



Short communication

Comparison of four methods of genotyping IL28B polymorphisms in chronic hepatitis C patients



Nathália Delvaux*, Vanessa Duarte da Costa, Maristella Matos da Costa, Elisabeth Lampe

Laboratory of Viral Hepatitis, Oswaldo Cruz Institute, FIOCRUZ, Avenida Brasil, 4365 – Mangueiras, Código, 21040-900 Rio de Janeiro, RJ, Brazil

A B S T R A C T

Article history:

Received 26 November 2014
Received in revised form 1 April 2015
Accepted 2 April 2015
Available online 9 April 2015

Keywords:

IL28B
SNP
HCV
Brazil
ARMS-PCR

Background: Single nucleotide polymorphisms (SNPs) of the interleukin 28B (IL28B) gene are associated with viral clearance and treatment response in hepatitis C virus (HCV) infection; however, most of the available SNP genotyping methods are expensive.

Aims: This study sought to evaluate the cost effectiveness of four methods used to genotype the rs12979860 and rs8099917 SNPs of the IL28B gene.

Methods: Tetra-primer amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR), restriction fragment length polymorphism (RFLP), quantitative (q) PCR and direct sequencing methods were evaluated in terms of specificity, cost and run time in 281 blood samples obtained from chronic HCV patients.

Results: In ARMS-PCR method, the primers designed to target both SNPs produced PCR fragments of specific sizes that distinguished the alleles of rs12979860 and rs8099917. In RFLP, the band profile allowed the distinction between genotypes. The qPCR was the faster and easier to perform. Validation by nucleotide sequencing showed 100% agreement among the three methods. The cost for a single reaction was lowest for ARMS-PCR, followed in turn by RFLP, qPCR and sequencing.

Conclusions: The methodology described for the ARMS-PCR showed the most favorable cost–benefit ratio. Moreover, this approach is fast and simple, requiring only equipment that is commonly used in molecular diagnosis, which is an essential parameter for use in developing countries where laboratories have scarce financial resources.

© 2015 Elsevier B.V. All rights reserved.

The treatment of chronic hepatitis C virus (HCV) infection, despite recent progress following the introduction of direct-acting antiviral (DAA) regimens, remains a great challenge in terms of cost effectiveness. Thus, the rapid identification of sustained virologic response (SVR) predictors remains a major target in HCV research. Two single nucleotide polymorphisms (SNPs) rs12979860 and rs8099917, in close proximity to the interleukin 28B (IL28B) gene, have reported associations with viral clearance and treatment response to HCV infection (Ge et al., 2009; Tanaka et al., 2009). The IL28B gene encodes interleukin 28, a cytokine belonging to the IFN- λ family that is involved in the regulation of the immune response against viral infections (Dellgren et al., 2009; Li et al., 2009). The SVR rate with PEG-IFN/RBV therapy in HCV-1 infected patients is two-fold higher in individuals with the rs12979860 CC genotype than

the CT or TT (70–80% vs. 30–40%) (Chen et al., 2011; Thompson et al., 2010). Similarly, the SVR is higher in individuals with the rs8099917 TT genotype compared to the TT or GT + GG genotype (81% vs. 59%) (Sakamoto et al., 2011). Several clinical studies have validated these findings and, IL28B genotyping has become an important tool to assist in clinical decisions regarding the most appropriate therapeutic regimen. Furthermore, IL28B genotyping has shown that it may be useful as a first-generation DAA approach for identifying patients who can be treated successfully with a shorter and simpler treatment scheme (Jacobson et al., 2011; Poordad et al., 2011). Additionally, the impact of the IL28B CC genotype (rs12979860) was observed in HCV-1a infected patients undergoing IFN-free combination therapy (58 to 84% among patients with IL28B CC vs. 33 to 64% in patients with non-CC genotypes) (Chu et al., 2011; Zeuzem et al., 2012, 2013).

IL28B genotyping can be determined using diverse methods, such as DNA sequencing, Taqman assays, PCR-RFLP, and DNA high-performance liquid Chromatography (DHPLC) (Fiorina et al., 2012; Medrano and de Oliveira, 2014). These methods have

* Corresponding author. Tel.: +55 21 2562 1894.

E-mail addresses: nmdr@ioc.fiocruz.br, nathaliandr@yahoo.com.br (N. Delvaux).

Table 1
Primers used in the tetra-primer ARMS-PCR and RFLP techniques.

