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TNF-α polymorphisms affect persistence and progression of HBV infection

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Abstract

Background: Hepatitis B virus (HBV) infections are a major threat worldwide. Disease progression and outcome is diverse and depends on host genetic background. Recently, a high rate of HBV reactivation in individuals receiving tumor necrosis factor-α (TNF-α) antagonists showed the importance of this cytokine in HBV infection control. Here, we investigated the influence of TNF-α promoter polymorphisms on susceptibility to chronic HBV infection (CHB), liver injury progression and outcomes.

Methods: A total of 231 patients with CHB constituted the study group and 100 healthy volunteers—the local control group. *TNF-α* −1031T/C, −863C/A, −857C/T, −308G/A, and −238G/A were genotyped using MALDI-TOF mass spectrometry.

Results: *TNF-α* −1031C and −863A alleles were observed more frequently in CHB group than in healthy controls. Carriers of *TNF-α* −1031C and −863A variant alleles had lower baseline levels of serum HBV DNA and lower liver necroinflammatory activity than dominant homozygotes. A −857CT genotype predisposed to higher necroinflammatory activity. No associations between *TNF-α* variants and liver fibrosis were found.

Conclusion: This study indicates that *TNF-α* −863A and −1031C alleles are associated with increased susceptibility to CHB in individuals from northern Poland. The same variants determine the course of CHB, lowering viremia and reducing necroinflammatory activity of the liver.

KEYWORDS
chronic hepatitis B, host factor, mass spectrometry, single-nucleotide polymorphism, tumor necrosis factor-α

1 | INTRODUCTION

Hepatitis B virus (HBV) infections are a major epidemiological threat worldwide with a toll of more than 800,000 deaths annually, mostly due to hepatocellular carcinoma (HCC) and complications of cirrhosis (Lozano et al., 2012). HBV is a noncytopathic virus that targets the hepatocytes and might trigger the host immune response, in particular cytopathic activity of the cytotoxic T lymphocyte (CTL) response, to induce liver inflammation and fibrosis (Suhail

et al., 2014). Disease progression and outcome is diverse, and largely depends on host genetic background (Thursz, Yee, & Khakoo, 2011). In most cases, the HBV infection is self-limiting: about 95% of older children and adults clear the virus spontaneously. The remaining individuals develop chronic HBV infection (CHB) that can progress to end-stage liver disease in up to 30% of patients (WHO, 2018, Hepatitis B Key facts). Virus clearance is mediated by the immune response, including secretion of type 1 inflammatory cytokines, such as interferon-γ, interleukin-2, and tumor necrosis factor-α (TNF-α). In this activity, $CD4^+$ T cells (Franco, Guidotti, Hobbs, Pasquetto, & Chisari, 1997), natural killer (NK) cells, natural killer T (NKT) cells as well as CTL (Chisari, Isogawa, & Wieland., 2010) are involved, ensuring self-limitation of the HBV infection in most immunologically competent adults.

TNF-α is a pleiotropic, proinflammatory cytokine involved in various signaling pathways associated with inflammation, proliferation, and apoptosis. It plays a key role in response to, among many others, HBV infection. The blockade of TNF-α pathway with anti-TNF agents, broadly used in treatment of autoimmune conditions such as psoriasis or inflammatory bowel disease, results in HBV reactivation in CHB patients (Pérez-Alvarez et al., 2011; Perrillo, Gish, & Falck-Ytter, 2015). Recent studies using animal models confirmed these observations, showing the importance of TNF-α as an innate immunity effector for decreasing viral load and HBV clearance (Chyuan et al., 2015; Tzeng et al., 2014).

In individuals with chronic HBV infection, the progression of hepatic injury to cirrhosis and HCC is influenced by the dynamics of viral replication. The events of hepatitis B flares, during which a strong immune response to HBV infection provokes necroinflammatory changes that contribute to liver damage, are preceded by elevated serum HBV DNA levels (Chang & Liaw, 2014). TNF-α, therefore, by inhibiting HBV DNA replication (Romero & Lavine, 1996a), can modulate hepatic outcome of CHB. A recent meta-analysis provided evidence that genetic polymorphisms within *TNF-α* are associated with the risk of HBV-induced HCC (Xiao et al., 2016).

