concern about exposure to potentially toxic metal(loid)s contained in the daily diet. Rice and fish are among the most widely consumed commodities in the world. Rice is a valuable source of complex carbohydrates and B vitamins and fish meat is rich in essential fatty acids and trace elements, especially selenium. Rice consumption provides energy, whereas fish consumption improves cardiovascular health, and during pregnancy, it benefits foetal growth and neurological development. At the same time, rice and fish are recognized as the main dietary sources of highly toxic forms of arsenic and mercury. Therefore, vulnerable population groups, such as women during the reproductive period and young children should avoid the consumption of predatory fish species that accumulate high levels of mercury. We study the levels of essential and main toxic metal(loid)s in white and brown rice and in several fish species, mainly of Adriatic origin, which are available on the Croatian market and commonly consumed in our population. Based on the obtained data, we evaluate the risks and benefits to consumers' health. The results obtained to date will be presented at the Symposium.

Acknowledgement: Our study has been funded by the Croatian Science Foundation (HRZZ-IP-2016-06-1998) and the Ministry of Science and Education of the Republic of Croatia (through the Institutional Funding made available to the Institute for Medical Research and Occupational Health in Zagreb).

Antonija Sulimanec is a postdoctoral researcher employed in the Division of Occupational and Environmental Health, Institute for Medical Research and Occupational Health in Zagreb. Her work is in the area of nutritional toxicology and focuses on the evaluation of health benefits and potential risks from dietary metal exposure in humans, especially in the vulnerable population groups of women during reproductive age and young children. She was an active collaborator in several national research projects. In addition, she is active in scientific popularization activities of the institutional project IMImobil.

Research on polybrominated diphenyl ethers in Croatia

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Polybrominated diphenyl ethers (PBDEs) are ubiquitous toxic substances classified as Persistent Organic Pollutants (POPs), but unlike the other classes, they are indoor pollutants. Research conducted at IMROH in the last five years resulted in the development of analytical methods that were used for the determination of seven PBDE congeners in different matrices, primarily indoor dust and human milk. Data on PBDE levels and distribution obtained for the collected samples are the first of their kind in Croatia, and were further used to assess the exposure of the general population to PBDEs. According to the results of our research, the PBDE contamination status of indoor dust in Croatia is at the lower end of reported values worldwide. Presence of PBDEs was investigated in dust samples from several indoor environments (households, workplaces, kindergartens, schools, cars) in order to obtain data about which of them contributed the most to human exposure to PBDEs in dust. Our results indicate the highest contribution to overall dust intake (considering the central case scenario) from intake of dust in homes for both children and adults. Exceptionally, in a few cases, high mass fractions of PBDEs were detected in car dust samples; therefore, ingestion of this dust prevailed as the main PBDE exposure route. On the positive side, lower mass fractions detected in newer and/or recently renovated households, as well as in dust from newly manufactured cars, confirmed the benefit of banning the production and use of PBDEs. Research on a second important matrix, human milk, revealed that although very low levels were detected in it, infants received the highest PBDE dose compared to other population groups. The PBDE levels obtained in matched samples of house dust and human milk indicated that PBDEs are not as accumulative in humans as some other POPs, e.g. PCBs, and that the detected concentrations did not pose a risk to human health. Also, there were no significant differences in PBDE levels in milk samples of first-time mothers through a ten-year time period (2010 vs. 2020). Additionally, PBDEs were also analysed in two animal species. The first was fish, which is important from two aspects, as an indicator of the pollution of the aquatic environment, and as part of human nutrition. The other is brown bear, and this is one of few studies to examine PBDE levels in terrestrial mammals. Our future objectives are the introduction of analysis of additional analytes, such as BDE-209 or novel brominated flame retardants, as representatives of compounds that came into use after the ban on the use of PBDEs, but also new matrices, in order to obtain a more complete data set on PBDE pollution.

Acknowledgement: This research was supported by the Croatian Science Foundation grant UIP-2017-05-6713.

