Received: 7 April 2019 | Revised: 19 May 2019 | Accepted: 30 May 2019

DOI: 10.1111/tid.13124

ORIGINAL ARTICLE

WILEY

IL28B rs12979860 T allele protects against CMV disease in liver transplant recipients in the post-prophylaxis and late period

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Funding information
The study was supported by the project (Ministry of Health, Czech Republic) for development of research organization 00023001 (IKEM, Prague, Czech Republic)—Institutional support

Abstract

Background: Cytomegalovirus (CMV) disease represents a serious complication in liver transplant (OLT) recipients. CMV prophylaxis reduces incidence of CMV disease in the early post-transplant period (on-prophylaxis disease, OPD) but may postpone its manifestation after the completion of prophylaxis. Post-prophylaxis disease (PPD) incidence after prophylaxis cessation may be modified by genetic factors.

Methods: We analyzed impact of *IL28B* rs1297986 variants on CMV disease incidence in 743 adult OLT recipients receiving universal prophylaxis.

Results: One hundred and forty-four (19.4%) patients had at least one CMV disease episode. One hundred and two of them (70.8%) had at least one OPD and 36 (25%) patients had PPD, six (4.2%) patients had both. The rate of *IL28B* T allele carriers was lower in PPD group (38.9%) in comparison with OPD group (66.7%, $P = 0.005$) and group without CMV disease (61.4%, $P = 0.009$). The impact of *IL28B* genotype on the risk of CMV OPD was significant neither in the allelic (TT + CT vs CC, $P = 0.32$) nor in the recessive model (TT vs CT + CC, $P = 0.79$). Contrarily, in the PPD group, T allele (TT + CT vs CC) had a protective effect, OR 0.4 (95% CI 0.2-0.8, $P = 0.008$). Further risk factors of PPD were age <55 years and valganciclovir prophylaxis, whereas the risk factors of OPD were age <55 years, cyclosporine A therapy and pre-transplant CMV serostatus (donor +/recipient −).

Conclusions: *IL28B* rs12979860 T allele carriers had a lower risk of CMV PPD.

KEYWORDS
cytomegalovirus, gene variants, *IL28B*, immunosuppression, interferon lambda 4, liver transplantation

1 | INTRODUCTION

Human cytomegalovirus (CMV) belongs to the *Betaherpesvirinae* subfamily.[1] CMV represents frequent opportunistic infection among solid organ transplant (SOT) recipients increasing their morbidity and mortality, particularly in patients with no pre-existing CMV-specific immunity.[2,3]

Two major types of CMV infection are known to occur in SOT recipients. Primary infection is observed in CMV seronegative recipients (R−) who receive an allograft from a CMV seropositive,

latently infected donor (D+). Reactivation of CMV occurs in CMV seropositive recipients after administration of immunosuppressive treatment.

CMV infection occurrence peaks 2-3 months post-transplant, but the onset can be delayed when CMV prophylaxis is administered in the early post-transplant period. Late onset CMV infection can develop after prophylaxis cessation and is more common in high-risk (D+/R–) recipients.[4-6]

Two approaches have been designed to minimize the risk of CMV infection. First, the preemptive treatment strategy, when antivirals are administered only to patients with positive CMV viraemia in order to prevent progression to CMV disease. Regular monitoring of CMV replication every 1-2 weeks is necessary.[7] The second option is universal administration of CMV prophylaxis for 3-6 months post-transplant. This approach postpones the onset of CMV disease (post-prophylaxis, former delayed onset)[8] and thus is effective in reducing the CMV incidence in the early post-transplant period, especially in the high-risk group (D+/R–).[9]

Several risk factors increasing susceptibility to CMV infection have been described and involve pre-transplant donor and recipient serostatus, type and dose of immunosuppression, coinfection with immunomodulating viruses, bacteria, or fungi. One of the major risk factors for CMV is occurrence of an acute cellular rejection (ACR) episode.[2,10-12]

Potent antiviral prophylaxis significantly decreases the number of CMV episodes in the early post-transplant period. However, some recipients still develop CMV disease on-prophylaxis in spite of the absence of the risk factors mentioned above.[13] In SOT recipients, the interaction between virus and innate immune system seems to play a crucial role since CMV-specific adaptive immune control is inhibited by immunosuppressants.[14] Several studies reported hypotheses of the role of innate immunity in the control of CMV infection and variants in certain genes involved.[15-21] Interferon-λs (IFNLs), also called type III IFNs, which are mainly secreted by dendritic cells and macrophages, are thought to have potent antiviral and immunomodulatory activities by activation of JAK/STAT pathway and inducing expression of interferon-stimulated genes (ISGs).[22,23] These properties may partially overlap with those induced by type I IFNs.[24]

Genome-wide association studies (GWAS) have identified a single nucleotide polymorphism (SNPs) on chromosome 19q13.13 near *IL28B* gene rs12979860 (C/T), which was first described in association with spontaneous or treatment-induced clearance of hepatitis C virus (HCV).[25] This SNP is assumed to impact the binding of transcription factors and methylation sites and is in linkage disequilibrium with other SNPs. The subsequently discovered *IFNL4* ss469415590 (TT/ΔG), which is located 367 bp upstream of *IL28B*, is well correlated with rs12979860 in Europeans and perfectly correlated in Asians; ss469415590 [ΔG] is a frameshift variant (also known as rs368234815) creating a novel gene encoding INFL4.[23] These SNPs are in an almost complete and perfect linkage disequilibrium.[26] However, association with other viral infections, such as chronic hepatitis B, remains to be elucidated.[27-29] Several studies have been published to describe the association of these SNPs with the outcome of CMV infection in SOT and hematopoietic stem cell recipients; the results, however, remain controversial.[19-21,30]

The aim of the study was to validate the impact of *IL28B rs12979860* variants on risk of CMV infection in a cohort of liver transplant recipients.

2 | METHODS

2.1 | Study design

Data were extracted from the electronic patient database containing records of all scheduled and unscheduled patients' visits. The primary selection criterion was liver transplantation (OLT) for liver cirrhosis with or without hepatocellular carcinoma in adult age. Children, adolescents and also adult patients transplanted for primary liver disease other than liver cirrhosis (acute liver failure, polycystic liver disease, tumors in non-cirrhotic liver) were excluded. The second step included selection of patients who presented with typical symptoms and had at least one record of pp65 antigen positivity or blood CMV DNA level higher than analytical detection limit of the used method or at least one record of histologically proven tissue-invasive CMV disease. The visit records of the patients selected in the second step were reviewed in order to classify the type of clinical presentation of CMV disease (viral syndrome or tissue-invasive disease, on-prophylaxis or post-prophylaxis onset).

2.2 | Study population

The study population identified in the first step of the search included 743 adult Caucasian patients who have undergone OLT in our center from April 1996 to December 2015. The cohort consisted of 477 males and 266 females of the mean age of 55 years (19-74). The median follow-up after OLT was 85 months (1-242). One hundred and fifty-three of them had a hepatocellular carcinoma complying with Milan criteria. The records of all patients were included in the study search from OLT until the date of death or their last visit to our center before June 30, 2016. The pre-transplant and demographic characteristics of the studied patients are summarized in Table 1 and Table 2.

The most frequent indication for OLT in our group of patients was alcoholic liver disease, followed by viral hepatitis, non-alcoholic steatohepatitis, cholestatic liver disease, and liver cirrhosis of different etiology (metabolic, cryptogenic). The backbone of the immunosuppressive maintenance regimens were calcineurin inhibitors: either tacrolimus (84.0% patients) or cyclosporine A (13.1% patients) was used, together with azathioprine or mycophenolate mophetil and prednisolone, according to the period of transplantation. Only 2.9% of the patients were on sirolimus at the time of CMV disease manifestation.

