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Functional polymorphisms in the *CYP1A1*, *ACE*, and *IL-6* genes contribute to susceptibility to community-acquired and nosocomial pneumonia

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SUMMARY

Objectives: To establish the contribution of genetic host factors to the risk of community-acquired pneumonia (CAP) and nosocomial pneumonia (NP) in the population of the Russian Federation.
Methods: A total of 796 subjects (CAP: 334 patients, 134 controls; NP: 216 critically ill patients with NP, 105 critically ill patients without NP) were included in two case–control studies. We analyzed 13 polymorphisms in 11 genes (*IL-6*, *TNF-α*, *MBL2*, *CCR5*, *NOS3*, *CYP1A1* (three sites), *GSTM1*, *GSTT1*, *ABCB1*, *ACE*, and *MTHFR*) using a tetra-primer allele-specific PCR method.
Results: Individual single nucleotide polymorphism (SNP) analysis revealed a strong association between *CYP1A1* rs2606345 and CAP ($p = 3.9 \times 10^{-5}$, odds ratio (OR) 0.42, 95% confidence interval (CI) 0.27–0.63). Three genes (*CYP1A1*, *ACE*, and *IL-6*) were identified that account for part of the increase in vulnerability to both diseases, CAP and NP. The carriage of three predisposing genotypes versus protective genotypes increased the CAP risk ($p = 0.001$, OR 7.01, 95% CI 1.99–24.70) and NP risk ($p = 0.028$, OR 4.34, 95% CI 1.15–16.45).
Conclusions: Genetic predisposition to CAP and NP is attributed to the cumulative contribution of polymorphisms at the *CYP1A1*, *IL-6*, and *ACE* genes, independently of age, gender, causative pathogen, and the use of mechanical ventilation, in patients in the Russian Federation.

1. Introduction

Community-acquired pneumonia (CAP) and nosocomial pneumonia (NP) are frequent infectious problems that show no tendency to a significant decrease. Susceptibility to CAP and critical CAP complications has been studied systematically by several groups of investigators, and apparent success has been achieved in the identification of polymorphic variants of some host genes associated with the diversity in the response to CAP. These are genes critical to the CAP innate immune system,[1–16] genes controlling lung defense against inhaled microorganisms[4,17] and inhibition of fibrinolysis,[18] and some others. Genetic variations in several genes (*MBL2*, *MASP-2*, and *PAI-1*) reported to be associated with CAP have also been studied in NP patients,[19–23] but to our knowledge there are no publications investigating both CAP and NP for the same single nucleotide polymorphisms (SNPs).

To evaluate candidate host genetic variants predisposing to CAP and NP, we selected 13 polymorphic variants assigned to 11 different genes. The selection of the genes was based on their association with molecular pathways probably implicated in CAP pathogenesis and on literature data. Genes involved in the immune and inflammation response are directly relevant to the human host defense (*IL-6* rs1800795, *TNF-α* rs1800629, *MBL2* rs7096206, *CCR5* rs333, and *NOS3* rs1799983). Xenobiotic detoxification genes were also included in our study as they encode enzymes involved in the detoxification and excretion of a broad range of compounds, including products of oxidative stress and drugs, thereby participating in the general resistance to exogenous and endogenous harmful metabolites, as well as in the biotransformation of drugs (*CYP1A1* rs2606345, rs4646903, rs1048943, *GSTM1* Ins/del, *GSTT1* Ins/del, and *ABCB1* rs1045642). Two additional genes might have an impact on CAP initiation and progression – the gene of the renin–angiotensin system *ACE* (rs4340) and the gene-candidate for occlusive vascular disease and hyperhomocysteinemia *MTHFR* (rs1801133), both disorders possibly affecting augmentation of inflammation processes.

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2. Methods

2.1. Patients and controls

From January 2008 to October 2011 a total of 334 Russian Federation patients with CAP, hospitalized at the clinical bases of the V. A. Negovsky Research Institute of General Reanimatology, Moscow, Russia, were included in the study. The diagnosis of CAP was based on the presence of acute symptoms resulting from a lower respiratory tract infection, confirmed by clinical (cough, sputum production, auscultatory findings consistent with pneumonia, a temperature of $>38\,^{\circ}C$ or $<35\,^{\circ}C$), radiological (an infiltrate on chest X-rays), and laboratory (a white blood cell count of $>10 \times 10^9$ cells/l or $<4 \times 10^9$ cells/l) data. Hospitalization was ascertained based on participant self-report and active surveillance by medical personnel.

The control group consisted of 141 unrelated healthy volunteers without a previous history of pneumonia.

We selected a group of accident victims who had sustained severe, mostly life-threatening physical trauma and patients with acute diseases requiring extensive surgery for the NP study. Clinical data were collected prospectively. The NP study included 216 patients with nosocomial pneumonia and 105 patients who did not develop NP during their stay in the hospital. Eighty (76.2%) patients without NP and 205 (94.9%) patients with NP were intubated and ventilated. The durations of mechanical ventilation were significantly longer in cases than in controls: 50% of NP patients were mechanically ventilated over 15 days compared with only 7% of patients without NP. The diagnosis of pneumonia was made on the basis of the appearance of a new infiltrate on chest X-ray in the presence of cough, fever, and leukocytosis.

Exclusion criteria for the CAP and NP groups consisted of age <18 years, lack of informed consent, defined immunodeficiency, corticosteroid administration <6 weeks prior, history of recent hospitalization (<30 days), chronic respiratory diseases (asthma, chronic obstructive pulmonary disease, tuberculosis, obstructive pneumonia caused by neoplasia), malignancy, 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.

The ethnicity of the population in the present study was primarily (≥97%) Caucasian, mainly Slavs (81.6–86.1%), among them Russians (65.7–74.5%) (see tables; more detailed information is available in the **Supplementary Material**).

Respiratory fluid samples obtained by endotracheal aspiration were taken for microbiological documentation of CAP and NP. The bacteriological examination consisted of Gram stain smears and cultures. Bronchoalveolar lavage (BAL) fluid was stained and cultured for aerobic and anaerobic bacteria, fungi (*Aspergillus* and *Candida* species), and mycobacteria. A positive quantitative culture was defined when bacteria were cultured from BAL samples at a concentration of 1×10^5 CFU/ml or more.

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.