Darija Klinčić started her scientific career in 2006 at IMROH, where she currently works as a senior scientific associate. She graduated, and later got her PhD, in the field of analytical chemistry. The main focus of her work are the development and application of analytical procedures for the detection of organic pollutants, primarily those classified as persistent organic pollutants (POPs). Moving away from polychlorinated biphenyls and organochlorine pesticides, in the last five years she established a small research group focused on the investigation of polybrominated diphenyl ethers (PBDEs) in different environmental and biological matrices. The results of these investigations provided the first contamination status on brominated flame retardants in Croatia and this part of Europe.

Impact of air pollutants on genome stability and health-related biomarkers

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Air pollution is the presence of harmful substances in the Earth's atmosphere, primarily caused by human activities like industrial processes, vehicle emissions, and deforestation. Common pollutants include particulate matter, nitrogen oxides, sulphur dioxide, and ozone. These pollutants can cause severe health effects, contribute to climate change, and harm ecosystems. Efforts to reduce air pollution involve regulations, technological advancements, and promoting sustainable practices to mitigate its impact on both human health and the environment. Therefore, we aimed to explore how air pollution can affect genomic instability and consequently our health by determining possible associations between air pollutants and biomarkers of exposure and effects. In the first part of the study, we retrospectively evaluated genomic instability in the general population (N=130) living in Zagreb, Croatia. We associated these genomic instabilities measured in blood with the comet and micronucleus assays with air pollution levels in the period from 2011 to 2015. There was no observed significant positive association between assayed parameters apart from benzo(a)pyrene (B[a]P), which showed a significant negative association. Our results also showed that the measured air pollution parameters were mainly below regulatory limits, except for B[a]P. In the second part of the study, we investigated the possible effects of air pollution and BTEX (benzene, toluene, ethylbenzene, o-, m-, and p-xylene) exposure on genomic instability using the comet and micronucleus assays on blood as well as on buccal cells of the general population (N=60) living in Zagreb (Croatia) during colder and warmer periods from 2021 to 2022. All measured outdoor air pollutants agreed with previously reported values and were below the regulatory limit, except for PM₁₀ particles and B[a]P bound to PM₁₀, which exceeded those levels. Again, we did not observe a notable impact of air pollutants on the tested parameters. Given the transboundary nature of air pollution, our results might be regionally important. Since air pollution is considered a significant health issue, and air pollution is often site-specific, more studies using biomarkers of exposure, as well as using different biomarkers of effect could be expected. This will also lead to the development of appropriate models for the prediction of air pollution-induced effects on the human population.

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Goran Gajski, PhD is a Senior Scientific Associate at the Institute for Medical Research and Occupational Health in Zagreb, Croatia with a scientific background in biochemistry and molecular biology. In the field of genetic toxicology, he uses various methodological approaches, both *in vitro* and *in vivo*, on different cell and animal models to investigate the effects of various physical and chemical agents on organisms, tissues, cells, and cell structures with special emphasis on the DNA molecule. Moreover, the scope of his work also comprises human biomonitoring studies in different environmental and occupational settings. He was involved in several national and international projects as principal investigator and team member and has published more than 120 papers and book chapters that have been cited more than 4000 times with an *h*-index of 36 (by Google Scholar). For his work he received several national and international scientific awards, among other the Danubius Young Scientist Award issued by the Austrian Federal Ministry of Science and National Award for Science in the Field of Natural Sciences issued by the Croatian Parliament. Currently he is a chair of the International Comet Assay Group an affiliated group of the European Environmental Mutagenesis and Genomics Society. He is a European Registered Toxicologist (ERT) and Editorial Board member of the journal *Medicine*[®].

