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## Toll-like receptor 7 rs179008/Gln11Leu Gene Variants in Chronic Hepatitis C Virus Infection

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3 2 **Toll-like receptor 7 rs179008/Gln11Leu Gene Variants in Chronic Hepatitis C Virus**  
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6 **Infection**  
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26 12 *Running title:* TLR7 gene variants in chronic hepatitis C  
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**ABSTRACT**

Hepatitis C virus (HCV) infection affects an estimated 3% of the world's population. The natural outcome of infection and the natural course of disease are highly variable. Sensing of viral single-stranded RNA (ssRNA) by Toll-like receptor 7 (TLR7) is likely involved in early pathogen detection and host response to viral infections. This study analyzed epidemiological and clinical data from 136 patients with HCV infection with regard to rs179008/Gln11Leu, a non-synonymous polymorphism within exon 3 of the X-linked TLR7 gene, the variant allele of which is suggested to code for a functionally impaired protein. Allele-specific transcript quantification (ASTQ) analyses in heterozygous females revealed individual skewed mosaicism in peripheral blood mononuclear cells (PBMCs). Thus, analyses were restricted to homo- and hemizygous individuals. Among the clinical and histological parameters studied, the variant allele T was found to be solely associated with the presence of portal lymphoid aggregates. Whereas hepatic viral load and expression of genes known to be induced in chronic HCV infection were not found to differ in patients with wildtype or variant TLR7 rs179008 genotype, significant lower gene expression of interleukin-29 (IL-29)/lambda<sub>1</sub> interferon (IFN-λ<sub>1</sub>) and both of its receptor subunits was found for T homo- and hemizygous patients. Irrespective of the minor differences in disease phenotype including hepatic viral load, natural and alpha interferon (IFN-α)-mediated outcome of infection, and disease activity and progression, the significant differences in hepatic IL-29/IFN-λ<sub>1</sub> and IFN-λ receptor gene expression between TLR7 rs179008 T and A allele patients might have implications for responsiveness to future IFN-λ-based approaches.

**KEYS WORDS:** Toll-like receptor7 (TLR7); single-stranded RNA (ssRNA); hepatitis C virus (HCV); single nucleotide polymorphism (SNP); portal lymphoid aggregates

## INTRODUCTION

Chronic infection caused by hepatitis C virus (HCV), an enveloped single-stranded RNA (ssRNA) virus [Choo et al., 1989], develops in 70%–80% of patients [Schwabe et al., 2006]. Patients are at a high risk of developing severe disease as liver cirrhosis and hepatocellular carcinoma [Schwabe et al., 2006]. Toll-like receptors (TLRs) play a critical role in the innate immune sensing of the invasion of pathogenic microorganisms [Akira and Takeda, 2004]. Alpha interferon (IFN- $\alpha$ ) is an important antiviral cytokine produced principally by plasmacytoid dendritic cells (pDCs), which circulate in the blood at low frequency and even lower in chronic hepatitis C [Kanto et al., 2004], through the stimulation of TLR7 and TLR9 [Hornung et al., 2005; Ito et al., 2005]. TLR7 senses unmethylated viral ssRNA [Diebold et al., 2004; Heil et al., 2004]. The expression of TLR7 in humans is mainly confined to the endosome-lysosome membrane of pDCs (including hepatic pDCs), hepatic natural killer cells [Seki and Brenner, 2008], and B lymphocytes [Hornung et al., 2002]. When the virus or virus-infected apoptotic cells are taken up by phagocytes, viral RNA is released in the highly acidified phagolysosome by degradation enzymes, leading to ssRNA release and recognition by TLR7. Upon TLR7 stimulation, a complex cascade is formed, starting with myeloid differentiation factor 88 (MyD88) and ending with the production of IFN- $\alpha$ /IFN-inducible genes and proinflammatory cytokines through the phosphorylation of interferon regulatory factor 7 and the liberation of nuclear factor- $\kappa$ B, respectively (reviewed in [Akira et al., 2006; Schwabe et al., 2006; Seki and Brenner, 2008]).

Macrophages overexpressing HCV nonstructural proteins NS3, NS3/4A, NS4B, or NS5A show a strong suppression of TLR-MyD88-dependent signaling pathway. NS5A interacts with MyD88 to prevent cytokine production, such as interleukin-1 (IL-1), IL-6, and beta interferon (IFN- $\beta$ ) in response to TLR7 ligands [Abe et al., 2007]. In addition, pDCs from HCV patients have reduced deviation marker (HLA-DR) and IFN- $\alpha$  expression in response to