2.2. Genotyping

DNA was isolated from 200 μl of blood using gDNA purification kit Diatom DNA Prep 200 (Isogene Laboratory, Moscow, Russia). Genotyping was performed using an allele-specific tetra-primer PCR developed to genotype a relatively large number of samples in a cost-effective and time-saving manner. In this method, allele-specific DNA products are amplified by means of applying appropriately designed two-pair primers (four primers) into an ordinary PCR tube.[24] Amplification was carried out in an ABI thermal cycler using two external and two internal sequence-specific primers (**Supplementary Material**, Table S2) and PCR MasterMix tubes (Isogene Laboratory, Moscow, Russia), as previously described.[25] The PCR products were then analyzed in 2% agarose gel stained with ethidium bromide. For each SNP, 10% of randomly taken DNA samples (cases and controls) were genotyped twice and no discrepancies were observed.

2.3. Statistical analysis

Exact Hardy–Weinberg equilibrium (HWE) tests were performed for each SNP independently using the goodness-of-fit Chi-square test to compare the observed and expected genotype frequencies for the CAP, NP, and control groups. Logistic regression analysis adjusted for age, gender, ethnicity, and the use and duration of mechanical ventilation was performed to evaluate the associations between the studied gene polymorphisms and CAP or NP risk. Odds ratios (ORs) and 95% confidence intervals (CIs) were calculated using the SNPStats software.[26] If one allele of the SNP was underrepresented among patients it was considered a protective allele (OR < 1). Reciprocally the second allele in a two-allele system would be overrepresented among patients and it would be a risk allele (OR > 1). Three genetic models defined relative to the minor allele were considered: dominant (minor homozygotes + heterozygotes vs. major homozygotes), recessive (minor homozygotes vs. heterozygotes + major homozygotes), overdominant (minor homozygotes + major homozygotes vs. heterozygotes).

Multiple SNP statistical analysis was performed using WinSTAT software (Robert K. Fitch Software, Germany).

WINPEPI test power and sample size calculators were used to evaluate type II error.[27] To detect OR = 2.0 in our sample size for the CAP study, the test power for the additive model for relative minor allele frequency 0.06 (rs1048943G) is 39.1% and for minor allele frequency 0.47 (rs4340Ins) is 91.2% (two-sided Fisher's test). Test power in our NP study is 43.3% for relative minor allele frequency 0.1 (rs1048943G) and 79.1% for allele frequency 0.45 (rs4340Del).

3. Results

A total 334 patients with CAP (307 males and 27 females; age range 18–55 years, mean age 27 years) were included in CAP case–control study. One hundred forty-one unrelated healthy volunteers without a previous history of pneumonia were matched by age, sex, and ethnicity (130 males and 11 females; age range 18–52 years, mean age 29 years). The main demographic and clinical variables of the patients with CAP and controls are shown in Table 1.

The NP set comprised 216 patients with nosocomial pneumonia (176 males and 40 females; age range 18–82 years, mean age 43 years) and 105 patients (83 males and 22 females; age range 19–93 years, mean age 41 years) who did not develop NP during their stay in the hospital due to severe life-threatening conditions. Controls were matched by age, sex, ethnicity, and underlying disease (Table 2).

Microbiological confirmation was achieved in 233 CAP patients. *Streptococcus* species were isolated in 149 patients (64% of the patients with a known causative microorganism), and *Streptococcus pneumoniae* was the most common etiologic agent (143 patients, 61.4%). *Staphylococcus aureus* was identified in 55 patients with CAP (23.6%) (Table 1). Microbiological data were available for only 66 NP patients. Typical nosocomial pathogens that are known to play a role in the development of NP, including ventilator-associated pneumonia (VAP), were identified: Gram-negative bacilli (36 patients, 54.5%

Table 1
Characteristics of the community-acquired pneumonia (CAP) and control groups involved in the study

Feature	Control, *n* (%)	CAP, *n* (%)
Total number	141	334
Age, years	29.1 ± 0.6	26.9 ± 0.8
Sex		
Male	130 (92.2)	307 (91.9)
Female	11 (7.8)	27 (8.1)
Ethnicity		
Caucasians	138 (97.9)	324 (97.0)
Slavs	115 (81.6)	276 (82.6)
Russian	105 (74.5)	238 (71.3)
Progression		
Bilateral	-	98 (29.3)
Unilateral	-	236 (70.7)
Background diseases	-	
No	-	326 (97.6)
Yes[a]	-	8 (2.4)
Comorbidity	-	
No		303 (90.7)
Yes[b]		31 (9.3)
Causative pathogens[c]	-	233 (69.8)
Streptococcal infections	-	149 (44.6)
Streptococcus pneumoniae	-	143 (42.8)
Streptococcus pyogenes	-	3 (0.9)
Other Streptococcus	-	3 (0.9)
Staphylococcus aureus	-	55 (16.5)
Haemophilus influenzae	-	11 (3.3)
Mycoplasma pneumoniae	-	11 (3.3)
Other infectious pathogens	-	7 (2.1)
Duration of hospitalization, days	-	27.4 ± 10.2
Hospital mortality	-	12 (3.6)

[a] Ischemic cardiopathy, diabetes, obesity.
[b] Most frequently purulent antritis (51.61%).
[c] In patients with mixed CAP ($n = 43$; 12.9%) the most prevalent microorganism is specified.

Table 2
Characteristics of the nosocomial pneumonia (NP) and non-NP (control) groups involved in the study