SNP	Primer	Primer sequence (5'–3')	T _m (°C)	Amplicon (bp)
Tetra-primer ARMS-PCR				
rs12979860	ARMS 860F1 (Forward outer)	CCA GGG CCC CTA ACC TCT GCA CAG TCT G	76.0	277
	ARMS 860R1 (Reverse outer)	CTA TGT CAG CGC CCA CAA TTC CCA CCA C	76.0	
	ARMS 860F2T (Forward inner)	ACT GAA CCA GGG ACG TCC CCG AAG GAG T	75.0	198
	ARMS 860R2C (Reverse inner)	CGG AGT GCA ATT CAA CCC TGG TGC G	76.0	132
rs8099917	ARMS 917F1 (Forward outer)	CAT CAC CTA TAA CTT CAC CAT CCT CCT C	65.0	437
	ARMS 917R1 (Reverse outer)	GGT ATC AAC CCC ACC TCA AAT TAT CCT A	66.0	
	ARMS 917F2C (Forward inner)	CTT TTG TTT TCC TTT CTG TGA GCA GTG	65.0	197
	ARMS 917R2T (Reverse inner)	TAT ACA GCA TGG TTC CAA TTT GGG TAA A	66.0	295
RFLP and sequencing				
rs12979860	860F (Forward)	GCT TAT CGC ATA CGG CTA GG	60.0	242
	860R (Reverse)	AGG CTC AGG GTC AAT CAC AG	60.0	
rs8099917	917F (Forward)	TCA CCA TCC TCC TCT CAT CC	60.0	539
	917R (Reverse)	ACC CTC TCC CCT TCC TTT AG	59.0	

different characteristics, including equipment needs, cost and technical knowledge. However, some of the methods are excessively expensive, and their high cost of implementation limits their use, especially in developing countries (Ferreira et al., 2013; Galmozzi et al., 2011; Medrano and de Oliveira, 2014). Aiming to obtain a cheap and simple test to determine IL28B genotype, in house protocols for tetra-primer amplification-refractory mutation system-polymerase chain reaction (ARMS-PCR) and for restriction fragment length polymorphism (RFLP) were optimized in this study. Commercial qPCR method was also used and direct sequencing was used to validate these techniques. Additionally, the four methods used to determine the IL28B polymorphisms (rs12979860 and rs8099917) in patients with HCV infection were evaluated in terms of specificity, cost and run time.

ARMS-PCR, RFLP, quantitative (q) PCR and direct sequencing methods were carried out in 281 blood samples (108 males, aged 56.0 ± 10.9 years) obtained from chronic HCV patients (positive anti-HCV antibody and detectable HCV RNA in serum samples of patients with infection for more than 6 months). The local Ethical Committee (CEPN° 297.459) approved this study. Genomic DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Kit (Qiagen, Hilden, Germany), according to the manufacturer's directions, and stored at –20 °C.

In ARMS-PCR assay, to design the primers to target the two SNPs, we used the program developed by Ye et al. (2001), available at <http://primer1.soton.ac.uk/primer1.html>. The limiting of fragment sizes was chosen within the range of 100–300 bp with a ratio of allelic bands of 1.5. Default settings were used for the other parameters. For rs12979860, the forward primer (FIT 860) was designed to hybridize to the genomic sequence with the T allele, and the reverse primer (RIC 860) was designed to hybridize with sequences containing the C allele. The same procedure was conducted for rs8099917 (FIG 917 inner – G allele, RIT 917 inner – T allele). The primers designed for ARMS-PCR assay are showed in Table 1. PCRs for both SNPs were performed in a volume of 20 µL. Different amplification conditions (annealing temperatures, PCR cycle protocols and primer concentrations) were assessed to ensure proper formation of all the fragments. For rs12979860 the most appropriated PCR conditions were: 95 °C for 15 min followed by 35 cycles of 94 °C for 30 s, 64 °C for 30 s, 72 °C for 1 min and 72 °C for 10 min. For rs8099917, the best results were obtained with annealing temperature of 55 °C using the same cycling conditions. The PCR products were separated by standard electrophoresis on 2.5% agarose gels containing gel red dye (Biotium Inc., Hayward, CA), with a 100 bp molecular weight marker (Promega, Madison, Wisconsin, USA).

