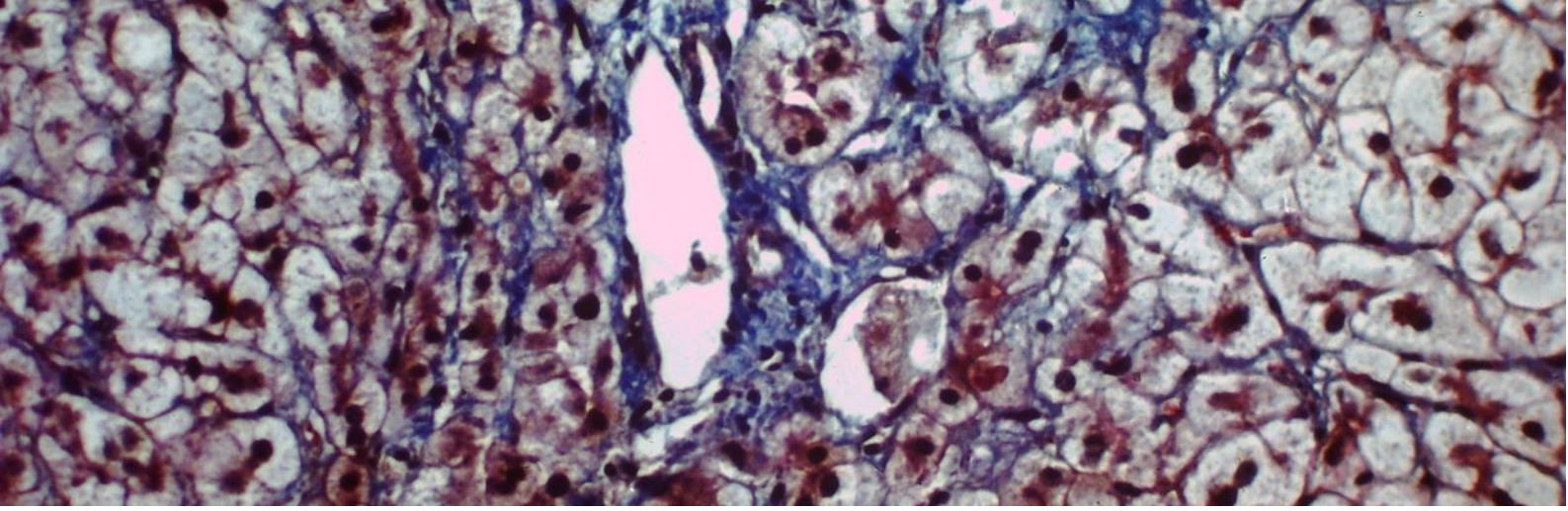

Interferon-stimulated gene expression in chronic hepatitis C virus genotype 1 infected patients treated with the NS3/4A protease inhibitor faldaprevir in combination with pegylated interferon alpha-2a/ribavirin

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Dedicated to my family

ABBREVIATIONS

ABI	Applied Biosystems
ALT	Alanine aminotransferase
BID	Twice daily
BT	Breakthrough
°C	Degree Celsius
cDNA	Complementary deoxyribonucleic acid
CI	Confidence interval
Ct	Cycle threshold
DAA	Direct-acting antiviral agent
DNA	Deoxyribonucleic acid
dsRNA	Double-stranded RNA
DVR	Delayed Virological Response
EVR	Early virological response
FC	Fold Change
FDV	Faldaprevir (former name BI 201335)
GT	Genotype
HCC	Hepatocellular carcinoma
HCV	Hepatitis C virus
ID	Identification
i.e.	id est (that is)
IFN	Interferon
IL28A, IL28B	Interleukin-28A, Interleukin-28B
IL29	Interleukin-29
IPS1	IFN-ß promoter stimulator 1
IRF3	Interferon regulatory factor 3
ISG	Interferon-stimulated gene
IU	International unit
JAK	Janus kinase
LI	Lead-in
MAVS	Mitochondrial antiviral signaling protein
mL	Milliliter
mRNA	Messenger RNA
NF-κB	Nuclear factor-kappa B
No.	Number
NS	Non-structural (viral protein)

n.s.	Not significant
NR	Null Response
OR	Odds Ratio
ORF	Open reading frame
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase Chain Reaction
PegIFN	Pegylated interferon α-2a
PR	Partial Response
PRR	Pattern-recognition receptors
QD	Once daily
qRT PCR	Quantative Real-Time PCR
r	Pearson correlation coefficient
RGT	Response guided treatment
RIG1	Retinoid acid inducible gene 1
RBV	Ribavirin
RNA	Ribonucleic acid
RVR	Rapid virological response
SD	Standard deviation
SDS	Sequence Detection System
SNP	Single-nucleotide polymorphism
SVR	Sustained virological response
TIR	Toll/Interleukin-1 receptor
TLR	Toll like receptor
TRIF	TIR -domain-containing adapter-inducing interferon-β
TE	Treatment-experienced
TN	Treatment-naïve
TYK	Tyrosine kinase
UTR	Untranslated regions
VISA	Virus-induced signaling adapter
VL	Viral load
ΔCt	Delta Ct
ΔΔCt	Delta Delta Ct

1. INTRODUCTION

1.1. Hepatitis C Virus (HCV)

Epidemiology

Hepatitis C virus infection (HCV) represents one of major public health problems in the world. It is estimated that approximately 130 – 210 million people worldwide (2.2 – 3.0%) are infected with HCV [49]. The prevalence of HCV infection varies substantially across geographic regions. In Europe the prevalence of HCV infection ranges from 0.4% to 6.0% with the highest prevalence in the Eastern Europe [25].