Characteristics	Patients without NP, *n* (%)	Patients with NP, *n* (%)
Total number	105	216
Age, years	41.0 ± 1.6	43.0 ± 2.0
Sex		
Male	83 (79.1)	176 (81.5)
Female	22 (20.9)	40 (18.5)
Ethnicity		
Caucasian	105 (100)	213 (98.6)
Slav	86 (81.9)	186 (86.1)
Russian	69 (65.7)	152 (70.4)
Background diseases		
No	89 (84.8)	156 (72.2)
Yes	16 (15.2)	60 (27.8)
Cardiovascular diseases[a]	3 (2.9)	19 (8.8)
Type 2 diabetes	2 (1.9)	7 (3.2)
Obesity	1 (0.95)	10 (4.6)
Neurological pathology	1 (0.95)	7 (3.2)
Gastric ulcer	2 (1.9)	4 (1.85)
Duodenal ulcer	1 (0.95)	5 (2.3)
Urolithiasis	2 (1.9)	4 (1.85)
Cholelithiasis	4 (3.8)	4 (1.85)
Underlying disease		
Severe combined trauma/wound	30/21 (48.6)	109/34 (66.2)
Pancreatonecrosis	8 (7.6)	15 (6.9)
Bowel obstruction	18 (17.1)	33 (15.3)
Acute phlegmonous and gangrenous appendicitis	2 (1.9)	2 (0.9)
Destructive cholecystitis	9 (8.6)	3 (1.4)
Hollow organ perforation	10 (9.5)	4 (1.85)
Purulent inflammatory diseases of the skin, subcutaneous tissue, and soft tissue	4 (3.8)	4 (1.85)
Renal carbuncle	3 (2.9)	5 (2.3)
Acute violation of cerebral circulation	(0.0)	3 (1.4)
Lacunar tonsillitis, retropharyngeal abscess	(0.0)	1 (0.5)
Suppurative submandibular lymphadenitis	(0.0)	1 (0.5)
Purulent sphenoiditis	(0.0)	1 (0.5)
Pericarditis	(0.0)	1 (0.5)
Complications (of basic disease)		
No	44 (41.9)	(0.0)
Yes	61 (58.1)	216 (100.0)
Major complications		
Nosocomial pneumonia	(0.0)	216 (100.0)
Peritonitis	28 (26.7)	49 (22.7)
Intra-abdominal abscesses	5 (4.8)	6 (2.8)
Anastomotic failure	(0.0)	8 (3.7)
Lung abscesses	(0.0)	17 (7.9)
Pleural empyema	(0.0)	13 (6.0)
Wound suppuration	3 (2.9)	3 (1.3)
Post-traumatic osteomyelitis	2 (1.9)	4 (1.85)
Secondary meningoencephalitis	2 (1.9)	4 (1.85)
Mediastinitis	1 (0.95)	8 (3.7)
Tracheoesophageal fistula	(0.0)	2 (0.9)
Severe sepsis	2 (1.9)	56 (25.9)
Septic shock	2 (1.9)	15 (6.9)
Multiple organ failure	5 (4.8)	74 (34.3)
Acute respiratory distress syndrome	3 (2.9)	21 (9.7)
Pulmonary embolism	3 (2.9)	5 (2.3)
Brain abscesses	1 (0.95)	(0.0)
Pleurisy	4 (3.8)	23 (10.7)
Combined complications	7 (6.7)	131 (60.7)
Infectious pathogens	-	66 (30.6)
Pseudomonas aeruginosa	-	7 (3.2)
Klebsiella pneumoniae	-	9 (4.2)
Staphylococcus aureus	-	12 (5.6)
Escherichia coli	-	7 (3.2)
Pseudomonas aeruginosa + *Acinetobacter baumannii*	-	13 (6.0)
Pseudomonas aeruginosa + methicillin-resistant *Staphylococcus aureus* + *Acinetobacter baumannii*	-	11 (5.1)
Klebsiella pneumoniae + *Staphylococcus aureus* + *Acinetobacter baumannii*	-	7 (3.2)
Use of mechanical ventilation		
No	25 (23.8)	11 (5.1)
Yes	80 (76.2)	205 (94.9)
Duration of mechanical ventilation, days	21.9 ± 9.2	5.3 ± 1.3
<5 days	59 (56.2)	40 (18.5)
5–15 days	14 (13.3)	57 (26.4)
>15 days	7 (6.7)	108 (5.0)
ICU admission		
No	4 (3.8)	9 (4.2)
Yes	101 (96.2)	207 (95.8)
Duration of hospitalization, days	41.3 ± 19.6	49.9 ± 18.9
Hospital mortality	3 (2.9)	80 (37.0)

ICU, intensive care unit.
[a] Ischemic cardiopathy, essential hypertension, widespread atherosclerosis, coronary artery bypass.

of the patients with a known causative microorganism), Gram-positive bacteria (12 patients, 18.2%), and mixed cases (18 patients, 27.3%) (Table 2).

3.1. Individual SNP analysis in the CAP association study

Thirteen polymorphic variants (11 SNPs and two copy number variations (CNV) – *GSTM1* and *GSTT1*) were genotyped in CAP and control subjects from the Russian population. SNPs were analyzed for HWE. In the control group the genotype frequencies of all studied variations were in HWE (Table 3). For rs2606345 and

Table 3
Distribution of genotypes among the community-acquired pneumonia (CAP) and control groups

