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Study of RBCs' membrane proteins and defining their potential biomarker role for tumourigenesis monitoring

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Abstract

Assessment of the causal relationship between ecological stress levels and the frequency of chronic oncological pathologies, developing new effective predictive markers of individual and population risk of population morbidity and identifying specific and non-specific mechanisms of individual and population sensitivity to external factors is one of the priority areas of modern medicine**.** Investigation of new potential biomarkers (erythrocyte membrane proteins characteristics) of virtually healthy residents of the ethnically homogeneous population for monitoring the cancer morbidity in the Sachkhere district as one of the most favourable regions (in terms of the territorial-economic, ecologic and geographic situation) of Georgia. The healthy volunteers from different villages (Chorvila, Sairkhe, Sareki) of Sachkhere District randomly were included in the study. Exclusion criteria were malignant tumours, nicotine users, excessive alcohol users, and severe chronic diseases. The spectrophotometric absorption of red blood cell (RBC) membrane proteins, their electrophoresis, glycophorin A expression level, and blood test clinical values in groups of volunteers from the different villages were investigated. The spectrophotometric absorption in RBCs' membrane proteins at 230nm was 14% higher and the level of dimerized Glycophorin A statistically significantly decreased by 15-20% in Sareki's inhabitants than in the inhabitants of Sairkhe and Chorvila. There were no statistically significant changes in the number of erythrocytes, platelets, leukocytes, lymphocytes, neutrophils, and haemoglobin; anisotropy of RBC distribution width (RDW) (variation in RBC volume and size), was found – in residents of Sareki it was higher than in Sairkhe and Chorvila. Each of the studied indicators belongs to the class of pleiotropic markers, only their complexity can be considered as an early predictor of oncological risk.

Keywords: Tumourigenesis, biomarker, RBC membrane proteins, Glycophorin A

1. Introduction

The wide prevalence of oncological diseases and their share in the mortality spectrum of the population raises the problem of environmental role, genome, and gene-environment interactions. Methodological difficulties in assessing the causal relationship between stress levels and the frequency of chronic pathologies in the population are associated with a low risk of developing the disease (relative risk ranges

from 1.2 to 2) and the length of the latent period. The study of this problem takes into account many accompanying exogenous or endogenous factors and large-scale, long-term, and costly studies [1].

Developing new effective predictive markers of individual and population risk of population morbidity and identifying specific and non-specific mechanisms of individual and population sensitivity to external factors is one of the priority areas of modern medicine.

One of the promising directions for solving the problem is a comprehensive study of morbidity and morbidity biomarkers based on so-called "outcome-based" assessments of health risks [2,3,4], which means: statistically reliable detection of areas with a high risk of cancer and at a later stage, identification of potential environmental risk factors. Restricting the study to territorial features gives a chance to reduce the degree of freedom of the studied parameters, which simplifies the task. If the environmental factors affecting the state of the system are characterized by a set of parameters x 1, x2,…in, (if x are independent variables), then the state of the system will be characterized by the function K (x1,..... x n), which reflects the intensity of the impact of the environment on a person (or population). Reducing the degree of freedom of studied parameters will give a chance to simplify some equations.

Red blood cells (RBCs) have traditionally been considered exclusively gas carriers. However, additional functions of erythrocytes as regulatory cells in the circulatory system have been established. Because RBCs are well equipped with the mechanisms needed for communication with surrounding molecules, they dynamically interact with the environment, regulate the biological activity of neighbouring cells [5], and have a systemic impact on the body. Many changes within RBCs, alterations of their structure and properties, also can be caused by interactions with plasma proteins or soluble factors (including drugs), released from activated cells, and also by tumour cells [6,7]. Consequently, they can be considered markers of specific diseases [8,9,10]. Altered RBCs have been described in several diseases such as diabetes and Alzheimer's, multiple sclerosis, and rheumatoid arthritis [11, 12]. Furthermore, it has been established that the interaction between RBCs and immune cells is important for the progression of atherosclerosis, in pathological environments they trigger the activation of the innate immune system, indicating the critical role of RBCs in inflammatory processes [13]. It has been reported that SARS-CoV-2 infection has a significant impact on RBC's membrane structure at the protein and lipid levels [14]. Changes in RBC membrane components have been identified in cancer patients [15,16,17,18,19].

