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Informativeness of Immunological Predictors of Prolonged and Chronic Critical Illness Outcome is Limited by Patient's Genotype

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Summary

The aim of the study was to determine the contribution of cellular immune system parameters and the *AQP4* (rs1058427) genetic polymorphism to the prognosis and outcome of patients with sequelae of severe brain injury (SBI), including patients who developed pneumonia.

Materials and Methods. The study included 464 intensive care unit (ICU) patients with prolonged or chronic critical illness (PCCI) admitted to the Federal Scientific and Clinical Center of Intensive Care Medicine and Rehabilitology (FSCCICMR) following SBI (strokes, traumatic brain and combined injuries, post-operative anoxic conditions, brain tumor surgery). Variants of the rs1058427 single-nucleotide polymorphism in the *AQP4* gene were detected in DNA isolated from whole blood with organic solvents and using genotyping with tetraprimer PCR followed by electrophoretic identification of the products.

Results. The entire cohort was divided into three groups of patients: those admitted without signs of pneumonia in the first 48 hours of hospitalization but who developed nosocomial pneumonia after 48 hours (group 1); admitted without signs of pneumonia, in whom no signs of pneumonia were detected throughout the hospitalization (group 2); with pneumonia diagnosed upon admission, which developed in the previous medical institution prior to transferring to the FSCCICMR (group 3). For the cohort combining groups 1 and 2 (admitted without signs of pneumonia), increased values of the neutrophil-to-lymphocyte ratio (NLR) (OR = 1.8, 95% CI: 1.1–3.9, $P = 0.0175$, χ^2, $N = 272$) and neutrophil count (OR = 2.1, 95% CI: 1.3–3.5, $P = 0.0038$, χ^2, $N = 272$) on the first day of hospitalization were associated with an increased risk of pneumonia. In the same cohort, elevated neutrophil counts (over 6×10^9/L) at admission significantly predicted adverse outcome, but only in the subgroup of patients with the *AQP4* rs1058427 GG major genotype (95% CI: 1.0–4.5, HR = 2.1, $P = 0.049$, log-rank test). In group 3 (patients with pneumonia diagnosed upon admission), a significant association with adverse outcome was found for both neutrophils and NLR (HR = 3.1, 95% CI: 1.3–6.9, $P = 0.019$, log-rank test, $N = 149$, and HR = 2.9, 95% CI: 1.3–6.6, $P = 0.026$, log-rank test, $N = 149$, respectively) in patients with *AQP4* GG genotype, not in alternative *AQP4* allele T carriers. Thus, the prognostic value of elevated neutrophil counts in patients with PCCI («immunophenotype») depends significantly on the genetic polymorphism of *AQP4*, a gene that controls the initiation of immune cell migration and is pathogenically significant for the development of the infectious process.

Conclusion. For patients with consequences of SBI in PCCI, an increase in neutrophil counts above 6×10^9/L upon hospitalization significantly predicts an adverse outcome only in patients homozygous for the *AQP4* rs1058427 G allele (GG genotype). The unique genetically restricted clinical and laboratory phenotype («gene-immunophenotype») could be considered in personalized critical care medicine as an example of a candidate predicting paradigm.

Keywords: chronic and prolonged critical illness; prognostic markers; nosocomial pneumonia; immune system cells; AQP4; genetic polymorphism; rs1058427

Conflict of interest. The authors declare no conflict of interest.

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Introduction

The number of patients in chronic or prolonged critical condition (CPCC) has doubled in recent decades and may double again in the next decade [1, 2]. These patients are also quite numerous among patients with the consequences of severe brain injury (SBI) requiring life support and hospitalized in intensive care units (ICUs) [3]. Persistent, long-term, prolonged critical conditions and chronical illness (PCCI), are defined as severe pathological conditions of patients requiring prolonged (at least 8–21 days) life support provided in intensive care units (ICUs). Current research has documented a growing number of such patients and a high incidence of in-hospital complications, accompanied by increased mortality. A separate group of patients with chronic brain injury includes those with sequelae of severe brain SBI resulting from trauma, hemorrhagic or ischemic stroke, prolonged brain surgery, intoxication, or hypoxia. These patients have survived the acute phase of the disease, ultimately resulting in a vegetative state or a state of low consciousness, and require prolonged intensive and rehabilitative treatment. The sequelae of SBI are accompanied by a cascade of pathological reactions not only in the brain (cerebral edema, cerebral hemodynamic disturbances, inflammatory complications, etc.). The cardiovascular, respiratory, digestive, water-balance, hormonal, and other systems are consistently and naturally involved in the pathological process. Impaired tissue nutrition and immune system function, changes in nutritional status, the development of hypotension, decreased tissue perfusion leading to hypoxia and multiple organ failure, the development of nosocomial infection, and the development of purulent-inflammatory complications complete the cycle of pathological reactions, increasing the likelihood of death [3].

Among the infectious complications developing with chronic coronary heart disease, pneumonia remains the most frequently diagnosed. The mortality rate of patients with pneumonia (etiologically unrelated to COVID-19) requiring intensive care unit (ICU) hospitalization is high, ranging from 13% to 30%. This high mortality rate may be due to frequent septic complications of severe pneumonia in ICU patients, resulting from persistent dysfunction of the innate and adaptive immune systems, which develops under conditions of prolonged bacterial infection and microbiota dysbiosis. Timely and appropriate use of antibiotics, high-tech methods of early diagnosis and treatment, and biomarkers help reduce mortality in severe pneumonia [4]. However, the informative value of the most commonly used biomarkers for pneumonia — procalcitonin and C-reactive protein — is only evident during treatment, when used to determine the duration of antibiotic use [4, 5]. Furthermore, personalized approaches to the effective treatment of critical conditions [1, 2, 6] require highly informative markers for disease prognosis. Such markers are unknown for the category of patients in the ICU. Therefore, the development of such markers for prognosticating the course and outcome of severe pneumonia in ICU patients is a pressing issue in critical care medicine.