The amplification results showed that the ARMS-PCR method distinguishes successfully between the two different SNPs. In the rs12979860 genotyping method, the outer primer pairs produced

a band of 277 bp. The homozygote TT could be distinguished by an additional band of 198 bp, the homozygote CC could be distinguished by a band of 132 bp and heterozygote C and T alleles produced fragments of 132 bp and 198 bp, respectively. The genotypes of rs8099917 could be differentiated according to the following band profiles: TT – 437 bp and 295 bp; GG – 437 bp and 197 bp; and TG – 437 bp, 295 bp and 197 bp. Fig. 1A shows an agarose gel representing ARMS-PCR profiles for SNP rs12979860, and Fig. 1B shows the results for SNP rs8099917.

For RFLP, two pairs of primers were designed for each IL28B gene SNP (Table 1). The primer pairs 860F/860R were designed to amplify a fragment of 242 bp for the rs12979860, and the primer pairs 917F/917R were designed to amplify a fragment of 539 bp for rs8099917. The PCRs were performed in separate tubes containing 0.2 pmol of the respective primer pairs. For rs12979860, the PCR conditions were as follows: 95 °C for 15 min, followed by 35 cycles of 95 °C for 1 min, 58 °C for 45 s, 72 °C for 45 s and 72 °C for 3 min. Amplified PCR products were digested with the restriction enzyme BstUI (10 U/rxn; New England Biolabs) at 37 °C for 3.5 h. For rs8099917, PCR was conducted as follows: 95 °C for 15 min; 20 cycles of 95 °C for 1 min, 50 °C for 45 s, and 72 °C for 45 s; and 25 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and 72 °C for 3 min. The amplified PCR products were digested with the restriction enzyme Tsp45I (2 U/rxn; New England Biolabs) at 65 °C for 3.5 h. The digested products of both SNPs were separated by standard electrophoresis on 3% agarose gels containing gel red dye (Biotium Inc., Hayward, CA), alongside 100 bp Molecular Weight (Promega, Madison, Wisconsin, USA). The 242 bp PCR fragment obtained with the primers designed for typing rs12979860 digested with BstUI produced the following band profiles: 3 fragments of 25 bp, 82 bp and 135 bp in individuals with the CC genotype; 2 fragments of 82 bp and 160 bp in individuals with the TT genotype; and 4 fragments of 25 bp, 82 bp, 135 bp and 160 bp in individuals with the heterozygote CT genotype (Fig. 1C). The enzymatic digestion of the 539 bp rs8099917 fragment with Tsp45I, produced a band profiles that allowed the distinction between the GG (39 bp, 214 bp and 286 bp), TG (39 bp, 214 bp, 286 bp and 325 bp) and TT (214 bp and 325 bp) genotypes (Fig. 1D). qPCR was performed using a commercial kit from Roche Life Technologies (TIB MOLBIOL GmbH, Berlin, Germany), according to the manufacturer's instructions. The genotyping results for rs12979860 and rs8099917 with the qPCR method could distinguish between the respective SNP genotypes according the fluorescence profile.

Direct sequencing after PCR amplification was done with the same primers as those used for the RFLP for each polymorphism. The PCR products were purified using the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and submitted to nucleotide sequencing reactions in both directions using the Big Dye