A switch to sirolimus-based regimen was performed in patients with persistent rejection, renal impairment and in patients

TABLE 1 Patients' demographic characteristics

	Total (n = 743)	Without CMV (n = 599)	OPD CMV (n = 102)	PPD CMV (n = 36)	OPD + PPD CMV (n = 6)	*P*-value
Sex, Male (%)	477 (64.2)	382 (63.8)	72 (70.6)	18 (50)	5 (83.3)	0.11
Age (y), median (range)	55 (19-74)	56 (19-74)	52 (19-69)	50 (21-62)	43 (25-64)	**<0.001**
CPT score at the time of LTx, median (range)	9 (6-15)	9 (6-15)	9 (5-14)	9 (5-13)	11 (6-13)	0.45
MELD score at the time of LTx, median (range)	15 (6-42)	15 (6-42)	15 (9-33)	15 (8-25)	14 (9-21)	0.52
Indication for LTx						
Alcoholic liver disease (%)	237 (35.0)	198 (36.6)	29 (31.2)	9 (25.0)	1 (16.7)	0.2
Viral hepatitis (%)	159 (23.6)	128 (23.7)	24 (25.8)	5 (13.9)	2 (33.3)	
Cholestatic liver disease (%)	157 (23.3)	114 (21.1)	25 (26.9)	16 (44.4)	2 (33.3)	
NASH and metabolic disease (%)	25 (3.7)	23 (4.3)	2 (2.1)	0 (0)	0 (0)	
Other (%)	97 (14.4)	77 (14.3)	13 (14.0)	6 (16.7)	1 (16.7)	
Immunosuppressive regimen						
Tacrolimus (%)	625 (84.1)	511 (85.3)	79 (77.4)	29 (80.5)	6 (100)	0.17
Cyclosporine (%)	97 (13.1)	69 (11.5)	22 (21.6)	6 (16.7)	0 (0)	
Sirolimus (%)	21 (2.8)	19 (3.2)	1 (1.0)	1 (2.8)	0 (0)	
CMV serostatus						
D+/R+	476 (64.1)	384 (64.1)	63 (61.8)	24 (66.7)	5 (83.3)	**0.04**
D+/R−	96 (12.9)	64 (10.7)	24 (23.5)	7 (19.4)	1 (16.7)	
D−/R+	138 (18.6)	123 (20.5)	11 (10.8)	4 (11.1)	0 (0)	
D−/R−	33 (4.4)	28 (4.7)	4 (3.9)	1 (2.8)	0 (0)	
CMV prophylaxis						
Valacyclovir or Acyclovir (%)	655 (88.2)	529 (88.3)	92 (90.2)	29 (80.6)	5 (83.3)	0.30
Valganciclovir or Ganciclovir (%)	88 (11.8)	70 (11.7)	10 (9.8)	7 (19.4)	1 (16.7)	
CMV disease						
Viral syndrome	89 (12.0)	0 (0)	65 (63.7)	21 (58.3)	3 (50.0)	0.69
Tissue-invasive disease	55 (7.4)	0 (0)	37 (36.3)	15 (41.7)	3 (50.0)	
Acute rejection episode (%)	87 (11.7)	72 (12.0)	12 (9.8)	2 (5.6)	1 (16.7)	0.67

Note: *P*-values express the differences between CMV and non-CMV groups.
P-values in bold are statistically significant.
Abbreviations: CMV, Cytomegalovirus; CPT, Child -Pugh-Turcotte; LTx, liver transplantation; MELD, Model for End-stage Liver Disease; OPD CMV, on-prophylaxis CMV disease; OPD + PPD CMV, both on-prophylaxis and post-prophylaxis CMV disease; PPD CMV, post-prophylaxis CMV disease.

TABLE 2 *IL28B* rs12979860 genotype frequencies

	Total (n = 743)	Without CMV (n = 599)	OPD CMV (n = 102)	PPD CMV (n = 36)	OPD + PPD CMV (n = 6)	*P*-value
IL28B rs12979860 genotype						
CC (%)	290 (39.0)	231 (38.6)	34 (33.3)	22 (61.1)	3 (50.0)	0.086
CT (%)	363 (48.9)	295 (49.2)	55 (53.9)	11 (30.6)	2 (33.3)	
TT (%)	90 (12.1)	73 (12.2)	13 (12.8)	3 (8.3)	1 (16.7)	

Note: *P*-values express the differences between CMV and non-CMV groups.
Abbreviations: CMV, Cytomegalovirus; OPD CMV, on-prophylaxis CMV disease, PPD CMV, post-prophylaxis CMV disease; OPD + PPD CMV, both on-prophylaxis and post-prophylaxis CMV disease.

with a de novo malignancy. Overview of the immunosuppressive regimens administered at the time of CMV disease episode is presented in Table 1. Seropositive for CMV pre-transplant were 617/743 (83.0%) of liver transplant recipients and 566/743 (76.9%) of donors.

2.3 | CMV disease definition

For the purpose of this study, CMV disease was defined as two different clinical manifestations: viral syndrome (a) defined as presence of clinical symptoms (fever, fatigue, malaise, or bone marrow

suppression) together with proven CMV replication by pp65 antigen positivity or DNA analysis or tissue-invasive disease (b) diagnosed by histological detection of CMV in the affected organ. Either laboratory assessment of CMV pp65 antigen or CMV DNA was performed on demand in all patients who presented in a regular or unscheduled visit with a symptom typical of CMV viral syndrome (fatigue, unexplained fever, neutropenia, or thrombocytopenia) or in patients who had a proven diagnosis of CMV tissue-invasive disease based on histological examination of the affected organ.[31]

The terminology of CMV disease onset in patients on universal prophylaxis in different periods after OLT followed the recommendation given by Razonable and Blumberg in 2015.[32] On-prophylaxis CMV disease (OPD) was defined as CMV disease manifestation during the period of prophylaxis, within the first 6 months post-transplant. Post-prophylaxis CMV disease (PPD) included cases of CMV disease in the immediate post-prophylaxis period (6 months following the antiviral prophylaxis completion) and cases of CMV disease in the late period (more than 6 months after completion of antiviral prophylaxis).

2.4 | Assessment of CMV replication

Cytomegalovirus pp65 antigen was detected using the monoclonal antibody indirect immunofluorescence method (CMV-vue Kit, Incstar Corp). CMV DNA was assessed from plasma and quantified by Real-time artus® CMV RG PCR (Quiagen, GmbH) with analytical detection limit of 57 copies/mL.[33,34] CMV tissue-invasive disease was diagnosed by identification of characteristic intracellular inclusion bodies on hematoxylin and eosin sections and/or identification of CMV-specific antigen by immunohistochemical staining (Ventana Medical Systems, Inc).

2.5 | CMV prophylaxis

Antiviral prophylaxis was given to the patients universally per protocol in the first 6 months after OLT. The antiviral prophylaxis was selected according to the patients' pre-transplant serostatus. Ganciclovir or valganciclovir was administered to seronegative recipients who received organs from seropositive donors (D+/R−, 83/743). Eleven of 83 patients, who started antiviral prophylaxis with ganciclovir or valganciclovir switched to valacyclovir or acyclovir in the first month after OLT for intolerance or leukopenia and were further evaluated as having prophylaxis with valacyclovir or acyclovir. Valacyclovir or acyclovir was primarily administered to the pre-transplant seropositive recipients regardless of serostatus of the donor (D+/R+ and D−/R+) and to the pre-transplant seronegative recipients who had received the liver grafts from seronegative donors (D−/R−). Treatment of the CMV disease episode consisted of intravenous administration of ganciclovir, foscarnet, or oral valganciclovir according to weight and kidney functions. Standard treatment period was 2 weeks.

2.6 | Genotyping of *IL28B* rs12979860

The *IL28B* (which was classified as an upstream variant of *IFNL4*) non-coding polymorphism rs12979860 C/T was genotyped by using the polymerase chain reaction and restriction fragment length polymorphism assay, as described by Fabris et al[35] using the primers 5′-GCTTATCGCATACGGCTAGG-3′(Fw) and 5′-AGGCTCAGGGTCAATCACAG-3′ (Rev). A 242 base-pair-long product was subsequently digested by the *Bsh*1236I enzyme. The length of the restriction products was assessed using agarose gel electrophoresis. In order to minimize genotyping errors, blank controls wells were left on the polymerase chain reaction plates and two operators, unaware of the status of the sample, performed the genotype assignment independently. The *IL28B* genotyping results are summarized in Table 2.

