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

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Research article

ON DETECTING OMIC-MARKERS OF NEGATIVE EFFECTS ASSOCIATED WITH COMBINED AEROGENIC EXPOSURE TO ALUMINUM AND FLUORIDE COMPOUNDS

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At present, it is relevant to study simultaneous combined impacts exerted by chemicals on developing adverse health effects. It is also becoming vital to search for molecular indicators of adverse effects with the altered expression level. This alteration makes it possible to determine peculiarities of molecular and cellular pathogenesis mechanisms regarding a number of non-communicable diseases under exposure to a mixture of chemicals.

Our research goal was to comparatively analyze and identify identical omic-markers of adverse effects under experimental and actual combined aerogenic exposure to aluminum and fluoride compounds. We substantiated molecular markers of prenosological changes by sequential implementation of an algorithm which included identifying altered proteins and peptides in blood plasma which were identical both under experimental and actual exposure; detecting and quantifying cause-effect relations between identical proteins and peptides and concentrations of aluminum and fluoride ion in urine.

The research results indicate that long-term combined aerogenic exposure to aluminum and fluoride compounds in low average daily doses (0.0005 mg/(kg·day) and 0.002 mg/(kg·day) accordingly) causes elevated concentrations of aluminum (by 2.8 times higher) and fluoride-ion (by 1.8 times higher) in exposed children's urine. This fact is verified by experimental research with its focus on combined exposure to the examined chemicals. We were able to substantiate identical omic-markers, J-chain of immunoglobulin and Kelch-like protein 4 (KLHL4 gene), under simultaneous exposure to aluminum and fluoride compounds both under experimental and actual combined aerogenic exposure. We proved a cause-effect relation between levels of identical proteins and concentrations of aluminum and fluoride ion in urine under simultaneous exposure to the mixture of the

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examined chemicals. Identified protein markers in blood plasma give an opportunity to predict future adverse effects including developing immunoglobulins A and M deficiency with subsequent humoral immunity failure when J-chain of immunoglobulin is expressed; occurring sclerotic and inflammatory changes in vascular walls when Kelch-like protein 4 is expressed. These predicted adverse effects can be estimated as resulting from simple summated (additive) toxic impacts exerted by aluminum and fluoride under simultaneous combined aerogenic exposure to both chemicals

Key words: *aluminum and fluoride ion in urine, risk of adverse effects, isolated and combined exposure, blood plasma proteomic profile, immune system, cardiovascular system, immunoglobulin J-chain and Kelch-like protein 4.*

At present, there is a significant trend in providing chemical safety in the Russian Federation and all over the world as well. This trend involves assessing adverse health effects produced by combined exposure to a mixture of contaminants even if each of them is hardly toxic when introduced separately. This aspect is outlined in guidelines issued by the World Health Organization (WHO) and the International Program on Chemical Safety (IPCS) [1, 2]. Nowadays people are often exposed to combined impacts exerted by a wide range of chemicals occurring in all media, ambient air included [3]. According to data provided by the WHO, ambient air pollution is a basic risk factor causing some non-communicable diseases, primarily, respiratory diseases, diseases of the cardiovascular and nervous systems [4]. A major contribution to ambient air pollution is made by industries, especially in places where a lot of industrial enterprises are concentrated. In some regions large metallurgical enterprises are located which mostly deal with aluminum production. In such regions ambient air contamination primarily occurs due to aluminum and fluoride compounds typical for such productions being introduced into the atmosphere, predominantly in gas and dust emissions. Long-term environmental aerogenic exposure to these compounds can result in damage to cellular membranes increasing their permeability; these chemicals can bind to proteins in blood and inhibit many enzyme systems and this can ultimately lead to various pathological changes in mechanisms which support homeostasis [5–7].