Seemingly, during cancer RBCs' proteomic composition is modified, probably, reflecting the systemic cancer disease, conditioned by the tumour microenvironment [18]. Thus, there are micro-environmental and systemic processes contributing to the regulation of RBCs' membrane structure.

RBCs are easily accessible, abundantly, and systematically distributed cells. Thus, RBCs' membrane proteins can be considered as potential biomarkers for monitoring various chronic and including tumour processes.

In the research cycle, we studied the patterns of spatial variability of the disease risk in ethnically homogeneous populations, living in different ecological stress zones of the Upper Imereti region (Georgia) and the potential distribution of morbidity in practically healthy subpopulations of this region [20,21]. Close cause-effect relationships were identified between the risk of disease development in populations and the distribution of nonspecific, potential biomarkers of risk [22,23]. In our early studies, high spatial heterogeneity of common oncological incidences in the Sachkhere district was revealed; for certain localizations of cancer, statistically significant high morbidity zones compared to background levels were identified. Also, in healthy residents of rural areas of the Sachkhere district, high variability in the interleukin content and the total activity of the non-enzymatic antioxidant system (TAA) [4,22] in blood serum, and the high frequency of epithelial micro-nuclear buccal cells [22] were observed.

The objectives: investigation of new potential biomarkers (erythrocyte membrane proteins characteristics) of virtually healthy residents of the ethnically homogeneous population for monitoring the cancer morbidity in the Sachkhere district as one of the most favourable regions (in terms of the territorial-economic, ecologic and geographic situation) of Georgia.

2. Methods

2.1. Patients

Healthy residents of the Sachkhere district (both sexes, 50-65 years old) living in the villages of Sareki,

Sairkhe, and Chorvila (total population of the districts: Sareki – 2076. Sairkhe - 2000, Chorvila - 1451) were examined (a total of 400 people) (Group I - residents of Sareki, 136 people (32 men, 104 women); Group II - residents of Sairkhe. 132 people (44 men, 88 women); Group III - residents of Chorvila, 132 people (20 men, 112 women).

The healthy persons randomly were included in the study. Exclusion criteria were malignant tumours, nicotine users, excessive alcohol users, and severe chronic diseases (severe forms of diabetes, stage 2-3 of chronic heart failure, chronic bronchitis, etc.).

All examined persons gave written informed consent for their participation in the study; they completed a questionnaire concerning general and lifestyle characteristics (e.g., age, gender, height, weight, smoking, and drinking), as well as personal and family medical history, and provided blood samples during their health checkup. In all patients' blood test clinical values were determined.

Our study plan was approved by the Ethics Committee of Tbilisi State Medical University of Georgia.

2.2. RBCs Preparation

Blood samples were used after all clinical analyses were completed. RBCs' membrane isolation was performed by the Hast method [24].

2.2.1. RBCs membrane isolation by the Hast method

Blood samples, collected in tubes containing anticoagulants were centrifuged at 3000g for 15 min. The obtained RBC sediment was washed 3 times with a 1: 4 volume of solution A, containing 130μM KCl, and 20μM Tris-HCl (pH-7.4). For hemolysis of the obtained RBCs sediment, the 1:10 volume of solution B, containing 5μM Tris-HCl, and 1mM EDTA, was added and the resulting mixture was left all night (for about 15 hours). The next day the suspension was centrifuged at 12000 g for 20 min. The obtained precipitate was washed again with solution "B" 2-3 times before bleaching. The precipitate was washed again with a 1:10 volume of "A" solution.

2.3. Protein analytical electrophoresis under dissociated conditions

The membrane protein content was quantified using the DC (detergent compatible) DC protein assay and was solubilized in Laemmli buffer [25]. Protein analytical electrophoresis was performed under dissociated conditions in a 12.5% gradient polyacrylamide gel with 1 mm thick and 6 ml volume with 0.1% sodium dodecyl sulfate SDS, by heating the samples for 10 min at 100◦C and loading 20 μg of membrane proteins on an 8% gel for protein staining by colloidal 0.2% Coomassie Blue G-250 [26]. A set of standard proteins (kDa) as electrophoresis markers were used.