The expression of *TNF-α* is influenced at both transcriptional and post-transcriptional levels by several genetic polymorphisms located within *TNF-α* promoter region: −238G/A (D'Alfonso & Richiardi, 1994), −308G/A (Wilson, Symons, McDowell, McDevitt, & Duff, 1997), and −1031T/C, −863C/A, −857C/T (Higuchi et al., 1998). The results of the studies investigating the clinical impact of these polymorphisms are, however, inconsistent (Kim et al., 2003; Niro et al., 2005; Xia, Zhou, Liu, Chen, & Chen, 2011; Zheng et al., 2012).

The purpose of this study was to investigate the possible influence of *TNF-α* promoter genetic polymorphisms on susceptibility to CHB in a population from northern Poland. Additionally, we analyzed associations between *TNF-α* promoter polymorphisms and key parameters of CHB outcome and progression of liver injury.

2 | MATERIALS AND METHODS

2.1 | Ethical compliance

The Local Independent Bioethics Committee at the Medical University of Gdansk approved the study protocol in compliance with the Declaration of Helsinki. Written informed consents were collected from all enrolled patients.

2.2 | Patients

A total of 231 patients with confirmed CHB, qualified for antiviral therapy, who were admitted to the Department of Infectious Diseases, Medical University of Gdansk, and the Hepatology Outpatients Clinic Pomeranian Centre for Infectious Diseases and Tuberculosis in Gdansk in 2014–2016, constituted the study group. A chronic HBV infection was recognized when HBsAg and anti-HBc antibodies (IgG type) were present for at least 24 months prior to enrolment. A liver biopsy was performed in HBV-infected subjects with viral load higher than 2000 IU/mL or increased ALT activity. CHB was recognized after liver specimen had been analyzed by the two independent pathologists.

All recruited patients underwent routine blood tests, including the activity of alanine aminotransferase (ALT), HBsAg, HBeAg, anti-HBe antibodies, and the quantification of HBV DNA. Liver biopsies were collected from 196 patients and assessed for inflammation activity and stages of fibrosis according to Scheuer scores.

A local control group included 100 blood donors with confirmed seronegativity to HIV, HBV, and hepatitis C virus (HCV) from the Gdansk Regional Centre of Blood Donations and Haemotherapy.

2.3 | SNP genotyping

Five single-nucleotide polymorphisms (SNPs) from the promoter region of *TNF-α* gene, shown earlier as important in regulation of cytokine expression, were selected for the study: −1031T/C (rs1799964), −863C/A (rs1800630), −857C/T (rs1799724), −308G/A (rs1800629), and −238G/A (rs361525). Isolation of genomic DNA from whole blood samples was done using QIAamp DNA Blood Mini Kit (QIAGEN, Germany), following manufacturer's protocols. SNP genotyping was performed using a MassARRAY MALDI-TOF MS platform with iPLEX Pro chemistry (Agena Bioscience, USA) following the routine protocol. Primers were designed with Agena Assay Design Suite v2. Primer sequences used for the genotyping are shown in Table S1. After desalting, the reaction products were dispensed on

a SpectroCHIP with a RS1000 Nanodispenser. The products were examined with an Analyzer 4 mass spectrometer, and recorded mass spectra were analyzed with Typer 4.0 software.

2.4 | Statistical analysis

STATISTICA 12 (StatSoft, USA), and R version 3.4.2 were used for statistical analysis. Deviations from Hardy–Weinberg equilibrium were assessed with the R HardyWeinberg package. For data visualization, GraphPad Prism 7 software was used. Chi-squared test and Fisher's exact test were applied to analyze the distribution of nominal variables. Quantitative variables were expressed as median values (unless stated otherwise) and compared with the Mann–Whitney *U* test or Kruskal–Wallis test. Logistic regression was conducted to determine the associations between analyzed variables adjusted for possible confounders. The online tool SNPstats (Sole, Guino, Valls, Iniesta, & Moreno, 2006). (https://www.snpstats.net/start.htm) was used to perform haplotype reconstruction from population genotype data and to calculate Linkage Disequilibrium (LD) parameters: D', r^2, and *p* between pairs of biallelic loci. All statistical tests were two-tailed. *p* values less than .05 were considered significant. A Benjamini–Hochberg procedure was applied to account for multiple testing.

3 | RESULTS

The study group included 231 patients with CHB, majority (59%) were male, with the mean age of 38.7 years. All individuals included in the study were Caucasian. Baseline characteristics of the study and control groups are shown in Table 1.