Examination of a proteomic blood plasma profile is a promising trend in research which provides an opportunity to effectively identify molecular and cellular mechanisms of changes in homeostasis caused by exposure to adverse risk factors. Identification, quantification and changes in levels of proteins (omic-markers) expressed in cellular and tissue structures under exposure to adverse risk factors, chemical ones included, are vital challenges in examining development of certain respiratory diseases, circulatory diseases, diseases of the nervous system etc. [8, 9]. Experimental research conducted on biological test models is a significant trend in molecular profiling aimed at searching for protein markers of negative effects, determining mechanisms and nature of interactions between chemical exposure factors which produce these negative effects both under separate and combined exposure [10]. Any changes in protein profiles which were determined by experiments should be verified with results produced by full-scale observations. This secures greater precision and objectivity of substantiated molecular protein markers. Foreign and domestic scientific data on alterations in peptides and genes coding their expression under exposure to a mixture of chemicals are rather controversial. Results produced by several experimental research works indicate that fluoride, when introduced separately, inhibits metal-containing enzymes and aluminum, in its turn, inhibits enzymes which are involved into energy metabolism in a cell [11]. Exposure to a mixture of these two chemicals makes the damage reverse and the chemicals inhibit each other's enzymes; this indicates that their toxic effects are antagonistic. Results produced by some other foreign studies show that mixed aluminum and fluoride act in the same way as they interact with the bilipid layer in cellular membranes, interfere with ion transportation, and induce conformation changes in guanosine triphosphate (GTP) which results in artificial activation of

guanosine diphosphate (GDP) and related Ras-proteins[1] [12–14]. These proteins are responsible for transferring signals from the extracellular space and participate in regulation of cellular proliferation[1].

Bearing in mind these controversial data on examining simultaneous combined exposure to different chemicals and its effects on the body, it seems advisable to search for molecular targets (proteins). Changes in their expression can be used as a tool for early prenosologic detection of non-communicable diseases. We can state that it is vital to establish relevant changes in proteins and peptides in a proteomic blood plasma profile in order to predict risks of developing adverse health effects produced by combined aerogenic exposure to chemical risk factors, including fluoride and aluminum compounds, both in experimental and actual conditions.

Our research goal was to comparatively analyze and identify identical omic-markers of negative effects both under experimental and actual combined aerogenic exposure to aluminum and fluoride compounds simultaneously.

Materials and methods. Our research objects were proteomic blood plasma profiles and protein peptides in children and experimental animals under combined exposure to aluminum and fluoride compounds.

We conducted our experiments on female Wistar rats, 12 animals overall. They were divided into 4 groups, 3 animals in each. Group No. 1 (test) was exposed separately to a standard sample (SS) of fluoride-ion in a dose equal to 20 mg/kg of body weight; Group No. 2 (test) was exposed separately to aluminum suspension based on isotonic sodium chloride solution in a dose equal to 1.67 mg/kg of body weight; Group No. 3 (test) was under combined exposure to a mixture of fluoride-ion and aluminum in doses outlined above; Group No. 4 was a control, animals were kept under the same conditions as three other groups but they were not exposed to the examined chemicals. The experimental exposures were one-time and intraperitoneal. Chemical doses introduced into experimental animals were equivalent to an actual aerogenic exposure allowing for the duration of exposure to chemicals, body weight, a period of exposure averaging, and species-related peculiarities.. Blood samples of the experimental animals were taken 24 hours after the exposure from the sublingual vein in a volume equal to 3 cm^3; urine samples were taken 24 hours after the exposure during one day in a DXL-D metabolic cage (3W Fengshi, China).

All the experiments conformed to the requirements fixed by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS No. 123) and the ethical committee of the Federal Scientific Center for Medical and Preventive Health Risk Management Technologies.

We performed profound screening examinations of 35 children aged 4–7 years. 25 children who underwent long-term combined aerogenic exposure to aluminum compounds (in a concentration equal to 0.0005 mg/(kg·day)) and fluoride compounds (0.002 mg/(kg·day)) were included into the test group. Other 10 children were not exposed to the examined chemicals and were included into the reference group. Children were included into the test group as per such a criterion as elevated aluminum and fluoride-ion concentrations in urine; children in the reference group had the examined chemicals in their urine in concentrations corresponding to minimal or reference values[2].

All the children were examined in conformity with the ethical principles stated in the WMA Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects, 2013) and the examinations were approved by the Committee on the biomedical ethics of the Federal Scientific Center for

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Medical and Preventive Health Risk Management Technologies. Legal representatives of all the participating children gave their informed voluntary consent to this participation. The conducted study didn't infringe on the rights of participating human subjects, didn't put their welfare in any danger and didn't cause any harm to their health.

Urine was analyzed to determine aluminum and fluoride-ion in it according to the methodical guidelines[3] using an ion-selective electrode with I-160M laboratory ionometer ("Antech" LLC, Belarus) and Agilent 7500cx mass spectrometer (Agilent Technologies Inc., USA) (by T.S. Ulanova, Doctor of Biological Sciences and Head of the Department for Chemical and Analytical Research Techniques).