The neutrophil-to-lymphocyte ratio (NLR) is a biomarker of the systemic inflammatory response used to assess the severity of the condition and predict outcome in various diseases: cerebrovascular accidents [7]; cardiovascular diseases [8]; bacterial, fungal infections and sepsis; community-acquired pneumonia; SARS-CoV-2 infection [9]; metabolic syndrome [10]; rheumatoid arthritis [11]; various types of cancer [12, 13]; decompensated liver cirrhosis [14]; severe trauma [15]. Quantitative NLR values are calculated based on a complete blood count by determining the ratio of the absolute number of neutrophils and lymphocytes in a unit of blood volume. The NLR value reflects a certain balance between the innate immune response (neutrophils) and adaptive immune reactions (lymphocytes) [16, 17]. Currently, the NLR is widely used as a reliable and readily available marker of immune system status in various infectious and non-infectious diseases. Critical illness and inflammation are characterized by a sharp increase in NLR values to 11–17, sometimes even above 30. Improved progression of sepsis and critical illness, as well as a reduced risk of mortality, are associated with a decrease in NLR values below 7. NLR helps differentiate more severe from milder illnesses. NLR is considered by many authors to be a popular and informatively promising biomarker of the balance of cellular immune systems, showing potential as a population predictor of adverse outcomes in various pathological conditions [16, 18–20]. Neutrophils are multifunctional granulocytic immune cells essential for providing first-line defense against invading pathogens. However, uncontrolled neutrophil activation can lead to severe, life-threatening complications caused by oxidative reactions, the production of oxygen and nitrogen radicals, cytokines, and other molecules that damage vascular endothelial cells. The binding of membrane-associated neutrophil structures to surface molecules of the vascular endothelium can disrupt the glycocalyx and compromise endothelial integrity. This contributes to tissue hypoperfusion, which is exacerbated by the penetration of activated neutrophils through the vascular wall [21, 22].

AQP4 is a protein that forms water channels in the cell membrane and is expressed in various tissues of the body, including the brain, kidneys, lungs, and the immune system. AQP4 expression promotes edema formation, determines the initial stages of immune cell migration, and maintains

the blood-brain barrier. *AQP4* plays a significant role in the development of pathological conditions. This protein controls the survival of neuronal cells and T cells. In vivo inhibition of AQP4 reduces the number of T lymphocytes in lymph nodes while simultaneously accumulating them in the liver. The 3' region of *AQP4* contains several functional single nucleotide substitutions. Polymorphisms in the 3' region of *AQP4* contribute to the development of cerebral edema after hemorrhagic stroke (*AQP4* T rs1058427) [23] and the outcome of traumatic brain injury (rs3763043) [24]. We previously demonstrated that the minor T allele of *AQP4* rs1058427, located in the 3' region of the gene, controls the favorable course and outcome of sepsis in a group of highly morbid intensive care unit (ICU) patients [24]. We also previously demonstrated that minor genotypes of *AQP4* rs1058427 increase the risk of developing pulmonary hypertension in patients with pleural empyema [25]. However, the possible influence of the 3' region polymorphism on the informativeness of cellular prognostic markers remained unclear. Since *AQP4* controls immune cell migration, and the *AQP4* rs1058427 polymorphism influences the prognosis of sepsis and the risk of complications in infectious diseases, we hypothesized that the prognostic value of cellular markers of the immune system may significantly depend on the *AQP4* genetic variant.

The aim of the study was to determine the contribution of cellular immune system parameters and the *AQP4* (rs1058427) genetic polymorphism to the prognosis and outcome of cerebral hemorrhage in patients with the consequences of severe brain injury, including the development of pneumonia.

Materials and Methods

We conducted an uncontrolled, prospective, observational, randomized study (decision of the Ethics Committee of the Federal Scientific and Clinical Center of Radiology and Radiology, Protocol No. 2.2.18 dated December 20, 2018). Patients were enrolled in the study between June 2018 and August 2021. According to our preliminary data, the mortality rate in patients with sequelae of cerebrovascular accidents admitted with pneumonia is approximately 13 percent, which was used to calculate the sample size. The formula for calculating the sample size was $\mathbf{n = (t2 \times P \times Q)/\triangle 2}$, where t is the critical value of the Student's *t*-test (at a significance level of 0.05, it is 1.96), $\triangle$ is the maximum permissible error (5%), **P** is the proportion of cases in which the studied characteristic is present (90), **Q** is the proportion of cases in which the studied parameter is not present (10), and **n** was 174. Considering that the frequency of the major genotype *AQP4* rs1058427 GG in the Moscow population is approximately 80 percent [24], we required at least 209 patients to recruit a sufficient number for calculations for patients with the major genotype. Half of the patients in our sample were admitted with pneumonia, therefore, to obtain the required number of patients with the major genotype *AQP4* rs1058427 GG, both with and without pneumonia, we required 418 patients in the cohort. Taking into account the possible lack of all data on the crucial for the study parameters (genotyping, course and outcome), it was decided to increase the planned cohort size by 10–12%. As a result, the entire cohort consisted of 467 patients. As a result of exclusion from the cohort due to incomplete data (loss of 2 blood samples for genotyping and lack of immunophenotype data during hospitalization (1 (patient), 464 ICU patients in PCCI as a consequence of cerebrovascular accidents (strokes, trauma, anoxic injuries, and brain tumors; Table 1) were included in the analysis. One hundred ninety-two patients in our sample were admitted with pneumonia. Upon admission, patients were assessed using the SOFA, Charlson, CIRS-G, and Glasgow Coma Scale scores. Allelic variants of *AQP4* rs1058427 were identified using tetraprimer polymerase chain reaction followed by electrophoretic separation and identification of stained products in the gel. The following primers were selected and synthesized at Evrogen LLC using the Primer-BLAST program (https://www.ncbi.nlm.nih.gov/tools/primer-blast/):

AQP4 1 for 5`-TATTGGCAAAACTGGGGATT-3`

AQP4 2 for 5`-CCCAATCTCTGCTCTCTCAA-3`

AQP4 2 rev. 5`-GATTATCAACAAATGTCACGAGAAG-3`

AQP4 1 rev 5`-TGCAACCATGTTGTACCTTG-3`

The normal or not normal distribution of quantitative variables was assessed using the Shapiro–Wilk test. Normally distributed variables were described using mean values (*M*), standard deviations (*SD*), and 95% confidence intervals (95% CI). Non-normally distributed quantitative data were described using medians (*Me*), lower and upper quartiles (IQR). Normally distributed variables, provided that variances were equal, were compared across groups using the Student t-test. Non-normally distributed variables were compared using the Mann–Whitney *U*-test. Categorical data were described indicating absolute values, the ratios of which in the compared groups were analyzed using four-field contingency tables using the χ^2 test with Yates's correction for sample continuity (for $N > 100$) or Fisher's exact test (FET) for $N < 100$. Statistical analysis was performed using a two-sided test with a significance level of $P < 0.05$. The odds ratio (*OR*) with a 95% confidence interval (95% CI) and the relative risk (*RR*) with a 95% confidence interval (95% CI) were used as a quantitative measure of effect when comparing relative indicators. The log-rank test was performed for survival analysis according to Kaplan–Meier. The results were presented as the hazard ratio (HR) with a 95% confidence interval (CI). The

results of the Cox regression analysis were presented as the hazard ratio (HR) with a 95% confidence interval (CI). To predict the probability of an unfavorable outcome (mortality), the ROC curve method and logistic regression analysis were used. According to the literature, the normal value of the NLR is 0.78–3.53 [26]. Based on these considerations, we assumed that a NLR value above 4 may be a risk factor, since this value is outside the normal range. ROC analysis was used to determine the cutoff point for the content of lymphocytes and neutrophils. The cutoff point was established based on the highest value of the Youden index and corresponded to the most optimal ratio of sensitivity (*Se*) and specificity (*Sp*). Multivariate regression analysis was performed using the backward stepwise Wald method. Differences were considered significant at $P > 0.05$. Statistical analysis was performed using SPSS Statistics (version 27), MedCalc (version 11.6), and SigmaStat (version 3.5).

