Clinical & Experimental Immunology
The Journal of Translational Immunology

Clinical and Experimental Immunology ORIGINAL ARTICLE doi:10.1111/cei.12592

Clinical associations of host genetic variations in the genes of cytokines in critically ill patients

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Accepted for publication 16 January 2015
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Summary

Host genetic variations may influence a changing profile of biochemical markers and outcome in patients with trauma/injury. The objective of this study was to assess clinical associations of single nucleotide polymorphisms (SNPs) in the genes of cytokines in critically ill patients. A total of 430 patients were genotyped for SNPs in the genes of pro- (*IL1B, IL6, IL8*) and anti-inflammatory (*IL4, IL10, IL13*) cytokines. The main end-points were sepsis, mortality and adult respiratory distress syndrome (ARDS). We evaluated the dynamic levels of bilirubin, blood urea nitrogen, creatine kinase, creatinine and lactate dehydrogenase in five points of measurements (between 1 and 14 days after admission) and correlated them with SNPs. High-producing alleles of proinflammatory cytokines protected patients against sepsis (*IL1B* −511A and *IL8* —251A) and mortality (*IL1B* −511A). High-producing alleles of anti-inflammatory cytokines *IL4* —589T and *IL13* 431A (144Gln) were less frequent in ARDS patients. The carriers of *IL6* —174C/C genotypes were prone to the increased levels of biochemical markers and acute kidney and liver insufficiency. Genotype-dependent differences in the levels of biochemical indicators gradually increased to a maximal value on the 14th day after admission. These findings suggest that genetic variability in pro- and anti-inflammatory cytokines may contribute to different clinical phenotypes in patients at high risk of critical illness.

Keywords: adult respiratory distress sybdrome, cytokines, genetic association studies, sepsis, single nucleotide polymorphism

Introduction

An unbalanced immune reaction is considered to be responsible for a substantial amount of poor outcome in severely injured patients. The protective role of proinflammatory cytokines in early innate defence against extracellular bacteria is well established, but it is recognized that excessive production of these cytokines may also be responsible for the development of severe complications [systemic inflammatory response syndrome, sepsis, adult respiratory distress syndrome (ARDS), multiple organ dysfunction syndrome (MODS)] [1]. However, an anti-inflammatory therapy in sepsis clinical trials was unsuccessful, and the assumption that poor outcome in patients with or at high risk of critical conditions derives from an uncontrolled proinflammatory response is questionable [2]. Having survived the initial, hyperinflammatory phase of sepsis, patients may suffer from immunosuppression due to the T helper type 2 (Th2) cell response in the later stages of sepsis [3].

Polymorphisms in cytokine genes may influence the corresponding proteins' quantity, activity and stability, and this may have an impact upon the development of critical conditions. Numerous assays were performed to seek the genetic markers in the genes of cytokines in association with sepsis and other severe acute complications (reviewed in [4–6]). Given the complexity and heterogeneity of the problem, it is not surprising that the results are contradictory. With regard to the most studied genetic variations, despite the total number of publications, data concerning the same single nucleotide polymorphisms (SNPs) in the same populations and clinical conditions are scarce. Moreover, some relevant genes and SNPs are unconsidered in the field.

Nowadays, numerous assays consider multiple biomarkers of sepsis and organ failure. For example, a review of sepsis biomarkers has mentioned 178 different biomarkers [7]. Parameters of routine biochemical tests may also be useful as biomarkers of sepsis and organ insufficiency, as they are measured sequentially to capture a changing profile which reflects the severity of illness and target organ damage. Bilirubin, blood urea nitrogen (BUN) and creatinine are included in multiple scoring systems for post-injury multiple organ failure [8]. Moreover, bilirubin is elevated in patients with severe infections, and its measurement may be performed to discriminate between patients with and without bacteraemia [9]. Hyperbilirubinaemia is both a risk factor and complication of sepsis, which is associated with a reduction of the bile flow in hepatocytes [9]. In people with severe sepsis, hyperbilirubinaemia has been correlated with worse outcomes and predicted the development of ARDS [10–13]. Serum creatinine levels are elevated not only in patients with renal failure, but also in patients with ARDS [14]. A high lactate dehydrogenase (LDH) level has been linked to infection as a marker for severe prognosis, in-hospital major complications and high mortality, probably related to tissue destruction from immune hyperactivity [12,15,16]. LDH activity level was shown to predict pathological conditions in the lungs, such as cell damage or inflammation [17]. Elevation of creatine kinase (CK) was associated with more complications in patients with pH1N1 influenza A infection admitted to the intensive care unit (ICU) for severe acute respiratory insufficiency (SARI) [18]. In animal experiments, concentrations of creatinine, BUN, CK and LDH were already increased markedly at the early stage of multiple organ dysfunction syndrome caused by trauma and infection [19]. It has also been shown that creatinine, BUN and CK levels were correlated negatively with the survival outcome in sepsis [20,21].