An algorithm which we applied to examine proteomic blood plasma profiles of children and experimental animals included several stages: taking samples; two-dimensional gel electrophoresis in polyacrylamide gel[4]; analysis of two-deminsional electrophoresis charts; spotting out significant protein stains as per their intensity. The subsequent mass-spectrometry analysis performed with UltiMate 3000 chromatographer (Germany) and ABSciex 4000 QTRAP tandem mass spectrometer with Nanospray 3 ion source (Canada) involved determining amino acid sequences of individual protein fragments, protein identification, and analysis of UniProt database with sampling as per Homo Sapience and Rattus norvegicus taxon. We determined genes which identified proteins corresponded to using HGNC database of human gene name[5].

Indicator values established in exposed children were comparatively estimated against the same indicators in non-exposed children; indicator values established in experimental animals in test groups were comparatively analyzed against the same values in the control. The research results are given as a simple mean ($\bar{X}$), standard error of mean (*SEM*) and standard deviation (*SD*). We applied Mann – Whitney test ($U \leq U_{cr}$) to check whether intergroup differences in variables were statistically significant with the significance level fixed as $p \leq 0.05$. All the data were statistically analyzed using Statistica 10 software package.

Omic-markers of negative effects in children which were associated with combined exposure to the examined chemicals were substantiated by the step-by-step implementation of an algorithm including the following: identifying identical proteins and peptides in blood plasma profiles under experimental and actual exposure; determining and quantifying cause-effect relations between identical proteins and peptides and aluminum and fluoride-ion concentrations in urine; predicting adverse effects based on analyzing data on molecular functions and biological roles played by identified peptides. We tested whether the resulting models were authentic and relevant using Fischer's test ($F \geq 3.96$) and determination coefficient (R^2) with the statistical significance being $p \leq 0.05$.

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Results and discussion. Our experiments revealed that an average fluoride-ion concentration was by 19.2 times higher in urine of rats exposed solely to this chemical in a concentration equal to 20 mg/kg of a body weight than in urine of rats from the control group ($p = 0.012$). Aluminum concentrations in urine of rats exposed to the chemical in a dose equal to 1.67 mg/kg of a body amounted to 0.057 ± ± 0.010 mg/dm^3 and was by 9.5 times higher than in the control group ($p = 0.012$) (Table 1).

Combined inhalation exposure to mixed fluoride-ion and aluminum in the same doses resulted in 5.6 times higher concentrations of fluoride-ion and 3.2 times higher concentrations of aluminum in urine of rats from Group No. 3 than in the control group ($p = 0.012$). It should be noted that bioaccumulation of fluoride-ion and aluminum in the body is more apparent under separate exposure than under combined one; this is confirmed by 3.0–3.3 times higher concentrations of aluminum and fluoride-ion in urine under separate exposure than under combined one ($p = 0.012$). This might indicate there is possible antagonism between these two chemicals when adverse effects are developing [11].

Our full-scale observations established that children from the test group who underwent long-term aerogenic exposure simultaneously to aluminum (0.0005 mg/(kg·day)) and fluoride (0.002 mg/(kg·day)) had 2.8 times higher concentrations of aluminum and 1.8 times higher concentrations of fluoride-ion in their urine than children in the reference group ($p = 0.006$–0.039).

We examined whether there were authentically different proteins in blood plasma of rats from the test groups and the control one. Overall, two-dimensional electrophoresis charts allowed identifying 10 protein fractions under separate exposure to fluoride; 13, under separate exposure to aluminum; and 13, under combined exposure to the mixture of the examined chemicals (Table 2).

We detected several proteins under combined exposure to the mixture of the examined chemicals which were identical to those detected in protein strains under separate exposure. In case of combined exposure and separate exposure to fluoride the identical proteins were plasma protease C1 inhibitor, zinc finger protein 644, nuclear receptor-coactivator 4, J chain of immunoglobulin, apolipoprotein A-I,