1 74 TLR7 ligand, which is associated with an impaired activation of naive CD4 T cells [Yonkers  
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4 75 et al., 2007]. TLR7 has been recently of particular medicinal chemistry interest because its  
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6 76 small molecule ligands may serve as immune stimulants by enhancing endogenous IFN- $\alpha$   
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8 77 production and thus, they may complement IFN- $\alpha$  therapy of chronic HCV infection,  
9  
10 78 especially in IFN- $\alpha$ -resistant patients. Horsmans and colleagues have applied a well-tolerated  
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12 79 intravenous isatoribine treatment with only few mild to moderate adverse events for one week  
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14 80 to chronic hepatitis C patients. It has resulted in viral load reduction regardless of the patients'  
15  
16 81 HCV genotype, an induction of the antiviral immunity marker 2'-, 5'- oligoadenylate  
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18 82 synthetase, and an increase in the levels of the gamma interferon (IFN- $\gamma$ )-inducible protein 10  
19  
20 83 (IP-10) and neopterin, a marker of macrophage activation [Horsmans et al., 2005]. Moreover,  
21  
22 84 a high-affinity ligand of TLR7, namely SM360320, has been found to inhibit HCV replication  
23  
24 85 both through type I IFN production by leukocytes, and direct activation of antiviral  
25  
26 86 mechanisms in infected hepatocytes [Lee et al., 2006].  
27  
28 87 TLR7 gene is located on the X-chromosome and contains three exons [Du et al., 2000].  
29  
30 88 Recently, the leucine (Leu) variant encoded by the T allele of the nonsynonymous single  
31  
32 89 nucleotide polymorphism (SNP) rs179008, which is located within TLR7 exon 3 and leads to  
33  
34 90 the replacement of the wild allele A-encoded glutamine (Gln) at codon 11 in the protein  
35  
36 91 (Gln11Leu), has been correlated with higher susceptibility to HCV infection and less chances  
37  
38 92 of response to an IFN- $\alpha$ -based therapy in chronic HCV-infected females [Schott et al., 2008].  
39  
40 93 Moreover, this variant has been associated with higher viral loads, accelerated progression to  
41  
42 94 advanced immune suppression in human immune deficiency virus (HIV) infection, increased  
43  
44 95 susceptibility to HIV-1 in women, and decreased IFN- $\alpha$  production after stimulation of  
45  
46 96 healthy peripheral blood mononuclear cells (PBMCs) with the TLR7 ligand Imiquimod [Oh et  
47  
48 97 al., 2009].  
49  
50 98 Taking the X-linked location into account, the present study aimed to investigate the  
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52 99 correlation between TLR7 rs179008 genotype and disease parameters in chronic hepatitis C,  
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1 100 including the natural outcome of infection, i.e. chronic versus self-limited course,  
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4 101 histological features, and the initial virological response to an IFN- $\alpha$ - based treatment on the  
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6 102 one hand, and hepatic expression of innate immunity genes on the other hand.  
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For Peer Review

## 103 PATIENTS AND METHODS

104

### 105 Patients

106 From a total of 144 mainly Caucasian chronic hepatitis C patients who consulted the Liver  
107 Unit of the Department of Gastroenterology and Endocrinology at the University Medical  
108 Center Goettingen (UMG), Germany, between 1993 and 2006, 136 with complete data sets  
109 (mean age  $45.0 \pm 12.3$ , median 44 years, 60 females) were enrolled in epidemiological,  
110 biochemical and histological analyses. Chronic infection was proven by detection of HCV-  
111 specific antibodies and HCV RNA in the patients' sera using a highly sensitive nested RT-  
112 PCR over a period of at least six months as described [Mihm et al., 1996a]. Before the start of  
113 therapy, liver biopsy procedures were performed and liver disease was confirmed in the  
114 course of a defined histological evaluation as described below. Biochemical liver disease  
115 parameters, i.e. serum activities of aspartate aminotransferase (AST), alanine  
116 aminotransferase (ALT), and gamma-glutamyl transferase ( $\gamma$ -GT) were recorded in parallel.  
117 Patients with concomitant non-C viral infections and those with continued alcohol or other  
118 drug abuse were excluded.

119 A total of 55 patients (mean age  $46.1 \pm 12.3$ , median 45 years, 25 females) were treated with  
120 IFN- $\alpha_{2a}$  (Roferon A; Hoffman-La Roche, Basel, Switzerland) at an initial dose of  $6 \times 10^6$  IU 3x  
121 per week for at least 4 months (mean, 7.7 months; range 4-12 months). Depending on well  
122 being and response parameters, both dose and duration were adapted individually. Initial  
123 virological response to therapy, which is defined as the elimination of HCV RNA below the  
124 limit of detectability during the first 4 months for a period of a least three consecutive months,  
125 was analyzed with regard to TLR7 rs179008 genotype.

126 Another group of 44 patients with self-limited HCV infection (mean age  $37.0 \pm 10.3$ , median  
127 36 years, 14 females) was studied in addition. Spontaneous elimination was assured by the  
128 presence of anti-HCV antibodies in the absence of detectable amounts of HCV RNA (for



1 129 detailed epidemiological and serological description of this cohort please refer to [Wietzke-  
2  
3 130 Braun et al., 2007]).

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5  
6 131 The study was approved by the local ethical committee and conformed to the ethical  
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8 132 guidelines of the 2000 Declaration of Helsinki. Patients gave their informed consent.

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### 11 134 **Determination of HCV Genotype**

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13 135 HCV genotyping was performed using the Innolipa HCV II line probe assay (Innogenetics,  
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16 136 Ghent, Belgium).

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### 21 138 **Histological Evaluation**

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24 139 Before the start of therapy, liver biopsies were taken from patients for histological evaluation.

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27 140 In brief, sections (5-10  $\mu\text{m}$ ) from formalin-fixed and paraffin-embedded liver biopsies were  
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30 141 stained with hematoxylin-eosin, trichrome, and Prussian blue. According to Desmet and  
31  
32 142 colleagues, necroinflammatory activity (grading, score 1 to 3) and architectural alterations  
33  
34 143 (staging, score 0 to 4) were scored separately [Desmet et al., 1994]. Other lesions typical of  
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36 144 hepatitis C such as the degree of steatosis (score 0 to 3), the presence or absence of portal  
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38 145 lymphoid aggregates, and the presence or absence of bile duct damage were studied  
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40 146 additionally as previously described [Mihm et al., 1997].

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### 45 148 **Preparation of PBMCs**

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48 149 PBMCs from approximately 30 ml of heparinized peripheral blood samples were prepared by  
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50 150 Ficoll density centrifugation using guanidinium isothiocyanate as described [Boyum, 1984].