The dynamics of the variations of biochemical parameters may depend particularly upon host genetic resistance to the infection. To the best of our knowledge, little or no research has been performed to date to understand the relationships between the genetic variability in the acute-phase response genes and the dynamic pattern of routine biochemical indicators. In light of these challenging issues, we performed an association study of the outcome in patients with severe trauma/injury and well-studied functional polymorphic variations in the genes of pro- (*IL1B*, *IL6*, *IL8*) and anti-inflammatory (*IL4*, *IL10*, *IL13*) cytokines. Single-nucleotide polymorphisms (SNPs) (*IL1B* −511 G>A, rs16944; *IL6* –174 G>C, rs1800795; *IL8* −251 T>A, rs4073; *IL4* −589 C>T, rs2243250; *IL10* −1082 G>A, rs1800896; *IL13* 431 A>G, 144Gln/Arg, rs20541) in the above genes have been shown to influence the level of the gene product (missense SNP in the *IL13* gene) or the activity of gene promoter (SNPs in other genes). In addition, these SNPs have been demonstrated to be associated with susceptibility to sepsis and/or organ failure (in particular, SNPs in *IL1B*, *IL6*, *IL8*, *IL10*) and a number of infectious and inflammatory diseases [22–26]. The main end-points studied in association with SNPs were sepsis, mortality and ARDS. The dynamic levels of biochemical markers (bilirubin, BUN, CK, creatinine and LDH), shown to be related to these end-points, were evaluated in five points of measurements (between 1 and 14 days after ICU admission) and correlated with gene polymorphisms. Based on the findings in the set of biochemical indicators, we performed additional genetic association studies of liver and renal insufficiency.

Materials and methods

Study subjects

From January 2008 to February 2013 we selected a group of accident victims with severe physical trauma and patients with acute diseases requiring extensive surgery. A total of 430 patients at risk of critical illness [81% males; among these, 193 patients (55%) were workers of the Rescue Service; mean age 42.12 ± 18.56 years], hospitalized at the clinical bases of V. A. Negovsky Research Institute of General Reanimatology, Moscow, Russia were included in the study. The severity of each patient was evaluated on the basis of the Acute Physiology and Chronic Health Evaluation (APACHE) II score within the first 24 h after ICU admission [27]. The Sequential Organ Failure Assessment (SOFA) score was calculated consecutively as an indicator of organ dysfunction [28]. Exclusion criteria for the group under study consisted of age less than 18 years, lack of informed consent, defined immunodeficiency, corticosteroid administration less than 6 weeks previously, final stage of chronic disease, decompensated heart failure [New York Heart Association (NYHA) Class IV], decompensated diabetes, severe neurological deficit (Glasgow Coma Scale ≤ 8), addiction, alcoholism, AIDS and pregnancy. Patients with chronic respiratory, kidney and liver diseases were also excluded. Treatment decisions for all study participants were standardized according to the conciliatory guidelines of the Russian Respiratory Society and Interregional Association of Clinical Microbiology and Antimicrobial Chemotherapy in all patients (http://webmed.irkutsk.ru/doc/pdf/cap.pdf). Sepsis and multiple organ dysfunction syndrome (particularly, renal and liver insufficiency) were diagnosed according the definitions of the ACCP-SCCM consensus conference on sepsis and organ failure [29] and SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definitions [30]. The diagnostics of the ARDS is similar to the ARDS Berlin definition [31].

All biochemical analyses were performed using Cobas 6000, ABX Pentra 400 and Mythic 22 analysers (Roche, Boulogne-Billancourt, France) according to the

manufacturer's instructions. Measurements were carried out within the first day of ICU admission and on the subsequent third, fifth, seventh and 14th days.

The study protocol was approved by the Ethics Committee of V. A. Negovsky Research Institute of General Reanimatology RAMS (with Institutional Review Board approval number 2/6/2012), and adhered to the tenets of the Declaration of Helsinki.

Genotyping

DNA was isolated from 200 μl of blood using the gDNA purification kit Diatom DNA Prep 200 (Isogene Laboratory, Moscow, Russia). The genotyping was performed with a PCR-CTPP (polymerase chain reaction with confronting two-pair primers) [32]. Amplification was carried out in an ABI thermal cycler using two external and two internal sequence-specific primers (Supporting information, Table S1) and tubes of PCR MasterMix (Isogene Laboratory, Moscow, Russia). The PCR products were analysed in 2% agarose gel stained with ethidium bromide. The CFX96 real-time PCR detection system with SYBR Green fluorescent dye was employed to genotype 10% of randomly taken DNA samples for each SNP once more. There was 100% concordance in genotype calling of duplicate samples.