Table 1

Fluoride-ion and aluminum concentrations under experimental and actual exposure

Group	Indicator	Fluoride-ion, mg/dm^3	Aluminum, mg/dm^3
Experimental research			
Test group of rats under separate exposure to chemicals	Simple mean ± standard error of mean, $\bar{X} \pm SEM$	6.800 ± 1.571	0.057 ± 0.010
	Standard deviation, *SD*	3.51	0.0230
	Validity of differences between mean values in test and control groups, *p*	0.012	0.012
Test group of rats under combined exposure to chemicals	Simple mean ± standard error of mean, $\bar{X} \pm SEM$	2.07 ± 0.271	0.019 ± 0.005
	Standard deviation, *SD*	0.606	0.012
	Validity of differences between mean values in test and control groups, *p*	0.012	0.012
Control group of rats	Simple mean ± standard error of mean, $\bar{X} \pm SEM$	0.354 ± 0.062	0.006 ± 0.001
	Standard deviation, *SD*	0.138	0.010
Full-scale observation			
Test group (children)	Simple mean ± standard error of mean, $\bar{X} \pm SEM$	0.687 ± 0.076	0.011 ± 0.003
	Standard deviation, *SD*	0.378	0.019
	Validity of differences between mean values in test and control groups, *p*	0.006	0.039
Reference group (children)	Simple mean ± standard error of mean, $\bar{X} \pm SEM$	0.374 ± 0.053	0.004 ± 0.001
	Standard deviation, *SD*	0.167	0.001

Table 2

Proteins and peptides in their structure which authentically differ from the control group and are identified in proteomic blood plasma profile of rats exposed separately to fluoride and aluminum and to the mixture of these chemicals

Sequences of amino acids residues in of a peptide	Peptide identification probability, %	Protein	Authenticity of a model showing "chemical – significant protein" relationship ($p \leq 0.05$)
Separate exposure to fluoride			
DSLNMWLCPR	47.5	Nuclear receptor coactivator 4	0.009
VDCLKTFGR	18.9	Laminin Subunit Alpha 3	0.070
CYTAVVPLVYGGETK	99.1	J chain of immunoglobulin	0.043
VSFLSALEEYTK	96.4	Apolipoprotein A-I	0.025
EAMGKLYNFSTSSR	94.1	Alpha-protein kinase 1	0.011
GWVTDGFSSLK	99.4	Apolipoprotein C-III	0.030
LLVVYPWTQR	97.2	Hemoglobin subunit gamma-2	0.025
FQPTLLTLPR	39.1	Plasma protease C1 inhibitor	0.010
NSAISPQK	75.4	Zink finger protein 644	0.301
Separate exposure to aluminum			
INGKPLPGATPAK	39.8	tRNA selenocysteine 1-associated protein 1	0.0001
GLCVATPVQLR	98.7	C4-B Complement	0.0001
QRIEALSLMHPSISFSLR	61.3	DNA mismatch repair protein Mlh3	0.002
NIVQNVR	28.9	Sideroflexin-3	0.029
LMAKAEDLR	69.8	Nck-associated protein 5	0.002
DDLIIDLLNEAK	36.8	V-type proton ATPhase of E 1 subunit	0.0001
EAMGKLYNFSTSSR	86.2	Alpha-protein kinase-1	0.004
QLCGCYLTR	82.3	Thrymethyllysine dioxygenase, mitochondria	0.0001
LPLLPPQLLADLETSSMFTGD LECQKLLMEAMK	97.6	Kelch-like protein 4	0.016
GLPDDHAGPIR	35.4	Alanyl-tRNA, editing protein Aarsd1	0.001
GLEEELQFSLGSK	97.3	C4-B complement	0.001
DESSLK	42.1	Probable E3 ubiquitin-protein lygase MID2	0.0001
EILSEVER	15.2	T-complex protein 1 subunit gamma	0.0001
Combined exposure to the mixture of fluoride and aluminum			
KMGEMATSGDR	41,9	MARVEL domain containing protein 2	0,002
FQPTLLTLPR	48.7	C1protease inhibitor in plasma	0.021
YMPYNHQHK	52.8	Acyl-KoA (8-3) desaturase	0.003
APETGGAPRAPGAGR	75.3	Serine/threonine-protein kinase LMTK3	0.037
NSAISPQK	64.8	Zinc finger protein 644	0.069
DSLNMWLCPR	28.9	Nuclear receptor coactivator 4	0.008
TSESGELHGLTTEEEFVEGIYK	98.9	Transthyretin	0.006
CYTAVVPLVYGGETK	99.8	J chain of immunoglobulin	0.018
VSFLSALEEYTK	96.3	Apolipoprotein A-I	0.060
EAMGKLYNFSTSSR	93.2	Alpha-protein kinase 1	0.0001
LPLLPPQLLADLETSSMFTGD	95.1	Kelch-like protein 4	0.004
LLVVYPWTQR	98.7	Hemoglobin subunit gamma-2	0.544
EILSEVER	78.9	T-complex protein 1 subunit gamma	0.012