2.7 | Statistical analyses

The results of statistical analyses are presented as means and standard deviations (SD), medians and ranges, or as frequencies, as appropriate. Mann-Whitney test, the chi-square, and Fisher's exact test were used for comparisons of the medians and frequencies, respectively. P-value < 0.05 was considered as statistically significant throughout the study.

The post-transplant survival rate was assessed by Kaplan-Meier analysis. Cox regression (proportional hazards model) was used to compare cumulative incidences between the individual groups, and the significance in different time points was assessed using confidence intervals.

In the study of predictors of CMV disease incidence, t test was used for comparison of the means, chi-square, and Fisher's exact test for comparison of frequencies. Fine-Gray subdistribution hazards model with two competing events was used to determine significant predictors of CMV disease occurrence.

Statistical analysis was performed using the R programming language v. 3.2.0 (www.r-project.org).

2.8 | Ethical standard

The study, approved by the Ethics Committee of Thomayer's Hospital and Institute for Clinical and Experimental Medicine, Prague, Czech Republic, was conducted as a part of Institutional grant support for development of research organization. Written informed consent for DNA sampling was obtained from all patients and the study conformed to the declaration of Helsinki Ethical Guidelines. The patients' informed consent for the clinical data analysis was not required by local law because of the retrospective design of the study and because the identification information had been removed.

3 | RESULTS

3.1 | CMV disease manifestation

A total number of 144/743 (19.4%) liver transplant recipients who presented with at least one episode of CMV disease were identified. Eighty-nine (61.8%) of these patients presented with CMV viral syndrome and 55 (38.2%) patients presented with CMV tissue-invasive disease. The most frequent presentation of tissue-invasive disease

was CMV hepatitis (34/55), followed by colitis (11/55). The 1-year cumulative incidence of CMV disease was 0.15. Regarding the period of CMV disease manifestation, at least one episode of OPD was diagnosed in 102/144 (70.8%) patients and 36/144 (25.0%) patients developed at least one episode of PPD. Both OPD and PPD were identified in 6/144 individuals (4.2%), who were subsequently excluded from further statistical assessment. After the cessation of CMV prophylaxis, 9/36 (25.0%) patients presented with PPD between 7th and 12th months post-transplant. In the second year post-transplant, a further 8/36 (22.2%) patients had a PPD. The period of PPD presentation ranged from 6 to 108 months post-transplant (median 25 months). The drug used for CMV prophylaxis (acyclovir or valacyclovir vs ganciclovir or valganciclovir) did not have an impact on the CMV disease incidence in the OPD group. On the other hand, in patients in the PPD group who received either valganciclovir or ganciclovir, the onset of the disease seemed to be postponed after the cessation of the prophylaxis (P = 0.03, OR 2.68, 95% CI 1.09-5.95). Ninety-two of 655 patients developed OPD on acyclovir or valacyclovir prophylaxis and 10/88 patients on ganciclovir or valganciclovir prophylaxis (P = 0.39). Twenty-nine of 655 patients who previously received acyclovir or valacyclovirir and 7/88 patients who previously received ganciclovir or valganciclovir presented with PPD (P = 0.08). The survival rate at 3, 5, and 10 years post-transplant was 97%, 93%, and 82%, respectively, in patients without an episode of CMV disease; 96%, 93%, and 85%, respectively, in patients with OPD and 100%, 97%, and 81%, respectively, in patients with PPD. The survival curves did not differ significantly in log-rank test (P = 0.86, Figure 1A). The fact that the patients suffered from any episode of CMV disease did not impact their survival.

3.2 | Association between *IL28B* genotype and CMV disease episode

IL28B rs12979860 genotype frequencies among patients with no episode of CMV disease and with at least one episode of OPD or PPD are summarized in Table 2. Genotype frequencies in the OPD group (33.3% carriers of CC and 66.7% carriers of CT or TT genotypes) did not differ from the genotype frequencies detected in the group without CMV disease (38.6% carriers of CC and 61.4% carriers of CT or TT, P = 0.32). The proportion of CC genotype carriers was significantly higher in the PPD subgroup (61.1% CC, 38.9% CT + TT) compared with the subgroup without CMV disease (P = 0.009, OR 0.43, 95% CI 0.23-0.81). The *IL28B* genotype had no impact on long-term survival of the patients when evaluating CC vs CT + TT genotype carriers, the survival 3, 5, and 10 years post-transplant according to genotypes was 97%, 93%, and 81%, respectively, in CC homozygotes, and 97%, 93%, and 84%, respectively, in the group of CT + TT carriers. The survival curves of CC vs CT + TT carriers did not differ significantly in log-rank test (P = 0.86, Figure 1B).

3.3 | Risk factors evaluation

In the OPD group, the impact of *IL28B* genotype on CMV disease incidence was significant neither in the allelic (CC vs CT + TT, P = 0.32) nor in the recessive model (CC + CT vs TT, P = 0.79). Age older than 55 years (P = 0.001), pre-transplant serostatus other than D+/R– (P < 0.001; OR 0.39, 95% CI 0.2-0.7) and immunosuppressive regimen based on tacrolimus (P = 0.01; OR 0.48, 95% CI 0.28-0.83) were identified as protective factors of OPD episode.

Contrarily, in the PPD subgroup, T allele carriage was protective against CMV disease episode with OR 0.4 (95% CI 0.20-0.80, P = 0.008). The age older than 55 years was also a protective factor (P = 0.02) whereas previous prophylaxis with valganciclovir or ganciclovir (P = 0.03; OR 2.75, 95% CI 1.1-6.23) was associated with a higher risk of the PPD.

The cumulative incidence curve of PPD episodes in the *IL28B* T allele carriers shows a less sharp shape in comparison with CC genotype carriers. The curves are divergent in the whole displayed period and significantly differ in log-rank test (P = 0.0069; Figure 2). The cumulative incidence curves of PPD are divergent only in the first 6 month after the cessation of antiviral prophylaxis, and then, the curves show a parallel course (Figure 3). The course of cumulative incidence curves showed that the effect antiviral prophylaxis on the risk of PPD disappeared after the 6 months after the cessation of antiviral prophylaxis, whereas the effect of the *IL28B* genotype was continuous.

3.4 | Multivariate analysis

Multivariate analysis was performed separately for OPD and PPD groups and the following factors with potential impact on CMV

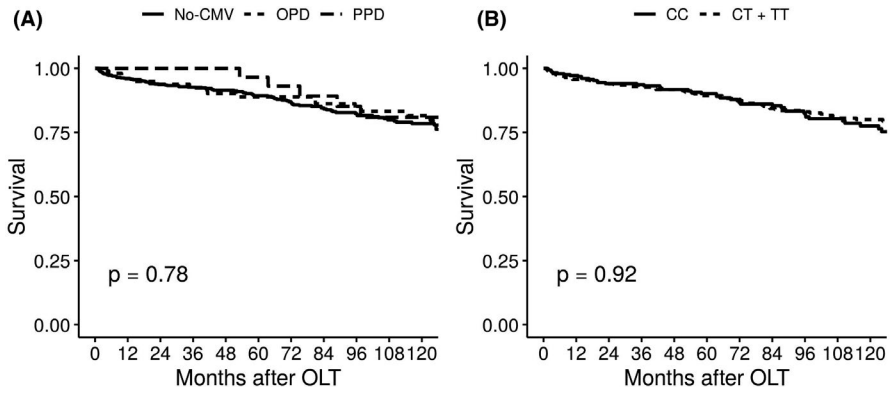

FIGURE 1 Kaplan-Meier survival analysis according to CMV disease onset (A) and *IL28B* genotype (B). CMV, Cytomegalovirus; OPD CMV, on-prophylaxis CMV disease, PPD CMV, post-prophylaxis CMV disease