DNA samples from all subjects included in the study were genotyped, with success rate of 100% for −1031T/C, −308G/A and −238G/A, 99.4% for −857C/T, and 99.1% for −863C/A. The distribution of genotypes followed Hardy–Weinberg equilibrium for both the CHB and control groups ($p > .05$), except for −863C/A for the CHB group ($p < .0001$). We calculated LD values for five analyzed SNPs of the *TNF-α* gene. A strong LD was present between *TNF-α* −1031T/C and four remaining SNPs: −238G/A (D' = 0.998, $r^2 = 0.174$, $p < .0001$), −308G/A (D' = 0.998, $r^2 = 0.0367$, $p < .0001$), −863C/A (D' = 0.843, $r^2 = 0.596$, $p < .0001$), and −857C/T (D' = 0.857, $r^2 = 0.0279$, $p < .0001$) as well as between rs1800629 and −863C/A (D' = 0.998, $r^2 = 0.0307$, $p < .0001$) and −308G/A and rs1799724 (D' = 0.549, $r^2 = 0.00918$, $p = .014$). Genotypic and allelic frequencies of the SNPs for the CHB patients and healthy controls are shown in Table 2. No statistically significant differences in genotypic distribution of *TNF-α* polymorphisms were observed between the CHB and the control group. However, the distribution of −1031T/C and −863C/A differed between the groups at the allelic level ($p = .026$ and .033 for −1031T/C and −863C/A, respectively).

Haplotype analysis revealed five distinct haplotypes of *TNF-α* polymorphisms which were represented with the estimated frequency of > 3%. A TGGCC (−1031, −238, −308; −863, −857) haplotype was the most commonly represented, with the frequency of >50%. The frequencies of haplotypes were similar between the CHB patients and the healthy control group (Table S2).

TNF-α −1031T/C and −863C/A polymorphisms influenced baseline HBV DNA levels in patients with CHB (Figure 1). The −1031TT homozygotes had significantly higher HBV DNA in both models of inheritance: codominant (17.3, 6.9, and 4.1 kIU/mL for TT, TC, and CC, respectively,

TABLE 1 Baseline demographic and clinical characteristics of the study and control groups

	CHB patients ($n = 231$)	Healthy controls ($n = 100$)	*p* value
Age, years	38.7 ± 1.0	27.3 ± 0.9	***<.00001***
Sex (% females)	41%	22%	***.00077***
Origin (% Caucasian)	100%	100%	*1*
ALT (IU/L)	75.4 ± 9.9	—	—
HBV DNA (kIU/mL)	17 379 ± 3 911	—	—
HBsAg (% positive)	100%	—	—
HBeAg (% positive)	29%	—	—
Anti-HBe (% positive)	80%	—	—
Liver inflammation grade[a]	1.5 (1–2)	—	—
Liver fibrosis stage[a]	1 (0.5–2)	—	—

Note: Data are shown as the mean ± standard error of the mean.
Abbreviations: ALT, alanine aminotransferase; CHB, chronic hepatitis B.
$p < .05$ are shown in bold.
[a]Median value (25th–75th percentile).

TABLE 2 Genotypic and allelic distribution of analyzed *TNFα* promoter polymorphisms among chronic hepatitis B (CHB) patients and healthy controls

		Genotypic distribution (%)				Allelic distribution (%)		
SNP ID	Genotype	CHB ($n = 231$)	Control ($n = 100$)	p	Allele	CHB ($n = 462$)	Control ($n = 200$)	p
−1031T/C (rs1799964)	TT	148 (64)	76 (76)	*.076*	T	370 (80)	175 (87.5)	*.026*
	TC	74 (32)	23 (23)		C	92 (20)	25 (12.5)	
	CC	9 (4)	1 (1)					
−863C/A (rs1800630)	CC	167 (73)	81 (82)	*.17*	C	379 (83)	177 (89)	*.033*
	CA	45 (20)	15 (15)		A	79 (17)	21 (11)	
	AA	17 (7)	3 (3)					
−857C/T (rs1799724)	CC	175 (76)	67 (68)	*.37*	C	398 (86)	161 (82)	*.19*
	TC	48 (21)	27 (28)		T	64 (14)	35 (18)	
	TT	8 (3)	4 (4)					
−308G/A (rs1800629)	GG	165 (71)	73 (73)	*.96*	G	393 (85)	172 (86)	*.81*
	GA	63 (27)	26 (26)		A	69 (15)	28 (14)	
	AA	3 (1)	1 (1)					
−238G/A (rs361525)	GG	212 (92)	95 (95)	*.36*	G	443 (96)	195 (97.5)	*.37*
	GA	19 (8)	5 (5)		A	19 (4)	5 (2.5)	
	AA	0 (0)	0 (0)					

Note: $p < .05$.

