MEDICAL AND BIOLOGICAL ASPECTS RELATED TO ASSESSMENT OF IMPACTS EXERTED BY RISK FACTORS

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Research article

MARKERS OF COMBINED AEROGENIC EXPOSURE TO METAL OXIDES AND TRANSFORMED PLASMA PROTEOMIC PROFILES IN CHILDREN

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Changes in homeostatic balance of the body, primarily at the cellular-molecular level, are a relevant research object in fundamental and applied studies. They can be eligible indicators for predicting negative effects under exposure to chemical risk factors.

The aim of this study was to substantiate markers of a transformed plasma proteomic profile in children. These markers should have prognostic value and an evidence-based association with combined aerogenic exposure to metal oxides (copper and nickel oxides used as an example). We propose an innovative methodical approach based on plasma proteomic profiling that includes the following: identification of identical proteins and genes encoding their expression; quantification of indicators within the 'identical protein – a chemical concentration in blood' system; prediction of negative effects as per indicators of homeostasis destabilization at the cellular-molecular level under chronic aerogenic exposure to chemicals. The proposed algorithm was tested by comparing changed proteins and peptides identified in plasma proteomic profiles of children exposed simultaneously to nickel and copper oxides in ambient air in actual conditions and small rodents under experimental combined and isolated exposure to the analyzed chemicals in levels equal to real ones.

Long-term aerogenic exposure simultaneously to copper and nickel oxides was established to create elevated nickel and copper levels in blood of exposed children substantiated as markers of exposure. They were up to 2.4 times higher against the same indicators in unexposed children and reference levels as well. The results of field observations were verified by elevated levels of the same chemicals in blood under experimental modeling of an equivalent combined exposure performed on biological models. APOBEC1 complement factor (the A1CF gene) was substantiated as an identical proteomic marker based on plasma proteomic profiling in experimental and field investigations. It has an evidence-based association with markers of exposure (nickel and copper simultaneously identified in blood). Lower expression of this protein under persistent combined aerogenic exposure to nickel and copper oxides makes it possible to predict such a negative effect as modification of low density lipoproteins with further induction of atherosclerotic changes in vessels, the latter being a risk factor of cardiovascular diseases.

Keywords: *proteomic markers, markers of exposure, children, biological model, the A1CF gene expression, prediction of negative effects.*

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It is important to investigate changes in homeostatic balance of the body at the earliest stages in their development, primarily at the cellular-molecular level. This is a promising area in both fundamental and applied research [1]. Proteomic analysis enriches theoretical knowledge on cellular-molecular mechanisms of negative effects thereby increasing the predictive potential of diagnostics when it comes down to some non-communicable diseases. Searching for informative molecular markers is a priority trend in fundamental research in the Russian Federation[1]. New knowledge on modification of etiopathogenesis of diseases caused by exposure to risk-inducing factors provides solid scientific grounds for identifying conditions, causes, occurrence, prevention and reduction of health risks and health harm [2].

Mechanisms of negative effects can be modified due to interaction between chemical risk factors and some genes that modulate expression of certain proteins responsible for providing functional activity of molecular-biological processes [3]. Homeostasis transforms at the cellular-molecular level against the persisting modifying effects produced by chemical factors and this leads to negative health outcomes thereby increasing a risk of a disease[2]. Analysis of genes coding for proteins that are influenced by chemical factors gives an insight into changes in biological functions and molecular networks occurring under chemical exposures. Investigation of such genetic-chemical interactions is a powerful information resource eligible for obtaining and developing knowledge about etiology and molecular mechanisms that underlie modification of processes associated with exposure to chemical risk factors [4, 5].

Proteins have a key role in providing vital activity of cells and the body as a whole. So, identified qualitative and quantitative changes in them can be potentially informative for the earliest detection of negative effects able to induce further substantial functional disorders of critical organs and systems.

Therefore, at present the most promising studies focus on searching for proteomic markers and their combinations as potential molecular targets. They reflect the functional state and characteristics of mechanisms with etiopathogenetic motivation as a response to chemical exposures. Identification of genetic-chemical interactions between expression of a protein marker and exposure factors makes it possible to predict negative health outcomes. This gave grounds for formulating the aim of the present study, which continues the research cycle performed by the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies. The cycle concentrates on using ‘omic’ technologies for substantiating informative molecular-cellular markers of negative effects [6, 7].