alpha-protein kinase 1, hemoglobin subunit gamma-2; in case of combined exposure and separate exposure to aluminum these proteins were alpha-protein kinase 1, Kelch-like protein 4, protein T-complex protein 1 subunit gamma. Growing contents of the aforementioned proteins, including apolipoprotein A-I, zinc finger protein 644 and hemoglobin subunit gamma-2, had a detected cause-effect relation with elevated concentrations of fluoride-

ion and aluminum in experimental animals' urine under exposure to their mixture (R^2 = 0.81–0.97; p = 0.0001–0.018).

We comparatively analyzed authentically different altered proteins in proteomic blood plasma profiles of children under actual aerogenic and those of against unexposed children. The analysis established 2 protein stains out of total 25 which were quantitatively and qualitatively similar to altered proteins detected under combined exposure to the mixture of fluoride and aluminum in experimental conditions. These two proteins were J chain of immunoglobulin (its expression is coded by JCHAIN gene) and Kelch-like protein 4 (expression coded by KLHL4 gene) (Table 3).

Thus, children from the test group had by 17.9–27.1 times greater volumes of proteins containing J chain of immunoglobulin and Kelch-like protein 4 than children from the reference group (p = 0.0001). This was comparable to changes in volumes of these protein stains detected in the experiment. A probability that these detected peptides would be identified varied from 98.7 % to 99.2 %. Figure shows an example two-dimensional electrophoresis chart showing one of the examined peptides.

We proved that the growth in relative volumes of Kelch-like protein 4 and J chain of immunoglobulin depended on elevated aluminum and fluoride-ion concentrations in urine under combined exposure to both chemicals in a mixture. Model parameters for Kelch-like protein 4 were as follows: R^2 = 0.07; b_0 = 1180.64; b_1 = 9141.60; b_2 = 190.10; (p = 0.046); for J chain of immunoglobulin, as follows: R^2 = 0.06; b_0 = 1804.56; b_1 = 5767.11; b_2 = 206.47; (p = 0.030).

Generalization and analysis of the existing scientific data on biological functions performed by the identified proteins in blood plasma make it possible to predict certain adverse health effects which may occur in the immune and cardiovascular system. Thus, the major function performed by J chain of

Table 3

Identical proteins detected under experimental and actual combined exposure to aluminum and fluoride ($p \leq 0.05$)

Protein	Relative protein stain volume, int Simple mean ± standard error of mean, $\bar{X} \pm SEM$			
	Experimental exposure		Actual exposure	
	Test group under combined exposure to both chemicals	Control	Test group	Reference group
Kelch-like protein 4	$4097 \pm 106^{p = 0.012}$	469 ± 224	$2548 \pm 57^{p = 0.0001}$	94 ± 36
J chain of immunoglobulin	$2635 \pm 52^{p = 0.007}$	1785 ± 86	$1807 \pm 38^{p = 0.0001}$	101 ± 34

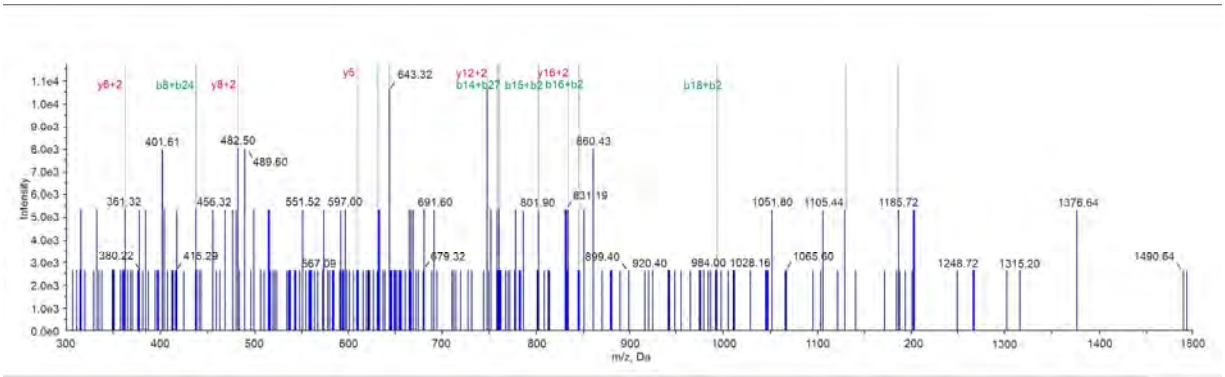

Figure. MS-identification of LPLLPPQLLADLETSSMFTGDLECQKLLMEAMK peptide (Kelch-like protein 4) in a child's blood plasma (SwissProt database)