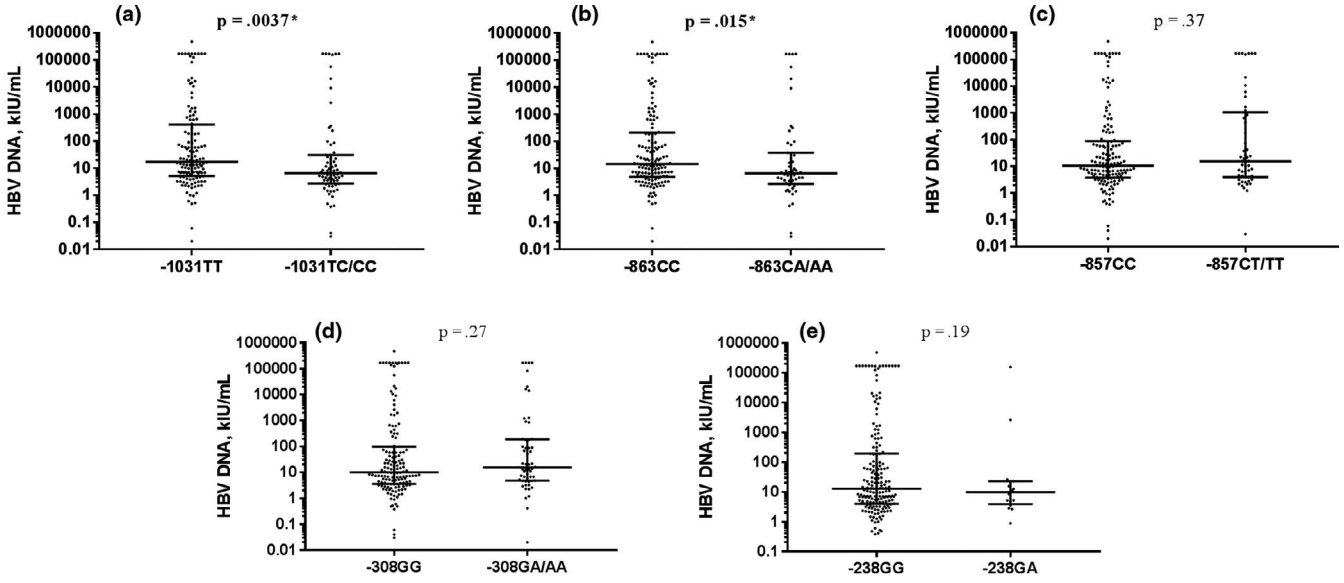

FIGURE 1 *TNF-α* promoter polymorphisms: −1031T/C (a), −863C/A (b), −857C/T (c), −308G/A (d), −238G/A (e) and baseline HBV DNA levels in patients with chronic hepatitis B. Lines represent median values and interquartile ranges. $p < .05$ are marked in bold. *p values remained significant after the adjustment for multiple testing

$p = .0046$) and dominant (17.3 and 6.5 kIU/mL for TT vs. TC, CC, respectively, $p = .0037$). Similarly, for −863C/A polymorphism, significantly higher HBV DNA levels were observed in CC homozygotes (codominant model: 14.6, 7.0, and 4.5 kIU/mL for CC, CA, and AA, respectively, $p = .021$; dominant model: 14.6 and 6.5 kIU/mL for CC vs. CA, AA, respectively, $p = .015$).