Statistical analysis

Deviation from Hardy–Weinberg equilibrium was assessed by χ^2 analysis. As all studied continuous variables did not assume a normal distribution, the Mann–Whitney *U*-non-parametric test was used to compare such variables. To evaluate associations between gene polymorphisms and studied end-points we performed logistic regression analysis using the SNPStats package [33], a free web-based tool designed specifically for genetic association studies. The association with disease is modelled depending on the response variable. For binary variables, unconditional logistic regression models are used. For quantitative response, linear regression models are used to examine the proportion of variation in the response explained by the SNPs. In analysis of the SNPs in relation to the response, SNPStats provides odds ratios (OR), confidence intervals (CI) and the *P*-values for multiple inheritance models (dominant, recessive, over-dominant, co-dominant and additive), as well as Akaike's information criterion (AIC) indicating the best inheritance genetic model for each specific polymorphism. Other quantitative or categorical variables may be added to the regression models for analysis as covariates and treated as potential confounders. In multivariate models, we adjusted for age, sex, APACHE II score and using (more than 24 h) mechanical ventilation. For the regression models we present OR for the minor allele. Therefore, $OR < 1{\cdot}0$ shows a protective effect of the minor allele, and $OR > 1{\cdot}0$ shows that the minor allele is a risk allele. The lowest AIC value was considered the best-fitting model for the fitted variant. For genotypes with minor allele frequencies, <10% only dominant and additive genetic models were evaluated.

The influence of multiple testing in the genotypical-dependent outcome (sepsis, mortality, ARDS) was evaluated applying Benjamini–Hochberg step-up false discovery rate (FDR) corrections (implemented in the WINPEPI computer programs [34]). The WINPEPI is a free and user-friendly resource comprising computer programs for a wide spectrum of statistical tools designed for epidemiologists and biomedical researchers. Procedures for adjusting *P*-values derived from multiple significance tests are supplied by one of the modules of the ETCETERA program of the WINPEPI package.

The association study between genetic and biochemical variables was considered exploratory, not requiring corrections for multiple comparisons [35].

The WINPEPI test power calculator (in the COMPARE2 program) was used to evaluate the test power. *Post-hoc* power calculations were generated using a detected OR assuming the specified model of risk, 5% type 1 error rate and 80% power. In the sepsis study, statistical power was 66·75% (*IL1B* rs16944; OR = 0·27) and 86·11% (*IL8* rs4073; OR = 0·44); in the hospital mortality study, statistical power was 94·96% (*IL1B* rs16944; OR = 0·41); in the ARDS study, statistical power was 71·45% (*IL4* rs2243250; OR = 0·41) and 41·50% (*IL13* rs20541; OR = 0·55).

Results

Characteristics of the study population

Disease progression in 430 Caucasian patients from the European region of Russian Federation was evaluated in association with genotype and laboratory data. The baseline characteristics of the study subjects are shown in Table 1. Within the first 24 h after ICU admission, the APACHE II and SOFA scores did not differ between patients with and without trauma (APACHE II: 17·98 ± 4·38 and 18·20 ± 4·14, respectively, $P = 0{\cdot}82$; SOFA: 11·55 ± 3·64 and 11·59 ± 4·03, respectively, $P = 0{\cdot}92$). We calculated pairwise associations for binary variables and found the following Pearson's correlation coefficients (r_φ) between clinical end-points: sepsis and mortality, $r_\varphi = 0{\cdot}34$; sepsis and ARDS, $r_\varphi = 0{\cdot}21$; mortality and ARDS, $r_\varphi = 0{\cdot}30$.

The dynamics of changes in SOFA score and laboratory parameters

The dynamics of changes in SOFA score and biochemical blood plasma values in relation to all studied clinical phenotypes is given in Supporting information, Table S2.

Table 1. Characteristics of the patients included in the study

Characteristics	All patients, *n* (portion) mean ± s.d.
Total number	430
Age (years)	42·12 ± 18·56
Male sex (*n*, %)	349 (0·81)
Pre-existing conditions	
• Cardiovascular diseases	45 (0·11)
• Diabetes	25 (0·06)
• Obesity	24 (0·06)
ICU admission	402 (0·94)
ICU length of stay (d)	14·82 ± 23·46
Patients on mechanical ventilator	153 (0·36)
APACHE II score[a]	18·08 ± 4·27
SOFA score[a]	11·57 ± 3·83
Diagnosis at admission	
• Severe combined trauma/wounding	236 (0·55)
• Bowel obstruction	28 (0·07)
• Inflammatory diseases of the abdominal cavity and retroperitoneal space complicated by destruction	106 (0·25)
• Purulent-inflammatory diseases of the skin, subcutaneous tissue	44 (0.10)
• Other	19 (0·04)
Critical conditions	
• Sepsis	80 (0.19)
• ARDS	75 (0·17)
Hospital mortality	95 (0·22)

[a]Within the first 24 h after admission. ARDS = adult respiratory distress syndrome; ICU = intensive care unit; APACHE = Acute Physiology and Chronic Health Evaluation; SOFA = Sequential Organ Failure Assessment; s.d. = standard deviation.

The mean SOFA score on ICU admission and during the ICU stay was, in general, higher in all studied cases (sepsis, mortality, ARDS, renal and hepatic insufficiency) compared with matched controls. As expected, the maximum differences were found in the mortality set. Normal levels of studied biochemical parameters are considered to be in the range of 3·4–17·1 mmol/L for total bilirubin, 1.7–8.3 mmol/L for BUN, 26·0–174·0 U/L for CK, 53·0–124·0 mmol/L for creatinine and 89·0–221·0 U/L for LDH. In non-survivors, all studied biochemical parameters increased gradually within 2 weeks. In comparison with survivors, the maximum differences were found on the 14th day. Patients with ARDS periodically had higher levels of BUN, creatinine and bilirubin. In patients with renal insufficiency, BUN and creatinine were elevated from the 3rd and 5th to the 14th days, respectively. With few exceptions in patients with liver insufficiency, levels of bilirubin, BUN and creatinine tended to increase over time. In the group of patients with liver insufficiency a very high level of LDH was found by the end of the second week.