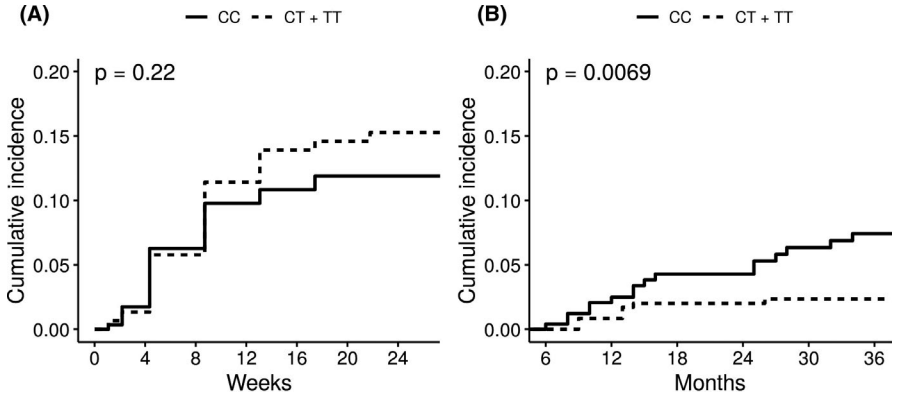

FIGURE 2 Cumulative incidence of OPD (A) and PPD (B) CMV disease according to *IL28B* genotype. CMV, Cytomegalovirus; OPD CMV, on-prophylaxis CMV disease, PPD CMV, post-prophylaxis CMV disease

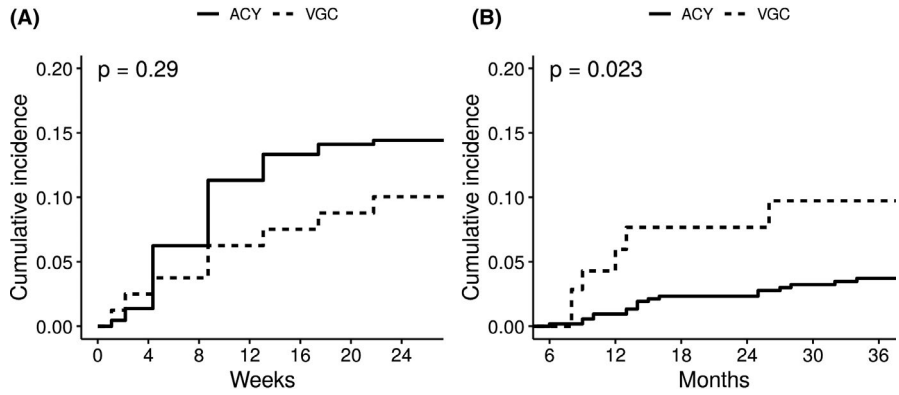

FIGURE 3 Cumulative incidence of OPD (A) and PPD (B) CMV disease according to antiviral prophylaxis. ACY, Valacyclovir or Acyclovir prophylaxis; CMV, Cytomegalovirus; OPD CMV, on-prophylaxis CMV disease, PPD CMV, post-prophylaxis CMV disease; VGC, Valganciclovir or Ganciclovir prophylaxis

disease incidence were considered: sex, age, pre-transplant CPT and MELD score, immunosuppressive regimen (tacrolimus or cyclosporine A), pre-transplant CMV donor and recipient serostatus, acute cellular rejection episode, type of administered CMV prophylaxis and *IL28B* rs12979860 genotype. Age older than 55 years (P = 0.01; HR 0.60, 95% CI 0.40-0.89), CMV serostatus other than D+/R− (P = 0.01; HR 0.59, 95% CI 0.39-0.90), and immunosuppressive regimen based on tacrolimus (P = 0.03; HR 0.58, 95% CI 0.36-0.93) remained significant protective factors of an OPD episode in the multivariate analysis, whereas in the PPD group, *IL28B* T allele carriage (P = 0.01; HR 0.41, 95% CI 0.21-0.8) was a protective factor and CMV prophylaxis with (val)ganciclovir (P = 0.009; HR 0.27, 95% CI 0.11-0.71) remained a significant risk factor of a PPD episode (Figure 3).

4 | DISCUSSION

We identified *IL28B* T allele carriage as a protective factor against CMV PPD, that is T allele carriers ran a lower risk of CMV disease after cessation of antiviral prophylaxis. Contrarily, we did not find any impact of *IL28B* genotype on CMV OPD presentation in patients on antiviral prophylaxis. Our results were in accordance with the results of other studies in this point. In the Swiss Transplant Cohort Study, Manuel et al[30] described a group of 840 SOT recipients, 373 of whom were on universal prophylaxis, and the remaining 467 were regularly checked for CMV infection and treated preemptively. The effect of *IFNL4* genotype (resp. *IL28B*) was pronounced only in the preemptive approach group but not in the universal prophylaxis group. Similar results were obtained in the study on kidney transplant recipients: the effect of *IL28B* was confirmed only in patients without antiviral prophylaxis.[17] However, the role of the minor allele (T allele in *IL28B rs12979860* or ΔG allele in *IFNL4 ss469415590*) in the control of CMV infection remains controversial across the studies published so far. In the stem cells transplant recipients, the carriers of the minor T allele showed a more robust CMV-specific T-cell response,[18] but the corresponding clinical impact of the minor allele was found only in one half of the published studies. While in 2 studies involving solid organ transplant recipients and hematopoietic stem cell transplant recipients, the carriage of the minor allele was associated with a reduced incidence of CMV replication episodes after transplantation.[20,21] Contrarily, in the Swiss Transplant Cohort Study, the SOT recipients, homozygotes ΔG/ΔG in *IFNL4 ss469415590*, were prone to have a higher cumulative incidence of CMV replication and HIV-infected carriers of the *IFNL4* minor allele had a higher incidence of CMV retinitis.[19] Our results were compatible with the studies reporting the protective effect of the minor allele against CMV infection. In our opinion, it is the protective role of T allele in *IL28B* gene (corresponding with the ΔG allele in *IFNL4*) which is compatible with the function of interferon-λ4 and with mechanism of immune-mediated control of CMV infection.

The *IL28B* rs12979860 polymorphism, located 3kB upstream of the *IFNL3/IL28B*, strongly predicted response to pegylated interferon-α-based therapy in chronic hepatitis C.[18,25,36,37] The SNP is in

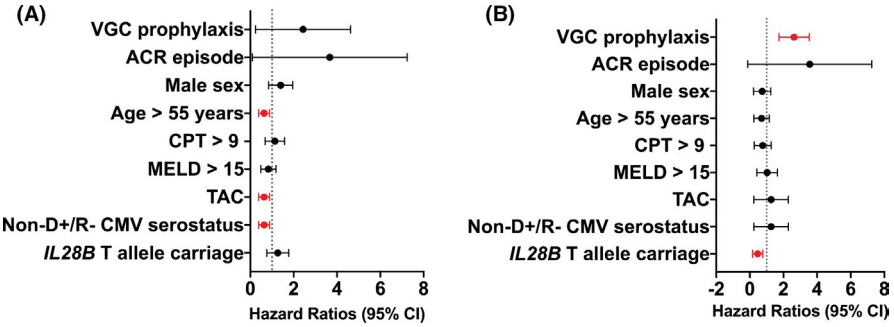

FIGURE 4 Multivariate analysis evaluation of risk factors for on-prophylaxis (A) and post-prophylaxis (B) CMV disease. ACR, acute cellular rejection; CMV, Cytomegalovirus; CPT, Child-Pugh-Turcotte score; D/R, donor/recipient CMV serostatus; MELD, model for End-stage Liver Disease; TAC, tacrolimus-based immunosuppression; VGC, Valganciclovir or Ganciclovir prophylaxis

almost complete and perfect linkage disequilibrium with the coding frameshift dinucleotide variant *IFNL4* rs368234815 in the European and Asian population.[38,39] Therefore, the genotyping of marker polymorphism *IL28B* rs12979860 provides comparable information with the genotyping of functional polymorphism *IFNL4* rs368234815 in these populations. HCV-infected individuals expressing functional IFNL4 from both alleles (*IL28B* rs12979860 TT genotype carriers) present with pre-treatment upregulation of interferon-sensitive genes (ISGs) in hepatocytes but they are refractory to further stimulation of ISGs after administration of exogenous interferon-α.[40] Individuals with non-functional IFNL4 (*IL28B* rs12979860 CC genotype carriers) had low baseline ISGs expression but they are prone to further ISGs stimulation by exogenous interferon-α, which represents a crucial step in the interferon-α induced HCV clearance.[41,42] The mechanism and reason of this condition have not been elucidated so for.