Natural History

The majority of acute HCV infections are asymptomatic. The incubation period until the first symptoms (if any) is on average 7 weeks ranging from 3 to 12 weeks. The transition from the acute to chronic hepatitis C infection and subsequent progression to the advanced liver disease is frequently asymptomatic or accompanied by non-specific symptoms. Up to 85% of patients develop chronic infection. These patients tend to have fewer symptoms as compared to those spontaneously resolved after the acute infection. Most common symptom of the HCV infection is intermittent fatigue. Serum ALT levels are continuously or intermittently elevated, but do not correlate with the disease activity [40].

At least 20% of patients with chronic HCV infection progresses to liver cirrhosis within 20 years and a significant proportion of them develop hepatocellular carcinoma (HCC). About 4% of patients with liver cirrhosis per year develop decompensation with the death rate from 15% in industrialized to 30% in developing countries [49].

Virus Structure

Hepatitis C virus, discovered in 1989 [18], is an enveloped single-stranded RNA (ribonucleic acid) virus of positive polarity which belongs to Hepacivirus in the family Flaviviridae. The genome is comprised of a positive-stranded RNA molecule of about 9600 nucleotides containing a single open reading frame (ORF) which encodes a large polyprotein of about 3,000 amino acids. The ORF is flanked by 5′ and 3′ untranslated regions (UTR) of 341 and 230 nucleotides in length, respectively [18, 44, 66].

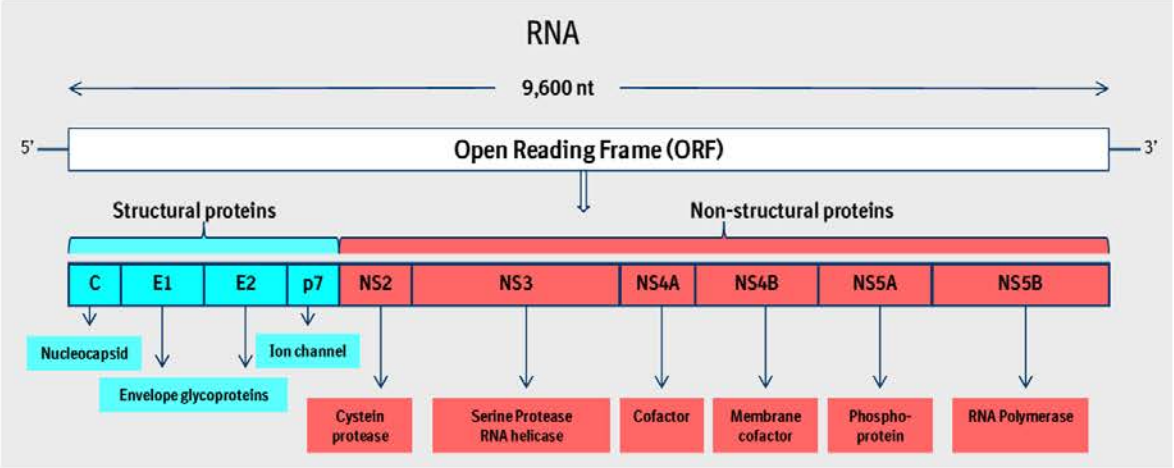

Figure 1: Schematic structure of HCV genome and the encoded proteins

HCV=Hepatitis C Virus; RNA=Ribonucleic acid; NS=Non-structural; nt=nucleotide.

One-third of the HCV genome encodes the structural proteins (core [C] protein, and glycoproteins E1 and E2). After the structural proteins the small integral membrane protein p7 is located. The rest of genome encodes the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) which play different roles in the virus life cycle and in particular HCV RNA replication [52, 62, 69].

Structural proteins consist of core protein that forms nucleocapsid and envelope glycoproteins E1 and E2 that are essential for viral replication [21, 62, 66, 69].

Non-structural proteins are involved in the protein synthesis and viral replication. NS2-NS3 proteinase is dedicated to the cleavage of NS2/NS3 site. NS3-NS4A complex consists of NS3 serine proteinase domain (189 amino acids) and NS helicase domain (442 amino acids). NS3 serine proteinase domain associates with the NS4A cofactor which consists of 54 amino acids [62, 66, 69]. Importantly, NS3/NS4 protease is able to cleave the toll like receptor 3 (TLR3) adaptor (TRIF) and mitochondrial antiviral signaling protein (MAVS) [16, 50, 60]. The ability to restore the interferon regulatory factor 3 (IRF3) signalling by NS3/NS4 protease inhibitors was seen in vitro [52]. The ability of faldaprevir to restore this signalling pathway by inhibiting NS3 protease will be investigated in this study. A mitochondrial antiviral signaling protein (MAVS), also called IPS1, Cardif or VISA mediates the activation of nuclear factor-kappa B (NF-κB) and IRF3 in response to viral infection [60, 78]. Cleaved MAVS results in less induction of Jak-STAT pathway and an inverse correlation between the cleavage of MAVS and the expression levels of interferon-stimulated genes (ISGs) was reported [12].The gene encoding this protein and other genes of this pathway are included in the list of genes analysed in this study.

Six genotypes (1, 2, 3, 4, 5 and 6) with variable number of subtypes (a, b, c etc) became a standard system for the nomenclature of HCV genotypes [46, 79, 80]. The most common subtype variants in Western countries are genotype 1 (subtypes 1a and 1b) and genotype 2 (subtypes a, b and c) [46]. Genotypes (GT) 1a and 1b account for about 60% of global infections. GT1a predominates in Northern Europe and North America, GT1b in Southern and Eastern Europe and Japan [88].

1.2. Current standard of care (interferon-based therapy)

Until recently, HCV antiviral drugs were associated with limited efficacy and significant toxicity. Treatment for 24 to 48 weeks with pegylated interferon-α (PegIFN) injections combined with ribavirin (RBV) represented the standard treatment. However, the combination of PegIFN/RBV induced a sustained virological response (SVR) in only 45% of patients with HCV GT1 infection [28, 57]. For patients chronically infected with HCV GT1 who failed PegIFN/RBV treatment, retreatment options were limited. Retreatment with PegIFN/RBV achieved SVR rates of approximately 36% in patients who relapsed and in 14% of patients who failed to respond to their previous regimen [42, 72].