Of analyzed *TNF-α* polymorphisms, −1031T/C, −863C/A, and −857C/T affected liver necroinflammatory activity (Figure 2). The −1031TT homozygotes had significantly higher liver inflammation (1.5 vs. 1.0 for TT vs. TC, CC, respectively, $p = .0022$). For −863C/A, liver inflammation was significantly higher in CC homozygotes (1.5 and 1 for CC vs. CA, AA, respectively, $p = .016$). −857CC genotype

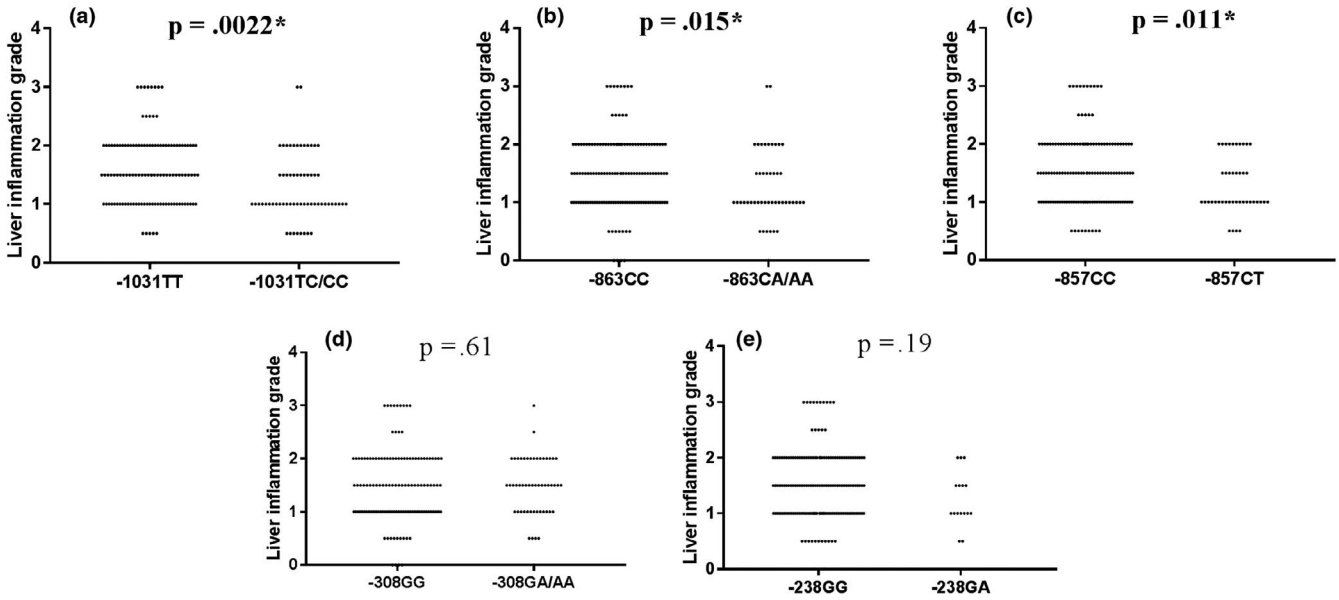

FIGURE 2 *TNF-α* promoter polymorphisms: −1031T/C (a), −863C/A (b), −857C/T (c), −308G/A (d), −238G/A (e), and liver necroinflammatory activity in patients with chronic hepatitis B. $p < .05$ are marked in bold. *p values remained significant after the adjustment for multiple testing

was associated with higher liver inflammation grade (1.5 and 1 for CC vs. CT, TT, $p = .011$). By contrast, no effect of *TNF-α* polymorphisms on liver fibrosis progression was observed, in both analyses: unadjusted and adjusted for sex and age (Table 3).

4 | DISCUSSION

TNF-α is a multifunctional cytokine that plays a pivotal role in inflammation, immune response, as well as cell proliferation, differentiation, and apoptosis. Levels of TNF-α are marginal in healthy cells, however, its production is strongly induced in case of tissue injury, bacterial and viral infections, or cancer. On the contrary, any dysregulation in TNF-α might disrupt the process of infection control. TNF-α can also provoke excessive immune response causing various autoimmune conditions such as multiple sclerosis, rheumatoid arthritis, psoriasis, or inflammatory bowel disease. The introduction of treatment strategies involving TNF-α antagonists not only revolutionized clinical management of these disorders (Kalliolias & Ivashkiv, 2016), but also shed light on the importance of TNF-α pathways in the immunological control of latent infections such as tuberculosis, HIV, HCV, and HBV (Rahier et al., 2014). High rates of HBV reactivation in patients receiving anti-TNF-α treatment (Pérez-Alvarez et al., 2011; Perrillo et al., 2015) provided rationale for further studies that redefined TNF-α as a key noncytopathic innate response mediator of HBV infection (Chyuan et al., 2015; Tzeng et al., 2014).