The data obtained by the analytical electrophoresis method were photographed and printed with a resolution of 2880 dpi on photographic paper 10x15 cm with an average density of 150 g/m^2. Each image was placed separately in a special device of the texture analysis system (TAS plus, Leitz, Germany) consisting of a special lamp on a tripod and a bed for graphic data material, connected to a computer system and a monitor of installation TAS plus system (which allows calculating quantitatively the areas indicated on the graphical pictures of electrophoresis results).

As described above, dark fragments in Fig. 1 indicate the content of the amount of protein that was obtained analytically by electrophoresis.

Fig.1 schematically shows the dotted lines framing the dark segment. This square describes the amount of protein on paper (the larger the area, the greater the amount of protein), which is measured in imaginary quantities dpi. dpi is an abbreviation for "Dots Per Inch" and means the number of dots (pixels), (which is "picture element" - is a point (minimal particle) of a digital image) per inch of image resolution, which equals 2.541 cm.

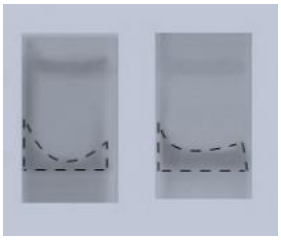


Fig.1 Two different samples belonging to different objects (persons). The dotted line shows the areas describing the protein quantitatively.

2.4. Erythrocytes' membranes proteins spectrophotometry

Ultraviolet/visible spectrophotometer "UVS-2800" (USA) was used to study the proteins separated from the blood erythrocyte membrane. The instrument was used to capture sample spectra to determine the presence of a sample structure and changes in it.

2.5. Statistical analysis

An analysis of variance (ANOVA) (SPSS-12 for Windows) was used for the comparative analysis of the data.

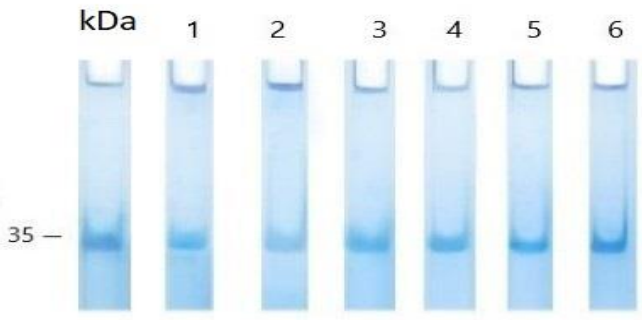
3. Results

3.1. Glycophorin A (GPA) expression level determination

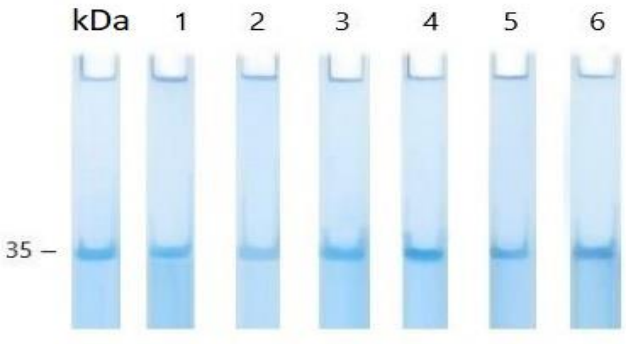
Table 1 and Fig. 1 show dimerized Glycophorin A (GPA) (35kDa) levels in erythrocytes from inhabitants of the Sachkhere region (villages Sareki, Sairkhe, and Chorvila). As seems from the data results, the level of GPA dimer in residents from Sareki village statistically significantly decreased by 15-20% in comparison to the level in residents of Sairkhe and Chorvila.