Genes and genotypes	Control subjects, *n* (%)	CAP, *n* (%)	Crude analysis		Adjusted analysis (adjusted by age, sex, and ethnicity)	
			p-Value[a] (genetic model)[b]	OR, 95% CI	*p*-Value[a] (genetic model)[b]	OR, 95% CI
Xenobiotic detoxification						
CYP1A1	*n* = 134	*n* = 334				
rs2606345	HWP[c] = 0.72	HWP = 0.0025				
T/T	46 (34.3)	186 (55.7)	3.9×10^{-5} (dom)	Reference	3.87×10^{-5} (dom)	Reference
T/G	63 (47.0)	**111 (33.2)**		0.42, 0.27–0.63		0.41, 0.27–0.63
G/G	25 (18.7)	**37 (11.1)**				
CYP1A1	*n* = 131	*n* = 323				
rs4646903	HWP = 0.22	HWP = 0.5				
T/T	98 (74.8)	265 (82.0)	0.086 (dom)	Reference	0.094 (dom)	Reference
T/C	33 (25.2)	57 (17.7)		0.65, 0.40–1.06		0.65, 0.40–1.07
C/C	0 (0.0)	1 (0.3)				
CYP1A1	*n* = 132	*n* = 323				
rs1048943	HWP = 1.0	HWP = 1.0				
Ile462Val						
A/A	124 (93.9)	291 (90.1)	0.17 (dom)	Reference	0.14 (dom)	Reference
A/G	8 (6.1)	32 (9.9)		1.70, 0.76–3.80		1.80, 0.80–4.05
G/G	0 (0.0)	0 (0.0)				
GSTM1	*n* = 140	*n* = 331				
Del(D/D)-Ins (I/*)	HWP (N/A)	HWP (NA)				
D/D	78 (55.7)	134 (40.5)	0.0024 (N/A)	Reference	0.0021 (N/A)	Reference
I/*	62 (44.3)	**197 (59.5)**		1.85, 1.24–2.76		1.90, 1.26–2.86
GSTT1	*n* = 140	*n* = 331				
Del(D/D)-Ins (I/*)	HWP (N/A)	HWP (N/A)				
I/*	118 (84.3)	268 (80.9)	0.39 (N/A)	Reference	0.42 (N/A)	Reference
D/D	22 (15.7)	63 (19.1)		0.79, 0.47–1.35		0.80, 0.46–1.38
ABCB1	*n* = 141	*n* = 333				
rs1045642	HWP = 0.31	HWP = 0.08				
Ile1145=						
T/T	47 (33.3)	140 (42.0)	0.074 (dom)	Reference	0.065 (dom)	Reference
T/C	63 (44.7)	140 (42.0)		0.69, 0.46–1.04		0.67, 0.44–1.03
C/C	31 (22.0)	53 (16.0)				
Immune response regulation and inflammation						
IL-6	*n* = 139	*n* = 322				
rs1800795	HWP = 0.061	HWP = 0.31				
G/G	37 (26.6)	103 (32.0)	0.033 (od)	Reference	0.053 (od)	Reference
G/C	80 (57.6)	**150 (46.6)**		0.64, 0.43–0.96		0.67, 0.44–1.01
C/C	22 (15.8)	69 (21.4)				
TNF-α	*n* = 139	*n* = 321				
rs1800629	HWP = 0.074	HWP = 0.8				
G/G	100 (71.9)	248 (77.3)	0.23 (dom)	Reference	0.27 (dom)	Reference
G/A	39 (28.1)	68 (21.2)		0.75, 0.48–1.19		0.77, 0.49–1.22
A/A	0 (0.0)	5 (1.5)				
MBL2	*n* = 120	*n* = 277				
rs7096206	HWP = 0.51	HWP = 0.39				
C/C	85 (70.8)	194 (70.0)	0.87 (dom)	Reference	0.93 (dom)	Reference
G/G	31 (25.8)	73 (26.4)		1.04, 0.65–1.66		1.02, 0.63–1.66
	4 (3.3)	10 (3.6)				
CCR5	*n* = 141	*n* = 319				
Del32	HWP = 1.0	HWP = 1.0				
rs333						
I/I	113 (80.1)	281 (88.1)	0.029 (dom)	Reference	0.0087 (dom)	Reference
I/D	27 (19.2)	**37 (11.6)**		0.55, 0.32–0.93		0.47, 0.27–0.82
D/D	1 (0.7)	**1 (0.3)**				
NOS3	*n* = 141	*n* = 321				
rs1799983	HWP = 0.15	HWP = 0.76				
Asp298Glu						
G/G	67 (47.5)	185 (57.6)	0.031 (od)	Reference	0.016 (od)	Reference
G/T	66 (46.8)	**116 (36.1)**		0.64, 0.43–0.96		0.60, 0.40–0.91
T/T	8 (5.7)	20 (6.2)				

Table 3 (*Continued*)

Genes and genotypes	Control subjects, *n* (%)	CAP, *n* (%)	Crude analysis		Adjusted analysis (adjusted by age, sex, and ethnicity)	
			p-Value[a] (genetic model)[b]	OR, 95% CI	*p*-Value[a] (genetic model)[b]	OR, 95% CI
Renin–angiotensin system						
ACE	*n* = 141	*n* = 321				
rs4340	HWP = 1.0	HWP = 0.014				
Alu-287 bp						
D/D	30 (21.3)	105 (32.8)	0.011 (dom)	Reference	0.0052 (dom)	Reference
I/D	70 (49.7)	**137 (42.8)**		0.55, 0.35–0.88		0.52, 0.32–0.83
I/I	41 (29.1)	**78 (24.4)**				
Occlusive vascular disease						
MTHFR	*n* = 131	*n* = 316				
rs1801133	HWP = 0.32	HWP = 0.53				
Ala222Val						
C/C	56 (42.8)	134 (42.4)	0.44 (rec)	Reference	0.49 (rec)	Reference
C/T	64 (48.9)	148 (46.8)		1.32, 0.64–2.68		1.29, 0.62–2.66
T/T	11 (8.4)	34 (10.8)				

OR, odds ratio; CI, confidence interval; N/A, Not applicable.
Genotypes associated with a response in accordance with OR (protective if OR < 1, susceptible if OR > 1) are in bold-type.
[a] Significant *p*-values <0.05.
[b] The genetic model: rec, recessive; dom, dominant; od, overdominant (for the overdominant model the reference genotype is major homozygote + minor homozygote).
[c] Hardy–Weinberg probability (a probability for the deviation between observed and expected numbers for the dominant homozygotes, the heterozygotes, and the recessive homozygotes in a two-allele system).

rs4340, there was considerable deviation from HWE in patients; a more detailed analysis showed that this was linked to CAP predisposition. We revealed CAP susceptibility genes in the crude analysis (*CYP1A1* rs2606345, *GSTM1*, *IL-6*, *NOS3*, *CCR5*, and *ACE*). Multivariate analysis adjusted for age, gender, and ethnicity showed that the abovementioned association signals became more pronounced, with only *IL-6* demonstrating a marginally significant *p*-value. In the CAP group we observed significant over-representation of the *CYP1A1* rs2606345T/T and *ACE* rs4340 D/D risk genotypes relative to the control group.

3.2. Individual SNP analysis in the NP association study

The same polymorphic variants were genotyped in two other groups from the Russian population: 216 patients with NP and 105 patients who did not develop NP during their stay in the hospital (Table 4). Genotype frequencies of the studied polymorphisms in the control group were in HWE. A significant *IL-6* heterozygote deficiency against the null hypothesis of HWE was observed in the NP group. These data are in line with the protective effect of heterozygotes found by a logistic regression analysis for the overdominant model. The *ACE* gene also showed an association with NP in the crude model. Regression analysis adjusted for age, sex, ethnicity, and duration of mechanical ventilation (if applied) showed that these findings remained significant for *IL-6* and *ACE*. Another gene, *CYP1A1* (rs2606345), appeared as a susceptibility gene with a marginally significant association with NP ($p = 0.051$).