The aim of the present study was to identify markers of a transformed plasma proteomic profile in children. These markers should have prognostic value and an evidenced association with combined aerogenic exposure to metal oxides (copper and nickel oxides used as an example)

Materials and methods. We applied an innovative methodical approach to identify and substantiate molecular protein markers changes in which were associated

[1] Programma fundamental'nykh nauchnykh issledovanii v Rossiiskoi Federatsii na dolgosrochnyi period (2021–2030 gody): Rasporyazhenie Pravitel'stva Rossiiskoi Federatsii ot 31.12.2020 № 3684-r [The program of long-term fundamental research in the Russian Federation (2021–2030): the RF Government Order issued on December 31, 2020 No. 3684-r]. *KonsultantPlus*. Available at: https://www.consultant.ru/document/cons_doc_LAW_373604/ (January 21, 2023) (in Russian).

[2] Drozdova E.V., Dudchik N.V., Sychik S.I., Shevlyakov V.V. Integrated toxicity assessment of environmental factors and objects using alternative biological test models: methodology and technology. Minsk, Transtekhnika Belorussian Scientific Research Institute of Transport Publ., 2017, 216 p. (in Russian).

with exposure to airborne copper and nickel oxides. The approach included the following stages:

– confirming actual aerogenic exposure based on identifying the relevant indicators within the 'chemical levels in the air – chemical levels in biological media' system;

– comparing protein stains with statistically significantly different intensities based on proteomic plasma profiling and identification of distinguished proteins;

– identifying proteins and peptides that were identical as per the results of experimental and field studies under combined exposure to the analyzed chemicals;

– quantifying parameters of cause-effect relations between identical proteins and peptides and chemical levels in biological media;

– predicting negative health outcomes based on building and analyzing a biotransformation molecular matrix of identical proteins together with identifying their functions, biological processes and expression in tissues.

The suggested algorithm was tested by comparing changed proteins and peptides identified in proteomic plasma profiles of children under actual combined exposure to airborne nickel and copper oxides and small rodents (a biological model) under experimental combined and isolated exposure equivalent to actual levels of the analyzed chemicals in the air.

The experimental studies were performed on female Wistar rats. Twenty four animals were divided into 4 groups, 6 rats in each: the test group No. 1 was exposed to isolated standard nickel oxide in a dose equal to 0.38 mg/kg; the test group No. 2 was exposed to isolated copper oxide in a dose equal to 1.23 mg/kg; the test group No. 3 was exposed to a combination of copper and nickel oxides in the aforementioned doses; the group No. 4 was a reference one kept under the same conditions as the test groups but without any exposure to the analyzed chemicals. The animals in the experiment were exposed to doses of chemicals equivalent to the actual chronic aerogenic exposure considering animals' body weight and species peculiarities. Blood samples were collected from the rats 24 hours after the exposure. Blood was taken from the sublingual vein in a volume of 3 cm^3.

The experiments were performed in conformity with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123)[3] and the requirements fixed by the Ethics Committee of the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies.

Plasma proteomic profiles were identified for 45 children aged 4–7 years. Twenty-five of them lived under long-term exposure to airborne nickel oxide (0.0034 mg/(kg·day)) and copper oxide (0.0016 mg/(kg·day)) and were included in the test group; the remaining 20 children were not exposed to the analyzed chemicals (the reference group). Children were included into the test group in case they had elevated (≥ 1.2 Rfl) cooper and nickel levels in blood; levels of the analyzed chemicals equaled minimal or reference values in blood of the children from the reference group (nickel Rfl = 0.01 mg/dm^3, copper Rfl = 0.9 mg/dm^3)[4].

The children were examined in conformity with the ethical principles stated in the

[3] European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123); the edition of the CED Protocol No. 170 dated December 02, 2005. Strasbourg, 1986, 11 p. Available at: https://www.biosoil.ru/files/docs/bioethics/info/European%20Convention%20for%20the%20Protection%20of%20Vertebrate%20Animals%20used%20for%20Experimental%20and%20Other%20Scientific%20Purposes.pdf (February 01, 2023).

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Declaration of Helsinki (64th WMA General Assembly, 2013[5]) provided that their legal representatives gave their voluntary informed consent to their participation in the study. All the studies conformed to the requirements fixed by the Ethics Committee of the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies (the Meeting Report No. 1 dated February 04, 2021).

Copper and nickel levels in blood were identified in conformity with the Methodical guidelines MUK 4.1.3230-14[6] using Agilent 7500cx mass spectrometer (Agilent Technologies, USA).