Results

Our study cohort included patients with SBT (Table 1).

Figure 1 presents patient mortality data by day of hospitalization.

Mortality in the entire cohort varied by the day of hospitalization (Fig. 1, *a*), with the maximum increases in the group of patients admitted without pneumonia in whom pneumonia was diagnosed during hospitalization (Fig. 1, *c*). In this group, mortality was characterized by a significant increase in mortality from days 10 to 30 of hospitalization, with peaks of up to 5 cases per day (Fig. 1, *c*), which could reflect the incidence of nosocomial pneumonia in patients predisposed to its development. In the group

Table 1. Demographic and clinical parameters of patients, *N* = 464.

Parameters	Values
Women, *N* (%)	186 (40%)
Age, *Me* (IQR)	58 (43–67)
SOFA scale score at admission, *Me* (IQR)	2 (1–3)
Diabetes mellitus, type II, *N* (%)	35 (7%)
Charlson Comorbidity Index (CCI), *Me* (IQR)	8 (6–10)
Comorbidity by Cumulative Rating Index Scale (CIRS) scale, *Me* (IQR)	14 (11–18)
Glasgow Coma Scale (SCG), *Me* (IQR)	14 (10–15)
Length of hospital stay, days, *Me* (IQR)	46 (30–66)
Lethality, *N* (%)	63 (14%)
Sequelae of ischemic stroke, *N* (%)	156 (34%)
Sequelae of hemorrhagic stroke, *N* (%)	114 (25%)
Sequelae of anoxic brain injury, *N* (%)	26 (6%)
Sequelae of traumatic brain injury, *N* (%)	99 (21%)
Brain tumors, *N* (%)	44 (9%)
Sequelae of severe COVID-19, *N* (%)	10 (2%)
Other*, *N* (%)	15 (3%)

Note. * — mixed genesis encephalopathy, consequences of previous neuroinfection, cerebrovascular disease, encephalomyelopolyneuropathy, other cerebrovascular diseases.

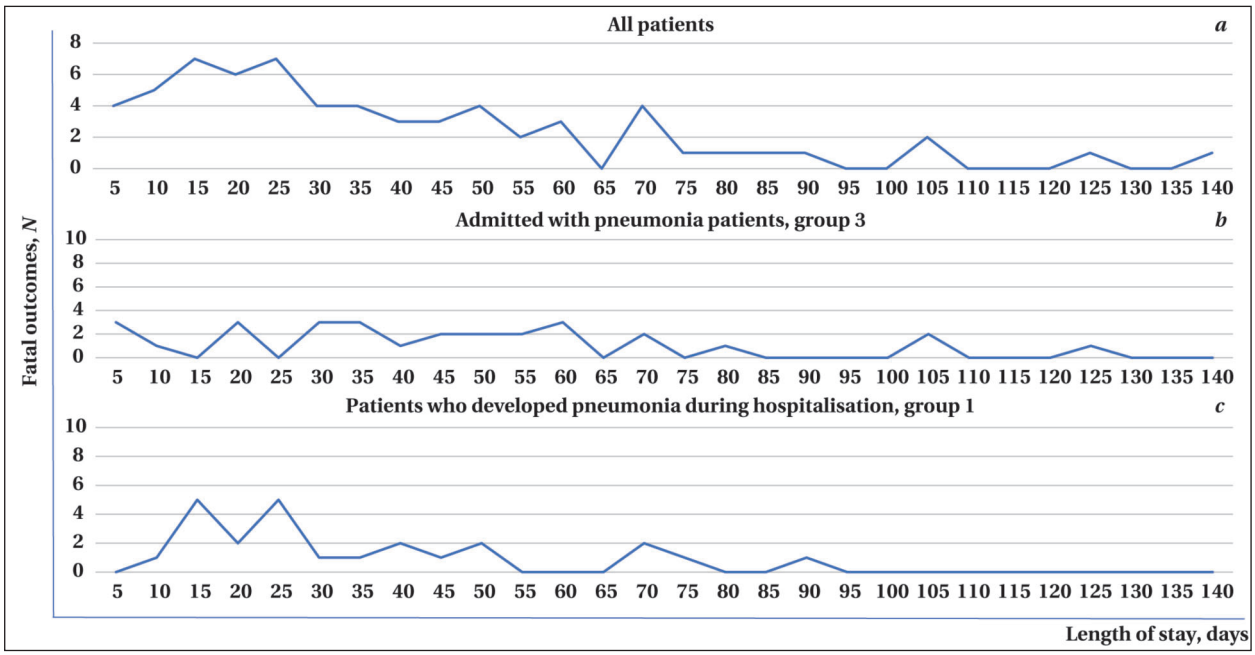

Fig. 1. Mortality of patients with sequelae of SBI by day of hospitalization.

of patients hospitalized with pneumonia (Fig. 1, *b*), peaks of higher mortality during the first month of hospitalization were virtually absent or were more smoothed out (not exceeding 3 patients per day).

It was of interest to identify early (on admittance) clinical and laboratory parameters associated with adverse outcomes in groups of patients with pneumonia and those predisposed to its development during the first weeks of hospitalization. As shown in Table 2, univariate logistic regression analysis of all patient data (N=464) revealed seven parameters out of nine that were significantly associated with mortality. Adjustment for the most common covariates in multivariate analysis revealed that the greatest contribution to mortality was attributed to two clinical parameters — the CCI and the GCS — as well as one integrated clinical and laboratory immunological parameter — the NLR (Table 2).

Calculations showed that a 1-point increase in CCI values was associated with a 26% increase in the odds of death, a 1-point decrease in the GCS was associated with a 15% increase in the odds of death, and a 1-point increase in the NLR was associated with a 3.8% increase in the odds of death. A similar analysis was conducted by dividing the entire cohort of patients into two groups, each differing in genotype: GG versus GT + TT, based on the results of genotyping the G and T alleles of *AQP4* rs1058427.

As can be seen from Table 3, in multivariate analysis, the same predictors of death persisted in patients with the GG genotype as in the entire sample (CCI, GCS, and NLR). For carriers of the *AQP4* T allele (GT and TT genotypes, N=92), similar information content was found only for the CCI values (Table 4). Using multivariate logistic regression, we found that for carriers of the minor T allele, both CCI and SOFA scores were significant predictors of mortality in ICU patients (Table 4).