We also evaluated changes in biochemical parameters in sepsis patients with and without acute trauma (Supporting information, Table S3). In general, the results seem to be consistent with the expectations. CK and LDH are expressed in many different organs and tissues and their levels were higher in trauma patients. After acute trauma, the levels of CK were elevated on the first and seventh days after admission to the ICU, and the levels of LDH were higher on the seventh day, compared to non-trauma patients with sepsis. BUN, creatinine and bilirubin are key biomarkers of renal and liver failure and their concentrations increased gradually in non-trauma subjects, but the only significant increment was observed for serum bilirubin on the 7th day. SOFA scores did not differ between trauma and non-trauma patients with sepsis.

Available data concerning changes in other biochemical parameters linked with renal and liver insufficiency are presented in Supporting information, Table S4. The most profound effect was found for AST concentrations in patients with liver insufficiency on the 14th day.

Genetic association study of selected candidate genes in sepsis, ARDS and mortality sets

All genotypes were in Hardy–Weinberg equilibrium (Supporting information, Table S5). Within the first day of ICU admission, APACHE II scores were significantly higher for the carriers of the *IL6* −174 C/C genotype. Higher SOFA scores correlated with the *IL6* −174 C/C genotype and with the *IL8* −251 A allele (additive model) (Supporting information, Table S6).

Genotype association results significant after correction for multiple testing are presented in Table 2; non-significant data are given in Supporting information, Table S7. Alleles of proinflammatory cytokines *IL1B* −511A and *IL8* −251A were protective against the development of sepsis. The protective effect of the same *IL1B* allele was found in relation to hospital mortality. In the ARDS set alleles of anti-inflammatory cytokines *IL4* −589T and *IL13* 431A were less frequent in the cases than in the controls.

Correlation between genotypes and biochemical parameters

Dynamic changes in SOFA scores and biochemical blood plasma values were assessed in relation to the studied SNPs. Addressing the SOFA score, it should be noted that within the first day on ICU admission, similar associations were found in the whole sample (n = 430) and in the observational group (n = 88) for the *IL6* and *IL8* SNPs (Supporting information, Tables S6 and S8, Fig. 1). The majority of the dynamic associations was not stable and might have arisen by chance (Supporting information, Table S8). The most prominent associations were found for SNPs *IL8* −251 T>A and *IL6* −174 G>C (Figs

Table 2. The distribution of genotypes among patients with/without sepsis, ARDS and survivors/non-survivors

Genes and genotypes		Controls	Cases	*P*-value (genetic model), OR (95% CI)
		Number (%)		
Sepsis				
IL1B		*n*=334	*n*=78	
	G/G	146 (43·7)	42 (53·9)	0·014 (rec)[a]
−511 G>A	G/A	145 (43·4)	33 (42·3)	0·27 (0·08–0·92)
	A/A	43 (12·9)	3 (3·8)	
IL8		*n*=342	*n*=79	
	T/T	98 (28·6)	37 (46·8)	0·0023 (dom)[b]
−251 T>A	T/A	183 (53·5)	33 (41·8)	0·44 (0·26–0·75)
	A/A	61 (17·8)	9 (11·4)	
Hospital mortality				
IL1B		*n*=321	*n*=91	
	G/G	136 (42·4)	52 (57·1)	0·0019 (dom)[c]
−511 G>A	G/A	147 (45·8)	31 (34·1)	0·41 (0·23–0·73)
	A/A	38 (11·8)	8 (8·8)	
ARDS				
IL4		*n*=345	*n*=72	
	C/C	198 (57·4)	51 (70·8)	0·01 (dom)[d]
−589 C>T	C/T	134 (38·8)	20 (27·8)	0·48 (0·27–0·86)
	T/T	13 (3·8)	1 (1·4)	
IL13		*n*=347	*n*=74	
	C/C	179 (51·6)	51 (68·9)	0·008 (add)[e]
431 A>G*	C/T	141 (40·6)	20 (27·0)	0·55 (0·35–0·88)
	T/T	27 (7·8)	3 (4·0)	

The analysis is adjusted for age, sex, Acute Physiology and Chronic Health Evaluation II (APACHE II) score and using (more than 24 h) of mechanical ventilation. ARDS = adult respiratory distress syndrome; OR = odds ratio; CI = confidence interval. The choice of each genetic model was based on Akaike information criterion (AIC) value. The genetic model: rec = recessive: dom = dominant; add = additive. **IL13* rs20541 (431 A>G) alleles are reported in reverse orientation to genome (A allele is T); http://www·ncbi·nlm·nih·gov/projects/SNP/snp_ref·cgi?rs=20541. Benjamini–Hochberg step-up false discovery rate (FDR)-adjusted *P*-values: [a]$P = 0·042$; [b]$P = 0·014$; [c]$P = 0·011$; [d]$P = 0·030$; [e]$P = 0·030$.