Current practice guidelines reflect a flexible approach to management of chronic HCV infection and take into account the development of direct acting antivirals (DAAs) as well as identification of several single-nucleotide polymorphisms (SNPs) [34, 64]. The HCV protease inhibitors telaprevir (Incivek™) and boceprevir (Victrelis™) have been approved in many countries throughout the world, for use in combination with PegIFN/RBV. Monotherapy with either of these DAA agents has led to the rapid emergence of viral mutants with resistance to the respective compound and cross resistance to other compounds of the same class [45].

Treatment with telaprevir for 8 or 12 weeks, in combination with PegIFN/RBV, followed by PegIFN/RBV alone for 12 or 36 weeks achieved SVR rates of 69% and 75%, respectively, in treatment-naïve patients with HCV GT1 infection [41]. Boceprevir, when given for 24 or 44 weeks, in combination with PegIFN/RBV, achieved similar SVR rates of 66% or 76%, respectively [71]. Thus, the combination of a protease inhibitor with PegIFN/RBV represents one of the latest treatment options for patients with HCV GT1 infection.

1.3. Treatment goals, definitions and response criteria

The primary goal of the treatment of HCV infection is the elimination of virus and achievement of undetectable HCV RNA 24 weeks after the end of treatment which is called SVR (Sustained Virological Response). SVR is defined as an undetectable HCV RNA level (<50 IU/ml) 24 weeks after treatment completion. It is the accepted surrogate parameter for virologic cure of the disease [24].

The following definitions and response criteria are used for the monitoring of the HCV treatment.

Table 1: Virological response definitions
Source: EASL 2011 Practice Guideline [24]

Abbreviation	Term	Definition
SVR	Sustained virological response	Undectable HCV RNA level (<50 IU/ml), 24 weeks after treatment
RVR	Rapid virological response	Undectable HCV RNA in a sensitive assay (lower limit of detection 50 IU/ml) at week 4 of therapy, maintained up to end of treatment
EVR	Early virological response	HCV RNA detectable at week 4 but undetectable at week 12, maintained up to end of treatment
DVR	Delayed virological response	More than 2 $\log_{10}$ drop but detectable HCV RNA at week 12, HCV RNA undetectable at week 24, maintained up to end of treatment
NR	Null response	Less than 2 $\log_{10}$ IU/ml decrease in HCV RNA level from baseline at 12 weeks of therapy
PR	Partial nonresponse	More than 2 $\log_{10}$ IU/ml decrease in HCV RNA level from baseline at 12 weeks of therapy, but detectable HCV RNA at weeks 12 and 24
BT	Breakthrough	Reappearance of HCV RNA at any time during treatment after virological response

Predictors of treatment response

The response guided treatment (RGT) concepts became an important part of the management of chronic HCV infected patients. This flexible approach may help to shorten the treatment duration and better predict the chances to achieve SVR [27, 74]. RGTs are considered during the triple therapies of DAAs with PegIFN/RBV and will be essential for the new treatment combinations. IL28B genetic polymorphism represents one of the best biomarkers predicting the treatment response (see section 1.4).

1.4. Host response to HCV infection

Pattern-Recognition Receptors (PRR)

Several classes of pattern-recognition receptors (PRRs) are described including toll-like receptors (TLR) which recognize distinct microbial and viral components and directly activate immune cells [2]. 12 different TLRs are characterized that are located in different types of cells and responsible for detection of microbial components, known as pathogen associated molecular patterns (PAMPs) [2, 87]. TLR3 specifically recognizes dsRNA which also senses HCV [51]. TLR7 and TLR8 are specific for ssRNA [2, 87].
TLR3, activated by HCV RNA, induces the cascade of reactions including activation of interferon regulatory factor 3 (IRF3) and nuclear factor-kappa B (NF-κB) [86]. There is another type of PRR, newly reported RIG1 (retinoid acid inducible gene 1) like receptors that bind nucleic acid [29, 30, 87]. In hepatocytes these two independent pathways of retinoic-acid-inducible gene 1 (RIG1) and TLR3 signalling comprise major pathways of host defence triggering by dsRNA [29]. In this study we characterize in vivo the changes of selected interferon-stimulated genes (ISGs), like the genes encoding TLR3 and RIG1. This should contribute to the understanding the host response processes and may help to optimize the treatment of HCV infected patients. A discovery of additional predictive biomarker, like IL28B, may help to optimize RGTs and shorten the treatment duration.

Interferons (IFN) and Interleukin-28B (IL28B)

Interferons (IFN) are divided into 3 types based on the receptor binding properties. Type I interferon includes IFN-a, IFN-β, IFN-ε, IFN-κ, IFN-ω . Type II includes IFN- γ. The type III group consists of IFN-like cytokines: interleukin-28A (IL28A), IL28B, and IL29 [67, 68]. Genetic polymorphism on the chromosome 19 near the gene encoding IL28B was reported by several independent pharmacogenetic studies to be strongly associated with the changed rate to achieve treatment response. Among various analyzed SNPs, rs12979860 SNP is predictive of better response and rs8099917 SNP is better predictive of non-response to PegIFN/RBV treatment. The rs12979860 CC genotype is associated with a more than 2 fold increased rate of SVR after PegIFN/RBV treatment than unfavorable CT or TT genotypes [33, 83, 84].