Moreover, an increase in the CCI values by 1 point corresponded to an increase in the chance of death by 40.8% (corr. OR = 1.408; 95% CI = 1.077; 1.842), and an increase in the SOFA scale value by 1 point corresponded to an increase in the chance of death by 47% (corr. OR = 1.474; 95% CI = 1.043; 2.08). However, in contrast to patients with the *AQP4* GG genotype, in carriers of the T allele, the clinical and laboratory indicator of NLR was not a prognostic biomarker of death in either univariate or multivariate analyses (Table 4). Similar data confirming the selective prognostic value of immune

Table 2. Relationship between mortality rate and demographic indicators, assessment scales, and cellular parameters (logistic regression analysis).

Parameters	Univariate analysis		Multivariate analysis*		Exact Fisher test
	Odds Ratio (OR) (95% CI)	*P*	Corr. Odds Ratio (OR) (95% CI)	*P*	Odds Ratio (OR) (95% CI)
CCI	1.25(1.134–1.371)	<0.001	1.26 (1.138–1.394)	<0.001	2.2 (1.4–3.4)
GCS	0.852 (0.787–0.923)	<0.001	0.849 (0.78–0.93)	<0.001	3.0 (1.8–5.0)
CIRS	1.108 (1.056–1.164)	<0.001	—	—	2.4 (1.5–3.8)
SOFA	1.22 (1.101–1.351)	<0.001	—	—	3.0 (1.8–5.0)
NLR	1.22 (1.101–1.351)	<0.001	1.038 (1.003–1.074)	0.035	1.8 (1.1–2.8)
Neutrophils	1.058 (1.002–1.116)	0.042	—	—	2.4 (1.5–4.0)
Lymphocytes	0.928 (0.748–0.153)	0.5**	—	—	
Sex	0.84*** (0.484–1.456)	0.533**	—	—	
Age	1.034 (1.016–1.053)	<0.001	—	—	2.2 (1.4–3.4)

Note. Hear and in tables 3, 4: CCA — Charlson comorbidity index; GCS — Glasgow coma scale; NLR — neutrophil-to-lymphocyte ratio. Indicators not included in the multivariate regression analysis model: CIRS, SOFA, neutrophils, lymphocytes, gender, and age. * — Hosmer–Lemeshow goodness-of-fit test, P=0.799; Chi-square = 4.6; DF = 8, percentage of correctly classified cases 86.3. ** — regression is not statistically significant, P>0.05; *** — OR is presented for women

Table 3. Dependence of the incidence of death on demographic indicators, assessment scales and cellular parameters on the first day of hospitalization, patients with the *AQP4* rs1058427 GG genotype (logistic regression analysis).

Parameters	Univariate analysis		Multivariate analysis*		Exact Fisher test
	Odds Ratio (OR) (95% CI)	*P*	Corr. Odds Ratio (OR) (95% CI)	*P*	Odds Ratio (OR) (95% CI)
CCI	1.23 (1.1–1.37)	< 0.001	1.243 (1.112–1.388)	< 0.001	2.4 (1.5–4.0)
GCS	0.86 (0.79–0.937)	0.001	0.858 (0.78–0.94)	0.001	2.7 (1.6–4.8)
CIRS	1.101 (1.044–1.162)	< 0.001	—	—	2.1 (1.3–3.5)
SOFA	1.198 (1.075–1.34)	0.001	—	—	2.7 (1.6–4.8)
NLR	1.057 (1.02–1.096)	0.002	1.041 (1.002–1.08)	0.037	2.0 (1.1–3.3)
Neutrophils	1.11 (1.03–1.195)	0.005	—	—	2.5 (1.5–4.3)
Lymphocytes	0.96 (0.79–1.18)	0.7**	—	—	
Sex	0.76*** (0.4–1.4)	0.382**	—	—	
Age	1.032 (1.013–1.053)	0.001	—	—	2.5 (1.5–4.1)

Note. N=372. Parameters not included in the multivariate regression analysis model: CIRS, SOFA, neutrophils, lymphocytes, gender, age. * — Hosmer–Lemeshow goodness-of-fit test, p = 0.756; Chi-square = 5.02; DF = 8, percentage of correctly classified cases 85.9; ** — regression is not statistically significant; P>0.05; *** — OR is presented for women.

Table 4. Relationship between mortality rate and demographic indicators, assessment scales, and cellular parameters on the first day of hospitalization, patients with the *AQP4* rs1058427 GT and TT genotypes (logistic regression analysis).

Parameters	Univariate analysis		Multivariate analysis*		Exact Fisher test
	Odds Ratio (OR) (95% CI)	*P*	Corr. Odds Ratio (OR) (95% CI)	*P*	Odds Ratio (OR) (95% CI)
CCI	1.35 (1.06–1.7)	0.016	1.408 (1.077–1.842)	0.012	3.1 (1.0–9.9)
GCS	0.8 (0.63–1.012)	0.063**	—	—	
CIRS	1.14 (1.02–1.28)	0.022	—	—	2.5 (1.0–6.7)
SOFA	1.4 (1.02–1.9)	0.036	1.474 (1.043–2.081)	0.028	5.6 (1.3–24.5)
NLR	1.06 (0.97–1.15)	0.21**	—	—	
Neutrophils	1.01 (0.936–1.097)	0.748**	—	—	
Lymphocytes	0.59 (0.23–1.53)	0.278**	—	—	

Note. *N* = 92. Indicators not included in the multivariate regression analysis model: GCS, CIRS, NLR, neutrophils, and lymphocytes. * — Hosmer–Lemeshow goodness-of-fit test, *P* = 0.64; Chi-square = 6.08; DF = 8, percentage of correctly classified cases=85.6. ** — regression not statistically significant, *P* > 0.05.

Table 5. Correlation coefficients of predictors included in the multivariate logistic regression model.

		CCI, *r*, *P*	GCS, *r*, *P*	NLR, *r*, *P*	SOFA, *r*, *P*
All patients, *N* = 464	CCI	—	–0.031, 0.505	**0.130** (0.0056)**	—
	GCS	–0.031 (0.505)	—	**–0.236** (0.0001)**	—
	NLR	**0.130** (0.0056)**	**–0.236** (0.0001)**	—	—
AQP4 GG carriers, *N* = 372	CCI	—	–0.029 (0.58)	**0.167** (0.00135)**	—
	GCS	–0.029 (0.58)	—	**–0.210** (0.0001)**	—
	NLR	**0.167** (0.00135)**	**–0.210** (0.0001)**	—	—
AQP4 GT or TT carriers, *N* = 92	CCI	—	—	—	–0.005 (0.962)
	SOFA	–0.005 (0.962)	—	—	—

Note. ** — Spearman correlation is significant at the 0.01 level.