immunoglobulin is to initiate IgM and IgA polymerization [15]. J chain inclusion into polymeric IgA and pentameric IgM gives these antibodies high valence of antigen-binding sites and zero potential of the complement activation. These alterations make for immunoglobulins to be capable to agglutinate bacteria and viruses thus preventing them from initiating a cascade of inflammatory reactions. Thereby secretory antibodies create "the first line" of protection against pathogens and adverse chemicals which penetrate the body through mucosa [16]. Relative volumes of the protein strain containing J chain of immunoglobulin grow under combined exposure to aluminum and fluoride both in experimental and actual conditions. This may indicate that the immune response is undergoing certain adaptive restructuring with further developing IgA and IgM deficiency which is typical for chronic inflammation [17].

Proteins from Kelch family play a major role in variable cellular processes including cytoskeleton organization and ion channels flickering. This process is accompanied with an ion channel transferring from its open state to a closed one [18, 19]. Ion channels (potassium- and calcium-dependent) detected in endotheliocytes are involved into electrolyte transportation. Any failure in it can make for decreasing vessel lumens and growing peripheral vascular resistance. In this regard we can assume that the excessive expression of this gene and, consequently, excessive synthesis of Kelch-like protein induces mechanisms which regulate the vascular tonus [20]. We established a direct relation between growing Kelch-like protein levels and elevated aluminum and fluoride concentrations in urine under combined exposure to the mixture of these two chemicals both in experimental and actual conditions. This can indicate that the mixture of the examined chemicals produces negative health effects such as developing sclerotic and inflammatory changes in vascular walls under combined exposure to them.

Therefore, it is vital to identify alterations in such proteins in blood plasma as Kelch-like protein 4 and J chain of immunoglobulin since it provides an opportunity to perform early diagnostics, to predict, assess, and mitigate risks of developing diseases of the cardiovascular and nervous system, blood and blood-making organs associated with long-term combined aerogenic exposure simultaneously to fluoride and aluminum compounds.

Conclusions:

1. Long-term combined aerogenic exposure simultaneously to aluminum and fluoride compounds in low average daily doses (0.0005 mg/(kg·day) and 0.002 mg/(kg·day) respectively) makes for elevated aluminum concentrations (by 2.8 times higher) and fluoride-ion concentrations (by 1.8 times higher) in urine of exposed children against non-exposed ones. This is verified by results produced in experimental studies on combined exposure to the mixture of the examined chemicals.

2. We substantiated identical omic-markers, J chain of immunoglobulin (JCHAIN gene) and Kelch-like protein 4 (KLHL4 gene) which are expressed both under experimental and actual combined aerogenic exposure simultaneously to aluminum and fluoride compounds.

3. We proved the cause-effect relation between elevated levels of the identical proteins and aluminum and fluoride-ion concentrations in urine under simultaneous exposure to both chemicals in a mixture.

4. The identified protein markers in blood plasma allow predicting certain adverse health effects under persisting combined aerogenic exposure to aluminum and fluoride compounds. These effects might be less active humoral immunity (when J chain of immunoglobulin is expressed) and disorders in the vascular wall tonus (when Kelch-like protein 4 is expressed).

5. We established cellular and molecular mechanisms determining involvement of the transformed proteomic profile into developing adverse effects. When J chain of immunoglobulin was expressed, this involvement

was characterized by growing valence of antigen-binding centers IgA and IgM and zero potential of the activated complement that determined adaptive restructuring of the immune response. When Kelch-like protein 4 was expressed, the process was characterized with sequential closing of potassium- and calcium-dependent ion channels in endotheliocytes, failure in electrolyte transportation, smaller vascular lumens and growing vascular resistance.

6. These predicted negative effects can be considered a result of simple summated (additive) toxic impacts exerted by aluminum and fluoride under combined aerogenic exposure to them in a mixture both in experimental and actual conditions.

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