Interferon-Stimulated Genes (ISGs)

Activation of IRF3 and NF-κB triggers the cascade of the signalling reactions which lead to the induction of many interferon-stimulated genes (ISGs). Overall, hundreds of ISGs are

described to date [36, 39, 43, 65, 76, 85, 86]. They are the large group of genes that play various functions to limit infection, including the influence on the HCV lifecycle in the infected cells as well as the triggering the host defence mechanisms in the healthy neighbour cells limiting the cell to cell spread of the virus. Some of them, their effectors and functions are well characterized, but many of them are still not sufficiently investigated. Many studies have been performed to date to investigate different expressions of ISGs during HCV infection and to distinguish non-responders from good responders to the treatment with PegIFN/RBV [36, 39, 43, 65, 76, 85, 86]. In our study we investigate ISGs during the triple therapy with faldaprevir in combination with PegIFN/RBV.

1.5. Faldaprevir (FDV)

Faldaprevir is a low molecular weight, peptidomimetic linear inhibitor of the HCV serine protease for the treatment of patients with chronic HCV infection. Faldaprevir was optimised to inhibit HCV GT1a/1b NS3/NS4A protease activity, which is required for the maturation of HCV viral polyprotein during the HCV replication cycle.

In phase 1b studies, faldaprevir combined with PegIFN/RBV demonstrated strong antiviral responses and was well tolerated in treatment-naïve (TN) and treatment-experienced (TE) HCV GT1 infected patients [57]. In a phase 2b study of faldaprevir, up to 84% of treatment-naïve GT1 patients achieved SVR and the safety and tolerability profile of faldaprevir was found to be favorable [81]. Moreover, up to 87% of patients achieved the criterion of a maintained rapid virologic response (HCV RNA <25 IU/mL at week 4 and undetectable from week 8 to week 20) and qualified for shortened treatment duration with 24 weeks overall treatment [81]. In patients who had never achieved undectable HCV RNA during prior treatment with PegIFN/RBV (non-responders), rates of SVR were up to 41% [82].

1.6. SILEN-C1&2 Trials

SILEN-C1&2 (**S**afety and ant**I**vira**L** **E**ffect of faldaprevir i**N** hepatitis **C**) were the phase II, multi-centre, randomised, double-blind, placebo-controlled trials which investigated antiviral effect, safety and PK of 120 mg or 240 mg QD FDV in treatment-naïve (TN) patients (SILEN-C1) and of 240mg QD or 240 mg BID FDV in treatment-experienced (TE) patients (SILEN-C2) for 24 weeks in combination with 24 or 48 weeks of PegIFN/RBV with or without a 3-day lead-in (LI) with PegIFN/RBV [19].

429 treatment-naïve and 290 treatment-experienced HCV infected patients were randomized in these trials. The optional substudy for the investigation of the interferon-stimulated gene expression was offered to the patients participating in SILEN-C1&2 trials. The patients who gave the specially designated informed consent for the participation in this optional ISG substudy were included into this thesis. Prior to start, the clinical trial protocol SILEN-C1&2 which included the optional ISG substudy, the patient information and informed consent form, and other locally required documents were reviewed by the Independent Ethics Committees and/or Institutional Review Boards and Competent Authorities of the participating countries and gave favorable opinion for the study.

1.7. Objectives and hypothesis

The objective of this thesis was to investigate the expression of selected ISGs in PBMC (peripheral blood mononuclear cell) mRNA (a) prior and (b) during the treatment with NS3/NS4A protease inhibitor faldaprevir plus PegIFN/RBV as compared to PegIFN/RBV alone in different sub-groups of treatment-naïve and treatment-experienced HCV GT1a and 1b patients and evaluate their predictive role for the treatment success (SVR).
Hypotheses of this thesis were:

- Baseline and on-treatment expression of various ISGs may have predictive role for the treatment success (SVR)
- Different expression and induction of ISGs may differentiate treatment response to PegIFN/RBV plus faldaprevir
- Differences in induction of ISGs in faldaprevir groups may confirm in vivo a role of protease inhibitor in reactivation of NS3/NS4A disrupted IFN signaling

2. PATIENTS, MATERIALS UND METHODS

This thesis has been performed in three consecutive steps.
First, we selected 95 most representative ISGs based on the literature review (Step 1: Gene selection by literature review).
Secondly, we included 30 patients (see section 2.3.2) and tested all 95 genes in these patients (Step 2: SVR-oriented exploratory screening).
Finally, we tested 15 genes selected during the SVR-oriented exploratory screening in all patients eligible for this study (N=263, Step 3: confirmatory gene expression analysis).

2.1. Patients

This investigation was added to the double-blind, parallel-group and placebo controlled, multi-centre, phase IIb clinical trials SILEN-C1 and SILEN-C2 (Co-ordinating Investigator: M. Sulkowski, M.D., Johns Hopkins University, Baltimore, MD, USA; Sponsor: Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany, Medical Trial Leader: Y. Datsenko, Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach an der Riss, Germany; ClinicalTrials.gov Identifier: NCT00774397).
Overall, 429 treatment-naïve (SILEN-C1) and 290 treatment-experienced (SILEN-C2) patients were randomized by 100 trial sites in 15 countries (Argentina, Australia, Austria, Canada, Czech Republic, France, Germany, Great Britain, Korea, Netherlands, Portugal, Romania, Spain, Switzerland and USA). 364 patients from these trials have signed the specially designated informed consent for the optional substudy to investigate the expression and induction of interferon-stimulated genes (ISG substudy) and were considered for this investigation. 293 patients were eligible for the analysis.

Diagnosis and main criteria for inclusion

ISG Substudy inclusion criteria: Availability of the respective baseline blood sample and at least one on-treatment blood sample (4 hours, day 4 or day 8).

SILEN-C1 main inclusion criteria

Treatment-naïve female and male patients, aged 18 to 65 years, with chronic HCV infection with genotype 1 (1a, 1b, or mixed 1a/1b), with an HCV viral load $\geq$100 000 IU/mL at screening. No prior therapy with interferon, pegylated interferon, or ribavirin was allowed. Patients with HCV of mixed GT, hepatitis B virus, human immunodeficiency

virus, decompensated liver disease, or hyperbilirubinemia (>1.5 X upper limit of normal) were excluded; patients with Gilbert's polymorphism were accepted.