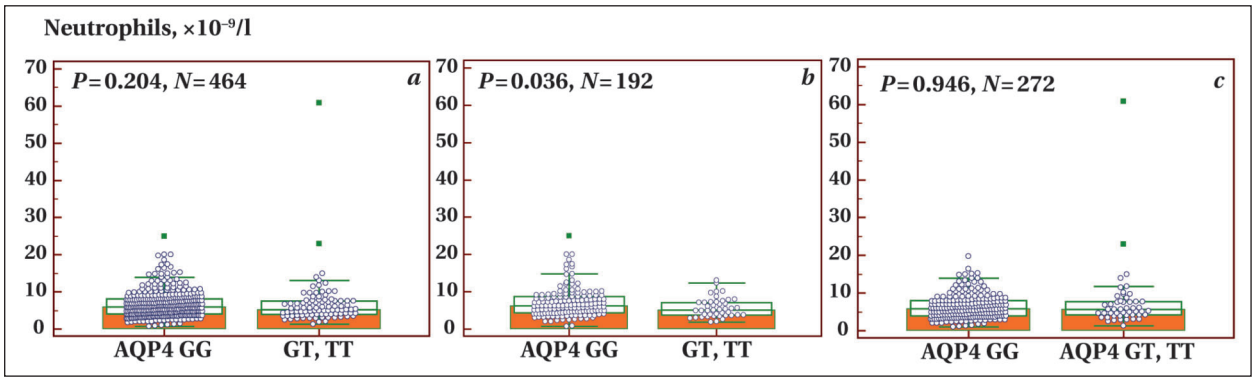

Fig. 2. Blood neutrophil counts in patients admitted to the PKC with different *AQP4* rs1058427 genotypes, depending on the presence of pneumonia on admission.
Note. *a* — all patients. The median (IQR) value for carriers of the major *AQP4* GG genotype was 6.0×10^{-9} per liter (4.1; 8.2), the mean (±σ) value was 6.6×10^{-9} per liter (±3.5). The IQR value for carriers of the minor *AQP4* GT and TT genotypes was 5.2×10^{-9} per liter (3.9; 7.6), and the mean (σ) value was 6.7×10^{-9} per liter (±6.6). *b* — patients admitted with pneumonia. The IQR for carriers of the major *AQP4* GG genotype was 6.2×10^{-9} per liter (4.4; 8.7), and the IQR for carriers of the minor *AQP4* GT and TT genotypes was 5.1×10^{-9} per liter (3.7; 7.2), while the IQR for carriers of the minor *AQP4* GT and TT genotypes was 5.7×10^{-9} per liter (±2.6). *c* — patients admitted without pneumonia. The IQR for carriers of the major *AQP4* GG genotype was 5.8×10^{-9} per liter (3.9; 8.0), and the IQR for carriers of the minor *AQP4* GT and TT genotypes was 6.3×10^{-9} per liter (±3.2). The IQR for carriers of the minor *AQP4* GT and TT genotypes was 5.6×10^{-9} per liter (4.2; 7.7), and the IQR for carriers of the minor *AQP4* σ genotype was 7.6×10^{-9} per liter (±8.6). The ordinate axis shows the neutrophil count, $\times10^{-9}$ per liter. *a, b, c* — Mann–Whitney test.

system cellular markers (neutrophils count and NLR values, but not lymphocytes count) in patients with the *AQP4* rs1058427 GG genotype were also obtained using Cox regression (Appendix). It should be noted that correlations between predictors included in the multivariate regression models remained weak or absent both when analyzing the entire patient cohort and when stratified by *AQP4* rs1058427 genotype (Table 5).

Neutrophil counts did not differ significantly between the overall sample and the subgroup of patients admitted to the hospital without pneumonia (Fig. 2, *a, c*). However, differences were found in the subgroup of patients admitted with pneumonia: carriers of the minor *AQP4* T allele had lower neutrophil counts (median values 6.2 (GG) and 5.1×10^{9}/L (GT, TT), *P* = 0.036, Mann–Whitney test, *N* = 192, Fig. 2, *b*).