Table 1
GPA levels in RBCs' membranes from residents of villages Sareki, Sairkhe and Chorvila

Parameter	Sareki	Sairkhe	Chorvila
Glycophorin A	0.46±0.03	0.54±0,05	0.58±0.04



A



B

Fig. 2. Expression levels of glycophorin A (electrophoretic absorbance), in RBCs' membranes from healthy residents from Sachkhere region's villages Sairkhe (A) and Sareki (B) villages

3.2. Spectrophotometric absorption of RBCs' membranes proteins

Fig. 3 shows the absorption spectrum of RBCs' membrane at a length of 230 nm and the average intensity of absorption in inhabitants of the Chorvilla, Sairkhe, and Sareki (Fig. 4). As can be seen from Fig.4, the average intensity of absorption at 230 nm in RBCs' membrane proteins from inhabitants of Sairkhe did not differ from this parameter in the inhabitants of Chorvila, in Sareki's inhabitants it was 14% higher than in inhabitants of Sairkhe and Chorvila.

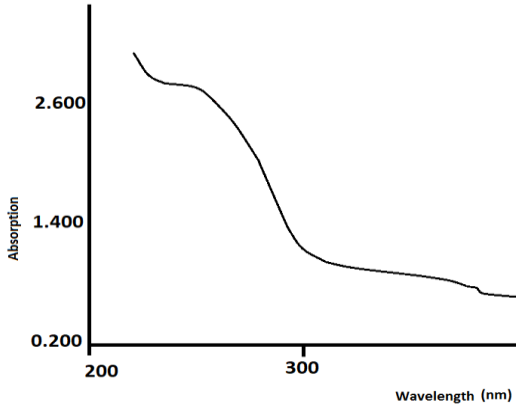


Fig. 3. The absorption spectrum of RBCs' membrane proteins at 200-300 nm

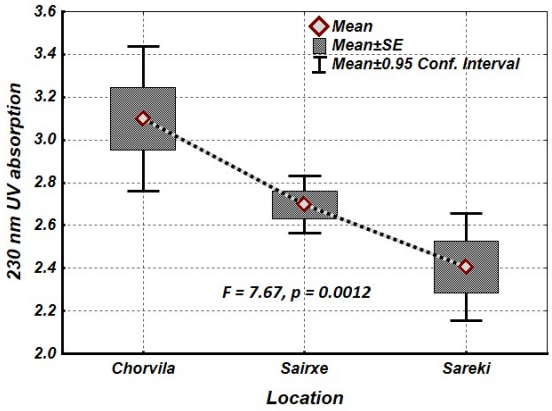
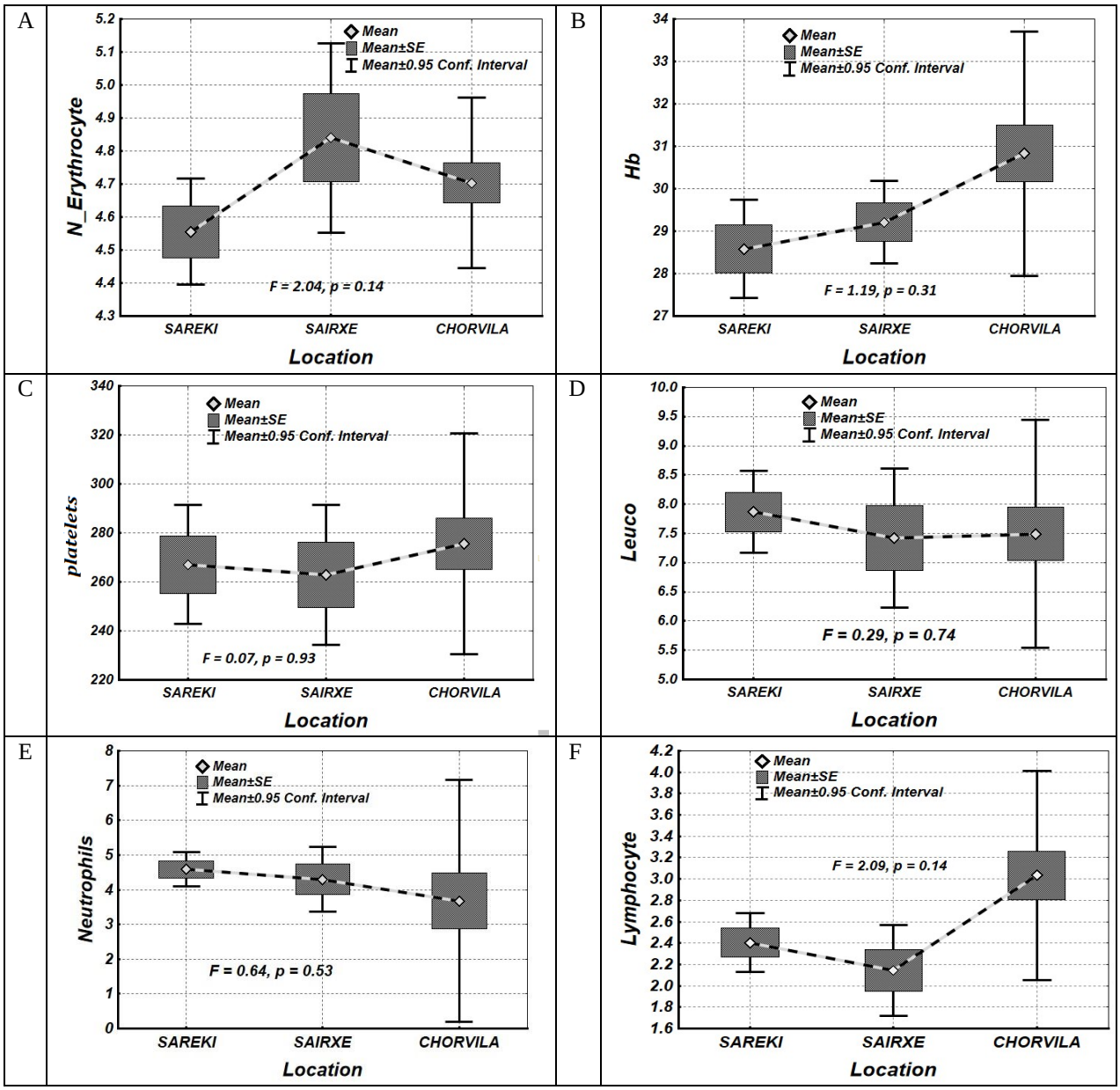


Fig. 4. The absorption intensity of RBCs' membrane proteins at the wavelength of 230 nm (I-Chorvila, II-Sairkhe, III-Sareki)

3.3. Blood test clinical values

According to the results of standard clinical analyzes in the population of the villages of Sachkhere district (Chorvila, Sairkhe, Sareki), there were no statistically significant changes in the number of erythrocytes, platelets, leukocytes, lymphocytes and neutrophils, hemoglobin levels; anisotropy of RBC distribution width (RDW), which measures the variation in RBC volume and size, was found, in residents of Sareki it was higher than in Sairkhe and Chorvila (Fig. 5A-G).