1, 2). The *IL8* −251 A allele correlated with higher SOFA score during the entire observation time (Fig. 1). The carriers of the *IL6* C/C genotype appeared to have elevated bilirubin levels from the first to the 14th day. LDH, CK and BUN levels were initially higher in patients with *IL6* G allele. However, over time, these associations inverted and the carriers of the *IL6* C/C genotype had significantly elevated concentrations of LDH, CK and BUN by the end of the second week (Fig. 2). Creatinine level also tended to increase in patients with the *IL6* C/C genotype, but the differences did not reach statistical significance (Supporting information, Table S8).

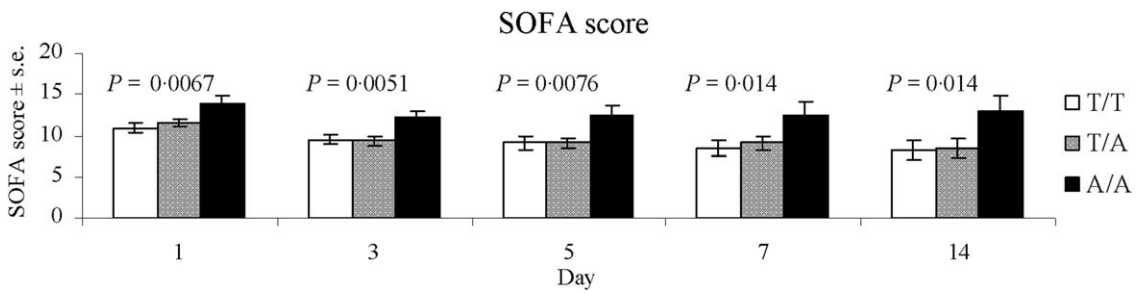

Fig. 1. Dynamic changes in sequential organ failure assessment (SOFA) score in the carriers of different *IL8* genotypes. Mean values and standard errors (s.e.) of SOFA scores are plotted by *IL8* —251 T>A genotypes in five points of measurements between 1 and 14 days after admission to the intensive care unit. Bars represent mean SOFA values for patients carrying specified genotype. *P*-values are given for recessive model (A/A *versus* T/T-T/A).