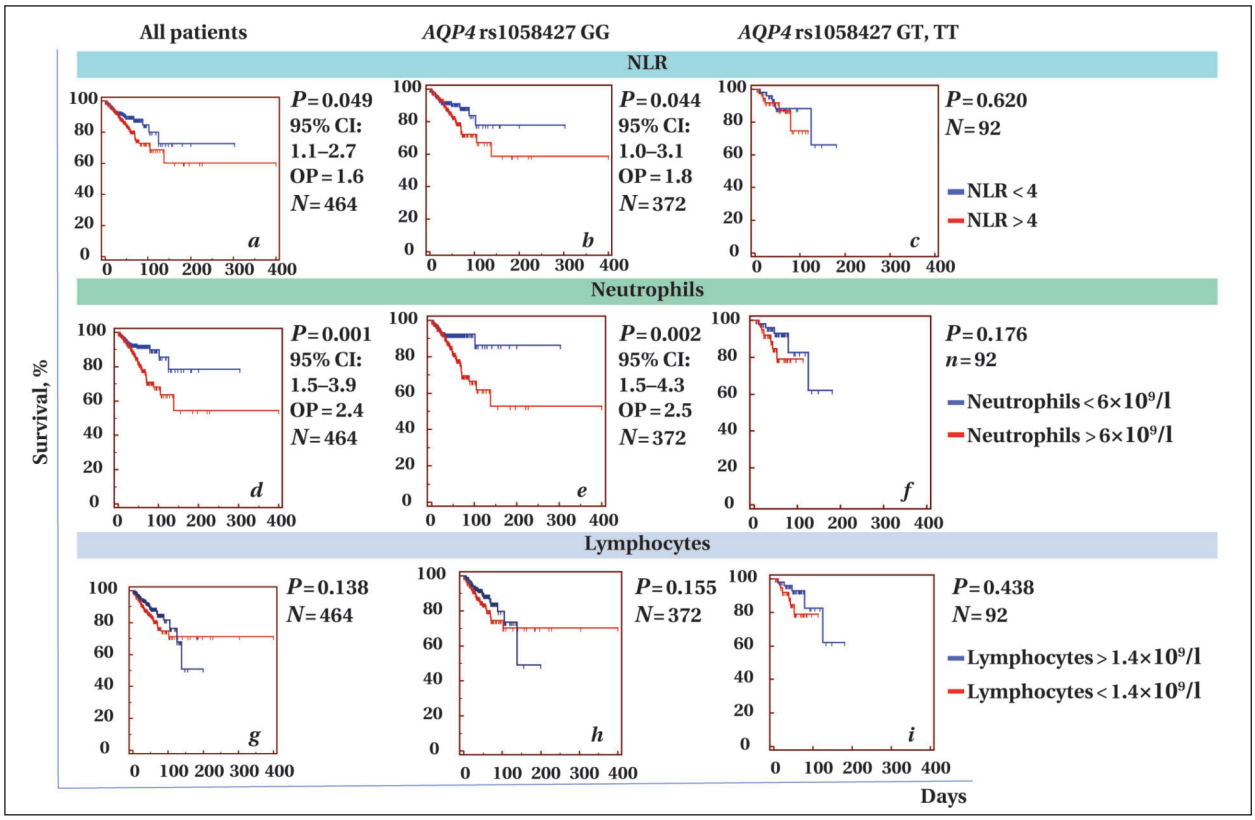

Fig. 3. Association of NL, neutrophil, and lymphocyte counts with outcome (entire cohort of patients in the PCCI).
Note. Here and in Fig. 4–5: *a* — NLR values, all patients; *b* — NLR values, patients with the *AQP4* rs1058427 GG genotype; *c* — NLR values, patients with the *AQP4* rs1058427 GT, TT genotypes; *d* — neutrophil count, all patients; *e* — neutrophil count, patients with the *AQP4* rs1058427 GG genotype; *f* — neutrophil count, patients with the *AQP4* rs1058427 GT, TT genotypes; *g* — lymphocyte count, all patients of the cohort; *h*— lymphocyte count, patients with the *AQP4* rs1058427 GG genotype; *i* — lymphocyte count, patients with the *AQP4*.

In subsequent analysis, using the log-rank test, we found that the value of NLR on the first day of hospitalization predicted outcome for patients with all *AQP4* rs1058427 genotypes ($P=0.049$, log-rank test, 95% CI: 1.1–2.7, HR = 1.6, $N=464$, Fig. 3, *a*). For patients with the major AQP4 rs1058427 GG genotype, the association remained ($P=0.044$, 95% CI: 1.0–3.1, HR = 1.8, $N=372$, Fig. 3, *b*). However, in the subgroup of patients carrying the minor T allele (GT and TT genotypes), no trend toward an association between the NLR value and adverse outcome was observed ($P=0.621$, $N=92$, Fig. 3, *c*).

Over 40% of patients in our sample were admitted to the hospital with pneumonia. The frequencies of the AQP4 rs1058427 genotypes did not differ in patients admitted with pneumonia (GG — 78%. GT — 21%, TT — 1%, consistent with the Hardy-Weinberg law, P = 0.657, $\chi^2=0.2$, $N=192$) and without pneumonia (GG — 82%. GT — 16%, TT — 2%, consistent with the Hardy–Weinberg law, $P=0.116$, $\chi^2=2.5$, $N=272$). For the group of patients admitted without pneumonia, the value of NLR as a prognostic marker was not statistically significant ($P=0.240$, $N=92$, Fig. 4, *a*). When subgroups of patients with different *AQP4* rs1058427 genotypes were analyzed separately, no patterns were found (Fig. 4, *b*, *c*). Only an increased neutrophil count significantly predicted an unfavorable outcome for patients admitted without pneumonia ($P=0.010$, log-rank test, 95% CI: 1.5–4.9, HR = 2.5, $N=272$, Fig. 4, *d*). However, when the patient genotype was taken into account, the association between an increased neutrophil count and an unfavorable prognosis was significant only for patients with the major *AQP4* rs1058427 GG genotype ($P=0.049$, log-rank test, 95% CI: 1.0–4.5, HR = 2.1, $N=223$, Fig. 4, *e*). For minor allele carriers, no such association was observed ($P=0.100$, $N=49$, Fig. 3, *f*). Circulating lymphocyte counts at admission for the subgroup of patients admitted without pneumonia were not associated with outcome regardless of *AQP4* rs1058427 genotypes (Fig. 4, *g*, *h*, *i*).

It should be concluded that a significant association with adverse outcomes for the immunophenotype of elevated neutrophil counts (over 6×10^9/L) in patients admitted to the PCC without signs of pneumonia is observed only when combined with the major *AQP4* rs1058427 GG genotype. Thus, the presence of a combined «geno-immunophenotype» such as the *AQP4* rs1058427 GG genotype and a

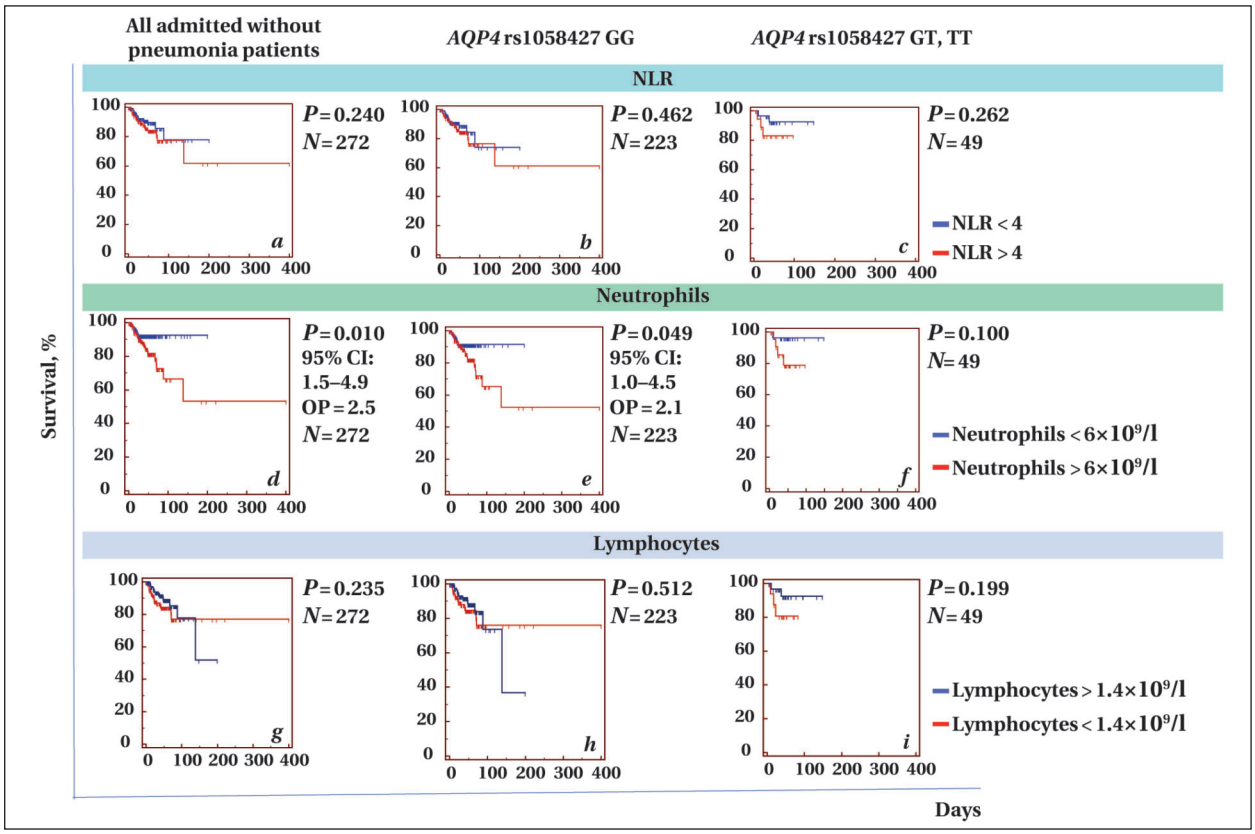

Fig. 4. Association of neutrophil counts, neutrophil counts, and lymphocyte counts with outcome in patients admitted without pneumonia.