3.3. Bonferroni correction for multiple comparisons

To exclude false-positive associations with disease, we used the Bonferroni correction for multiple comparisons. Significant *p*-values were multiplied by the number of studied polymorphisms. The *p*-value after Bonferroni correction remained significant for the *CYP1A1* gene (rs2606345): *p*bonf = 0.00051, and for the *GSTM1* gene: *p*bonf = 0.031, in the CAP case–control study.

3.4. CAP and NP association studies in Slavs

Individuals with ancestors other than Slav were excluded from the case and control groups (**Supplementary Material**, Table S3). Multivariate analysis adjusted for age, gender (and use of mechanical ventilation in the NP set) showed that *CYP1A1* TG–GG carriers (rs2606345) were significantly less likely to develop CAP and NP. The frequency of the *ACE* II-ID genotypes was significantly greater among controls compared with CAP patients. In the NP set, *ACE* I/I homozygous individuals also showed a lower disease incidence. A protective effect for *IL-6* heterozygotes was revealed in the CAP (trend $p = 0.056$) and NP studies. Significant differences in the frequency distributions among CAP cases and controls were also observed for *GSTM1* and *NOS3* genes.

3.5. Analysis of independent prognostic risk factors of CAP and NP

Since the inflammatory response and the immune response may vary depending on the microorganism, the role of the SNPs showing *p*-values of <0.1 in the CAP set was analyzed in the groups of CAP patients with known causative microorganisms. A total of 149 patients with streptococcal infections and 55 patients with staphylococcal infections were further analyzed (Table 5). Multivariate analysis adjusted for age, gender, and ethnicity confirmed that *CYP1A1* T/G–G/G genotypes and *ACE* Ins I/I-I/D genotypes were the independent protective factors for streptococcal CAP and for staphylococcal CAP. A marginally significant association was also found between the *IL-6* gene and streptococcal CAP risk. The small sample size in the staphylococcal CAP group results in wide confidence intervals and a *p*-value that is outside the significance level ($p = 0.067$). Multivariate regression also identified *GSTM1*, *CCR5*, and *NOS3* genotypes as having significant effects on streptococcal CAP susceptibility. In contrast we found not even a trend ($p < 0.15$) for an association of these polymorphic variants with staphylococcal CAP (Table 5).

Additionally we compared the distributions of *CYP1A1* (rs2606345), *ACE*, and *IL-6* genotypes in the subgroups of patients with streptococcal CAP and staphylococcal CAP. The frequencies of genotypes did not differ significantly in patients with different causative microorganisms (Chi-square = 0.95, $p = 0.62$; Chi-square = 1.9, $p = 0.39$; Chi-square = 1.1, $p = 0.59$, respectively).

With regard to the specific NP causative microorganisms we could not perform the association analysis because of the small numbers of patients in the groups. We compared the genotype distributions among NP patients with Gram-negative bacilli (36

Table 4
Distribution of genotypes among the nosocomial pneumonia (NP) and control groups

Genes and genotypes	Non-NP, *n* (%)	NP, *n* (%)	Crude analysis		Adjusted analysis (adjusted by age, sex, ethnicity, and duration of mechanical ventilation)	
			p-Value[a] (genetic model)[b]	OR, 95% CI	*p*-Value[a] (genetic model)[b]	OR, 95% CI
Xenobiotic detoxification						
CYP1A1	*n* = 102	*n* = 209				
rs2606345	HWP[c] = 0.22	HWP = 0.88				
T/T	32 (31.4)	88 (42.1)	0.066 (dom)	Reference	0.051 (dom)	Reference
T/G	56 (54.9)	**97 (46.4)**		0.63, 0.38–1.04		0.60, 0.35–1.01
G/G	14 (13.7)	**24 (11.4)**				
CYP1A1	*n* = 94	*n* = 186				
rs4646903	HWP = 0.59	HWP = 0.48				
T/T	74 (78.7)	144 (77.4)	0.8 (dom)	Reference	0.92 (dom)	Reference
T/C	20 (21.3)	41 (22.0)		1.08, 0.59–1.97		1.04, 0.47–2.29
C/C	0 (0.0)	1 (0.5)				
CYP1A1	*n* = 95	*n* = 186				
rs1048943	HWP = 1.0	HWP = 1.0				
Ile462Val						
A/A	85 (89.5)	171 (91.9)	0.5 (dom)	Reference	0.17 (dom)	Reference
A/G	10 (10.5)	15 (8.1)		0.75, 0.32–1.73		0.39, 0.10–1.53
G/G	0 (0.0)	0 (0.0)				
GSTM1	*n* = 104	*n* = 208				
Del(D/D)-Ins (I/*)	HWP (N/A)	HWP (N/A)				
D/D	48 (46.2)	94 (45.2)	0.87 (N/A)	Reference	0.50 (N/A)	Reference
I/*	56 (53.8)	114 (54.8)		1.04, 0.65–1.67		1.22, 0.68–2.21
GSTT1	*n* = 104	*n* = 208				
Del(D/D)-Ins (I/*)	HWP (N/A)	HWP (N/A)				
D/D	13 (12.5)	31 (14.9)	0.56 (N/A)	Reference	0.47 (N/A)	Reference
I/*	91 (87.5)	177 (85.1)		0.82, 0.41–1.63		0.74, 0.32–1.71
ABCB1	*n* = 105	*n* = 216				
rs1045642	HWP = 0.23	HWP = 0.32				
Ile1145 =						
T/T	38 (36.2)	72 (33.3)	0.17 (rec)	Reference	0.35 (rec)	Reference
T/C	45 (42.9)	112 (51.9)		0.66, 0.36–1.20		0.70, 0.34–1.46
C/C	22 (20.9)	32 (14.8)				
Immune response regulation and inflammation						
IL-6	*n* = 100	*n* = 206				
rs1800795	HWP = 0.14	HWP = 0.009				
G/G	32 (32.0)	83 (40.3)	0.006 (od)	Reference	0.04 (od)	Reference
G/C	56 (56.0)	**81 (39.3)**		0.51, 0.31–0.83		0.53, 0.29–0.98
C/C	12 (12.0)	42 (20.4)				
TNF-α	*n* = 105	*n* = 208				
rs1800629	HWP = 0.35	HWP = 0.23				
G/G	81 (77.1)	167 (80.3)	0.52 (dom)	Reference	0.38 (dom)	Reference
G/A	24 (22.9)	41 (19.7)		0.83, 0.47–1.46		0.71, 0.33–1.52
A/A	0 (0.0)	0 (0.0)				
MBL2	*n* = 80	*n* = 151				
rs7096206	HWP = 0.34	HWP = 0.55				
C/C	59 (73.8)	107 (70.9)	0.64 (dom)	Reference	0.91 (dom)	Reference
C/G	21 (26.2)	39 (25.8)		1.16, 0.31–2.12		0.95, 0.41–2.23
G/G	0 (0.0)	5 (3.3)				
CCR5	*n* = 105	*n* = 208				
Del32 rs333	HWP = 0.59	HWP = 0.021				
I/I	85 (80.9)	174 (83.7)	0.55 (dom)	Reference	0.19 (dom)	Reference
I/D	20 (19.1)	29 (13.9)		0.83, 0.45–1.53		0.60, 0.28–1.29
D/D	0 (0.0)	5 (2.4)				
NOS3	*n* = 105	*n* = 209				
rs1799983	HWP = 0.79	HWP = 0.33				
Asp298Glu						
G/G	59 (56.2)	98 (46.9)	0.12 (dom)	Reference	0.52 (dom)	Reference
G/T	41 (39.0)	95 (45.4)		1.45, 0.91–2.33		1.21, 0.68–2.17
T/T	5 (4.8)	16 (7.7)				