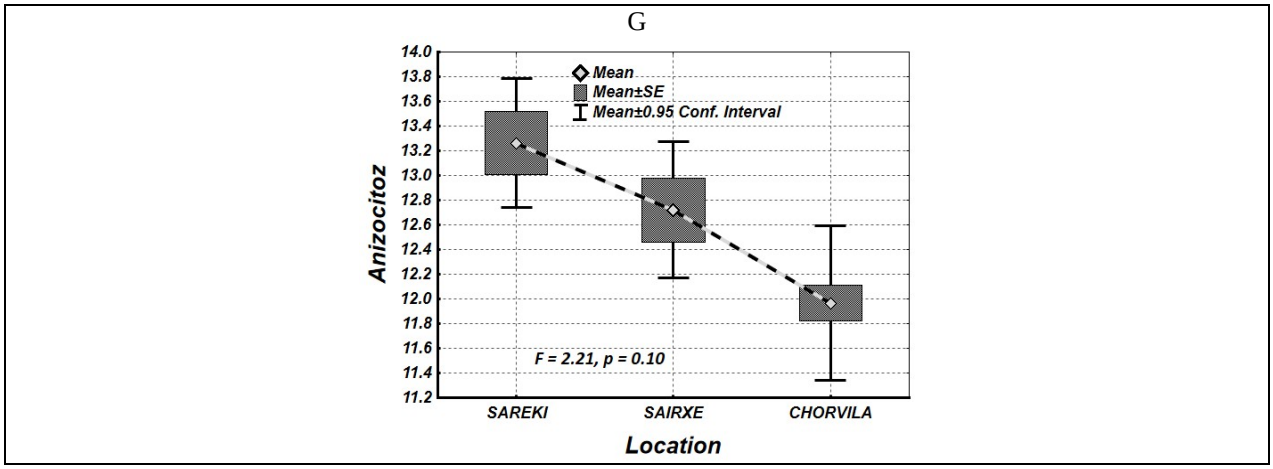


Fig. 5. Clinical blood test parameters in the residents of the Sachkhere region's villages (Chorvila, Sairkhe, Sareki)

erythrocytes, platelets, leukocytes, lymphocytes and neutrophils, hemoglobin levels; anisotropy of RBC distribution width (RDW), which measures the variation in RBC volume and size, was found, in residents of Sareki it was higher than in Sairkhe and Chorvila (Fig. 5A-G).

4. Discussion

According to the oncology database of Sachkhere District Hospital-Polyclinic Association (2011-2020) in the population of the studied villages of Sachkhere district (villages Chorva, Sairkhe, Sareki) the following cancer incidence was observed: Chorvila - 129.1±30.40 per 100000 population per year, Sairkhe - 130±24.57 per 100000 population per year and Sareki - 235.4±38.96 per 100000 population per year. Analysis of study results follows, that between the cancer incidence in villages Chorvila and Sairxe significant difference in incidence was not revealed ($p = 0.730$), whereas the cancer incidence in Sareki was statistically significantly higher (by 82%) as in Chorvila and Sairkhe ($p = 0.002$; $p = 0.004$) [4].

In order to develop new effective prognostic markers of individual and population risk of oncological morbidity in the population and to identify specific and non-specific mechanisms of individual and population sensitivity to external factors, we investigated some characteristics of RBCs in virtually healthy residents of the ethnically homogeneous population of the Sachkhere district are presented.

RBCs account for nearly > 70% of the total cell count in the average adult [27]. Erythropoiesis begins with the differentiation of multipotent hematopoietic stem cells in the bone marrow, which then gives rise to erythroid-committed precursors. In the last stages of the process, the nucleus and other organelles are extruded, and these enucleated reticulocytes are released into the bloodstream to complete their maturation process in a tightly regulated process. It has been suggested that an increment in immature RBCs in the circulation could be the underlying reason for the anisotropy in RBCs, and the rise in the Red blood cell Distribution Width (RDW) value, which reflects the variation in the volume and size of circulated RBCs. The anisotropic increase in RBCs' distribution width (RDW) was detected in the population of the Sachkhere villages (Chorvila, Sareki, Sairkhe) - in Sareki it was higher than in Sairkhe and Chorvila (Fig 5G). Abnormal Red blood cell Distribution Width (RDW), has been associated with poor prognosis in cancer and chronic diseases [28,29,30,31], meta-analysis has shown that RDW may be a potential prognostic marker in cancer patients [32].

We wondered if there was a difference in the RBC membranes' protein composition in residents of Sachkhere district villages (Chorvila, Sareki, Sairkhe). Study results have shown that in RBC membranes of residents from Sareki village the level of dimerized form of GPA was statistically significantly lower (by 15-20%) in comparison to its level in residents of Sairkhe and Chorvila.