Air quality in Croatia – levels, legislation, and recent research

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The first measurements of ambient air quality in Croatia were carried out in the 1960s, when scientists from the Institute for Medical Research and Occupational Health (IMROH) started measuring SO₂ and black smoke at three locations in Zagreb. Over the years, the number of monitoring stations has been increasing, as well as the number of measured pollutants. Today, there are thirty monitoring stations within the national air quality monitoring network and additional local and special purpose stations. Air quality is assessed annually according to the Croatian Air Pollution Act and Regulation on Levels of Pollutants in Ambient Air. Since 2011, Croatian legislation has been fully harmonized with the European Union (EU) legislation. Although decreasing trends of pollutant concentrations are observed at most of the stations in Croatia, at some locations air is still assessed as polluted regarding PM_{2.5}, PM₁₀, benzo(a)pyrene (BaP) in PM₁₀, NO₂, and O₃. In 2021, based on the last scientific evidence, the World Health Organization (WHO) published new global air quality guidelines with stricter recommendations on the guidelines levels for these pollutants. In 2022, the EU Commission proposed a revision of the Ambient Air Quality Directives, setting air quality standards that are more closely aligned with the latest WHO recommendations. IMROH's Division of Environmental Hygiene is a testing laboratory accredited in accordance with the requirements of the ISO/IEC 17025 standard for 14 measuring methods in the field of ambient air quality and is a national reference laboratory for measurements of particulate matter and its content. In addition to the professional work of ambient air quality monitoring, the Division continuously conducts scientific research, studying spatial and temporal variability, interactions and assessment of the possible impact of polluted air on health and the environment. An overview of recent and current projects and activities of the Division will be presented.

Gordana Pehnec, PhD graduated at the Department of Chemistry, Faculty of Science of the University of Zagreb. In 1997, she started working at the Environmental Hygiene Unit of the IMROH. She received her doctorate in 2007 at the Chemistry Department, Faculty of Science. Since the beginning of her employment at IMROH, she has participated in scientific research and professional work in the field of air quality, including optimization and development of measuring methods, the study of atmospheric oxidants (ozone, peroxides), and carcinogenic pollutants related to airborne particulate matter. She participated in several projects in the field of air quality research and was the leader of the IMROH project team in the EU project "Expansion and modernization of the state network for continuous air quality monitoring-AIRQ", within the Operational Program "Competitiveness and Cohesion" 2014-2020. She teaches courses within postgraduate studies at the University of Zagreb and was mentor of several master's and doctoral theses. She has actively participated in the organization of national and international meetings and various science popularization activities and was president of the organising committees of three national conferences and president of the scientific committee of one international conference. From 2015 to 2023, she was the head of the IMROH Environmental Hygiene Unit and in 2023 she became the head of Division of Environmental Hygiene. From 2011 to 2019, she was the president of the Croatian Air pollution Prevention Association (CAPP). In 2023, she was elected president of the European Federation of Clean Air and Environmental Protection Associations – EFCA.

Hair analysis as a toxicological tool for the detection of psychoactive substances

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According to data provided by the World Health Organization in 2020, about 270 million people (or about 5.5 % of the global population aged 15-64) had used psychoactive drugs in the previous year. Advances in the sensitivity of analytical techniques have enabled analyses of substances of abuse in alternative biological matrices such as hair. The unambiguous identification of a specific substance must be carried out by a combination of chromatographic and mass spectrometric techniques able to distinguish among similar structures and detect low psychoactive substances levels in hair. Hair analysis is a reliable tool for detecting long-term exposure to illegal drugs over a period from a few weeks to a few months, depending on the length of the hair used for analysis. Compared to conventional biological samples such as blood or urine, hair is preferable as a biological sample because of the stability of incorporated psychoactive substances, easy sampling without violating individual privacy, and a longer window of detection for drug abuse. Since 1999, over 1900 hair samples have been analysed using gas chromatography mass spectrometry (GC-MS) at the Institute for Medical Research and Occupational Health, Croatia for the presence of cocaine, opioids, and amphetamine-type stimulants. A total of 20 % of the tested samples were positive for one or more illegal psychoactive substances. Ecstasy and cocaine were the most frequently detected substances. In this lecture, the main drawbacks in hair analysis such as false positive results as well as several clinical and forensic cases will be discussed.