Table 4 (*Continued*)

Genes and genotypes	Non-NP, *n* (%)	NP, *n* (%)	Crude analysis		Adjusted analysis (adjusted by age, sex, ethnicity, and duration of mechanical ventilation)	
			p-Value[a] (genetic model)[b]	OR, 95% CI	*p*-Value[a] (genetic model)[b]	OR, 95% CI
Renin–angiotensin system						
ACE	*n* = 105	*n* = 208				
rs4340	HWP = 0.24	HWP = 0.89				
Alu-287 bp						
D/D	24 (22.9)	59 (28.4)	0.044 (rec)	Reference	0.03 (rec)	Reference
I/D	46 (43.8)	102 (49.0)		0.58, 0.35–0.98		0.47, 0.24–0.93
I/I	35 (33.3)	**47 (22.6)**				
Occlusive vascular disease						
MTHFR	*n* = 94	*n* = 184				
rs1801133	HWP = 0.48	HWP = 0.5				
Ala222Val						
C/C	41 (43.6)	83 (45.11)	0.81 (dom)	Reference	0.81 (dom)	Reference
C/T	45 (47.9)	85 (46.20)		0.94, 0.57–1.55		1.08, 0.57–2.07
T/T	8 (8.5)	16 (8.70)				

OR, odds ratio; CI, confidence interval; N/A,.
Genotypes associated with a response in accordance with OR (protective if OR < 1, susceptible if OR > 1) are in bold-type.
[a] Significant *p*-values <0.05.
[b] The genetic model: rec, recessive; dom, dominant; od, overdominant (for the overdominant model the reference genotype is major homozygote + minor homozygote).
[c] Hardy–Weinberg probability (a probability for the deviation between observed and expected numbers for the dominant homozygotes, the heterozygotes, and the recessive homozygotes in a two-allele system).

patients, 54.5% of the patients with a known causative microorganism), Gram-positive bacteria (*S. aureus*; 12 patients, 18.2%), and mixed Gram-negative and Gram-positive infections (18 patients, 27.3%). Three SNPs showing *p*-values of <0.1 in the NP set (Table 4) were analyzed. The distribution of *CYP1A1* (rs2606345), *ACE*, and *IL-6* genotypes did not differ among the three groups of NP patients with different infectious pathogens (Chi-square = 1.6, $p = 0.81$; Chi-square = 4.7, $p = 0.32$; Chi-square = 0.4, $p = 0.98$, respectively).

Mechanical ventilation is the key risk factor for the development of NP. There was no significant difference in the distribution of genotype frequency among the four groups of NP patients with different durations of mechanical ventilation (no ventilation, less than 5 days, 5–15 days, more than 15 days): for *CYP1A1* (rs2606345) Chi-square = 1.6, $p = 0.95$; for *ACE* Chi-square = 5.6, $p = 0.48$; for *IL-6* Chi-square = 2.5, $p = 0.87$. As there were mechanically ventilated patients in both NP groups (cases and non-NP patients), we then analyzed the genotype–covariate (duration of mechanical ventilation) interaction. The interaction *p*-value was insignificant: for *CYP1A1* (rs2606345) $p_{interaction} = 0.7$, for *ACE* $p_{interaction} = 0.14$, for *IL-6* $p_{interaction} = 0.10$.

NP patients were grouped by age ≥65 years ($n = 37$) and <65 years ($n = 179$). The difference in the distribution of genotypes was non-significant in the age subgroups: for *CYP1A1* (rs2606345) Chi-square = 2.56, $p = 0.28$; for *ACE* Chi-square = 0.72, $p = 0.70$; for *IL-6* Chi-square = 3.29, $p = 0.19$. The interaction analysis also showed no significant interaction between genotype and age: for *CYP1A1* (rs2606345) $p_{interaction} = 0.32$, for *ACE* $p_{interaction} = 0.10$, for *IL-6* $p_{interaction} = 0.97$.

3.6. Multiplicative genetic model and CAP and NP

We further explored the multiplicative genetic model of the genes demonstrating an association with both CAP and NP risk in all sets with a *p*-value of <0.1. Figure 1 shows the effect of having three risk genotypes on modulating the CAP and NP risk. The carriage of three predisposing genotypes (*CYP1A1* rs2606345T/T and *ACE* rs4340D/D and *IL-6* rs1800795G/G+C/C) versus protective genotypes (*CYP1A1* rs2606345G/G+T/G and *ACE* rs4340I/I+I/D and *IL-6* rs1800795G/C) increased the CAP risk ($p = 0.001$, OR 7.01, 95% CI 1.99–24.70) and the NP risk ($p = 0.028$, OR 4.34, 95% CI 1.15–16.45). The prognostic value of this genetic model is characterized by a sensitivity of 36.2%, specificity of 92.5%, and balancing accuracy of 64.4% for CAP, and a sensitivity of 32.3%, specificity of 90.0%, and balancing accuracy of 61.5% for NP.