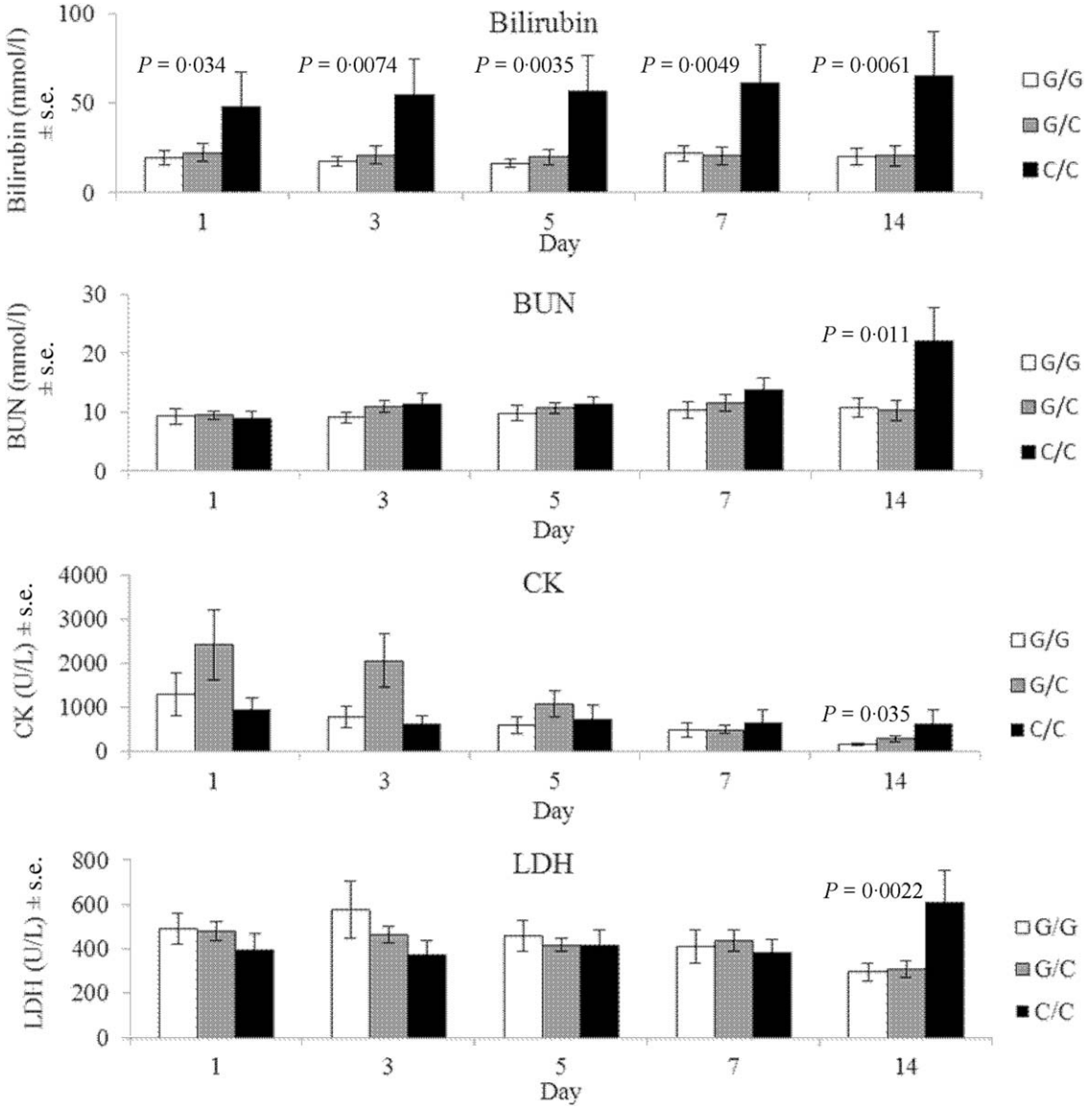

Fig. 2. Dynamic changes in biochemical indicators in the carriers of different *IL6* genotypes. Mean values and standard errors (s.e.) of bilirubin, blood urea nitrogen (BUN), creatine kinase (CK) and lactate dehydrogenase (LDH) values are plotted by the *IL6* —174 G>C genotypes in five points of measurements between 1 and 14 days after admission to the intensive care unit. Bars represent mean indicator values for patients carrying specified genotype. Significant *P*-values are given for recessive model (C/C *versus* G/G-G/C). The analysis is adjusted for age, sex, APACHE II score, and using (more than 24 h) of mechanical ventilation.

Biochemical characteristics and genotype–phenotype correlation

The revealed genotypical associations with biochemical traits were used to analyse additional clinical phenotypes. The groups with renal and liver insufficiency were small. For this reason, they were not included in the initial genetic association study. Based on the results of bilirubin and BUN associations with *IL6*, we explored an interaction between the *IL6* polymorphism and liver and renal insufficiency (Table 3). *IL6* C/C genotype appeared to be associated with liver insufficiency. A marginally significant association was also found for renal insufficiency under the same genetic model.

Discussion

In this study we investigated functional polymorphic variants in the genes of cytokines in relation to clinical phenotypes in patients with critical illness.

Alleles of proinflammatory cytokines *IL1B* −511A and *IL8* −251A were associated with protection against sepsis. In active inflammation, interleukin IL-1B is known to

Table 3. The distribution of *IL6* genotypes among patients with/-without renal and liver insufficiency

Genotypes		Controls Number (%)	Cases	*P*-value (genetic model),[c] OR (95% CI)
Renal insufficiency				
		n = 387	*n* = 38	
IL6	G/G	137 (35·4)	14 (36·8)	0·05 (rec)
−174 G>C	G/C	181 (46·8)	12 (31·6)	2·21 (1·00–4·78)
	C/C	69 (17·8)	12 (31·6)	
Liver insufficiency				
IL6		*n* = 400	*n* = 25	
	G/G	141 (35·4)	10 (40·0)	0·012 (rec)
−174 G>C	G/C	188 (47·0)	5 (20·0)	3·10 (1·33–7·23)
	C/C	71 (17·8)	10 (40·0)	

The analysis is adjusted for age, sex, Acute Physiology And Chronic Health Evaluation II (APACHE II) score, and using (more than 24 h) of mechanical ventilation. OR = odds ratio; CI = confidence interval. The genetic model: rec = recessive.

serve as triggering cytokine for the cytokine cascades. *In-vitro* studies have shown that allele A in the promoter (upstream of the transcriptional start site) SNP −511 G>A is related to increased lipopolysaccharide (LPS)-induced IL-1B protein secretion [36]. In a recent meta-analysis, the SNP −511A allele was less frequent in patients with sepsis compared to controls among Caucasians (one study) and Asians (four studies), although the results failed to reach statistical significance [37]. The same direction of the effect was observed in our study for the same genetic model. Sepsis is a risk factor for mortality, and the revealed association of the SNP −511A allele with improved survival supports our findings in the group of patients with sepsis. These data are in line with the results of other studies of bacterial infections. The *IL1B* −511A allele has been protective against human leptospirosis [38], *Helicobacter pylori* eradication failure [39] and susceptibility to bacteraemia within the first year after kidney transplantation [40].

IL-8 is a member of the chemokine family that initiates and enhances inflammation by activation and chemotaxis of immune cells. The results of the studies of genotype-specific influence on *IL8* gene expression are controversial. Higher LPS-stimulated expression of *IL8* mRNA was shown for the *IL8* SNP −251A allele in Caucasians [41,42], but for the allele T in Chinese [43,44]. The *IL8* transcription is also influenced by haplotype and is tissue-specific [45,46]. The protective effect of the *IL8* −251A allele in acute infections has been observed in acute suppurative apical periodontitis, but not chronic non-suppurative apical periodontitis [47] and respiratory syncytial virus (RSV) bronchiolitis [42].