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### 53 152 **Isolation of Genomic DNA (gDNA) and Total Cellular RNA**

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56 153 gDNA was purified from PBMCs or from 2 ml samples of serum using the QIAamp DNA  
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58 154 Mini or Midi Kits, respectively, following the blood and body fluid spin protocol (Qiagen,

1 155 Hilden, Germany). The concentration and the purity of the DNA isolated from PBMCs were  
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4 156 determined photometrically by reading the absorbance levels at 260 nm and 280 nm. The  
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6 157 integrity of gDNA was ascertained through electrophoresis using a 0.6% agarose gel.  
7

8 158 Total cellular RNA was prepared from available freshly isolated PBMCs and homogenized  
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10 159 liver tissue samples by CsCl density gradient ultracentrifugation essentially as described  
11  
12 160 [Mihm et al., 1996b].  
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15 161

### 162 **Reverse Transcription**

163 To get complementary DNA (cDNA), an amount of 1 µg of total cellular RNA was reverse  
164 transcribed by using random hexamers (6 µM) for priming as described previously [Mihm et  
165 al., 1996b].  
166

### 167 **Genotyping for the Variant Position rs179008/Gln11Leu**

168 Allelic discrimination of the TLR7 exon 3-located SNP was performed by the commercially  
169 available TaqMan genotyping assay C\_2259574\_10 (Applied Biosystems, Foster City, CA).  
170 Reactions of 10 µl containing 4 ng of PBMCs-derived gDNA- or an aliquot corresponding to  
171 6.7 µl serum- were performed in the sequence detection system StepOne-Plus (Applied  
172 Biosystems, Darmstadt, Germany) according to the supplier's instructions.  
173

### 174 **Allele-Specific Transcript Quantitation (ASTQ) of TLR7 rs179008 Variants**

175 Discrimination and quantitation of TLR7 rs179008 transcript variants (A and T) was achieved  
176 by applying the commercially available TaqMan genotyping assay C\_2259574\_10 (Applied  
177 Biosystems, Foster City, CA) on cDNA samples (3.2 ng). Heterozygote gDNA and  
178 homozygote gDNA and cDNA samples served as controls.  
179

### 180 **Quantification of Hepatic Gene Expression**

1 181 Competitive RT-PCR was applied to quantify mRNA transcripts of HCV, the IFN- $\alpha/\beta$   
2  
3 182 inducible antiviral myxovirus resistance protein-1 gene (MxA), IFN- $\alpha$  and, as a reference  
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5 183 gene, albumin, essentially as described [Mihm et al., 2004], and transcripts of IP-10, the gene  
6  
7 184 encoding IFN- $\alpha/\beta$ -inducible p44 [Patzwahl et al., 2001], IFN- $\gamma$  [Mihm et al., 1996b]. The  
8  
9 185 relative number of interleukin-29 (IL-29)/lamda<sub>1</sub> interferon (IFN- $\lambda_1$ ), IFN- $\lambda$  receptor subunits  
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11 186 (IL-10R $\beta$  and IL28R $\alpha$ ), and IFN- $\alpha/\beta$  receptor 2 (IFNAR2) mRNA transcripts was calculated  
12  
13 187 by real-time RT-PCR using the sequence detection system ABI prism 7000 following the  
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15 188 supplier' instructions (Applied Biosystems, Darmstadt, Germany) as described [Doyle et al.,  
16  
17 189 2006; Mihm et al., 2004]. Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH)  
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19 190 transcripts served as a housekeeping gene, using a commercially available TaqMan gene  
20  
21 191 expression Assay on Demand (Hs 99999905 ml) (Applied Biosystems). Comparable results  
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23 192 were found when relating the targets to  $\beta$ -actin transcripts (data not shown).  
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### 194 **Statistical Analysis**

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34 195 Females and males were analyzed both separately (data shown in the text where necessary),  
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36 196 and combined due to the ASTQ results. Quantitative parameters were described by mean and  
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38 197 standard deviation if the data are normally distributed, or median and inter-quartile range  
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40 198 (IQR) if the distribution is not normal.  $\chi^2$ -test and independent samples *t*-test were applied  
41  
42 199 where applicable. The level of significance was set to a screening level of 0.05. All tests were  
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44 200 performed by using PC STATISTIK software package version 4.0 (Hoffmann-Software,  
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46 201 Giessen, Germany).  
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## 202 RESULTS

### 203 Genotyping of HCV with Regard to TLR7 rs179008

204 A total of 136 patients with chronic hepatitis C (60 females/76 males) were genotyped for the  
205 bi-allelic SNP rs179008/Gln11Leu within exon 3 of the X-linked TLR7 gene (Table I).  
206 Genotype distribution in women followed Hardy-Weinberg equilibrium ( $P = 0.318$ ). The  
207 frequency of the minor allele (MAF) was found to be close to that given for Caucasians in  
208 public data bases and to be similar in females and males ( $P = 0.175$ ).

### 210 ASTQ

211 Due to the X-linked location of TLR7, in females, the random inactivation of one X  
212 chromosome leads to cellular mosaicism with two populations of cells differing in the  
213 parental origin of the active X [Fish, 2008]. In heterozygous females, the presence of two  
214 kinds of cells might set up a competition between them [Migeon, 2006]. Non-random  
215 inactivation (skewing) has been implicated for discrete cell populations, e.g. dendritic cells  
216 [Fish, 2008; Migeon, 2006]. In order to find out whether heterozygous females should be  
217 assigned either to the wildtype, the variant genotype or to be considered as a separate, i.e. true  
218 heterozygous group, ASTQ was performed to quantify the relative proportion of A and T  
219 allele transcript variants in RNA preparation from freshly isolated PBMCs (Fig. 1). gDNA, a  
220 natural source of equal amounts of A and T sequences served as a control. RNA preparations  
221 from 3 heterozygous women were found to contain nearly equal numbers of both alleles'  
222 transcripts, whereas material from 4 heterozygous females was found to contain an excess of  
223 either A or T, 2 women each, respectively (Fig. 1A).

224 Because of the limited amount of available samples, 5 heterozygous females were further  
225 identified among a total of 42 healthy blood donors. ASTQ with these 5 samples yielded  
226 comparable results (equal expression in 2 samples, an excess of A in 3 samples) (data not  
227 shown).