3.7. Analysis of genotype frequencies in NP subgroups with or without a poor outcome

Since the majority of NP patients also had sepsis/septic shock, acute respiratory distress syndrome (ARDS), peritonitis, or multiple organ failure (MOF), we divided our NP group into two subgroups: absence of a poor outcome ($n = 85$) and presence of a poor outcome ($n = 131$). We compared the distribution of *CYP1A1* rs2606345, *IL-6*, and *ACE* genotypes in these subgroups to get an answer to the question "Are our findings indeed linked only to pneumonia, and not to a poor outcome?" The frequencies of genotypes did not differ in NP patients with and without a poor outcome, even at a trend level (**Supplementary Material**, Table S4). Therefore we can conclude that the observed associations are indeed between gene polymorphisms and pneumonia per se.

4. Discussion

In this study we found that the *CYP1A1*, *ACE*, and *IL-6* variant genotypes were significantly associated with CAP and NP. The relevance of the observed association was indirectly confirmed by a similar character of association in the CAP and NP groups. These findings were reaffirmed by departures from HWE for the genes associated with CAP and NP. Alleles within genotypes that confer greater susceptibilities are represented in the sample at disproportionally high rates. Disequilibrium is expected to be greatest at the disease-susceptibility locus itself, since this is the factor that determines the selection criterion.[28]

The main finding of this study was the association of the gene *CYP1A1* (rs2606345), encoding an enzyme of the phase I detoxification system, with CAP. The same trend was also revealed for NP. *CYP1A1* is the actively studied pulmonary *CYP* gene regulating enzyme, expressed mainly in the epithelium of the

Table 5
The distribution of genotypes among CAP patients with different causative microorganisms and controls

Genes and genotypes	Control subjects, *n* (%)	CAP with streptococcal infection, *n* (%)	Adjusted for age, sex, and ethnicity OR (95% CI) *p*-Value[a] (genetic model)[b]	CAP with staphylococcal infection, *n* (%)	Adjusted for age, sex and ethnicity OR (95% CI) *p*-Value[a] (genetic model)[b]
CYP1A1 rs2606345	*n* = 134	*n* = 149		*n* = 46	
T/T	46 (34.3)	84 (56.4)	Reference	25 (54.4)	Reference
T/G	63 (47.0)	**46 (30.9)**	0.36 (0.21–0.61)	**17 (37.0)**	0.39 (0.26–0.99)
G/G	25 (18.7)	**19 (12.8)**	$p = 9.1 \times 10^{-5}$ (dom)	**4 (8.7)**	$p = 0.021$ (dom)
CYP1A1 rs4646903	*n* = 131	*n* = 148		*n* = 44	
T/T	98 (74.8)	120 (81.1)	Reference	38 (86.4)	Reference
T/C	33 (25.2)	28 (18.9)	0.62 (0.33–1.18)	5 (11.4)	0.35 (0.11–1.14)
C/C	0 (0.0)	0 (0.0)	$p = 0.15$ (dom)	1 (2.3)	$p = 0.06$ (dom)
GSTM1 Del(D/D)-Ins (I/*)	*n* = 140	*n* = 149		*n* = 46	
D/D	78 (55.7)	57 (38.3)	Reference	23 (52.3)	Reference
I/*	62 (44.3)	**92 (61.7)**	2.23 (1.33–3.76) $p = 0.0022$ (N/A)	21 (47.7)	1.14 (0.52–2.50) $p = 0.75$ (N/A)
ABCB1 rs1045642 Ile1145=	*n* = 141	*n* = 147		*n* = 46	
T/T	47 (33.3)	63 (42.9)	Reference	15 (32.6)	Reference
T/C	63 (44.7)	60 (40.8)	0.72 (0.43–1.21)	24 (52.2)	0.73 (0.26–2.09)
C/C	31 (22.0)	24 (16.3)	$p = 0.21$ (dom)	7 (15.2)	$p = 0.56$ (rec)
IL-6 rs1800795	*n* = 139	*n* = 148		*n* = 44	
G/G	37 (26.6)	47 (31.8)	Reference	14 (31.8)	Reference
G/C	80 (57.5)	**68 (46.0)**	0.60 (0.36–1.0)	23 (52.3)	0.45 (0.19–1.05)
C/C	22 (15.8)	33 (22.3)	$p = 0.05$ (od)	7 (15.9)	$p = 0.067$ (dom)
CCR5 Del32 rs333	*n* = 141	*n* = 147		*n* = 43	
I/I	113 (80.1)	132 (89.8)	Reference	32 (74.4)	Reference
I/D	27 (19.1)	**15 (10.2)**	0.35 (0.16–0.76)	11 (25.6)	1.33 (0.52–3.43)
D/D	1 (0.7)	**0 (0.0)**	$p = 0.0056$ (dom)	0 (0.0)	$p = 0.56$
NOS3 rs1799983 Asp298Glu	*n* = 141	*n* = 142		*n* = 44	
G/G	67 (47.5)	86 (60.6)	Reference	21 (46.7)	Reference
G/T	66 (46.8)	**49 (34.5)**	0.55 (0.33–0.92)	23 (51.1)	0.83 (0.38–1.82)
T/T	8 (5.7)	7 (4.9)	$p = 0.023$ (od)	1 (2.2)	$p = 0.65$ (dom)
ACE rs4340 Alu-287 bp	*n* = 141	*n* = 148		*n* = 43	
D/D	30 (21.3)	51 (34.5)	Reference	14 (32.6)	Reference
I/D	70 (49.6)	**59 (39.9)**	0.49 (0.28–0.86)	**17 (39.5)**	0.41 (0.18–0.98)
I/I	41 (29.1)	**38 (25.7)**	$p = 0.012$ (dom)	**12 (27.9)**	$p = 0.047$ (dom)

OR, odds ratio; CI, confidence interval; N/A,.
Genotypes associated with a response in accordance with OR (protective if OR < 1, susceptible if OR > 1) are in bold-type.
[a] Significant *p*-values <0.05.
[b] The genetic model: rec, recessive; dom, dominant; od, overdominant (for the overdominant model the reference genotype is major homozygote + minor homozygote).