Sepsis is a condition caused by a hyperinflammatory immune response to infection. In the 1990s, sepsis was believed to be associated with an exacerbated production of mainly proinflammatory cytokines. Later, it became evident that a tightly regulated balance in the cytokine network, which comprises proinflammatory cytokines, anti-inflammatory cytokines and soluble inhibitors of proinflammatory cytokines, is crucial to protect from sepsis [48]. The data today have shown that deficiencies in the production of proinflammatory cytokines result in defective activation of the host defence against infectious pathogens. Innate deficiency in cytokine release leads to a rapid accumulation of the microorganisms and may be followed by secondary systemic inflammatory and anti-inflammatory reactions [49]. The results of the current study support this assumption, as high-producing alleles of proinflammatory cytokines *IL1B* −511A and *IL8* −251A protected against sepsis development.

IL-8 is considered one of the most important cytokines in the pathogenesis of ARDS, with the −251A allele being associated with the acute lung injury (ALI) susceptibility and related outcomes (references in [50]). The opposite direction of the *IL8* genetic associations in acute infections (including sepsis in the current study) and ALI/ARDS may be linked to pathophysiological differences between sepsis-related ARDS and non-sepsis-related ARDS [51]. In our sample, only 12 people (∼3%) developed ARDS as a sequela from sepsis and, in line with literature data, the risk allele A (−251 T>A) was found more frequently in patients with ARDS than in non-ARDS controls, although the results were non-significant (P = 0·15, OR = 1·54, 95% CI = 0·84–2·80, dominant model) due to the insufficient statistical power of the test (26·67% at the two-sided significance level 0·05). *IL-8* may be produced early in the inflammatory response, and may persist for a relatively long period of time [52]. A profound proinflammatory response might have a beneficial role in activating host defence, but a prolonged proinflammatory state can promote organ damage [1]. We found that a high-producing *IL8* −251A allele was associated with higher SOFA scores, and this association is consistent with the above statement.

Variant alleles of anti-inflammatory cytokines *IL4* −589T and *IL13* 431A were associated with protection against ARDS. Type 2 cytokines IL-4 and IL-13 are two closely related cytokines that play simultaneously overlapping and distinct roles in type 2 immunity [53]. Although these cytokines are usually considered as inducers of humoral response through the immunoglobulin (Ig)-mediated allergic/inflammatory pathway, they can also influence humoral responses during infections with extracellular pathogens [54,55] and inhibit secretion of proinflammatory cytokines in response to bacterial toxins [56,57]. The genes for *IL4* and *IL13* are located in a cluster of cytokine genes in the region 5q23-31 (also including *IL3*, *IL5*, *IL9*, *IL15*, *GMCSF* and interferon regulatory factor) [23]. The *IL4* promoter variant −589C>T allele T

is considered a high-producer allele [58]. −589C>T SNP is linked tightly to 18 other SNPs, among which is −33C>T (rs2070874). Haplotype TT comprising both variant alleles in linkage disequilibrium was associated with threefold higher transcriptional activity *in vitro* and *in vivo* [59].

The *IL13* missense SNP (431 A>G) is also described as functional, with allele A being associated with higher IL-13 production [60]. This allele is in linkage disequilibrium with variant allele T of promoter SNP −1055C>T (rs1800925), which is also associated with higher IL-13 expression in patients with allergic asthma and non-atopic controls [61].

The balance between proinflammatory and anti-inflammatory mediators may determine the extent of lung injury. Anti-inflammatory cytokines may protect against lung injury [62]. Although Th2 cytokines have been characterized not only as major contributors to allergy but also as mediators of response to pathogens, there have been very few studies conducted on the role of *IL4* and *IL13* SNPs in infectious and non-allergic inflammation diseases. The *IL4* −589T allele protected against HIV-1 disease progression by reducing virus load [63], and was less frequent in patients with tuberculous infection than in controls [64]. The carriers of the *IL13* 431A allele had a reduced risk of severe malaria [65]. For the first time, we observed the protective effect of high-producing alleles of anti-inflammatory cytokines *IL4* −589T and *IL13* 431A in ARDS, and these results seem to have a biological rationale.