Although interferons play a crucial role in immune-mediated clearance of both HCV and CMV, there are significant differences between these infections. The most important point is the type of the infected cells: whereas the primary replication site of HCV are hepatocytes, expressing the interferon type III receptor, CMV is predominantly found in peripheral blood mononuclear cells (PBMC) not expressing the interferon type III receptor, even in otherwise healthy individuals.[43] Since type I and II interferons and the consequent ISGs upregulation are of paramount importance in the CMV immune-mediated control,[44,45] CMV-infected cells express the IE1 protein, which is abundantly produced in the immediate early phase of human CMV replication and acts as an antagonist of the innate immune defense against the virus by inhibiting interferon type I and II signaling.[46-48] Inhibition of interferon type III signaling in epithelial cells by IE1 has not been reported to our best knowledge; however, the binding of IE1 to STAT2 may have the same effect as in the type I interferon signaling inhibition. Therefore, we speculate that individuals with preserved IFNL4 are able to control better the CMV replication by a hitherto unknown mechanism circumventing the receptor-mediated pathway.

Our cohort may be considered as heterogenous regarding the method used for the diagnosis of CMV replication. The reason to evaluate together patients diagnosed using pp65 and CMV DNA was based on studies confirming that both method had a similar diagnostic value for the diagnosis of CMV disease.[33,34,49] Other drawback may be a low sample size in PPD group, nevertheless the results are statistically significant. The retrospective arrangement of our study may be also considered as a limitation, but we were able to obtain accurate data owing to the fact that all the liver transplant recipients were regularly followed up at our center and admitted to hospital with any infectious complication. We possess a complete series of medical records in all liver transplant recipients. The quality of the obtained data is supported by the fact that clinical characteristics of our cohort were compatible with previously described groups of liver transplant recipients, to whom universal prophylaxis was administered. The identified number of cases of CMV disease corresponded to a cumulative 1-year incidence of 0.16. The cumulative 1-year incidence of CMV disease of 0.16 is higher than reported in prospective studies with universal CMV prophylaxis.[50] The high proportion of seropositive donors (76.9%) and recipients (83.0%) in our cohort may explain the fact of the relatively high 1-year cumulative incidence of CMV disease. In concordance with other studies, the patients with D+/R− serostatus had a higher incidence of the CMV OPD. The superiority of ganciclovir and valganciclovir to acyclovir and valacyclovir in the prophylaxis of CMV OPD was confirmed neither in our cohort nor in previous studies in liver or kidney transplant recipients.[51,52] On the other hand, patients in the PPD group who received valganciclovir or ganciclovir seemed to have a delayed onset of the disease until the termination of the prophylaxis in our cohort. A higher risk of late onset CMV disease is considered to be a disadvantage of the universal prophylaxis approach according to the recent meta-analysis comparing universal prophylaxis and preemptive treatment.[53] However, the postponed onset of CMV infection may represent a milder complication for the patient in comparison with a CMV episode in the early post-transplant period.

In conclusion, our results suggest that knowledge of recipient's genetic markers might lead to a better CMV prophylaxis individualization. Regular assessment of CMV viraemia after the cessation of universal prophylaxis in *IL28B* CC genotype carriers, who are at the highest risk of post-prophylaxis CMV disease, could represent an individualized approach reducing the incidence of the post-prophylaxis CMV disease.

ACKNOWLEDGEMENT

We thank Lucie Budisova for her excellent technical assistance.

AUTHOR CONTRIBUTIONS

Jan Sperl, designed the study; Klara Chmelova, Sona Frankova, Pavel Trunecka and Renata Senkerikova provided clinical and laboratory data; Magdalena Neroldova, Milan Jirsa and Klara Chmelova genotyped DNA samples; Jan Sperl, Sona Frankova and Klara Chmelova analyzed the data and wrote the manuscript; Eva Sticova evaluated biopsy samples; Sona Frankova and Dusan Merta performed statistical analysis; Milan Jirsa, Pavel Trunecka and Julius Spicak revised the manuscript.

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How to cite this article: Chmelova K, Frankova S, Jirsa M, et al. *IL28B* rs12979860 T allele protects against CMV disease in liver transplant recipients in the post-prophylaxis and late period. *Transpl Infect Dis*. 2019;00:e13124. https://doi.org/10.1111/tid.13124

PLOS ONE

RESEARCH ARTICLE

PNPLA3 rs738409 G allele carriers with genotype 1b HCV cirrhosis have lower viral load but develop liver failure at younger age

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Citation: Senkerikova R, Frankova S, Jirsa M, Kreidlova M, Merta D, Neroldova M, et al. (2019) *PNPLA3* rs738409 G allele carriers with genotype 1b HCV cirrhosis have lower viral load but develop liver failure at younger age. PLoS ONE 14(9): e0222609. https://doi.org/10.1371/journal.pone.0222609

Editor: Pavel Strnad, Medizinische Fakultat der RWTH Aachen, GERMANY

Received: May 19, 2019

Accepted: September 3, 2019

Published: September 17, 2019

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The study was supported by the project (Ministry of Health, Czech Republic) for development of research organization 00023001 (Institute for Clinical and Experimental Medicine, Prague, Czech Republic) - institutional support. The funder had no role in study design, data collection

Abstract

Background

PNPLA3 rs738409 minor allele c.444G represents a risk factor for liver steatosis and fibrosis progression also in chronic hepatitis C (HCV). We investigated its impact on the timing of liver transplantation (LT) in patients with genotype 1b HCV cirrhosis.

Methods

We genotyped and evaluated 172 LT candidates with liver cirrhosis owing to chronic HCV infection, genotype 1b. One hundred patients needed LT for chronic liver failure (CLF) and 72 for a small hepatocellular carcinoma (HCC) in the cirrhotic liver without CLF. Population controls (n = 647) were selected from the Czech cross-sectional study MONICA.

Results

The CLF patients were younger (53.5 ± 7.2 vs. 59.6 ± 6.6, $P < 0.001$) with more advanced liver disease than HCC patients (Child-Pugh's score 9.1 ± 1.8 vs. 7.1 ± 1.9, $P < 0.001$, MELD 14.1 ± 3.9 vs. 11.1 ± 3.7, $P < 0.001$). *PNPLA3* G allele increased the risk of LT for CLF in both allelic and recessive models (CG + GG vs. CC: OR, 1.90; 95% CI, 1.017–3.472, $P = 0.045$ and GG vs. CC + CG: OR, 2.94; 95% CI, 1.032–7.513, $P = 0.042$). Multivariate analysis identified younger age ($P < 0.001$) and the G allele ($P < 0.05$) as risk factors for CLF. The genotype frequencies between the CLF group and MONICA study significantly differed in both, allelic and recessive model ($P = 0.004$, OR 1.87, 95% CI 1.222–2.875; $P < 0.001$, OR 3.33, 95% CI 1.824–6.084, respectively). The OR values almost doubled in the recessive model compared with the allelic model suggesting the additive effect of allele G. In contrast, genotype frequencies in the HCC group were similar to the MONICA study in both models.

and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The authors have declared that no competing interests exist.

Abbreviations: AFP, alpha-fetoprotein; ALT, alanine-aminotransferase; BMI, body mass index; CI, confidence interval; CLF, chronic liver failure; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HDL, high density lipoprotein cholesterol; INR, International normalized ratio; LDL, low density lipoprotein cholesterol; LT, liver transplantation; MELD, Model for End-Stage Liver Disease; NASH, non-alcoholic steatohepatitis; OR, odds ratio; PNPLA3, patatin-like phospholipase domain containing 3; SD, standard deviation.