1 228 On the basis of these findings we decided to restrict analyses to the comparison between A  
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3 229 and T homo- and hemizygous individuals.  
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### 8 231 **Epidemiological and Biochemical Characteristics**

10 232 Demographic analysis revealed a slight trend of the variant allele carriers to be older than  
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12 233 those who carry the wild type allele ( $47.8 \pm 15.0$  vs.  $42.5 \pm 11.2$ , respectively,  $P = 0.069$ ). This  
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14 234 difference, however, was clearer in males ( $P = 0.083$ ) (Table I).  
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17 235 As expected for a European population, most patients (80.1%) were infected with HCV type-1  
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19 236 (including subtypes 1b, 1a and 1a+1b), while 19.9% were infected with HCV non-1  
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21 237 (including mainly subtypes 3a, and a minority of 2a and 2b). No significant difference among  
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23 238 the distribution of HCV types (or subtypes, data not shown) infections according to patients'  
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25 239 TLR7 rs179008 genotype was found (Table I).  
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28 240 AST, ALT, and  $\gamma$ -GT serum activities were recorded as indicators of liver injury in chronic  
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30 241 hepatitis C. Although T homo- and hemizygotes seemed to have higher AST and ALT but  
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32 242 lower  $\gamma$ -GT serum levels than the A counterparts, the proportion of A or T homo- and  
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34 243 hemizygote patients among those with markedly elevated transaminase activities, i.e. greater  
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36 244 than 2-fold of the upper normal limit, was not found to be significantly different (Table I).  
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### 44 246 **Genotyping of Individuals with Self-limited HCV Infection**

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46 247 The T allele is suggested to confer enhanced susceptibility to chronic HCV infection as MAF  
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48 248 has been found to be significantly lower in healthy individuals [Schott et al., 2008]. To  
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50 249 address the question whether this enhanced susceptibility is due to a higher incidence of  
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52 250 infection or to an impaired capacity to self-eliminate the virus, TLR7 rs179008 genotype  
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54 251 distribution of the chronic hepatitis C patients was compared to a group of 44 patients with  
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56 252 self-limited HCV infection [Wietzke-Braun et al., 2007]. The proportion of T homo- and  
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58 253 hemizygous individuals or even of heterozygous female patients was not found to be  
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1 254 significantly higher in chronic HCV patients (15.4% and 14.7%, respectively) in  
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4 255 comparison to patients with self-limited HCV infection (11.4% and 13.6%, respectively)  
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6 256 (Table I).  
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### 10 258 **Hepatitis C Histological Manifestations**

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13 259 To investigate whether a functionally impaired TLR7 protein might be related to histological  
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15 260 features of chronic hepatitis C, liver biopsy specimens were taken and evaluated histologically  
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17 261 with regard to hepatitis activity, fibrosis progression, steatosis stage, portal lymphoid  
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19 262 aggregates and bile duct damage (Table II). TLR7 rs179008 genotype distribution showed no  
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21 263 association with the first three characteristics. Nevertheless, a higher frequency of the minor  
22  
23 264 allele T among patients with portal lymphoid aggregates was observed ( $P = 0.013$ ), this  
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25 265 difference, however, was only valid in males ( $P = 0.032$ ) (Table II). Whereas only 30.5% of A  
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27 266 carriers were found to have bile duct damage, 52.4% of T carriers did have the lesion ( $P =$   
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29 267 0.051) (Table II).  
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34 268 Noteworthy, it was reported recently that patients homozygous for the variant allele T of the  
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36 269 endotoxin receptor CD14 SNP rs2569190/C-159T have more frequent portal lymphoid  
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38 270 aggregates than C carriers [Askar et al., 2009]. Interestingly, analyzing CD14 C-159T  
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40 271 genotype separately in the two genders revealed that the significant association was only  
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42 272 confined to males ( $P = 0.004$ ) (unpublished observation).  
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### 48 274 **Response to an IFN- $\alpha_{2a}$ Monotherapy**

49  
50 275 A total of 55 patients were treated with IFN- $\alpha_{2a}$  as described in the patients and methods  
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52 276 section. The initial virological response to therapy is defined as the elimination of HCV-RNA  
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54 277 below the limit of detectability during the first 4 months for a period of at least 3 consecutive  
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56 278 months. Some patients kept undetectable viral RNA levels till completing therapy i.e. end-of-  
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58 279 treatment response, or even for a period of at least continuous six months after the last dose of  
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1 280 IFN- $\alpha_{2a}$ , i.e. sustained virological response. While 22 (63%) of the A carriers responded, at  
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4 281 least initially, to the therapy, only 3 (30%) of the T counterparts (and 30% of the heterozygous  
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6 282 females) were responders ( $P = 0.069$ ) (Table III). Similar results were found considering a  
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8 283 larger cohort of 145 patients treated with an IFN- $\alpha$ -based therapy (data not shown).  
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10 284

### 13 285 **Hepatic Gene Expression**

15 286 To examine whether a functionally impaired TLR7 protein might be related to hepatic gene  
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17 287 expression in chronic HCV infection, innate immunity gene transcripts were quantified in  
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19 288 freshly derived liver tissue samples. Data were related both to GAPDH and to albumin as  
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21 289 reference genes. When A homo- and hemizygous samples were compared to T homo- and  
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23 290 hemizygous samples, no significant difference was found with regard to the amount of hepatic  
24  
25 291 viral RNA (Fig. 2A), or the genes that have been shown to be enhanced in chronic HCV  
26  
27 292 infection when compared to healthy liver tissue as IP-10, p44, MxA, or IFN- $\gamma$  [Mihm et al.,  
28  
29 293 2004] (Fig. 2B). In contrast, T homo- and hemizygotes were found to express significant  
30  
31 294 lower amounts of IL-29/IFN- $\lambda_1$  ( $P = 0.015$ ), IL-10 receptor beta (IL-10RB) ( $P = 0.001$ ) and  
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33 295 IL-28 receptor alpha (IL-28R $\alpha$ ) ( $P = 0.003$ ), which constitute the two components of IFN- $\lambda$   
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35 296 heterodimeric receptor, as well as lower amounts of IFN- $\alpha$  and IFNAR $_2$  (Fig. 2B).  
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**DISCUSSION**