SILEN-C2 main inclusion criteria

Treatment-experienced patients with confirmed virologic failure during or after at least 12 weeks of combination treatment with an approved dose of PegIFN α-2a or α-2b combined with RBV. Per protocol, virologic failure was defined as either a <1 log_{10} maximum reduction in HCV RNA any time during treatment (null-response), or a maximal reduction in HCV RNA at any time point ≥1 log_{10} but never having achieved HCV RNA below the level of detection (partial-response). Relapsers, who experienced HCV RNA suppression during, and rebound after the end, of prior HCV treatment, were excluded. All other inclusion and exclusion criteria were identical in SILEN-C 1 and SILEN-C2 trials.

SILEN-C Trial Designs

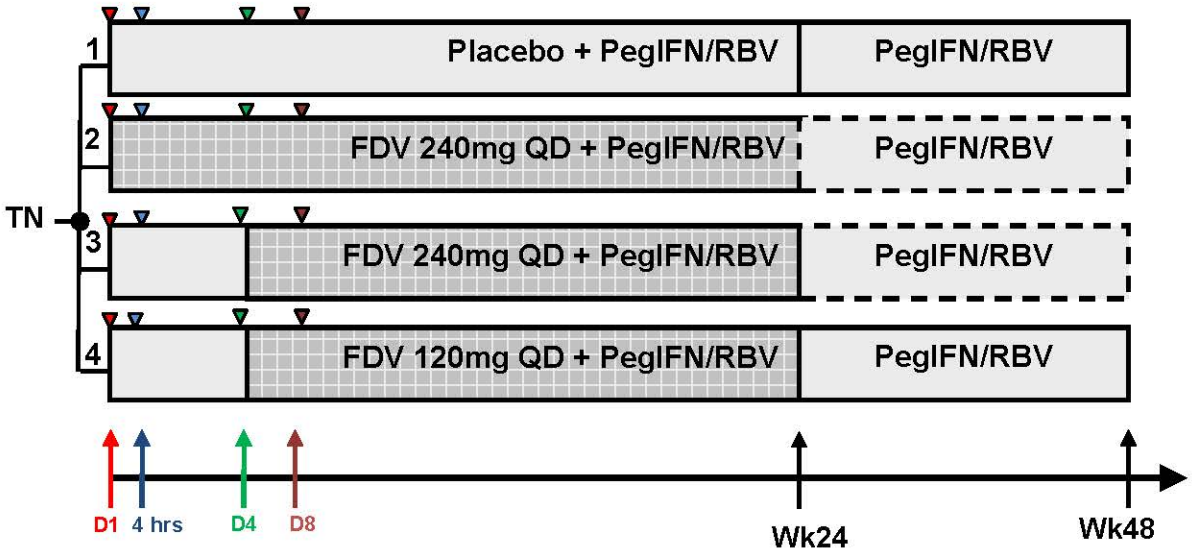

Figure 2: SILEN-C1 Trial Design

TN=treatment-naïve patients; D=day, Wk=week; QD=once daily; PegIFN/RBV=pegylated interferon α-2a/ribavirin; FDV=faldaprevir.
ISG substudy included blood sampling on Days 1, 4, and 8 of treatment. Blood collections on Day 1 included two sampling time points: one pre-dose and one 4 (+/-1) hours post dosing in all groups. Blood sampling at days 4 and 8 was performed prior to the scheduled dose at that day.

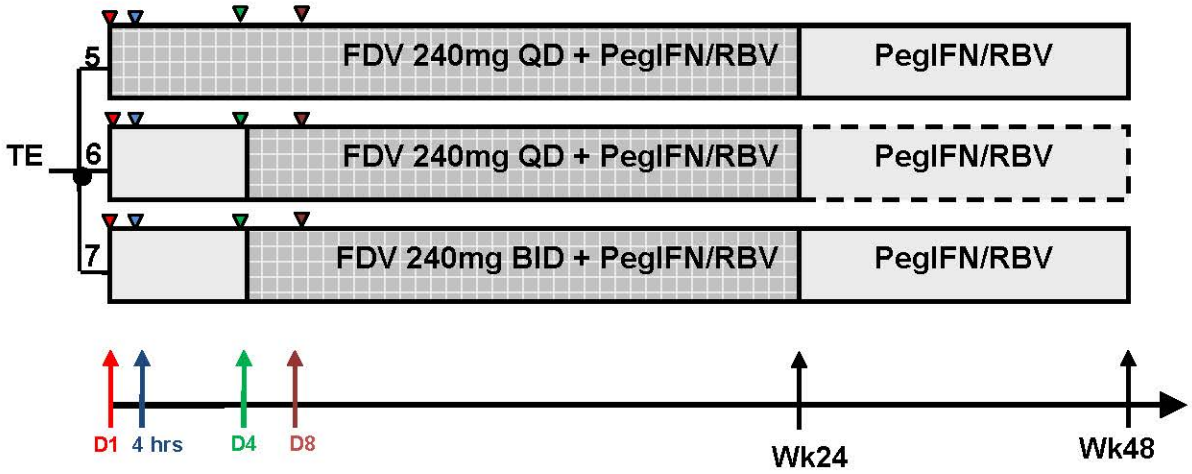

Figure 3: SILEN-C2 Trial Design

TE=treatment-experienced patients; D=day, Wk=week; QD=once daily; BID=twice daily; PegIFN/RBV=pegylated interferon α-2a/ribavirin; FDV=faldaprevir.
ISG substudy included blood sampling on Days 1, 4, and 8 of treatment. Blood collections on Day 1 included two sampling time points: one pre-dose and one 4 (+/-1) hours post dosing in all groups. Blood sampling at days 4 and 8 was performed prior to the scheduled dose at that day.