Analysis of plasma proteomic profile included sampling, two-dimensional electrophoresis in polyacrylamide gel[7], analysis of two-dimensional electrophoretogram, spotting out significant protein stains as per their intensity, mass-spectrometric analysis performed with UltiMate 3000 chromatographer (Germany) and ABSciex 4000 QTRAP tandem mass spectrometer with Nanospray 3 ionization source (Canada). Proteins were identified based on the UniProt[8] database with the selection as per *Homo Sapience* taxon and *Rattus norvegicus* taxon. Genes coding for identified proteins were established using the HUGO Gene Nomenclature Committee database (HGNC)[9] and The Rat Genome Database (RGD)[10]. Biological functions of proteins were described using The Gene Ontology[11]; data on phylogenetics and functional genomics were taken from the PhyloGenes[12]; the obtained data on protein expression in tissues were analyzed using Tissue expression database[13] and The Human Protein Atlas[14]. Data about probable etiopathogenetic mechanisms of predicted negative health outcomes associated with chemical exposures were obtained and analyzed by using Comparative Toxicogenomics[15] and DisGeNET[16].

Indicator values identified in the exposed children were compared with the same indicators in the unexposed ones; in the animal experiments, the test groups were compared with the reference one. Descriptive statistics of quantitative variables was given with the mean value (M) and error of mean (m). Statistical significance of differences between compared groups was determined with the Mann – Whitney test ($U \leq Ucr$), the levels of significance being $p \leq 0.05$. All the data were statistically analyzed with Statistica 10.

Molecular markers of negative effects were substantiated based on created models of 'chemical levels in blood – statistically significant intensity of a protein stain' relation-

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[16] DisGeNET. Available at: https://www.disgenet.org/dbinfo (January 27, 2023).

ships. The latter were described with a multiple liner regression as per the formula (2):

$$y_j = b_{0j} + \sum_i b_{ij} x_i \quad (2)$$

where y_j is a dependent variable (intensity of the j^{th} protein stain, int);

x_i is an independent variable, i^{th} influencing factor (chemical level in blood, mg/dm^3);

b_{0j}, b_{ij} are the model coefficients.

Validity and relevance of the created models were compared with dispersion analysis using the Fisher's F-test, determination coefficient (R^2), and the Student's t-test, the statistical significance being $p \leq 0.05$.

Results and discussion. Comparative analysis of copper and nickel levels in blood of the experimental animals established authentic statistical differences between the groups. Isolated exposure to nickel created its levels in blood equal to 0.014 ± 0.002 mg/dm^3; this level was 0.008 ± 0.001 mg/dm^3 under combined exposure to both analyzed chemicals. These concentrations were 2.9 times and 1.7 times higher accordingly than in the reference group (p = 0.001–0.012). Copper level in blood equaled 2.323 ± 0.060 mg/dm^3 under isolated exposure and 2.006 ± 0.047 mg/dm^3 under combined exposure, being 1.5 times and 1.3 times higher accordingly than the same indicator in the reference group (p = 0.0001–0.002). Nickel and copper levels in blood were 1.2 times and 1.8 times higher accordingly under isolated exposure than under combined one (p = 0.002–0.022).

Actual simultaneous exposure to copper and nickel oxides created elevated blood levels of the analyzed chemicals in the exposed children; these levels were 1.2–2.4 times higher against the same indicators in the unexposed children (p = 0.032–0.033) and 1.2–1.3 times higher than the reference levels. The established and parameterized association of copper and nickel in blood and levels of these chemicals in ambient air (copper a_0 = 0.515, a_1 = 752.32; nickel a_0 = 0.005, a_1 = 145.36, p = 0.05) substantiates copper and nickel levels in blood as relevant markers of aerogenic exposure.

We compared the results obtained by densitometry of plasma proteomic profiles in the experimental animals under isolated and combined exposure to the analyzed chemicals. The comparison identified 8 proteins identical in all the experimental groups (Table 1).

We performed densitometry to study and compare proteins in plasma proteomic profiles of the exposed and unexposed children. As a result, we identified 20 stains with authentically different intensity and a proven association with elevated nickel and copper levels in blood (Table 2).