The TLR7 rs179008/Gln11Leu is located in the signal sequence of TLR7, adjacent to the typical basic residues in the N-terminal part of this sequence. Signal peptide degeneracy modulates posttranslational modification, localization, quantity and thus the functionality of the affected protein [Hegde and Bernstein, 2006]. In the studied cohort of 136 chronic hepatitis C patients, no significant association was found between TLR7 rs179008 and any of the epidemiological or biochemical characteristics, inflammation activity (grading) or fibrosis progression (staging). These results are in concordance with previous findings [Schott et al., 2007]. Considering the presence of portal lymphoid aggregates, a significant higher frequency of the T hemizygoty was found among male patients. Portal lymphoid aggregates are defined as densely packed collection of small lymphocytes within the portal tract with or without the formation of a germinal center [Luo et al., 1999]. Their presence, which is suggested to play an immunological albeit indeterminate role in chronic HCV liver injury similar to the mechanism of autoimmune hepatitis [Hino et al., 1992; Mosnier et al., 1993], has been found to be significantly correlated with hepatic inflammatory activity and bile duct damage [Askar et al., 2009; Freni et al., 1995; Luo et al., 1999; Wong et al., 1996]. Interestingly, the common allele A trended to be low-frequented among patients with bile duct damage ( $P = 0.051$ ) (Table II).

Portal lymphoid aggregates have been recently found to be more frequent among patients homozygote for the T allele of CD14 rs2569190/C-159T [Askar et al., 2009], this association, however, was valid only in males as it is for TLR7 rs179008 in the present study. Noteworthy, sex itself is neither found to be associated with portal lymphoid aggregates [Askar et al., 2009; Luo et al., 1999; Mihm et al., 1997], nor with CD14 rs2569190 genotype distribution [Askar et al., 2009]. Freni et al. described the cellular composition of this manifestation as a core of B cells -which do express TLR7- mixed with many T helper/inducer lymphocytes, and an outer ring prominently formed by T suppressor/cytotoxic lymphocytes, and a rarely



1 323 identifiable germinal center [Freni et al., 1995]. Taken together, being the first observation  
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3 324 of its kind, although its real biological mechanism is still to be found out, replication in an  
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6 325 independent larger cohort, and correction of multiple testing are required, TLR7 rs179008  
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8 326 might have a role in the formation of portal lymphoid aggregates.  
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11 327 TLR7 rs179008 T allele has been found recently to be over-represented and predictive of  
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13 328 unfavourable outcome of IFN- $\alpha$  therapy in female patients with chronic HCV infection  
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15 329 [Schott et al., 2008]. In the present study, we investigated the distribution of this SNP among  
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17 330 patients who spontaneously resolved HCV infection. Comparing the two cohorts with regard  
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19 331 to TLR7 rs179008 genotype did not reveal any significant difference (Table I). Unfortunately,  
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21 332 this analysis lacks statistical power due to the small cohort of self-limited individuals, yet it  
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23 333 did not give any preliminary indication for an altered capacity of resolving the infection  
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25 334 spontaneously. Moreover, the observed slight (but still non-significant) trend of the TT/T  
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27 335 patients to be non-responders to a mono- or a combined IFN- $\alpha$ - based therapy and to have  
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29 336 lower hepatic expression of both IFN- $\alpha$  and IFNAR<sub>2</sub> might confirm - in general - the previous  
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31 337 findings of Schott et al [2007] with cautious limitations due to the novel ASTQ analyses in  
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33 338 this study that let us omit female heterozygotes from our analyses.  
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35 339 The impaired receptor appeared not to affect HCV hepatic viral load, accordingly, no further  
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37 340 effect was observed on p44, MxA, IFN- $\gamma$  or IP-10, genes known to be upregulated in chronic  
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39 341 HCV infection in the absence of hepatic type I IFN induction [Mihm et al., 2004]. The minor  
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41 342 allele T, however, was found to be significantly associated with lower hepatic mRNA  
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43 343 expression of IL-29/IFN- $\lambda_1$  and both IL-10R $\beta$  and IL-28R $\alpha$  (Fig. 2B). This suggests that,  
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45 344 rather than being useful in forecasting the current IFN- $\alpha$ - based therapy outcomes, genotyping  
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47 345 for TLR7 rs179008 might be predictive for response to IL-29/IFN- $\lambda_1$  based therapy  
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49 346 approaches [Sheppard et al., 2003] currently being in phase 2 of clinical development.  
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51 347 Three independent genome-wide association studies (GWASs) have reported recently  
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53 348 on several SNPs in the intergenic region between the genes coding for IL-28A/IFN- $\lambda_2$  and IL-

1 349 28B/IFN- $\lambda_3$  on chromosome 19 to be associated with response outcomes to an IFN- $\alpha$ -  
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4 350 based therapy [Ge et al., 2009; Suppiah et al., 2009; Tanaka et al., 2009] and with  
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6 351 spontaneous clearance of HCV [Thomas et al., 2009]. The minor non-responder allele of  
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8 352 rs8099917 in IL-28B/IFN- $\lambda_3$  gene has been found, moreover, to be associated with lower IL-  
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11 353 28 mRNA expression in PBMCs [Suppiah et al., 2009; Tanaka et al., 2009]. These GWASs  
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13 354 have identified as well many SNPs in several genes to be of minor predictability for IFN- $\alpha$ -  
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15 355 based therapy outcomes, TLR7, however, not to be among them.

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18 356 Taken together, despite of significant decreased hepatic gene expression in TLR7 rs179008 T  
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20 357 compared to A allele patients, that might be due to improper virus sensing and that might  
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22 358 affect responsiveness to IL-29/IFN- $\lambda_1$  rather than IFN- $\alpha$ , differences in phenotype of disease  
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24 359 including hepatic viral load, natural outcome of infection, and disease activity and progression  
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27 360 appear to be minor with the exception of the presence of portal lymphoid aggregates in T  
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30 361 hemizygous males. Further investigations will elucidate the impact of this polymorphism on  
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32 362 responsiveness to endogenous and probably exogenous IFN- $\lambda$ .