neutrophil count exceeding 6×10⁹/L in a patient without signs of pneumonia upon hospitalization determines a high risk of mortality. Another group of patients was further analyzed — those diagnosed with pneumonia upon admission (within the first 48 hours of hospitalization). In the group of patients of all genotypes admitted with pneumonia, a statistically insignificant trend towards increased mortality was revealed in patients whose NLR values exceeded 4 on the day of admission ($P = 0.096$, log-rank test, Fig. 5, *a*). Statistically significant association between increased NLR value and mortality in this group of patients was observed only in carriers of the homozygous G allele ($P = 0.026$, log-rank test, HR = 2.9, 95% CI: 1.3–6.6, $N = 149$, Fig. 5, *b*). In the subgroup of carriers of the minor T allele, no trend towards increased mortality was even revealed with an NLR value of more than 4 ($P = 0.65$, $N = 43$, Fig. 5, *c*).

An increase in the blood neutrophil count above 6×10⁹/L predicted an unfavorable outcome for all patients admitted with pneumonia ($P = 0.034$, log-rank test, HR = 2.3, 95% CI: 1.1–4.7, $N = 192$, Fig. 5, *d*). However, a separate subgroup analysis of patients with different *AQP4* rs1058427 genotypes revealed a similar pattern to that found for ANP: differences were significant only for patients with the *AQP4* rs1058427 GG genotype ($P = 0.019$, log-rank test, HR = 3.1, 95% CI: 1.3–6.9, $N = 149$, Fig. 5, *e*). For patients with alternative genotypes GT and TT, prognosis did not differ depending on the blood neutrophil count ($P = 0.760$, $N = 43$, Fig. 4, *f*). A reduced lymphocyte count in our sample was not associated with an unfavorable prognosis for either the subgroups with the *AQP4* rs1058427 GG genotype ($P = 0.167$, $N = 149$, Fig. 5, *h*) or for the subgroup of carriers of the minor T allele ($P = 0.649$, $N = 43$, Fig. 5, *i*).

Thus, both the increased blood neutrophil count and its secondary marker — an elevated NLR value during hospitalization of patients with pneumonia — indicate an increased risk of an unfavorable disease course not in any patients, but only in patients with the *AQP4* rs1058427 GG genotype. It should be assumed that, as in the previous group of patients (hospitalized without signs of pneumonia), the *AQP4* rs1058427 allele complements the prognostic value of the immunophenotype (in the presence of pneumonia, these are two related indicators, NLR and an elevated neutrophil count), forming in concert a unique, combined gene-immunophenotype.

Discussion

A logistic regression analysis revealed that, of simple laboratory parameters, only elevated neutrophil counts (6×10⁹/L) and derived marker values — NLR (exceeding the norm, i. e., more than 4) —

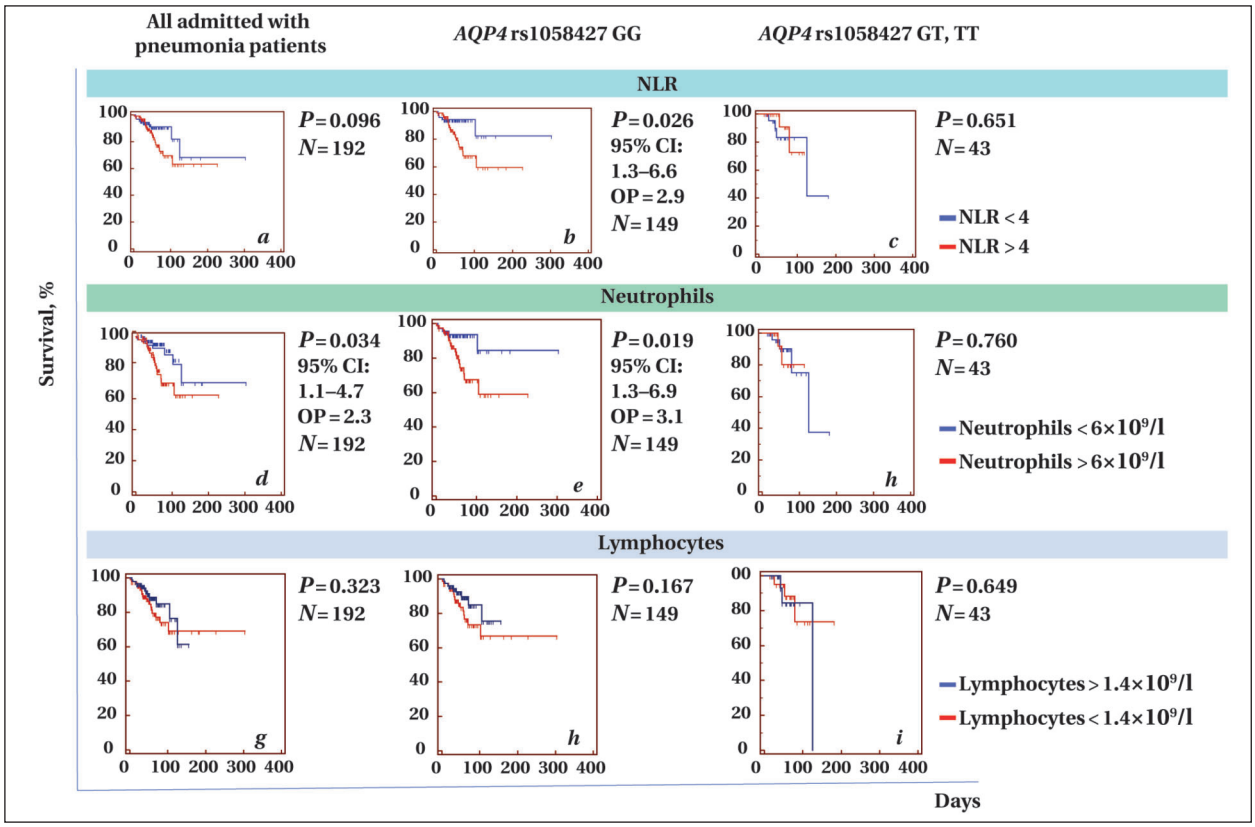

Fig. 5. Association of NLR, neutrophil and lymphocyte counts with outcome in patients admitted with pneumonia.

were associated with an unfavorable outcome in patients with the consequences of SBI diagnosed with PCCI (Table 2). A log-rank test confirmed the unfavorable prognosis when analyzing the entire cohort of patients using both parameters separately as prognostic biomarkers (Fig. 3). However, further analysis dividing the entire cohort into genetically distinct subgroups revealed limitations in the prognostic value of cellular immune parameters associated with genetic variability in patients for the *AQP4* gene alleles.