To understand further the genetic susceptibility to severe complications of trauma/injury, routine biochemical parameters were evaluated and related to the development of critical conditions and genetic variability in the studied genes. The *IL6* –174 C/C genotype was linked consistently with the elevated levels of biochemical indicators: bilirubin, BUN, CK and LDH, but the only stable association from the first to the 14th day was found for bilirubin. IL-6 is a multi-functional cytokine with both pro- and anti-inflammatory activities and a central role in host defence [66]. It is produced by a large quantity of cell types and has many functions, including stimulation of the hepatic acute-phase proteins such as C-reactive protein (CRP) and fibrinogen. This function is reflected in one of the alternative names for IL-6: hepatocyte stimulatory factor (HSF) (http://omim.org/entry/147620). The liver is a crucial organ in the first line of host defence [67], and IL-6 is the critical cytokine during the hepatic acute-phase reaction [68]. It is secreted by hepatic Kupffer cells (liver macrophages) in response to liposaccharide or tumour necrosis factor (TNF) [69]. IL-6 is known to inhibit an endotoxin-associated increase in TNF-α [70] and to protect hepatocytes from TNF-α-induced hepatic injury [71,72]. Moreover, IL-6 increases the production of anti-inflammatory cytokines IL-1RA and IL-10 and stress-related anti-inflammatory hormone cortisol [73]. Many of the hepatoprotective effects of IL-6 are mediated through the activation of signal transducer and activator of transcription-3 (STAT-3) and mitogen-activated protein kinase (MAPK) signalling pathways [69,74]. Apart from the acute-phase response reaction, peaking within 2–3 days, IL-6 exerts anti-oxidant capacity in hepatocytes and protects liver from drug-induced hepatoxicity [75–77]. SNPs in the promoter region of the *IL6* gene may be responsible for variations in transcription that subsequently affect serum levels. The best-characterized of these polymorphisms is a promoter SNP rs1800795 at position −174, upstream of the transcription start site, involving transversion of guanine for cytosine. Several studies of the associations of this SNP with severe acute conditions have shown that G allele is favourable for the disease outcome. Patients with the −174 G/G genotype demonstrated an improved survival in sepsis [78,79] and had a reduced risk of septic shock [80,81] and extrapulmonary pneumococcal dissemination [82]. In subjects with pneumococcal community-acquired pneumonia the −174 G/G genotype was protective against septic shock, ARDS, MODS and mortality [25]. By contrast, non-significant associations with severity or outcome have been observed in two other studies of critical conditions [83,84]. Although certain discrepancies also exist in relation to an assessment of genotype influence on gene expression, the *IL6* −174 G allele is usually considered a high-producing allele [80,83,85–88]. The evidence for polymorphisms in the *IL6* gene affecting the risk of organ failure is scarce. In addressing this theme, additional to the above-mentioned study by Martín-Loeches *et al.* [25], investigation by Verduijn *et al.* [89] has shown that the *IL6* −174 C/C genotype is associated with mortality and technique failure in European peritoneal dialysis patients. In our study, we also found correlations between the *IL6* –174 C/C genotype and liver and kidney insufficiency, the conditions connected closely to the levels of bilirubin, BUN and creatinine. Other clinical phenotypes and SOFA scores after 1 week of ICU stay were not associated with the *IL6* polymorphic variant, although initially the carriers of the *IL6* –174 C/C genotype had higher APACHE II and SOFA scores. While the severity of the state in the early period after injury depends upon the severity of the damage and the intensity of the acute-phase response, the development of different complications and critical conditions over time may be affected by genetically mediated associations of certain metabolic pathways. In our sample, it is the *IL6* association with the level of biochemical parameters, notably bilirubin, and liver and kidney insufficiency in patients without the initially compromised liver and kidney functions.

The study has several limitations. Our sample size was modest, and the study is powered to detect only relatively large effect sizes (minimum detectable OR ∼1·6–1·9; reciprocal OR∼ 0·52–0·63). Although having an

exploratory character, an association study between genetic and biochemical variables is limited by the multiple comparison issue. Our patients were mainly men, and the results may not be generalizable to women. The results require replication, but the revealed associations are important to understand the pathways and mechanisms of the development and progression of acute severe complications in patients with trauma/injury.

In summary, first, we present new results about the protective role of high-producing alleles of anti-inflammatory cytokines *IL4* −589T and *IL13* 431A in patients with ARDS.

Secondly, for the first time, we tested correlations between SNPs in the genes of cytokines and the dynamics of routine biochemical parameters. Our data brought novel observations that higher concentrations of bilirubin are associated routinely with the *IL6* −174C/C genotype. This genotype also appeared to be linked with the increased levels of BUN, CK and LDH, but only up to the end of the second week. It is an interesting challenge for future research directions to determine whether stronger hepatotoxic effects in the carriers of the −174C/C genotype were causal and/or synergistic for these last associations. The tested approach seems to be useful in genetic association studies, as biochemical indicators, being the intermediate metabolites, may have greater reliability and demonstrate larger effect sizes than those reported to disease itself [90].

Thirdly, we observed the protective effects of high-producing alleles −511A *IL1B* and −251A *IL8* in patients with sepsis. At the same time, −251A allele of the *IL8* gene was associated consistently with higher SOFA score. The associations of polymorphic variations in the genes of proinflammatory cytokines *IL1B* and *IL8* in patients with acute infectious, including sepsis, have been studied previously with inconsistent results. Our data are in line with the premise that a powerful proinflammatory response can have beneficial effects on sepsis development, but may increase the risk of subsequent organ damage [1,91].

It is assumed that, for infectious diseases, genetics is more important than for cancer or cardiovascular diseases [92]. The PIRO concept [92], which attempts to characterize sepsis across four components (predisposition, infection, response, organ dysfunction) is designed to classify states of sepsis. PIRO recognizes that the incidence and outcome of sepsis are influenced by genetic susceptibility, which has been suggested as a risk stratification tool and an inclusion criterion for therapeutic trials.

Acknowledgements

This work was supported by grants from the Presidium of the Russian Academy of Sciences program 'Fundamental Sciences for Medicine'—2012, 2013, 2014.

Disclosures

There are no conflicts of interest.

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