Pretransplant viral load was significantly lower in GG than in CC + CG genotypes (median, IQR; 162,500 (61,550–319,000) IU/ml vs. 570,000 (172,000–1,595,000) IU/ml, $P < 0.0009$).

Conclusions

Our results suggest that *PNPLA3* rs738409 G allele carriage may be associated with a faster progression of HCV cirrhosis to chronic liver failure.

Introduction

Adequate timing of liver transplantation (LT) represents one of the main factors determining favourable posttransplant outcome. Prediction of the patients' prognosis based on the known natural course of a particular liver disease is crucial in the evaluation process [1]. The natural course of liver diseases may be altered by genetic factors. Single nucleotide polymorphism rs738409 c.444C>G (p.Ile148Met) in the patatin-like phospholipase domain-containing protein 3 (*PNPLA3*) is nowadays one of the most important genetic factors with an impact on progression of several liver diseases of different etiology [2].

Association between liver fat content as a quantitative trait and *PNPLA3* rs738409 genotype was described in a large genome-wide association mapping study [3] in 2008 and confirmed in a more detailed study [4] by the same group of authors in 2014. More than fifty studies demonstrating that the *PNPLA3* rs738409 G allele is a risk factor for non-alcoholic steatohepatitis (NASH), liver cirrhosis in NASH or alcoholic liver disease have been published in the past decade [5–11]. The same allele was also identified as a risk factor for liver fibrosis and cirrhosis in HCV-monoinfected individuals [12–16] and in those with HCV/HIV coinfection [17–20] and it also turned out to be a predisposing factor of hepatocellular carcinoma (HCC) [21–24]. In a recent study, the increased risk of HCC and *PNPLA3* G allele was found only in alcoholic liver disease, but not in non-alcoholic fatty liver disease or viral hepatitis B and C [25].

Whereas the impact of the G allele on the liver fibrosis progression in chronic hepatitis C seems to be well known, its impact on chronic liver failure (CLF) progression and the need of LT has not been described so far. In this study, we aimed to investigate the impact of *PNPLA3* genotypes on the risk of CLF in a homogenous group of cirrhotic patients infected with HCV genotype 1b.

Patients and methods

Study design and eligibility of patients

We retrospectively evaluated 172 adult patients with HCV-related cirrhosis caused by HCV genotype 1b with Child-Pugh's class A, B and C who underwent LT between January 1995 and August 2018 at our center. One hundred patients were enlisted for LT and transplanted for CLF (CLF group) using standard criteria evaluating liver dysfunction according to the Child-Pugh's and MELD score and 72 patients were transplanted for a small HCC (HCC group). Fifty-two patients fulfilled Milan criteria, remaining 20 complied with San Francisco or up-to-seven criteria based on pre-transplant imaging techniques results [26–28]. The diagnosis of HCC was confirmed in the liver explants using standard histological staining techniques. Neither patients with HBsAg positivity nor those with HBcAb positivity were included. Patients combining HCV infection with excessive alcohol consumption (60 g per day in males and 40 g per day in females) were also excluded. None of HCV-infected patients had obtained antiviral

treatment in the year preceding LT in accordance with our centre anti-HCV treatment policy: very short times in the liver transplant waiting list, 80–90 days, do not allow for a safe entire treatment course before LT, even in the era of direct acting antivirals. The patients were treated after LT according to the period of transplantation, using an interferon-based regimen until 2014 or a direct acting antivirals combination thereafter. Demographic, clinical, laboratory and histological data were collected from the internal hospital and outpatient database (S1 Table).

Genotype frequencies in both CLF and HCC groups were compared with the *PNPLA3* genotype frequencies in 647 subjects 0.566/0.372/0.062 (CC/CG/GG) reported in the Czech cross-sectional population study MONICA [29], genotyping data were taken from Trunecka et al. [30].

HCV viral load and HCV genotype assessment

HCV viral loads (serum HCV RNA levels) were determined in blood samples taken from HCV-infected patients within 24 hours before LT (last value unaffected by immunosuppression or antiviral therapy). In 133 patients, serum HCV RNA level was assessed according to the period of sampling by the Roche COBAS® AmpliPrep/COBAS® TaqMan® HCV Quantitative Test v1.0 or v2.0 (Roche Molecular Systems Inc., South Branchburg, NJ).

In the 39 remaining patients, only an in-house quantitative method was used and therefore those results were not included in the statistical analysis. HCV genotype was assessed using the SIEMENS Versant® HCV Genotype 2.0 Assay (LiPA) (Siemens Healthcare Diagnostics Inc., Tarrytown, NY).

Genotyping

DNA was isolated from the peripheral blood using the Qiagen QIAamp kit (Qiagen, Hilden, Germany). All patients were genotyped for the *PNPLA3* rs738409 c.444C>G polymorphism by the TaqMan predesigned SNP genotyping assay No. C_7241_10 (Thermo Fisher Scientific, Waltham, MA). Genotyping was performed according to the manufacturer's protocol using the Applied Biosystems ABI 7300 Real-Time PCR instrument (Thermo Fischer Scientific). No significant deviation from the Hardy-Weinberg equilibrium was observed in *PNPLA3* genotypes distribution within the CLF and HCC patient groups.

Statistical analysis

Continuous variables are presented as means and standard deviations, whereas categorical variables are expressed as frequencies (%). Categorical data were analyzed using the chi-square test. For continuous data, Student's t-test or the non-parametric Mann-Whitney test were used appropriately. Genotype frequencies were determined and tested for consistency with the Hardy-Weinberg equilibrium using the chi-square test. Testing for genetic associations was performed as described in [31]. Risk factors were examined using multivariate logistic regression analysis. All statistical analyses were two-sided and P value of < 0.05 was considered statistically significant throughout the study. Statistical analysis was performed using the R programming language version 3.2.0 (www.r-project.org).

Ethics statement

This study was approved by the Ethics Committee of the Thomayer Hospital and Institute for Clinical and Experimental Medicine, Prague, Czech Republic, and was carried out in compliance with the Helsinki Declaration. The patients' informed consent was not required by local

law because of the retrospective design of the study and the use of data from which the patients' identification information had been removed. All study participants gave written consent to the storage of blood samples and agreed to using blood for future research including genetic testing. The written consent was obtained before enlistment for LT.

Results

Demographic, clinical data and laboratory data

Demographic, clinical and laboratory data of the CLF and HCC groups are shown in Table 1. Patients transplanted for CLF were younger with a higher proportion of males and suffered from more advanced liver disease according to the Child-Pugh's and MELD score in comparison with the HCC group. Patients in CLF group had lower AFP levels and lower total cholesterol, HDL and serum triglycerides levels.

Pretransplant viral load

Pretransplant viral load was known in 133 of 172 HCV cirrhotic patients. HCV patients with known pretransplant viral load included 66 of 82 patients with the *PNPLA3* rs738409

Table 1. Demographic, clinical and laboratory data of subgroups with CLF and HCC.

Variables	CLF group n = 100	HCC group n = 72	*P* value
Males (n)	68 (68.0%)	38 (52.8%)	0.0428
Age (years)	53.5 ± 7.2	59.6 ± 6.6	< 0.001
BMI (kg/m^2)	26.2 ± 4.2	26.8 ± 3.7	0.175
Type 2 diabetes mellitus	27 (27.0)	25 (34.7)	0.277
Child-Pugh's class			< 0.001
A	6 (6.0)	37 (51.4)	
B	48 (48.0)	27 (37.5)	
C	46 (46.0)	8 (11.1)	
Child-Pugh's score (points)	9.1 ± 1.8	7.1 ± 1.9	< 0.001
MELD score (points)	14.1 ± 3.9	11.1 ± 3.7	< 0.001
Ascites			< 0.001
None	44 (44.0)	53 (73.6)	
Small	32 (32.0)	14 (19.5)	
Large	24 (24.0)	5 (6.9)	
AFP (μg/l)	34.5 ± 50.1	337.1 ± 926.8	< 0.001
Total bilirubin (μmol/l)	51.8 ± 77.3	35.6 ± 46.7	< 0.001
Albumin (g/l)	29.0 ± 6.5	33.5 ± 6.8	< 0.001
ALT (μkat/l)	1.3 ± 0.9	1.5 ± 1.2	0.117
Total cholesterol (mmol/l)	3.4 ± 1.0	3.7 ± 1.0	0.004
HDL cholesterol (mmol/l)	0.9 ± 0.4	1.1 ± 0.4	0.037
LDL cholesterol (mmol/l)	1.9 ± 0.8	2.1 ± 0.7	0.080
Triglycerides (mmol/l)	1.1 ± 0.5	1.3 ± 0.7	0.009
Prothrombin time (INR)	1.4 ± 0.3	1.2 ± 0.2	< 0.001

Data are given as number, number (%), or mean ± SD.