Selection of Patients

30 patients from SILEN-C1 and SILEN-C2 trials were selected for the SVR-oriented exploratory screening. 263 patients were included into the confirmatory gene expression analysis. Other patients (N=71) who signed informed consent, but were not included into the analysis had either withdrawn informed consent or were excluded due to the missing baseline blood sample or all 3 on-treatment blood samples (see Table 2).

Table 2: Patients overview

FDV=faldaprevir; PegIFN/RBV=pegylated interferon α-2a/ribavirin; QD=once daily; BID=twice daily,
*LI - lead-in (3-day lead-in period of PegIFN/RBV), N=number of patients
** - Number of patients who signed informed consent regardless of the availability of samples and quality of RNA at any visit.

Group		ISG Substudy Informed Consent**(N)	SVR-oriented exploratory screening (N)	Confirmatory gene expression analysis (N)
1	Placebo + PegIFN/RBV	40	10	23
2	FDV 240 mg QD + PegIFN/RBV	78	10	54
3	FDV 240 mg QD LI* + PegIFN/RBV	74	-	56
4	FDV 120 mg QD LI* + PegIFN/RBV	42	-	38
5	FDV 240 mg QD + PegIFN/RBV	32	10	47
6	FDV 240 mg LI* + PegIFN/RBV	63	-	16
7	FDV 240 mg BID LI* + PegIFN/RBV	35	-	29
Total		**364**	**30**	**263**

2.2. Materials

2.2.1. Blood samples

Blood samples were drawn into the PAXgene Blood RNA Tube. Two 2.5 ml PAXgene RNA Blood tubes were collected at pre-dose time point (baseline), 4 hours after the first PegIFN injection, at day 4 and day 8 (see Table 3). The samples (PBMC, type of the cells was not differentiated) were stored in a wire rack at room temperature (18°C– 25°C) for a minimum of 2 hours and maximum of 72 hours before transferring the rack to a freezer (-20°C or colder). The samples were shipped to the central laboratory in a temperature controlled shipment environment.

Table 3: Time points of samples collection

Baseline	4 hours	Day 4	Day 8
Prior to the treatment start	4 hours (+/- 1 hour) after the first dose of pegylated interferon α-2a.	3 days after the treatment start. Prior to dosing at day 4.	7 days of the treatment start. Prior to dosing at day 8.

2.2.2. Materials and assays for qRT-TaqMan PCR

The following materials were used for the RNA isolation, reverse transcription to DNA and Quantitative Real-Time (qRT) Polymerase Chain Reaction (PCR).

Table 4: Materials for qRT-TaqMan PCR

Material	**Source**
Nuclease-Free Water	Promega
Gene Expression MasterMix	Applied Biosystems
384-Well Clear Optical Reaction Plate	Applied Biosystems
Optival Adhesive Cover	Applied Biosystems

Table 5: Assays for qRT-TaqMan PCR

* - Assay Type: TaqMan Gene Expression Assay; Label: 5' FAM/3' MGB.

No.	Gene ID – Assay ID*	No.	Gene ID – Assay ID*	No.	Gene ID – Assay ID*
	18S-Hs99999901_s1 (housekeeper gene)	32	IFI44-Hs00197427_m1	64	MX1-Hs00895608_m1
1	ADAR-Hs00241666_m1	33	IFI44L-Hs00199115_m1	65	MX2-Hs00159418_m1
2	APOBEC3A-Hs00377444_m1	34	IFI6-Hs00242571_m1	66	NMI-Hs00190768_m1
3	ATF5-Hs01119208_m1	35	IFIH1-Hs01070332_m1	67	NT5C3-Hs00828759_g1
4	BLZF1-Hs00388707_m1	36	IFIT1-Hs01675197_m1	68	OAS1-Hs00973637_m1
5	C1S-Hs01043795_m1	37	IFIT2-Hs00533665_m1	69	OAS2-Hs00942643_m1
6	C3AR1-Hs00377780_m1	38	IFIT3-Hs00155468_m1	70	OAS3-Hs00196324_m1
7	CCL2-Hs00234140_m1	39	IFIT5-Hs00202721_m1	71	OASL-Hs00984390_m1
8	CCL8-Hs99999026_m1	40	IFITM1-Hs01652522_g1	72	PKLR-Hs00176075_m1
9	CCR1-Hs00174298_m1	41	IFITM2-Hs04194297_g1	73	PLSCR1-Hs01062171_m1
10	CD38-Hs01120071_m1	42	IFITM3-Hs01635484_gH	74	PML-Hs00971694_m1
11	CDK18-Hs00384387_m1	43	IFNA4-Hs01681284_sH	75	RNF125-Hs00215201_m1
12	CHMP5-Hs00603789_mH	44	IFNAR2-Hs01022060_m1	76	RPL22-Hs01865331_s1
13	CXCL10-Hs00171042_m1	45	IL18BP-Hs00271720_m1	77	RPLP2-Hs01115130_g1
14	CXCL11-Hs00171138_m1	46	IL1RN-Hs00893626_m1	78	RPS28-Hs02597258_g1
15	CXCL9-Hs00171065_m1	47	IL28RA-Hs00417120_m1	79	RSAD2-Hs00369813_m1
16	DDIT4-Hs01111686_g1	48	IRF1-Hs00233698_m1	80	SAMD4A-Hs00324455_m1
17	DDX58-Hs00204833_m1	49	IRF2-Hs01082884_m1	81	SERPING1-Hs00163781_m1
18	DDX60-Hs00214153_m1	50	IRF3-Hs01547283_m1	82	SIGLEC1-Hs00988063_m1
19	DHX58-Hs00225561_m1	51	IRF7-Hs00185375_m1	83	SOCS1-Hs00864158_g1
20	DUSP1-Hs00610256_g1	52	IRF9-Hs00196051_m1	84	SP100-Hs00162109_m1
21	EIF3L-Hs00275016_m1	53	ISG15-Hs00192713_m1	85	SP110-Hs00893490_m1
22	EPHB2-Hs00362096_m1	54	ISG20-Hs00158122_m1	86	SSBP3-Hs00401909_m1
23	GAPDH-Hs99999905_m1	55	LAMP3-Hs00180880_m1	87	STAT1-Hs01013996_m1
24	GBP1-Hs00977005_m1	56	LAP3-Hs00429769_m1	88	STXBP5-Hs00291072_m1
25	HERC5-Hs00180943_m1	57	LGALS9-Hs00371321_m1	89	TBX3-Hs00195612_m1
26	HERC6-Hs00215555_m1	58	LY6E-Hs00158942_m1	90	TLR7-Hs00152971_m1
27	HESX1-Hs00172696_m1	59	MAP3K14-Hs00177695_m1	91	TNFSF10-Hs00921974_m1
28	HPSE-Hs00935036_m1	60	MAVS-Hs00920075_m1	92	TRIM5-Hs01552559_m1
29	IFI16-Hs00194261_m1	61	MB21D1-Hs00403553_m1	93	UBE2L6-Hs01125548_m1
30	IFI27-Hs01086370_m1	62	MOV10-Hs00253093_m1	94	USP13-Hs00187594_m1
31	IFI35-Hs00413458_m1	63	MS4A4A-Hs00254770_m1	95	USP18-Hs00276441_m1