Glycophorins (GPA, GPB, GPC, and GPD) are the RBCs' transmembrane proteins that, although not widely appreciated in clinical haematology, are important players in the fields of membrane biochemistry and cellular biology. Glycophorins play important role in the regulation of mechanical properties of RBCs'

membrane and in maintaining their shape. All the expressed glycophorins are 0-glycosylated proteins, which have three domains, (1) a cytoplasmic domain, containing a cluster of basic residues that are located near the plasma membrane, (2) a hydrophobic domain existing as a single α-helix spanning the lipid bilayer, and (3) an extracellular domain which is heavily glycosylated [33,34]. The end of the glycophorins' O-linked oligosaccharide is linked to sialic acid (N-acetylneuraminic acid (NeuAc)); at physiological pH, sialic acids are negatively charged conferring a negative charge to RBCs' membrane. Because of their high sialic acid content, RBCs' membrane glycoproteins account for approximately 60% of the membrane's negative surface charge. The negative surface charge of erythrocytes plays a crucial role in modulating RBC–RBC interactions and as well RBCs interactions with vascular endothelium and the other circulating blood cells [35,36].

The electrical properties of the erythrocyte's surface determine the aggregate stability and deformability of these cells. Due to electrostatic repulsion between like-charged cells and vessel walls, erythrocytes move freely through blood vessels and perform a transport function. Thus, the rheological status of blood and the intensity of microcirculation in the capillaries are formed, which provides organs and tissues with adequate blood circulation. Thus, they are formatters of the intensity of microcirculation, providing enough functioning capillaries and a developed microcirculation network. An electric characteristic of the erythrocytes' surface is determined by the electrophoretic mobility of their membrane proteins. The electrophoretic mobility of erythrocytes proteins, together with the magnitude of the surface charge and the activity of erythrocyte metabolism, which indicates the state of not only individual cells but also microcirculation, ultimately determines the state of organs and tissues and the whole organism. Since blood cells must have a stable charge to maintain an optimal state of homeostasis, their change can be considered an integral indicator of pathological changes in the body [37].

The above once again emphasizes the significance of the study and the need to determine the absorption spectrum and electrophoretic characterise of erythrocyte membrane proteins in practically healthy residents to determine their changes as potential biomarkers/predictors of the disease.

GPA-dimer is the major component of RBCs' membrane glycoproteins, representing 1.6% of total membrane proteins [38], and has a molecular mass of 29-36 kDa [33]. GPA expression is uniquely erythroid. The GPA molecule is about 20% p-sheet; one short stretch of p-sheet, composed of residues 90 through 93, plays important role in the formation of GPA dimers. GPA dimerization is regulated solely by the transmembrane domain, the distance between the dimerization elements of the transmembrane domain and the basic residues that form the cytoplasmic segment is important. The carbohydrate structures on the GPA extracellular domain do not affect dimerization because monomers with varying degrees of glycosylation are dimerized [39]. It was shown that the most crucial in GPA dimerization appears Glycin-Glycin interaction [40] because Glycine is particularly susceptible to oxidative damage by weak oxidants such as superoxide (through an exposed $\alpha C - H$ bond), which can lead to impaired GPA dimerization [41].

Folded and unfolded conformation of membrane proteins can be characterized according to absorption intensity at 230 nm, unfolded proteins have lower absorption at 230 nm than folded proteins [42]. As follows from the results of our study, the average intensity of absorption at 230nm in RBCs' membrane proteins of Sareki's inhabitants was 14% lower than in inhabitants of Sairkhe and Chorvila. This data indicates the lower protein folding and therefore GPA dimerization level in the RBCs membranes of Sareki's inhabitants.

Conformational changes (folding-unfolding) of membrane proteins are related to the molecular etiology of various diseases [43]. The function of proteins is determined by their specific three-dimensional structure, which ensures their functioning in cells of living organisms under physiological and stressful conditions. Any stressful situation in living organisms (microbial or viral infection, chronic diseases, carcinogenesis, etc.) is usually manifested by the accumulation of reactive oxygen compounds (ROS) and their aggressive interaction with biological macromolecules. ROS causes major metabolic, transcriptional, and proteomic changes and significantly impairs cellular homeostasis. Membrane proteins are one of the main cellular targets of ROS, which is largely due to the presence of side chains of oxidant-sensitive amino acids and weak, competitive interactions between proteins and lipids (membrane protein-lipid rafts) [44]. Under oxidative stress conditions, the lateral groups of polypeptide chains undergo modifications that directly affect their conformation (folding) [45, 46]. Thus, the decrease in the absorption intensity at 230 nm and GPA-dimers content in the RBCs membranes of the Sareki inhabitants, compared with the corresponding indicators in the inhabitants of Sairkhe and Chorvila, may be related to the presence of an excess oxidative factor on the territory of residence of this population. In our previous studies, high variability and unequal