Earlier, it has been shown that the major allele of *AQP4* rs1058427 is associated with the development of edema after hemorrhagic stroke [23] and the outcome of sepsis in highly morbid patients [24]. In current study, in another cohort of patients (patients with the consequences of cerebrovascular accidents, admitted to the hospital), for the first time we demonstrated the contribution of the same *AQP4* genetic region to the formation of a complex gene-immunophenotype of patients with a high risk of unfavorable outcome. These were the patients with the *AQP4* GG genotype and an immunophenotype characterized by an increased number of neutrophils or the NLR value who had a significantly higher risk of death. For patients with the major genotype *AQP4* GG, NLR values above 4 had predictive informativeness regardless of the diagnosis of pneumonia (Tables 3–5). Perhaps, this reflects the differentiated effect of the *AQP4* gene on the processes of migration of neutrophils from the bone marrow and lymphocytes from the lymph nodes, expressed during activation of the infectious process. In patients with the major genotype *AQP4* admitted with pneumonia, the number of neutrophils was higher (Fig. 2), which may reflect the causative reason for the association of both biomarkers with mortality. It can be assumed that the presence of the minor T allele in the regulatory 3`-untranslated region of the *AQP4* gene rs1058427 in patients with PCCI determines the reduced level of *AQP4* gene expression in neutrophil precursor cells and, as a consequence, their lower level of migration into the bloodstream.

Populations of immature neutrophils contain subpopulations of granulocytic myeloid-derived suppressor cells (G-MDSCs) [27, 28], and with a relatively reduced content of neutrophils in circulation, a decrease in the number of G-MDSCs should be expected. In this case, the reduced potential of proinflammatory and antibacterial activity of the entire neutrophil mass as key cells of innate immunity can be compensated by improved development of antibacterial adaptive immunity due to the expected reduced number of G-MDSCs, which are known suppressors of the functional activity of

adaptive immune cells [27, 28]. On the contrary, the association we identified between the *AQP4* GG genotype and an increased number of neutrophils in the bloodstream in patients with pneumonia may lead to a higher content of circulating G-MDSCs in the blood and, consequently, to a greater manifestation of their functional activity. As a result, the antibacterial immune system may become weaker, and the risk of a fatal outcome in pneumonia in PCCI patients may increase. NLR reflects the balance between innate immunity and adaptive immunity. Increased NLR values may indicate the preferential circulation of the proinflammatory innate immune cells and/or decreased number of lymphocytes. These alterations may reflect the immune potential to contribute to both neutrophil-induced tissue damage at sites of localized infection (including abscess formation [29]) and decreasing the antibacterial activity mediated by T and B cells of adaptive immunity.

We believe that, in addition to increased blood neutrophil counts and NLR values, a key component of the immunophenotype associated with an unfavorable course and outcome of pathological conditions, including PCCI, may be an increased number of G-MDSCs, which are capable of being activated by bacterial endotoxins [30] and are associated with an unfavorable outcome of sepsis [31].

An increase in blood neutrophil counts and, consequently, an increase in NLR values are observed in various conditions for which inflammation is a key factor in pathogenesis: bacterial and fungal infections, acute stroke, myocardial infarction, atherosclerosis, trauma, tumors, postoperative complications — that is, in conditions characterized by tissue damage, activation of the systemic inflammatory response, and the development of organ failure [15, 16, 18–20]. Increased neutrophil activation markers on the first day of hospitalization for COVID-19 predicted subsequent transfer of patients to the intensive care unit (ICU) and an unfavorable course of the disease [22].

Lower NLR values are generally associated with a favorable prognosis for pathological conditions, reflecting a favorable immune balance [31]. Assessing this balance by determining NLR helps predicting the outcome in mechanical ventilation-associated pneumonia patients [31, 32], as well as in pneumonia developing after severe stroke. The latter is characterized by immunosuppression resulting from the enhancement of two processes: apoptotic lymphocyte death and the release of maturing neutrophils from the bone marrow into the bloodstream due to stimulation of granulocyte maturation by growth factors [33, 7]. Ventilator-associated pneumonia is one of the most severe complications in patients with traumatic brain injury (TBI) and is considered a risk factor for adverse outcomes [35]. Elevated NLR values predict adverse outcomes in patients with TBI and correlate with lower GCS scores [36, 37], which is consistent with our results.

Our findings suggest that testing a patient's genetic polymorphism (in particular, *AQP4*) may be useful for more targeted use of NLR and neutrophil count as a biomarker immunophenotype characterizing existing immune system imbalance. The reasons for the selective prognostic value of elevated NLR and/or neutrophil counts only in patients with the *AQP4* rs1058427 GG genotype of PCCI patients may relate to the increased functional significance of the AQP4 molecule for immune system cells, neuroglia, epithelium, and endothelium — key «players» and cellular targets in various pathological conditions, including infectious and inflammatory processes [38–40].

These data substantiate the advisability of expanding the concept of immunophenotypes to «predictive geno-immunophenotypes», more specifically, taking into account the genetic variability of patients, characterizing their high risk of a fatal outcome from PCCI. The search for and application of such combined predictive immunophenotypes/genotypes as prognostic biomarkers useful for early stratification of patients into risk groups will complement the existing principles of phenotyping critically ill patients [41–45] through genotyping in order to improve treatment outcomes in the format of personalized critical care medicine.

Conclusion

For patients in PCCI with sequelae of SBI, a neutrophil count above 6×10^9/L upon hospitalization significantly predicts an adverse outcome only in patients homozygous for the *AQP4* rs1058427 G allele (GG genotype carriers). It is suggested to consider the polymorphic genetic markers as a conceptual tool and a biomarker source complementing existing principles of clinical and laboratory phenotyping of patients in predictive critical care medicine («predictive immunophenotype/genotype for critical illness»).

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Приложение

Таблица. Результаты анализа выживаемости, проведенного с помощью регрессии Кокса на основе значений клеточных маркеров в 1-й день госпитализации.

Показатели	Все пациенты			*AQP4* rs1058427 GG			*AQP4* rs1058427 GT, TT
	P	**ОР**	**95% ДИ**	***P***	**ОР**	**95% ДИ**	***P***
Лимфоциты	0.62				0.84		0.16
Нейтрофилы	**0.006**	1.0	1.0–1.1	**0.032**	1.1	1.0–1.2	0.32
ОНЛ	**0.0001**	1.0	1.0–1.1	**0.0001**	1.0	1.0–1.1	0.22