2.3. Methods

2.3.1. Gene selection by literature review

The literature search was performed in PubMed and SCOPUS databases using the following terms and their combination: *ISG, interferon-stimulated gene, gene expression, interferon signaling, HCV, Hepatitis C*, narrowing the selection by the year after 2001. Identified publications were reviewed for relatedness and inclusion criteria.
Key selection criteria were (valued in the descending order):

- Publications should be based on the in-vivo data.
- The data should be derived from the patients with HCV infection. Studies in experimental models were not considered for the primary selection.
- As our source of mRNA was PBMC, some publications should describe ISG expression in PBMC or the comparison of the gene expression in liver tissue and PBMC.
- Identified genes should have played a role in the treatment response (e.g. significant difference between responders and non-responders) to PegIFN/RBV treatment.
- Publication date after 2001.

Ingenuity® Systems Database

Selected genes were reviewed and analyzed through the use of IPA, Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com) which represents the software for modeling, analyzing and understanding complex biological and chemical systems based on the most current knowledge available on: genes, drugs, chemicals, protein families, normal cellular and disease processes, signaling and metabolic pathways.

Most common canonical pathways by measuring the significance of the association between the data set and the canonical pathway was reviewed to confirm the relevance of the gene set for our analysis. Additionally, we looked at the molecular relationships between molecules using the graphical representation (overlapping networks). Ingenuity IPA, Version 1440082, date 01-Dec-2012.

2.3.2. Selection of patients for the SVR-oriented exploratory screening

In order to get the groups with the most pronounced differences in the gene expression, we selected the half of patients with SVR to be compared with the patients without SVR.

For the balanced distribution across 7 available treatment arms from SILEN-C1 and SILEN-C2, we selected the patients from placebo arm to be compared with the patients from FDV 240 mg QD treatment arm (highest dose in the treatment-naïve patients). Secondly, we selected the patients from the FDV 240 mg QD arm of treatment-experienced patients arm to be compared with the FDV 240 mg QD arm of the treatment-naïve patients. The final selection comprised of 6 groups (see Table 6).

Table 6: Selection of the groups for the SVR-oriented exploratory screening

TN=treatment-naïve patients; TE=treatment-experienced patients; QD=once daily; PegIFN/RBV=pegylated interferon α-2a/ribavirin; FDV=faldaprevir; SVR=sustained virological response; Non-SVR=non-responder.

TN/TE	Treatment	Exploratory Screening Groups	
		SVR	Non-SVR
TN	Placebo + PegIFN/RBV	Group 1 (G1)	Group 2 (G2)
TN	FDV 240 mg QD + PegIFN/RBV	Group 3 (G3)	Group 4 (G4)
TE	FDV 240 mg QD + PegIFN/RBV	Group 5 (G5)	Group 6 (G6)

The sample size was based on the feasibility to perform the analysis of up to 100 genes at 4 time points per patient and gene. A total of 30 patients (5 patients per group) was feasible for this analysis and considered sufficient for this exploratory step.

The selection of the patients from each of the defined treatment arm was random, but based on the blood sample availability at each time point and highest quality of the extracted RNA. Patients with missing samples or the samples with the low quality of RNA were not included.

2.3.3. Gene expression analysis

RNA isolation and purification, reverse transcription and gene expression analysis via TaqMan® PCR was performed in the laboratories of Boehringer Ingelheim Pharma, Biberach by Dr. Patrick Baum, Franziska Wiech and Manuela Bulling.

2.3.3.1. RNA isolation and purification

RNA was isolated from whole blood samples using the total RNA Preparation PreAnalytix PAXGene Blood RNA Kit according to the manufacturer's instructions. PAXgene Blood RNA Tubes contain a proprietary reagent composition that protects RNA molecules from

degradation. After the samples were stabilized they were homogenized and a protein digestion was performed before they were transferred to spin columns. After the nucleic acids were bound to the matrix of the spin columns a DNA digestion was performed. Finally, the remaining RNA was eluted and the concentration as well as the purity was determined via absorbance measurement using a NanoDrop device.

2.3.3.2. Reverse transcription

For gene expression analysis, RNA was converted to single-stranded and more stable complementary deoxyribonucleic acid (cDNA) through reverse transcription using the High Capacity cDNA Reverse Transcription Kit according manufacturer's instructions [4]. RNA was quantitatively converted to cDNA using random primers to a final concentration of 50 ng/µL.