TABLE 3 *TNF-α* promoter polymorphisms and risk of severe (F3, F4) liver fibrosis ($n = 23$) versus no or mild (F0, F1) fibrosis ($n = 145$) in patients with CHB

		Univariate analysis			Multivariate analysis[a]		
SNP ID	**Genotype**	**OR**	**95% CI**	***p***	**OR**	**95% CI**	***p***
−1031T/C	TT versus TC, CC	1.08	0.43–2.74	*.87*	1.09	0.38–3.11	*.87*
−863C/A	CC versus CA, AA	1.18	0.40–3.46	*.77*	1.66	0.46–6.04	*.44*
−857C/T	CC versus CT, TT	1.32	0.46–3.84	*.60*	1.00	0.34–2.94	*.99*
−308G/A	GG versus GA, AA	0.55	0.22–1.39	*.21*	0.78	0.27–2.27	*.65*
−238G/A	GG versus GA, AA	2.54	0.31–20.51	*.38*	1.54	0.17–13.80	*.70*

Note: Abbreviation: CHB, chronic hepatitis B.
$p < .05$.
[a]Age and sex-adjusted.

Common genetic polymorphisms within the promoter region of *TNF-α*, known to significantly affect its expression, can be considered as factors determining the broad range of host response to HBV infection. Nevertheless, no significant associations between *TNF-α* polymorphisms, −238G/A, −308G/A, −1031T/C, −863C/A, and susceptibility to CHB were observed in Italians (Niro et al., 2005). Our study revealed no significant differences in the prevalence of *TNF-α* polymorphisms and susceptibility to CHB on the genotypic level. However, the prevalence of −1031C and −863A alleles was significantly higher in CHB than in healthy volunteers. Similarly to our findings, *TNF-α* −863C/A, but also −308G/A were associated with spontaneous virus clearance in a cohort of 1,400 Koreans, with −308AA/AG and −863CC genotypes, conferring high levels of TNF-α, more likely to resolve the HBV infection (Kim et al., 2003). Findings on *TNF-α* −863C/A and CHB risk in Chinese populations are contradictory. One study described association of *TNF-α* −863A variant with persistent HBV infection (Qiu et al., 2012). In contrary, no influence of *TNF-α* −863C/A on HBV persistence was observed for both genotypic and allelic levels on Chinese, describing the significant effect of another TNF-α variant, −308A (Xu et al., 2013). In turn, the role of *TNF-α* −863A polymorphism in susceptibility to CHB was confirmed in a meta-analysis by Xia *et al.* covering studies from both Asia and Europe (Xia et al., 2011). By contrast, a recent study on a Egyptian population showed significantly higher prevalence of *TNF-α* −863C variant in individuals with CHB, and no effect of −308G/A polymorphism on HBV persistence (Talaat et al., 2017). However, another meta-analysis found no effect of −863C/A on the risk of developing CHB infection (Zheng et al., 2012). The study described a significant association between −238G/A polymorphic site and CHB susceptibility with a −238A as a risk allele, but when the analysis was restricted to European population only (Zheng et al., 2012). Similarly, the study on a German population by Hohler et al. showed that the −238A variant was present more frequently in CHB individuals than in healthy controls or in patients with acute HBV (Höhler et al., 1998). A study on Tunisian population investigating *TNF-α* −238G/A and −308G/A polymorphisms showed that the prevalence of −308GA and AA as well as −238AA genotype was significantly higher in the CHB patients than in the healthy control group (Sghaier et al., 2015). By contrast, −238G, and additionally −863C variants were shown to predispose to HBV persistence in patients from India (Panigrahi et al., 2014). Taken together, although the previous findings on the role of *TNF-α* genetic background in CHB chronicity are inconsistent, the results of this and majority of other mentioned above studies (except for (Panigrahi et al., 2014; Talaat et al., 2017)) suggest that the −863A variant can act as a risk factor for persistent HBV infection. Such discrepancy in findings on the impact of host genetic background within *TNF-α* promoter region underline the complexity of not only pathways regulated by TNF-α, but also the whole immune response to the early HBV infection that imply the outcome of infection.