Table 1

Statistically significant proteins proven to be associated with experimental combined exposure

No.	Statistically significant proteins			Validity of the model for 'chemical – intensity of a protein stain under combined exposure' relationship ($p \leq 0.05$)
	Isolated exposure		Combined exposure	
	copper	nickel	copper + nickel	
1	2	3	4	5
1.	Telomerase protein component 1	-	Telomerase protein component 1	0.002
2.	Keratin, type II cytoskeletal 75	-	Keratin, type II cytoskeletal 75	0.0001
3.	**Peroxisomal 2,4-dienoyl-CoA reductase**	-	**Peroxisomal 2,4-dienoyl-CoA reductase**	0.002
4.	Advillin	-	Advillin	0.019

End of the Table 1

1	2	3	4	5
5.	Cytosolic aldehyde dehydrogenase 1	-	Cytosolic aldehyde dehydrogenase 1	0.008
6.	-	Elongation factor 1-γ	Elongation factor 1-γ	0.001
7.	-	Styrene carrier protein 2	Styrene carrier protein 2	0.002
8.	-	Myosin-6	Myosin-6	0.002
9.	-	Calcium-binding protein 7	Calcium-binding protein 7	0.002
10.	-	Vesicular transporter SEC22B	Vesicular transporter SEC22B	0.001
Statistically significant proteins identified under both isolated and combined experimental exposure				
11.	Hemoglobin subunit β-2			0.001
12.	Glycogen synthase, muscular			0.001
13.	**CAP-GLY** domain containing **linker 4**			0.002
14.	Vesicular transporter SEC22A			0.001
15.	Protein SDA1 homolog			0.003
16.	Transcription activator BRG1			0.001
17.	Ig kappa chain C region, allele			0.001
18.	APOBEC1 complement factor			0.006

Table 2

The parameters of the multi-factor relationship between changes in intensity of a protein stain and simultaneous copper and nickel levels in blood of the exposed children

No.	Protein in a stain	A change in intensity of a protein stain	The parameters of the 'exposure marker (nickel and copper in blood) – proteomic marker (intensity of a protein stain)' relationship				Validity of differences ($p \le 0.05$)
			b_0	Copper (b_1)	Nickel (b_2)	Determination coefficient (R^2)	
1.	Sodium/hydrogen exchanger 2	Decline	5291.9	-1458	-54,723	0.24	0.013
2.	Protein 33 with a spiral coil	Decline	5245.2	-1202.9	-63,777.4	0.21	0.025
3.	Myotubularin	Decline	1803	-1065.8	-39,425.8	0.23	0.016
4.	Coagulation factor V	Decline	439.2	-270.6	-7449.8	0.19	0.032
5.	Ornithine decarboxylase antizyme 2	Decline	1112.9	-616.7	-28,418.7	0.25	0.011
6.	RING finger protein unkempt homolog	Decline	2656.4	-821.9	-39,891.8	0.19	0.031
7.	Vitronectin	Decline	3092.7	-1860.8	-40,674.4	0.16	0.059
8.	Centrosomal protein 290kDa	Decline	2242.6	-830.3	-49,443.7	0.30	0.003
9.	Zinc finger protein 221 protein	Decline	2989.8	-1496.5	-49,250.8	0.24	0.013
10.	Apolipoprotein A-I	Decline	936.8	-660.6	1007.4	0.19	0.032
11.	ADAM-like, decysin 1	Decline	3739.1	-1020.1	-62,371.9	0.25	0.010
12.	Nuclear protein MDM1	Growth	1596.3	935.8	38,990	0.17	0.048
13.	APOBEC1 complement factor	Decline	3581.2	-384.5	-11,137.7	0.13	0.017
14.	DNAJ homolog subfamily C member 3	Growth	2231.2	1747.2	63,004.9	0.24	0.013
15.	WD repeat protein 64	Growth	1014.9	768.6	44,043.7	0.22	0.019
16.	L-type beta-4 voltage-gated calcium channel subunit	Decline	1391.3	-861.5	-22,471.4	0.25	0.010
17.	Leucine-rich repeats and immunoglobulin-0like domains, protein 3	Decline	2531.7	-1032.3	-40,311.9	0.19	0.033
18.	Protein-glutamine gamma-glutamyltransferase e	Growth	394.6	-35.5	32,116.8	0.19	0.032
19.	Pyroglutamyl-peptidase 1-like protein	Growth	1657.4	666.3	39,792.4	0.17	0.048
20.	Clathrin heavy chain 2	Decline	2605.8	-1031.1	-95,362.5	0.36	0.001