Abbreviations: CLF, chronic liver failure; HCC, hepatocellular carcinoma; BMI, body mass index; MELD, Model for End-Stage Liver Disease; AFP, alpha-fetoprotein; ALT, alanine-aminotransferase; HDL cholesterol, high density lipoprotein cholesterol; LDL cholesterol, low density lipoprotein cholesterol; INR, International normalized ratio.

https://doi.org/10.1371/journal.pone.0222609.t001

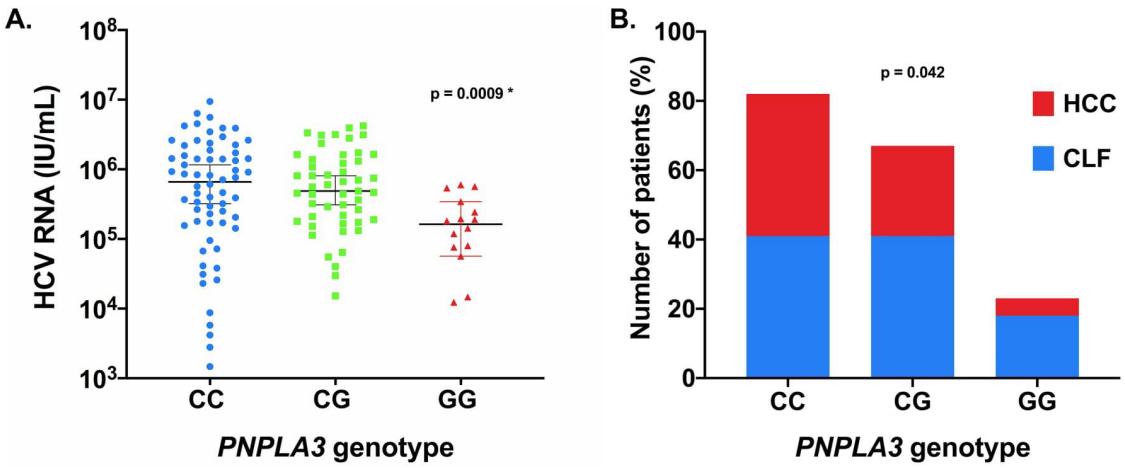

Fig 1. Impact of *PNPLA3* rs738409 genotypes on pre-transplant HCV RNA levels (panel 1*A*) and percentage of patients with CLF (panel 1*B*). Pre-transplant HCV viral load assessed in 133 of 172 patients. Data are given as medians and interquartile ranges. * p value for recessive model.

https://doi.org/10.1371/journal.pone.0222609.g001

CC genotype, 51 of 67 patients with the CG genotype and 16 of 23 patients with the GG genotype.

Similarly, pretransplant viral load was available in 68 of 100 patients with CLF and in 65 of 72 patients with HCC. *PNPLA3* GG homozygotes had a significantly lower pretransplant HCV viral load in comparison with the C allele carriers (median, interquartile range [IQR]; GG 162,500 (61,550–319,000) IU/ml vs. CC+CG 570,000 (172,000–1,595,000) IU/ml, $P < 0.001$, Fig 1*A*). Pre-transplant viral load was significantly lower in patients with CLF in comparison with patients with HCC (median [IQR]; CLF 292,500 (83,725–829,801) IU/ml vs. HCC 806,000 (237,000–1,680,000), $P = 0.008$).

PNPLA3 rs738409 genotype association with CLF

PNPLA3 genotype frequency differences between the CLF and HCC groups were found in both allelic and recessive models (Table 2*A*) ($p < 0.05$). Genotype frequencies between the CLF group and Czech cross-sectional population study MONICA significantly differed with $P = 0.004$ for the allelic model (OR 1.87, 95% CI 1.222–2.875, test power with $\alpha = 0.05$: 0.85) and $P < 0.001$ for the recessive model (OR 3.33, 95% CI 1.824–6.084 (Table 2*B*). The OR values almost doubled in the recessive model compared with the allelic model indicating the additive effect of allele G (Fig 1*B*). By contrast, genotype frequencies in the HCC group were the same as in the MONICA study in both models (Table 2*C*). Importantly, the minor allele frequency in the MONICA study (0.25) did not differ from the frequencies recorded in European population subsets of the GnomAD (0.23) and ExAC (0.23) databases [32].

The proportion of CLF in HCV cirrhotic patients grouped according to their *PNPLA3* rs738409 genotypes is shown in Fig 1*B*.

Risk factors for the need of liver transplantation

In multivariate logistic regression analysis, age and *PNPLA3* rs738409 genotype turned out to be significant determinants of the need of LT. Specifically, presence of the *PNPLA3* G allele increased the risk of LT in CLF 2.4-fold (Fig 2). Other investigated variables such as gender, BMI and type 2 diabetes mellitus did not influence the risk of LT.

Table 2. Genotype frequencies of *PNPLA3* rs738409 C>G polymorphism in the CLF group, HCC group and the MONICA study.

A	Locus	Genotype	CLF group (n = 100)	HCC group (n = 72)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (41%)	41 (57%)	1	-	-
		CG	41 (41%)	26 (36%)	1.90	1.017–3.472	0.045[a]
		GG	18 (18%)	5 (7%)	2.94	1.032–7.513	0.042[b]
B	Locus	Genotype	CLF group (n = 100)	MONICA (n = 647)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (41%)	366 (57%)	1	-	-
		CG	41 (41%)	241 (37%)	1.87	1.222–2.875	0.004[a]
		GG	18 (18%)	40 (6%)	3.33	1.824–6.084	< 0.001[b]
C	Locus	Genotype	HCC group (n = 72)	MONICA (n = 647)	OR	95% CI	*P* value
	PNPLA3 rs738409 c.444C>G	CC	41 (57%)	366 (57%)	1	-	-
		CG	26 (36%)	241 (37%)	0.98	0.602–1.610	0.951[a]
		GG	5 (7%)	40 (6%)	1.13	0.432–2.968	0.800[b]

[a] Allelic model (*PNPLA3* CG + GG vs. CC),

[b] Recessive model (*PNPLA3* GG vs. CC + CG)

Abbreviations: CLF, chronic liver failure; HCC, hepatocellular carcinoma; MONICA, MONItoring trends and determinants in Cardiovascular disease; OR, odds ratio; CI, confidence interval

https://doi.org/10.1371/journal.pone.0222609.t002

Discussion

The study was prompted by our long-term observation that the liver transplant candidates with HCV genotype 1b decompensated liver cirrhosis (or CLF) had significantly more advanced liver dysfunction and were younger that liver transplant candidates with a small HCC. A similar difference in the degree of liver dysfunction between liver transplant candidates indicated for HCV with or without HCC was reported by others [33–35]. However, the age difference between liver transplant candidates was not significant probably due to the fact