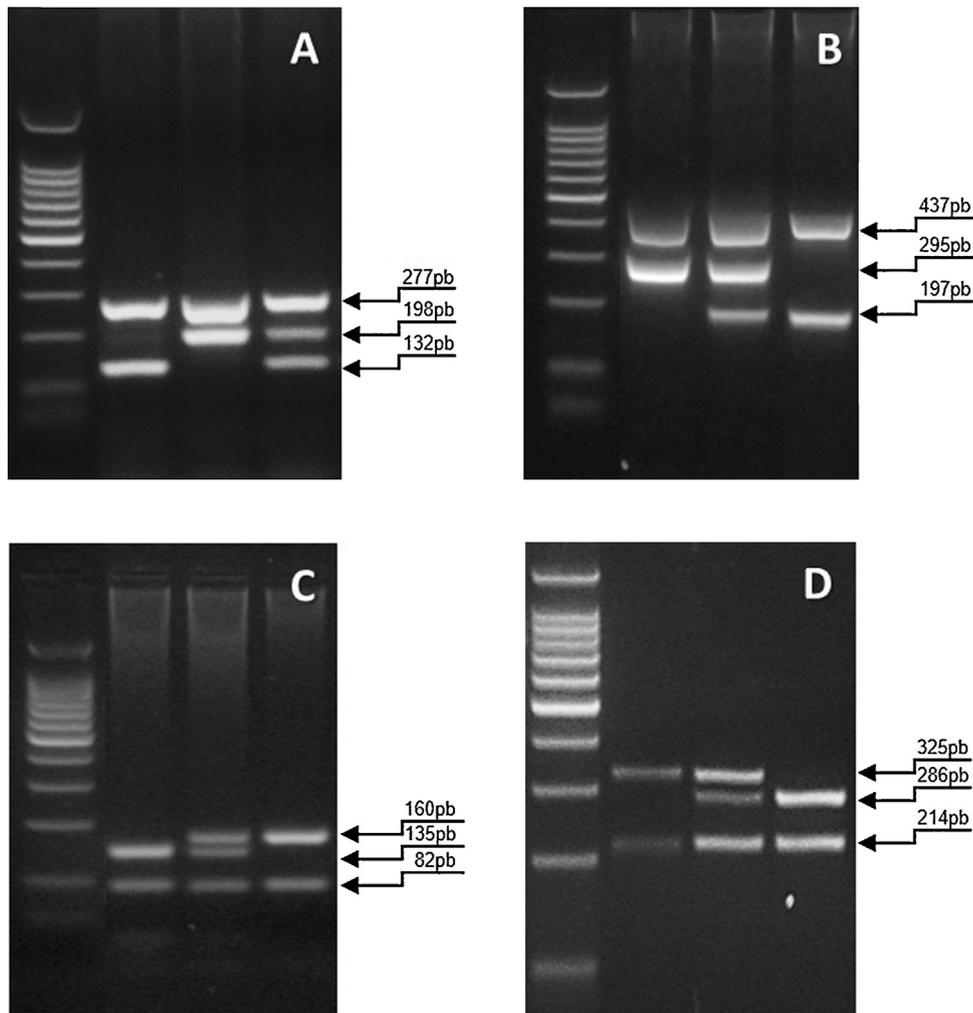


Fig. 1. (A) A 2.5% agarose gel showing ARMS PCR profiles for SNP rs12979860 (CC, TT and CT genotypes). (B) A 2.5% agarose gel showing ARMS PCR profiles for SNP rs8099917 (TT, TG and GG genotypes). (C) A 3% agarose gel showing RFLP profiles for SNP rs12979860 (CC, CT and TT genotypes). (D) A 3% agarose gel showing RFLP profiles for SNP rs8099917 (TT, TG and GG genotypes). Molecular Weight 100 bp (Promega).

Terminator kit 3.1 (Applied Biosystems, Foster City, CA, USA), according to the manufacturer’s instructions, followed by analysis on the ABI 3730 DNA automated sequencer (Applied Biosystems, Foster City, CA, USA). In sequencing chromatogram, sequences that showed single peaks were considered as homozygous, whereas those that showed double overlapping peaks were interpreted as heterozygous. All results obtained agreed 100% with the other three methods used in this study. The distribution of genotypes according to the methods used in this study is shown in [Table 2](#).

To estimate the cost of each technique, the price of reagents (enzymes, buffers, agarose, dyes, and other specific reagents for each procedure) and disposable materials (tips, polystyrene tubes, gloves) used in each of the methods was calculated based on

currently available commercial prices. Other indirect costs were not included, such as equipment maintenance and human resources. Thus, the price of a single reaction for ARMS-PCR, RFLP, qPCR and direct sequencing was, respectively: US\$19.40; US\$27.80; US\$22.90 and US\$202.80. The assay that requires more execution time was direct sequencing (2520 min or more), followed by RFLP (570 min), ARMS-PCR (300 min) and commercial qPCR (130 min).

ARMS-PCR methodology showed the lowest cost and easiest execution, as well as the second fastest run time. In addition, this method presents a wide convenience of execution; it requires only equipment that is routinely used in most laboratories that perform molecular biology assessments, such as a thermal cycler and electrophoresis apparatus. In this technique, the high specificity of the reaction relies on the 3’ terminus mismatch and the position-2 (second to the terminal) mismatch from the 3’ terminus of the same allele-specific primer. This last mismatch destabilizes the base pairing between the primers and their corresponding non-target templates and increases the specificity of the reaction by eliminating false-positive results (Ye et al., 2001). This method uses four primers in a single PCR reaction; two non-allele-specific primers (outer primers) amplify the region that comprises the SNP, and as the outer primer fragment is produced, it serves as a template for the two allele-specific primers (inner primers) that generate allele-specific fragments. By placing the outer primers at different distances from the SNP, the two allele-specific fragments can be

Table 2
Distribution of genotypes in 281 HCV chronic patients according to the methods used in this study.