Irena Brčić Karačonji received her BSc degree in Medical Biochemistry in 1998 at the Faculty of Pharmacy and Biochemistry, MSc degree in Toxicology in 2004 at the Faculty of Science, and PhD degree in Biomedicine and Health Science in 2010 again at the Faculty of Pharmacy and Biochemistry, University of Zagreb, Croatia. Since 2000 she has worked at the Institute for Medical Research and Occupational Health (Zagreb, Croatia). As an Assistant Professor at the Faculty of Health Studies, University of Rijeka she teaches students about psychoactive substances in food. Irena's research focuses on the development of gas chromatographic-mass spectrometric methods for determining bioactive compounds in food and biological samples and assessment of their toxicity. She is a European Registered Toxicologist (ERT), Associate Editor of the journal Archives of Industrial Hygiene and Toxicology, and Academic Editor of the journal Food Safety and Health.

PyrOPECh project – risk assessment for adverse effects of dietary pesticide exposure on neuropsychological and sexual development in pubertal boys

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Recent epidemiological studies and animal experiments indicate the potential adverse effects of exposure to pesticides on neuropsychological and sexual development in children, even at very low exposure levels. However, a limited number of studies explored these potential risks during puberty, with inconsistent results and significant methodological limitations, such as insufficiently assessed exposure and cross-sectional study design. The main objective of the project PyrOPECh (HRZZ IP-2019-04-7193) is to assess the risk of pyrethroid (PYR) and organophosphate (OP) insecticides exposure to neuropsychological development and hormonal status in prepubertal and pubertal boys in a prospective cohort study, while controlling for potential confounders and using only non-invasive methods. Children's exposure to pesticides is being assessed by biomonitoring (analysing PYR and OP urinary pesticide metabolites) in the 5th and 7th grade of elementary school and by dietary exposure assessment. Neuropsychological development is evaluated by testing different aspects of attention, memory, visuospatial ability, and processing speed and by assessing the characteristics of a child's sleep and daytime functioning. Pubertal development is evaluated by clinical examination (according to Tanner and Prader) and urinary gonadotropins and salivary sex hormone (testosterone and dehydroepiandrosterone sulphate) measurements. The project's design and methodology will be presented, separately for the first phase – Total Diet Study conducted in the Zagreb region (PyrOPECh-TDS) and for the second phase – a cohort study in more than 450 (pre)pubertal boys from the same region (PyrOPECh-Health). Preliminary results of PyrOPECh-TDS will be shown in a separate presentation, while this presentation will provide certain general demographic, health- and dietary-related characteristics of the PyrOPECh-Health study group.

Veda Marija Varnai, MD, PhD, Permanent Scientific Advisor, is employed at the Institute for Medical Research and Occupational Health, Zagreb, Croatia. Her main activities are in the framework of research projects (epidemiological research, data analysis, dissemination of the results); PhD mentorship; participating in the Croatian Poison Control Centre 24-h duty; evaluation of the mammalian toxicity within Regulation (EC) No 1107/2009 concerning the placing of plant protection products on EU market, since the Institute is appointed to perform these tasks by the National Competent Authority (Ministry of Agriculture); evaluation of the proposals for harmonised classification and labelling, proposals for restriction and authorisation, and occupational exposure limits, as a member of the European Chemicals Agency's (ECHA) Risk Assessment Committee, appointed by the National Competent Authority (Ministry of Health).

Insecticide residues in food samples from the Total Diet Study – Zagreb region: preliminary results