peripheral airways, i.e. bronchiolar, terminal bronchiolar, and alveolar epithelium, with high inter-individual variability.[29] It has been reported that SNP rs2606345, associated with CAP in our study, is located in the first intron of the gene, but has a functional character up-regulating CYP1A1 activity for the T or G allele correspondingly in the absence or in the presence of the specific substrates (Polycyclic aromatic hydrocarbon (PAH), polychlorinated biphenyls (PCBs), and other chemicals capable of binding to and activating the Ah receptor).[30,31]

In recent years, the genetic determinants of individual differences in *CYP1A1* expression and their association with lung carcinogenesis have been examined widely (10 meta-analyses according to the HuGE Navigator website, http://hugenavigator.net/HuGEwiki/index.php/HuGE_Navigator), but to our knowledge no works have examined the possible role of *CYP1A1* in lung inflammation. Meanwhile proinflammatory cytokines dramatically down-regulate CYP1A1 expression, disrupting the balance in expression of CYP family 1 enzymes, increasing the genotoxic effects of substrates and inducing apoptosis.[32] Proinflammatory cytokines inhibit apoptotic cell clearance in the lung, further exacerbating acute inflammation.[33] Thus we can suggest that genetically determined alterations in CYP1A1 activity could contribute to the pathogenesis of lung inflammation.

We also detected the protective effect of *ACE* insertion in both the CAP and NP groups. The involvement of the renin–angiotensin system in the pathogenesis and evolution of pneumonia has gained

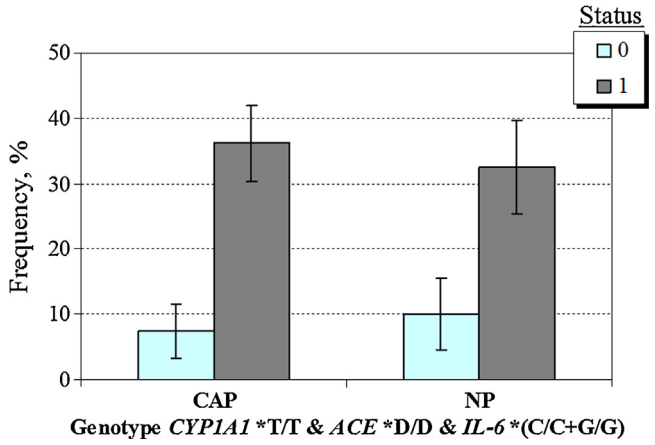

Figure 1. Relative frequency distribution by the multiplicative risk genotype in the community-acquired pneumonia (CAP) and nosocomial pneumonia (NP) sets. The mean and standard error (SE) of relative frequencies are plotted by the risk genotype, so that percentages of cases with CAP or NP (1) and matched control subjects (0) carrying the unfavorable genotype are shown.

substantial interest due to the possibility of the use of ACE inhibitors.[34] According to a meta-analysis, the *ACE* I/D polymorphism contributed to the risk of *ACE* insertion-related cough in East Asians but a negative association was observed in Caucasians.[35] The I/D polymorphism in intron 16 of the *ACE* gene is associated with plasma ACE levels being the highest for the D/D carriers[36] and with degradation of bradykinin in the circulation.[37] The D/D genotype carriers have higher serum levels of the proinflammatory angiotensin II.[38] By current data, the functional character of the I/D polymorphism might be indirect, being a result of linkage disequilibrium with an allele G2350A.[39] It was shown that the *ACE* D allele is an independent risk factor for fatal pneumonia in an elderly Asian population,[40] but not in an elderly Dutch white population.[41] We submit results confirming that the *ACE* D allele is a risk factor with low penetrance for CAP and NP in a population of the Russian Federation.

The carriage of *IL-6* −174 homozygous genotypes G/G and C/C (rs1800795) was associated with CAP and NP risk. According to Terry et al., the *IL-6* 174G/C polymorphism affects transcription by altering the serum levels of IL-6, with the C allele associated with significantly lower levels of plasma IL-6.[42] The results of the association studies are controversial as they show the deleterious effects of the minor allele *IL-6* 174C,[1,11] the absence of association,[43,44] and even suggest its protective role.[45] An interesting finding of the present study was the protective effect of the *IL-6* 174G>C (rs1800795) heterozygote, which was observed in both the CAP and NP studies. One of the many reasons for the inconsistent results in association studies might be molecular heterosis, which is rather common in humans.[46] Heterozygosity may facilitate the dynamic balance between proinflammatory and anti-inflammatory factors, thus providing better defense against exposure to a wider range of causative agents, exogenous and endogenous pathogens.

The study presented some limitations that should be addressed. All the polymorphisms were analyzed in the entire CAP and NP cohorts. The role of the SNPs showing *p*-values of <0.1 in the CAP set was also analyzed in the groups of CAP patients with known causative microorganisms. The microbial diversity of NP was higher and typical members of different phylogenetic lineages were detected. A wider range of pathogens and a large number of polymicrobial cases did not allow us to perform a stratification analysis of subgroups in the NP set, but the presented quantitative and qualitative spectrum of causative agents of NP is similar to that of other clinical series.[47]

In summary genotype analyses have not shown any significant difference regarding CAP and NP in models non-adjusted or adjusted for independent covariates (age, sex, ethnicity, and the use and duration of mechanical ventilation). In addition the frequencies of *CYP1A1*, *ACE*, and *IL-6* genotypes did not differ between CAP patients with specific causative pathogens, as well as between NP patients with known infectious agents. We next assessed the genotype distributions in NP patients by age and use and duration of mechanical ventilation and tested the genotype–covariate interaction. Genotype frequencies were not different in the subgroups and the genotype–covariate interaction was insignificant. This study revealed that individuals bearing *CYP1A1* rs2606345T/T, *ACE* rs4340Del/Del, and *IL-6* rs1800795G/G+C/C genotypes had a higher susceptibility to CAP and NP. Carriage of three risk genotypes in these genes imposed considerably on the risk of both CAP and NP, independently of age, gender, type of causative pathogen, and the use of mechanical ventilation in patients from the Russian Federation. The strongest new association was for *CYP1A1* (rs2606345) with susceptibility to CAP.

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Conflict of interest: The authors declare that they have no conflict of interest.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijid.2013.01.005.

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