Method	SNP rs12979860			SNP rs8099917		
	Genotypes			Genotypes		
	CC	CT	TT	TT	TG	GG
Tetra-primer ARMS-PCR	80	135	66	173	95	13
RFLP	80	135	66	173	95	13
Real-time PCR	80	135	66	173	95	13
Sequencing	80	135	66	173	95	13

distinguished by their different sizes in an agarose gel (Medrano and de Oliveira, 2014; Ye et al., 2001).

The RFLP technique was the second most expensive method and required a longer run-time. This method also requires two stages: PCR reaction and incubation with restriction enzymes. Furthermore, it requires two electrophoresis runs: one for analysis of the PCR amplicons and the other for analyzing the band profiles after enzymatic digestion that takes more than 3 h. In addition, restriction enzymes are high in cost and are extremely unstable. On the other hand, despite its higher cost, the equipments required for RFLP are routinely used in most molecular biology laboratories (thermal cycler, electrophoresis apparatus and water bath) and the training for this technique and interpretation of the results is not complex. Thus, even with disadvantages in several issues, the RFLP technique is, along with ARMS-PCR, very accessible to laboratories with few financial resources.

Among the four methods evaluated in this study, qPCR was the fastest and easiest to perform. Moreover, this method is sensitive and specific due to Taqman probe technology. qPCR was the second least expensive technique, even though it uses a commercial kit. However, this method requires a specific and expensive thermal cycler that is not available in many laboratories and requires additional training for the interpretation and analysis of data.

Direct sequencing is considered the gold standard and was used in this study to validate the results obtained with the other techniques. This method is the most time-consuming and laborious of the procedures evaluated. The number of steps required is large, including two PCR rounds, agarose gel electrophoresis, purification of PCR products, and diverse handling steps, until the sequence is obtained as a chromatogram. Also, it is necessary to have good knowledge of sequencing programs to analyze the data, especially in the heterozygous case. Moreover, the equipment is extremely expensive, and the maintenance cost is high because it demands several specific reagents. In the present work, the results of all three methods were 100% concordant with the results of direct sequencing.

In conclusion, all methods tested were specific for genotyping SNPs rs12979860 and rs8099917 of the IL28B gene. However, ARMS-PCR showed the best results according to the cost-benefit analysis. This approach represents a simple, fast and cost-effective method that involves a single PCR reaction followed by gel electrophoresis. Therefore, this technique is easy to use in a routine molecular diagnostic setting, with minimum equipment requirements. These results are particularly important for developing countries where laboratories generally have scarce financial resources.

Acknowledgments

We appreciate the contributions of Dr Maria L Abate, Dr Carlos E de Melo and Dr Antônio A Barone for the opportunity to complement our knowledge of the methodologies used. In addition, we wish to thank Geane L Flores, Islene Azevedo, Letícia P Scalioni, Moyra M Portilho and Selma XSL Pinheiro for technical assistance

in collecting blood samples. Also, authors thank to Oswaldo Cruz Foundation (FIOCRUZ); FAPERJ (grant No. 110.341/2012) and CNPq (grant No. 481564/2011-0) for financial support.