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1 373 **LEGEND TO FIGURE 1:**

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6 375 **TLR7 ASTQ in Heterozygous Female Hepatitis C Patients**

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11 377 (A) ASTQ carried out on corresponding gDNA and cDNA samples from 3 representative  
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13 378 TLR7 rs179008 heterozygous female patients revealed either nearly equal amounts of both  
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15 379 alleles (top), a 2.7-fold excess of the A allele variant (middle), or a 2.6-fold excess of the T  
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17 380 allele variant (bottom). Analyses were made in duplicate, therefore the mean  $\Delta$  CT is given  
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19 381 but one representative amplification plot is shown.

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22 382 (B) ASTQ carried out on one representative pair of corresponding gDNA and cDNA samples  
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24 383 from a TLR7 rs179008 homozygote T patient yielded only non-specific signal for the allele A  
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26 384 as defined by a 10-fold less fluorescence intensity in the plateau phase at the end of the  
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34 387 **LEGEND TO FIGURE 2:**

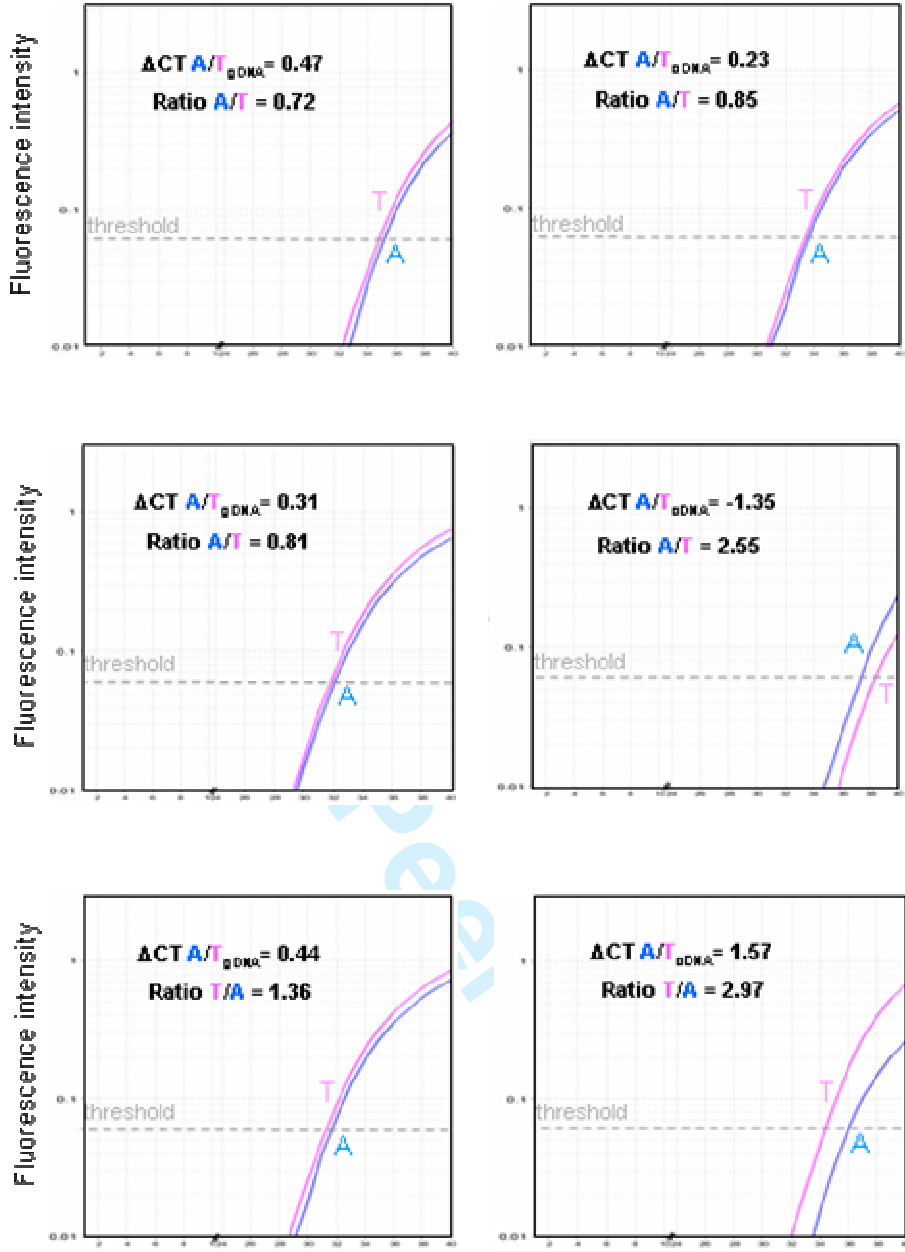
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39 389 **Hepatic Gene Expression in Chronic Hepatitis C Patients with Regard to TLR7**  
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41 390 **rs179008 Genotype**

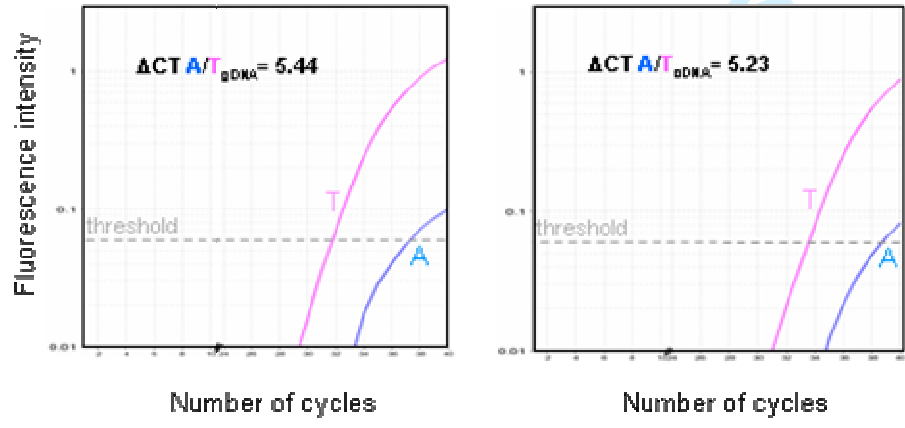
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45 392 Total cellular RNA from liver biopsy specimens taken from homo- and hemizygous patients  
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47 393 with chronic hepatitis C was quantified with respect to (A) HCV RNA, (B) p44, MxA, IP-10,  
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49 394 IFN- $\gamma$  and IFN- $\alpha$  in relation to albumin mRNA transcripts by using competitive quantitative  
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51 395 RT-PCR, and IL-10R $\beta$ , IL-28R $\alpha$ , IL-29 and IFNAR $_2$  in relation to GAPDH mRNA by using  
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53 396 quantitative real-time RT-PCR assays. Data are given as ratios of the target to reference gene  
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55 397  $\times 10^{-3}$ . Medians are indicated by horizontal bars. Levels of significance are given. Similar  
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57 398 results were obtained when data were related to  $\beta$ -actin (data not shown).  
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**A**