In contrast to *TNF-α* −863C/A variant, whose role in CHB is broadly investigated in the literature, the relevance of −1031T/C polymorphism for HBV persistence is far less described. No significant associations of −1031T/C with HBV clearance were observed in Italian (Niro et al., 2005) and Chinese (Chen et al., 2011; Du et al., 2006) populations as well as in the meta-analysis (Xia et al., 2011). The −1031T/C polymorphism significantly affects the levels of serum TNF-α: carriers of C variant have lower serum TNF-α than TT homozygotes (Cui et al., 2012). The clinical significance of −1031T/C variant is well recognized. Among many others, it is implicated in pathogenesis of ischemic stroke (Cui et al., 2012) and other acute cardiovascular conditions, (Sandoval-Pinto et al., 2016) asthma (Sun, Li, Jin, & Qiao, 2018), or autoimmune diseases (Ramírez-Bello et al., 2018). To the best of our knowledge, this is the first study demonstrating the possible association of *TNF-α* −1031C allele with increased risk of CHB.

TNF-α suppresses HBV lifecycle on multiple levels. It inhibits an early phase of infection involving HBV entry and replication in a activation-induced cytidine deaminase-dependent mechanism (Watashi et al., 2013). Additionally, it destabilizes HBV nucleocapsids and decrease levels of nuclear cccDNA (Puro & Schneider, 2007). TNF-α was also shown to inhibit HBV core promoter (Romero & Lavine, 1996a). Recently, antiviral effect of TNF-α was attributed to activity of p22-FLIP which restricted HBV lifecycle by inhibiting transcription and HBV DNA replication (Park et al., 2016). Our findings confirm the role of TNF-α in reducing HBV replication. We demonstrated that the levels of serum HBV DNA is significantly lower in individuals carrying rare alleles of *TNF-α* −1031T/C (TC and CC) and −863C/A (CA and AA) polymorphisms than in dominant homozygotes (−1031TT and −863CC). As −1031C and −863A were associated with lower CHB risk, one may speculate that lower viremia levels, in particular in early stage HBV infection, might impair immune response and affect rates of HBV clearance. A study of Dunn et al. (2009) on individuals with acute HBV infection showed that levels of HBV correlated with immunosuppressive IL-10 concentrations which attenuated NK and T-cell anti-HBV response (Dunn et al., 2009). Carriers of the same genetic variants experienced weaker liver necroinflammatory activity, which, the most presumably, reflected lower replicative activity of the virus in those individuals. A *TNF-α* −308A allele was shown to be associated with increased necroinflammatory activity of the liver (Ferreira et al., 2015). In the same study, no such effect was observed for −238G/A. Our study provided evidence for the role of *TNF-α* −1031TT, −863CC, and −857CC as risk factors for high necroinflammatory activity in the course of CHB.

As opposed to liver inflammation, the impact of *TNF-α* polymorphisms on the progression of liver injury to cirrhosis

and HCC has been broadly investigated. A *TNF-α* −308A allele, which predisposed to increased necroinflammatory activity, was also associated with risk of severe (F3, F4) liver fibrosis (Ferreira et al., 2015). The role for −308A, as well as −238A, as risk factors in HCC development in patients with CHB was also described by Sghaier et al (Sghaier et al., 2015). Similar finding was made in a meta-analysis from 2016, where −308A, −238A, as well as −863T alleles were shown to increase the incidence of HBV-induced HCC (Xiao et al., 2016). A study conducted on Italian patients showed that the −308GG genetic variant is a risk factor for decompensated cirrhosis (Niro et al., 2005). A study by Panigrahi et al. provided evidence that *TNF-α* −863CC genotype protects against liver disease progression toward cirrhosis and HCC, with no such effects for −857C/T and −238G/A polymorphisms (Panigrahi et al., 2014). A *TNF-α* −857C decreased risk of HBV-induced HCC, but this association was observed for women only (Chen et al., 2011). By contrast, in this study we observed no influence of *TNF-α* polymorphisms on liver disease progression toward cirrhosis. A similar finding regarding lack of association of *TNF-α* −238G/, −308G/A, −857C/T, −863C/A, and −1031T/C, with risk of liver fibrosis was described by Miyazoe et al. (Miyazoe et al., 2002).

To conclude, the results of our study indicate that *TNF-α* −863A and −1031C alleles might be associated with susceptibility to chronic HBV infection in patients with CHB from northern Poland. Moreover, we showed that the same variants determine CHB susceptibility and are associated with decreased viremia and lower necroinflammatory activity of the liver.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

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