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As part of the PyrOPECh study (<http://pyropech.imi.hr/>), which investigates potential adverse effects of low-level dietary exposure to pesticides on neuropsychological and sexual development in children during puberty, the Total Diet Study for the city of Zagreb and Zagreb County was conducted. Food samples were prepared “as consumed” in order to reflect practices in Croatian homes, homogenised, stored frozen at -20 °C, and shipped on dry ice to the collaborative laboratory in Ireland. Extraction was done using the miniLuke method. Pesticide residues in the samples were analysed by gas chromatography-high resolution accurate mass spectrometry (GC-HRAM) and triple quadrupole liquid chromatography (LC-QQQ). Quality Control on the analyses was carried out according to SANTE/11312/2021. Out of the 629 analysed composite samples, pesticide residues were quantified in 208 (33 %) food samples. Out of the 445 quantified pesticides in these 208 food samples, 27 (6 %) were above the Maximum Residue Level defined by the EU Regulation (MRL, i.e. the highest level of a pesticide residue that is legally tolerated in or on food or feed when pesticides are applied correctly). The largest number of quantified pesticide residues was found, as expected, in fruits and vegetables and their products. However, the highest proportion of residues above MRL was measured in foods of animal origin. Of the quantified pesticides, most belonged to the group of fungicides (62 %) and insecticides (23 %). Among the insecticides, neonicotinoids (33%) were the most prevalent (acetamiprid, imidacloprid, thiacloprid), followed by pyrethroids (17 %) (bifenthrin, cyhalothrin lambda, cypermethrin, deltamethrin, etofenprox, fenvalerate, permethrin, tetramethrin), organophosphates (11 %) (chlorpyrifos, coumaphos, dimethoate, isocarbofos, malathion, omethoate, phosmet), and carbamates (11 %) (methiocarb sulfoxide, oxamyl, pirimicarb, thiodicarb). Insecticide residues above the MRL were found in 8 food samples: oxamyl in peeled and unpeeled cucumber; methiocarb sulfoxide in kale; thiodicarb in raisins; tetramethrin and cyhalothrin lambda in processed meat products, and chlorpyrifos, malathion, pyridaben, and pyriproxifen in lemons. These findings were compared with the data obtained by the EU and national monitoring programme.

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Marija Macan, MSc Lab. Diag., is employed as an assistant and PhD student at the Institute of Medical Research and Occupational Health. Her dissertation research is part of the PyrOPECh project. She also participates in other research projects at the Institute, as well as in the Croatian Poison Control Centre's 24-hour service.

Oxime therapy diminishes the neurotoxic effect of organophosphorus compounds in mice

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Organophosphate (OP) compounds such as the nerve agent sarin inhibit the physiological function of acetylcholinesterase (AChE), promoting hypercholinergic activity leading to hypoxia, vasodepression, and respiratory arrest, which can result in death. The approved therapy for cholinergic toxidrome includes permanently charged oxime compounds that do not cross the blood-brain-barrier (BBB) easily making them inadequate for restoring the synaptic AChE activity. Consequently, the increased levels and residence time of the neurotransmitter acetylcholine lead to seizures and activation of glial cells resulting in neuroinflammation and brain damage. We hypothesized that treatment with uncharged, but ionizable oxime RS194B that crosses the BBB and reactivates OP-inhibited synaptic AChE could act protectively on the brain of mice exposed to sarin. We compared levels of specific proteins expressed in neuronal and glial cells of sarin-exposed mice with mice treated by two oximes – the centrally-active oxime RS194B and the one currently in use, pyridinium oxime 2PAM – and untreated control mice. The level of ionized calcium-binding adapter molecule 1 (IBA-1) protein was investigated as a measure of microglial response, and glial fibrillary acidic protein (GFAP) of astrogliosis, whereas neuronal cell viability was detected following neuronal nuclei antigen (NeuN) immunoreactivity. The obtained results show that RS194B therapy in mice diminished the sarin-induced neurotoxicity, especially within 1.5 h after sarin exposure, indicating the neuroprotective potential of RS194B oxime therapy.

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Nikolina Maček Hrvat, PhD, Scientific Associate, defended her PhD thesis in biochemistry in 2015. For the postdoctoral research she was trained at the Croatian Institute for Brain Research, School of Medicine, University of Zagreb. Her scope of interest is focused on studying the *in vitro* enzyme kinetics of the cholinesterase interactions with various organophosphates and newly developed oximes as the potential reactivators of the organophosphate-inhibited cholinesterase activity, *ex vivo* and *in vivo* antidotal oxime therapy and bioscavenging potential of exogenous cholinesterases in the event of organophosphate poisoning. Lately, her focus has been on studying organophosphate-induced neuroinflammation and the effect of oxime therapy on neuroprotection of mice exposed to organophosphates. She was collaborator on 8 scientific projects and a principal investigator of one on-going scientific project. She participated in over 20 scientific congresses and symposia, with poster presentations or lectures, and trained at numerous schools and workshops. For her work, she was honored by the award for a particularly outstanding graduate thesis in the interdisciplinary field of biomedical engineering and several fellowships for participation at scientific meetings.