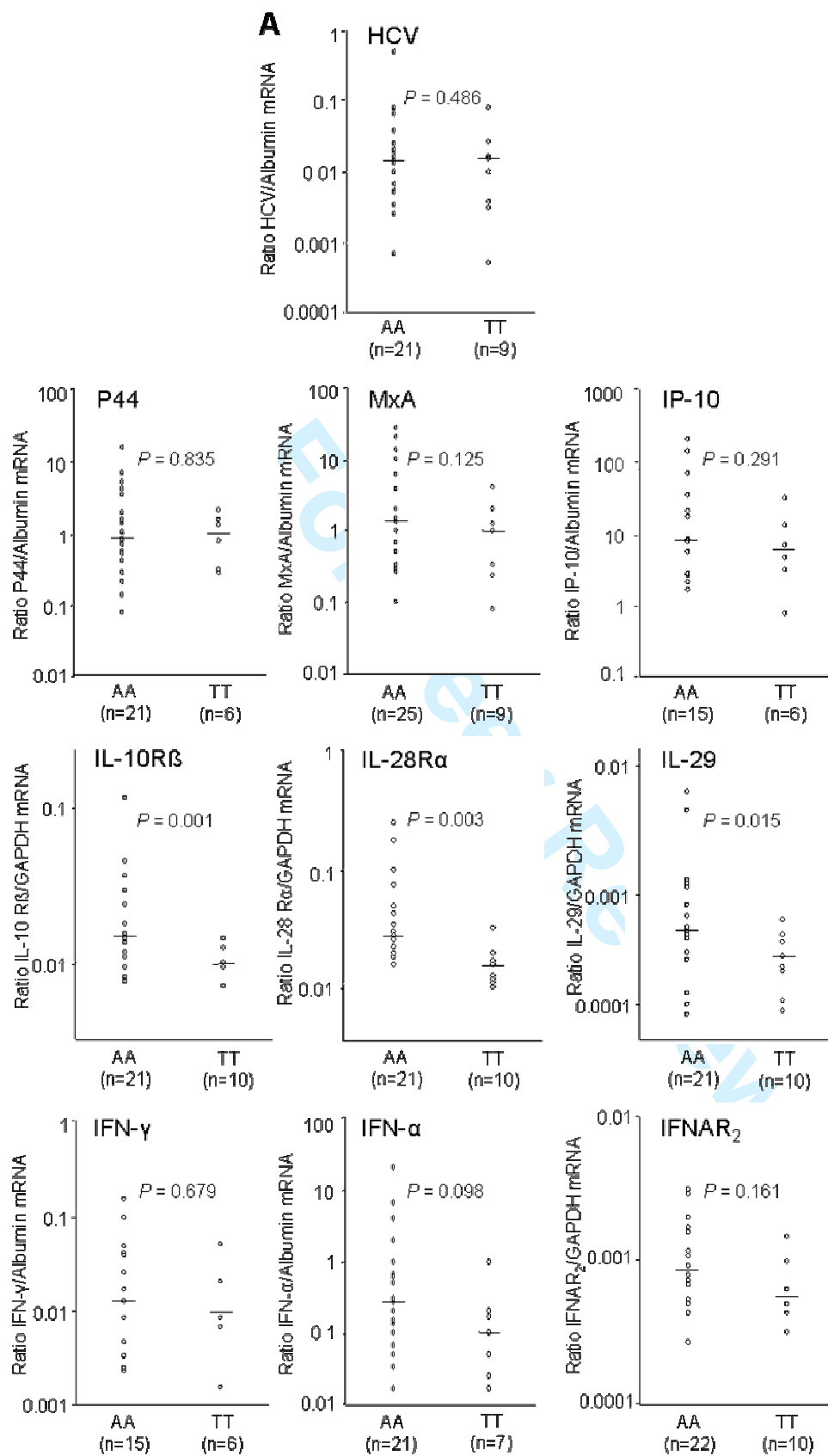
**B**



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Table I. TLR7 rs179008 Genotype Distribution in Patients with Chronic or Self-limited HCV Infection with Regard to Epidemiological and Biochemical Parameters