References

- Chen, J.Y., Lin, C.Y., Wang, C.M., Lin, Y.T., Kuo, S.N., Shiu, C.F., et al., 2011. IL28B genetic variations are associated with high sustained virological response (SVR) of interferon- α plus ribavirin therapy in Taiwanese chronic HCV infection. *Genes Immun.* 12 (4), 300–309, <http://dx.doi.org/10.1038/gene.2011.1>
- Chu, T., Kulkarni, R., Gane, E.J., Roberts, S.K., Stedman, C., Angus, P.W., et al., 2011. The effect of host IL28B genotype on early viral kinetics during interferon-free treatment in patients with chronic hepatitis C. *J. Hepatol.* 54 (S1), S521, <http://dx.doi.org/10.1053/j.gastro.2011.12.057>
- Dellgren, C., Gad, H.H., Hamming, O.J., Melchjorsen, J., Hartmann, R., 2009. Human interferon-lambda3 is a potent member of the type III interferon family. *Genes Immun.* 10 (2), 125–131, <http://dx.doi.org/10.1038/gene.2008.87>
- Ferreira, C.D.A.S., Abreu, R.M., da Silva, M.C., Ferreira, A.S., Nasser, P.D., Carrilho, F.J., et al., 2013. A fast and cost-effective method for identifying a polymorphism of interleukin 28B related to hepatitis C. *PLoS ONE* 8 (10), e78142, <http://dx.doi.org/10.1371/journal.pone.0078142>
- Fiorina, L., Paolucci, S., Papadimitriou, S., Baldanti, F., 2012. Comparison of three different methods for the evaluation of IL28 and ITPA polymorphisms in patients infected with HCV. *J. Virol. Methods* 184 (1–2), 103–105, <http://dx.doi.org/10.1016/j.jviromet.2012.05.006>
- Galmozzi, E., Del Menco, B., Ramezza, R., Dongiovanni, P., Fracanzani, A.L., Benedan, L., et al., 2011. A tetra-primer amplification refractory mutation system polymerase chain reaction for the evaluation of rs12979860 IL28B genotype. *J. Viral Hepat.* 18 (9), 628–630, <http://dx.doi.org/10.1111/j.1365-2893.2010.01349.x>
- Ge, D., Fellay, J., Thompson, A.J., Simon, J.S., Shianna, K.V., Urban, T.J., et al., 2009. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. *Nature* 461 (7262), 399–401, <http://dx.doi.org/10.1038/nature08309>
- Jacobson, I.M., McHutchison, J.G., Dusheiko, G., Di Bisceglie, A.M., Reddy, K.R., Bzowej, N.H., et al., 2011. Telaprevir for previously untreated chronic hepatitis C virus infection. *N. Engl. J. Med.* 364 (22), 2405–2416, <http://dx.doi.org/10.1056/NEJMoa1012912>
- Li, M., Liu, X., Zhou, Y., Su, S.B., 2009. Interferon-lambdas: the modulators of antiviral, antitumor, and immune responses. *J. Leukoc. Biol.* 86 (1), 23–32, <http://dx.doi.org/10.1189/jlb.1208761>
- Medrano, R.F., de Oliveira, C.A., 2014. Guidelines for the tetra-primer ARMS-PCR technique development. *Mol. Biotechnol.* 56 (7), 599–608, <http://dx.doi.org/10.1007/s12033-014-9734-4>
- Poordad, F., McCone Jr., J., Bacon, B.R., Bruno, S., Manns, M.P., Sulkowski, M.S., et al., 2011. Boceprevir for untreated chronic HCV genotype 1 infection. *N. Engl. J. Med.* 364 (13), 1195–1206, <http://dx.doi.org/10.1056/NEJMoa1010494>
- Sakamoto, N., Nakagawa, M., Tanaka, Y., Sekine-Osajima, Y., Ueyama, M., Kurosaki, M., et al., 2011. Association of IL28B variants with response to pegylated-interferon alpha plus ribavirin combination therapy reveals intersubgenotypic differences between genotypes 2a and 2b. *J. Med. Virol.* 83 (5), 871–878, <http://dx.doi.org/10.1002/jmv.22038>
- Tanaka, Y., Nishida, N., Sugiyama, M., Kurosaki, M., Matsuura, K., Sakamoto, N., et al., 2009. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. *Nat. Genet.* 41 (10), 1105–1109, <http://dx.doi.org/10.1038/ng.449>
- Thompson, A.J., Muir, A.J., Sulkowski, M.S., Ge, D., Fellay, J., Shianna, K.V., et al., 2010. Interleukin-28B polymorphism improves viral kinetics and is the strongest pre-treatment predictor of sustained virologic response in genotype 1 hepatitis C virus. *Gastroenterology* 139 (1), 120–129, <http://dx.doi.org/10.1053/j.gastro.2010.04.013>
- Ye, S., Dhillon, S., Ke, X., Collins, A.R., Day, I.N., 2001. An efficient procedure for genotyping single nucleotide polymorphisms. *Nucleic Acids Res.* 29 (17), E88, <http://dx.doi.org/10.1093/nar/29.17.e88>
- Zeuzem, S., Soriano, V., Asselah, T., Bronowicki, J.P., Lohse, A.W., Müllhaupt, B., et al., 2013. Faldaprevir and deleobuvir for HCV genotype 1 infection. *N. Engl. J. Med.* 369 (7), 630–639, <http://dx.doi.org/10.1056/NEJMoa1213557>
- Zeuzem, S., Soriano, V., Asselah, T., Bronowicki, J.P., Lohse, A.W., Müllhaupt, B., et al., 2012. SVR4 and SVR12 with an interferon-free regimen of BI 201335 and BI 207127, +/- ribavirin, in treatment-naïve patients with chronic genotype-1 HCV infection: interim results of SOUND-C2. *J. Hepatol.* 56 (Suppl. 2), S45.