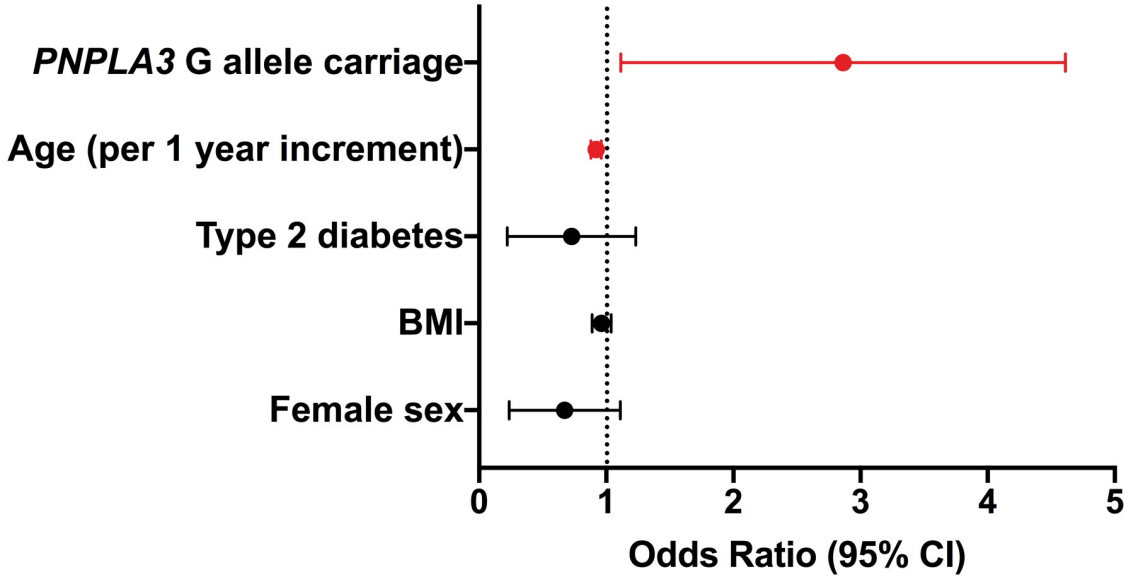

Fig 2. Risk factors for the need of liver transplantation: Multivariate analysis. Bars represent OR with 95% confidence interval.

https://doi.org/10.1371/journal.pone.0222609.g002

that the patients enrolled in these studies were infected with all HCV genotypes and HCV genotype may modify the risk of HCC [36].

To explain the age difference in our cohort, we assumed that the clinical difference between HCV liver transplant candidates with or without HCC might be caused by some genetic factor. A single nucleotide polymorphism *PNPLA3* rs738409 c.444C>G was identified as a risk factor for concurrent liver steatosis and a faster liver fibrosis progression in patients with chronic HCV infection in the past, but its impact on the need and timing of LT has not been evaluated. In our study, we identified further consequences of the carriage of the G allele: accelerated CLF development requiring LT at a younger age and lower pretransplant blood viral load. The CLF patients were younger than HCC patients and had a significantly higher frequency of *PNPLA3* allele G in comparison with HCC patients as well as with population controls.

The earlier need for LT suggests that the G allele carriage is a strong factor contributing to liver fibrosis progression. Consistently with our results, the recently published studies also presented the G allele carriage as a factor accelerating liver fibrosis progression in patients infected with chronic HCV infection. The meta-analysis by Fan and colleagues [12] showed that Caucasians with chronic HCV infection carrying the GG genotype have a more pronounced liver fibrosis and steatosis. In line with these findings, association of the GG and CG genotypes with progression of liver fibrosis was also demonstrated in a large cohort of HCV-infected patients in the HALT-C study [13].

In contrast to studies documenting association of the *PNPLA3* rs738409 genotype with the risk of HCC development in alcoholic liver disease and non-alcoholic fatty liver disease [21–23], no such association was found in HCV-infected subjects [13, 25]. This led us to initial misinterpretation of our data that the *PNPLA3* G allele was protective from HCC. However, since the G allele carriers underwent liver transplant for CLF at younger age, we realized that they were not able to develop HCC later in the course of the disease. Indeed, age is a well-known risk factor of HCC in patients with chronic HCV infection [37, 38].

As mentioned above, *PNPLA3* G allele carriers with chronic HCV infection have also more pronounced liver steatosis. We assume that in these subjects, lipid accumulation in hepatocytes with subsequent steatohepatitis accelerates progression of liver fibrosis caused by the underlying liver disease which is chronic HCV infection. Indeed, coincidence of chronic HCV infection with lipid accumulation and steatohepatitis results in more rapid development of CLF in comparison with HCV-infected individuals without steatohepatitis [12–16]. The hypothesis of two independent synergic processes leading to CLF (HCV infection and steatohepatitis) is further supported by Jimenez-Sousa et al. [15] who demonstrated a dose dependent effect of *PNPLA3* G allele on the progression of liver stiffness in HCV infected individuals. Finally, a dose dependent effect of *PNPLA3* G allele on the serum ALT activity has recently been described in a large study which included patients with chronic liver disease of various aetiologies [39]. When looking at our data, we realized that there is also a notable dose dependent effect of G allele in our cohort: the proportion of patients transplanted for CLF in the subgroups according to *PNPLA3* genotype increased with the number of G alleles (Fig 1B).

A relatively low number of subjects in the HCC group may be considered as the major disadvantage of our study. On the other hand, the comparison with a large number of population controls confirmed the same G allele frequency in the HCC group and population controls.

We also found that G allele carriers had a lower blood HCV viral load. This has been already known but it seems that the impact of the G allele on viral load is different in different HCV genotypes. Rembek et al. [40] reported a significantly lower viral load in GG homozygotes than in CG and CC genotype carriers infected with HCV genotype 2; however, the *PNPLA3* genotype had no impact on the viral load in subjects infected with HCV genotype 3. Contrarily, Eslam et al. [41] found no impact of the *PNPLA3* genotype on the viral load in a large

study group, but the authors included subjects with various HCV genotypes (1–4) and they did not evaluate subjects with different genotypes separately. Our study group was homogenous regarding HCV genotypes: all patients were infected with genotype 1b and this fact allowed us to observe the impact of *PNPLA3* gene variants on the blood viral load. The HCV replication, virus assembly and release is linked to the host cell lipid metabolism. Endoplasmic reticulum–derived membranous web represents the viral RNA replication complex site and lipid droplets serve as virion assembly sites [42, 43]. It has recently been reported that HCV induces complex remodeling of the host cell lipid metabolism in order to enhance both virus replication and virions assembly [44]. The mechanism by which the PNPLA3 variant protein alters lipid turnover in hepatocytes has also been elucidated: the variant protein accumulates on the surface of lipid droplets [45] and binds the cofactor CGI-58 of adipose triglyceride lipase (ATGL or PNPLA2) [46]. Both inactivated ATGL and the barrier of PNPLA3 variant protein on the surface of lipid droplets impede lipolysis of triglycerides and their trafficking in hepatocytes. We assume that changes in lipid metabolism in hepatocytes caused by the PNPLA3 variant protein may affect the HCV life cycle. We consider the lower blood viral load in G allele carriers as a manifestation of the altered lipid trafficking in hepatocytes, but its impact on liver fibrosis progression remains unclear since long-term lowering of viral load by administration of low doses of interferon alpha had no beneficial effect on liver fibrosis progression in the HALT-C study [47].

Conclusions

In conclusion, our results show that the pronounced liver steatosis and fibrosis in *PNPLA3* rs738409 G allele carriers with HCV genotype 1b cirrhosis may have a real impact on the timing and need of liver transplantation. The clinical consequence of G allele carriage could be a faster CLF development and need for liver transplantation at a younger age.

Supporting information

S1 Table. Patients' clinical and laboratory data.
(XLSX)

Acknowledgments

Thanks to Lucie Budisova for her excellent technical assistance.

Author Contributions

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Funding acquisition: Milan Jirsa.

Investigation: Renata Senkerikova, Sona Frankova, Milan Jirsa, Miluse Kreidlova.

Methodology: Milan Jirsa, Miluse Kreidlova, Dusan Merta, Magdalena Neroldova, Jan Sperl.

Project administration: Jan Sperl.

Software: Dusan Merta.

Supervision: Milan Jirsa, Julius Spicak, Jan Sperl.

Validation: Jan Sperl.

Writing – original draft: Renata Senkerikova, Sona Frankova, Dusan Merta, Klara Chmelova.

Writing – review & editing: Sona Frankova, Milan Jirsa, Miluse Kreidlova, Julius Spicak, Jan Sperl.

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