distribution of redox parameters of blood in the healthy populations of the Sachkhere district villages (Chorvila, Sarek, Sairkhe) were revealed [4,22,23]. In particular, the research results indicated the intensification of ROS production in the blood serum of the Sareki inhabitants compared to their levels in the blood serum of the inhabitants from Chorvila and Sairkhe. The important deviations in redox status of residents from Sareki toward oxidative can induce oxidative damage and alterations in the structure of the erythrocytes' membrane proteins, accompanied by disorders in GPA dimerization.

During normal conditions, in humans, low concentrations of complement-opsonized circulating inflammatory particles are bound to RBCs via complement receptor 1 (CR1). During inflammation, and excessive complement activation, nascent C3b, and C4b fragments bind irreversibly through thioester bonds to hydroxyl groups of heavily glycosylated GPA on circulating RBCs, which causes changes in membrane properties and behaviour [34, 47]. GPA ligation induces an NADPH oxidase-dependent increase in intracellular ROS production by RBCs, decreases GPA dimerization level and initiates the formation of GPA/band 3/ankyrin-spectrin complexes in the cytoplasmic side of their membranes, which directly impacts RBCs membrane deformability, increases its rigidity. That impairs RBCs' flow through the microcirculation, significantly reduces their lifespan, and supports their delivery to resident sinusoidal macrophages in the liver and spleen (a process known as immune-adherence clearance) [47].

Therefore, the mechanical and biochemical consequences following GPA ligation of the human RBCs membranes either by naturally occurring autoantibodies, complement fragments or oxidative stress, represent an initial signal triggering a cascade of events culminating with the premature removal of altered RBCs from circulation and alterations of RDW of RBCs.

In our early studies differences in the rheological properties of erythrocytes in the population of Sachkhere district villages (Sareki, Sairkhe and Chorvila) were found [46]. Statistically reliable alterations in indicators of oxidative status, levels of GPA dimerization in the RBCs membrane, and their resistance (rheological coefficient) in the blood of inhabitants of Sachkhere district villages allow us to suggest a causal relationship between these parameters. From this position, deviations in redox status can quite rightly be considered as a factor determining the RBCs' membrane proteins conformation and their rheological properties [47].

As follows from the results of our research, the most significant changes in the protein fraction of RBCs' membranes were revealed in residents of village Sareki. Given the relatively high level of cancer incidence of cancer in the population of this village, the alterations in RBCs' membrane proteins can be considered as potential biomarkers for monitoring tumourigenesis. It should also be noted that, since each of the studied indicators belongs to the class of pleiotropic markers, only their complex can be considered as an early predictor of oncological risk.

The fact that the behaviour of erythrocytes in the bloodstream indirectly depends on the level of electrophoretic properties was known earlier [48,49]. Due to its importance for the normal functioning of an organism, electrophoretic characteristics are one of the most rigid constants of its internal environment [50]. However, ensuring high stability of the electrophoretic properties of erythrocytes implies the existence of subtle mechanisms of its regulation. However, ensuring high stability of the electrophoretic properties of erythrocytes implies the existence of subtle mechanisms of its regulation. These mechanisms remain unclear, and their study is an urgent problem in biomedicine.

Despite modern theoretical and mathematical approaches and a number of experimental and clinical studies [47,52], evaluation of the clinical informativeness of such complex predictors of carcinogenic risk is the subject of further longitudinal studies in the investigated populations, however, already at this stage of the research, there are clear prospects of the obtained results in terms of developing an approach based on the synthesis of evidence of health risk in small geographical areas. Clarifying the reasons for the identified patterns and their significance requires more detailed studies.

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