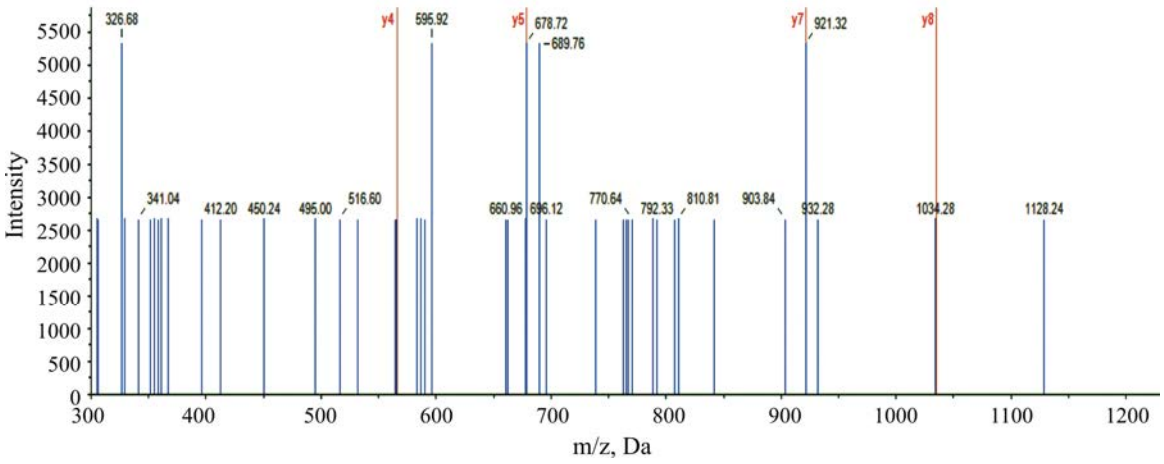

Figure. The spectrum of the SGPGLSGTQK peptide (APOBEC-1 complement factor) (SwissProt database) in a child's plasma

We performed comparative biotransformation analysis of phylogenetics of the identified proteins and genes coding for their expression under experimental and actual combined exposure. As a result, we established one identical protein, APOBEC1 complement factor, and the orthologous A1CF gene encoding it in the human body. The Figure provides the spectrum of the identical protein peptide.

APOBEC1 complement factor is a vital component in the enzymatic complex. It modifies mRNA of apolipoprotein B (ApoB), which is necessary for collecting low density lipoproteins (LDL) from lipids. In rats (Rattus norvegicus), APOBEC1 complement factor is widely spread in the liver [8], kidneys [9], intestines [10], thyroid gland, and the nervous system [11]. In the human body, this factor is expressed solely in GIT epithelial cells and small intestines [12, 13]. Several experimental studies have established that APOBEC-1 super-expression in the liver reduces ApoB levels effectively thereby regulating cholesterol metabolism [14]. Low A1CF gene expression is a reason why consumption of fats in high quantities is potentially hazardous for human health. As exogenous lipids are being absorbed, the ApoB level grows thereby inducing increased synthesis of low density lipoproteins [15], which may lead to atherosclerotic changes in the vessels [14, 16].

We identified and estimated a multi-factor relationship (R^2 = 0.19; b_0 = 3581.2; b_1 = -384.5; b_2 = -11,137.7; p = 0.017) between a decline in intensity of the APOBEC-1 complement factor in plasma and elevated nickel and copper levels as markers of exposure. This relationship is consistent with the results of research focusing on genetic-chemical interactions between the protein and analyzed chemicals. Exposure to copper and nickel has been shown to reduce the A1CF gene expression. This indicates the analyzed chemicals are able to modify molecular functions and biological processes of this protein [17]. Some experimental research established that exposure to nickel caused elevated triglycerides and LDL levels in blood serum and this can have some negative effects on lipid metabolism as a whole [18]. Exposure to airborne copper can induce higher production of reactive oxygen species and oxidative stress. As a result, LDL oxidative modification occurs; this induces a local immune response in vessel walls with subsequent atherosclerotic changes developing in them as a risk factor of cardiovascular diseases [19–21].

Conclusion. Our research has established that long-term combined exposure to airborne copper and nickel oxides creates elevated copper and nickel levels in blood of the exposed children. These levels are 1.2–2.4 times higher than the same indicators in the unexposed children and are also higher than the reference levels for these chemicals. Therefore, they are substantiated as marker of exposure. Results of

the field observations have been verified by 1.7–2.9 times higher levels of the same chemicals (against the reference group) established in experimental modeling of the equivalent combined exposure on a biological model (small rodents). We have substantiated APOBEC1 complement factor (the A1CF gene) as a proteomic marker identical under both experimental and actual exposure. It has a proven association with markers of exposure (simultaneous nickel and copper levels in blood). Declining expression of this protein under persistent exposure to airborne copper and nickel oxides makes it possible to predict developing negative health outcomes such as modification of low density lipoproteins with subsequent induction of atherosclerotic changes in the vessels, which are a risk factor of cardiovascular diseases.

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