	<i>n</i>	TLR7 rs179008 genotype					<i>P</i>	MAF
		Females			Males			
		AA	AT	TT	A	T		
<b>Patients with chronic HCV infection</b>								
<i>Number (%)</i>	136	34 (56.7)	20 (33.3)	6 (10.0)	61 (80.3)	15 (19.7)	0.175 <sup>b</sup>	0.267/0.197 <sup>a</sup>
<i>Age (Mean±SD)</i>		45.8 ± 10.7	46.6 ± 11.5	50.0 ± 13.6	40.7 ± 11.2	46.9 ± 15.9	0.069 <sup>c</sup>	
<b>HCV types</b>								
<i>HCV type-1 n (%)</i>	109	28 (53.9)	19 (36.5)	5 (9.6)	47 (82.5)	10 (17.5)	0.449 <sup>d</sup>	0.279/0.175 <sup>a</sup>
<i>HCV non-1 n (%)</i>	27	6 (75.0)	2 (25.0)	0 (0)	14 (73.7)	5 (26.3)		
<b>Biochemical Serum Parameters</b>								
<i>AST (Median, IQR)</i>		23.0, 15-38	18.5, 13.5-31.5	45, 16-80	29, 18-67	36, 18-52		
<i>No. Patients with elevated/normal AST<sup>e</sup></i>	136	12/22	6/14	3/3	28/33	7/8	0.411 <sup>d</sup>	
<i>ALT (Median, IQR)</i>		32.5, 22-60	31.5, 20.5-44	58, 22-90	58, 36.5-129	78, 47-92		
<i>No. Patients with elevated/normal ALT<sup>e</sup></i>	136	13/21	7/13	3/3	36/25	12/3	0.078 <sup>d</sup>	
<i>γ-GT (Median, IQR)</i>		19.5, 10-45	19.5, 13.5-41	17, 5-34	39, 23.5-62.5	24, 14-72		
<i>No. Patients with elevated/normal γ-GT<sup>e</sup></i>	136	10/24	5/15	2/4	17/44	5/10	0.419 <sup>d</sup>	
<b>Patients with self-limited HCV infection</b>								
<i>Number (%)</i>	44	7 (50.0)	6 (42.9)	1 (7.1)	26 (86.7)	4 (13.3)	0.766 <sup>f</sup>	0.286/0.133 <sup>a</sup>
<i>Age (Mean±SD)</i>		39.3 ± 10.4	38.0 ± 13.2	30	36.0 ± 9.9	40.5 ± 9.7	0.722 <sup>g</sup>	

<sup>a</sup>MAF is given for females/males, respectively, <sup>b</sup> $\chi^2$ -test was applied to compare MAF between females and males. <sup>c</sup>Independent samples *t*-test was applied, the trend was valid only in males ( $P = 0.083$ ), <sup>d</sup> $\chi^2$ -test was applied to compare A with T homo- and hemizygous patients (combined females males).

<sup>e</sup>Markedly elevated serum activities of transaminases (> 2-fold of the upper normal limit) were considered. Upper normal limits for females/males, respectively are: 15 U/L/19 U/L for AST, 19 U/L/23 U/L for ALT; and 18 U/L/28 U/L for  $\gamma$ -GT.

<sup>f</sup> $\chi^2$ - test was applied to compare TLR7 rs179008 genotype distribution in patients with chronic- and patients with self-limited HCV infection.

<sup>g</sup>Independent samples *t*-test was applied.

**Table II. Histological Manifestations of Chronic Hepatitis C Patients with Regard to TLR7 rs179008 Genotype**

Histological manifestations	TLR7 rs179008 genotype					<i>P</i> <sup>a</sup>
	Females n (%)			Males n (%)		
	AA	AT	TT	A	T	
<b>Hepatitis activity</b>						
Mild	20 (57.1)	12 (34.3)	3 (8.6)	35 (85.4)	6 (14.6)	0.156
Moderate	13 (59.1)	6 (27.3)	3(13.6)	22 (75.9)	7 (24.1)	
Severe	1 (33.3)	2 (66.7)	0 (0)	4 (66.7)	2 (33.3)	
<b>Fibrosis</b>						
Absent	5 (55.7)	3 (33.3)	1(11.1)	9 (100.0)	0 (0)	0.222
Mild	18 (56.3)	11 (34.4)	3 (9.4)	29 (80.6)	7 (19.4)	
Moderate	6 (54.6)	4 (36.4)	1 (9.1)	11 (37.3)	4 (26.7)	
Marked	4 (66.7)	1 (16.7)	1(16.7)	5 (62.5)	3 (37.5)	
Cirrhosis	1 (50.0)	1 (50.0)	0 (0)	7 (87.5)	1 (12.5)	
<b>Steatosis</b>						
Absent	15 (60.0)	7 (28.0)	3(12.0)	27 (77.1)	8 (22.9)	0.509
Mild	12 (50.0)	10 (41.7)	2 (8.3)	20 (83.3)	4 (16.7)	
Moderate	3 (42.9)	3 (42.9)	1 (14.3)	9 (75.0)	3 (25.0)	
Marked	4(100.0)	0 (0)	0 (0)	5 (100.0)	0 (0)	
<b>Portal lymphoid aggregates</b>						
Absent	25 (64.1)	11 (28.2)	3 (7.7)	39 (88.6)	5 (11.4)	0.013 <sup>b</sup>
Present	9 (42.9)	9 (42.9)	3(14.3)	22 (68.8)	10 (31.3)	
<b>Bile duct damage</b>						
Absent	26 (63.4)	12 (29.3)	3 (7.3)	40 (85.1)	7 (14.9)	0.051
Present	8 (42.1)	8 (42.1)	3(15.8)	21 (72.4)	8 (27.6)	

<sup>a</sup> $\chi^2$ -test was applied on A and T homo- and hemizygous patients, to compare mild vs. moderate and severe hepatitis activity, absent, mild vs. moderate and marked fibrosis and cirrhosis, and absent, mild vs. moderate and marked steatosis.

<sup>b</sup>The difference is valid only in males ( $P = 0.032$ ).



**Table III. Initial Virological Response to an IFN- $\alpha_{2a}$  Monotherapy in Chronic Hepatitis C Patients with Regard to TLR7 rs179008 Genotype**

	TLR7 rs179008 genotype					<i>P</i>
	Females			Males		
	AA	AT	TT	A	T	
<b>No. Non-responsive patients (%)</b>	6 (35.3)	7 (41.2)	4 (23.5)	7 (70.0)	3 (30.0)	0.069 <sup>a</sup>
<b>No. Responsive patients (%)</b>	4 (50.0)	3 (37.5)	1 (12.5)	18 (90.0)	2 (10.0)	

<sup>a</sup> $\chi^2$ -test was applied to compare A with T homo- and hemizygous patients.

For Peer Review

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