2.3.3.3. Gene expression analysis via TaqMan® PCR technology

cDNA was quantitatively amplified via PCR using gene specific primer and probe sets (Gene Expression Assays) and the Gene Expression Master Mix (Applied Biosystems) following the manufactuer's instructions [5, 6].

The principle of the TaqMan real-time detection is based on the fluorogenic 5' nuclease assay, where a PCR fragment is amplified with simultaneous detection of the degradation of a labeled probe. Probes are labeled at both ends with an allele-specific dye and a quencher. During the amplification reaction, the specifically hybridized probe is displaced by the DNA polymerase. This displacement occurs either as degradation through the 5' exonuclease activity of the polymerase in the case of a perfect match with the probe or without degradation in the case of a mismatch. Upon degradation, the quencher and dye are separated and the fluorescence signal increases. Fluorescence signals were recorded with the ABI PRISM 7900HT system (Applied Biosystems, ABI).

All reactions were performed in the ABI 7900HT Sequence Detection System for test samples and no template controls. Samples as well as controls were run in duplicates using the Sequence Detection System (SDS) 2.4 program.

2.3.3.4. Gene expression data processing and comparative Ct method

Overview

To determine Ct (Cycle threshold) values of samples and controls, initial analysis of raw data collected was done within the SDS (Sequence Detection System) run file. Ct values were then exported and further processed for calculation of mean and deviation of duplicates. Data were finally expressed as ΔCt values and used accordingly for the statistical analysis.

Analysis was performed using the ΔΔCt Method (comparative Ct Method which is well described in the literature [3, 7]), where the normalized Ct value (ΔCt) of sample of interest is compared to a control or calibrator, e.g. baseline sample. Here calibration takes place to subject's endogenous expression level before drug administration (baseline sample). Prior to that, the samples were normalized to a house-keeping-gene creating the ΔCt values. Afterwards, the ΔΔCt was calculated through subtraction of the ΔCt of the calibrator from ΔCt of sample of interest and further calculating the Relative Quantity. Mean Ct values, ΔCts and ΔΔCts values were calculated using Ms Excel.

Signal Detection

During a standard Real-Time-PCR run, a laser scans and excites the fluorescent dye emission in each of the wells after each cycle; a spectrograph and charge-coupled device camera spectrally resolve and collect the fluorescence emission from each sample. Based on the fluorescent intensity, the SDS 2.4 software calculated the cycle threshold (Ct) of all samples in relation to an internal dye standard.

Data Processing

Raw Data were analyzed within the Real-Time-PCR run file (SDS 2.4 software) to determine Ct value of each well. The Ct was defined as the number of cycles required for the fluorescent signal to exceed background levels. Therefore, the Ct is inversely proportional to copy number (i.e. low Ct = high copy number of transcripts). All Ct values greater than 36 or designated as 'Undetermined' by the software were assigned as 'Undetermined'.

Ct values were exported to Excel and mean and deviation of duplicates of each sample was calculated.

Mean Ct values generated for each gene of interest were then normalized to mean Ct values generated for house-keeping-gene (ΔCt). To determine the ΔCt for a sample, the

mean Ct of house-keeping-gene was subtracted from the mean Ct of gene of interest. ΔCt was reported for each sample.

ΔΔCt values were calculated from the ΔCt values: ΔΔCt = ΔCt test sample – ΔCt calibrator sample, where the test sample is defined as on-treatment (4h, day 4 or day 8), and the calibrator as the baseline sample.

Normalized amount of each gene of interest could then be used to compare relative amounts of this gene at different time points after treatment, using baseline sample as calibrator: ΔΔCt = ΔCt (4h, day 4 or day 8) - ΔCt (baseline). To express fold change (FC), the formula $2^{-\Delta\Delta Ct}$ was used. Fold changes were calculated for each gene and on-treatment time point.

2.3.4. SVR-oriented exploratory screening

We hypothesized the effect of SVR (comparison of the groups 1 and 3 versus groups 2 and 4) and FDV effect (comparison of the groups 3 and 4 versus groups 1 and 2) at all time points after baseline.

For the analysis, we used absolute mean ΔCt (for the baseline) and mean ΔΔCt (for the on-treatment time points) values per group without data transformation per patient and group showing the absolute numerical difference versus baseline at pre-defined on-treatment time point (see details in the section 2.3.3.4). We intentionally left out the analysis in a 2-fold change transformation (exponential transformation with the basis 2) as this approach was considered to be less sensitive for this exploratory screening.

The group interaction effect (SVR and FDV effect) was tested using the following formula: (mean ΔΔCt in G1+mean ΔΔCt in G4)−(mean ΔΔCt in G2+ mean ΔΔCt in G3) at 4 hours, day 4 and day 8.

The main effect of SVR was tested by comparing SVR versus non-SVR groups according to the following formula: (mean ΔΔCt in G1+mean ΔΔCt in G3)−(mean ΔΔCt in G2+mean ΔΔCt in G4) at 4 hours, day 4 and day 8. Most pronounced differences between SVR and non-SVR groups were captured.

The assessment of the main effect of FDV was calculated as follows: (mean ΔΔCt in G3+mean ΔΔCt in G4)−(mean ΔΔCt in G1+mean ΔΔCt in G2) at 4 hours, day 4 and 8.

Finally, we checked the most pronounced absolute difference of ΔCT at baseline across the groups according to the following formulas showing the maximum TN, TE and group interaction effects: (mean ΔCt in G1+mean ΔCt in G3)−(mean ΔCt in G2+mean ΔCt in G4), (mean ΔCt in G1+mean ΔCt in G5)−(mean ΔCt in G2+mean ΔCt in G6) and (mean

ΔCt in G1+mean ΔCt in G4)−(mean ΔCt in G2+mean ΔCt in G3). The analysis of the group differences was performed using Ms Excel.

2.3.5. Confirmatory gene expression analysis

The confirmatory gene expression analysis was performed in SAS Version 9.2 and R Version 2.15.2 and programmed by Nanshi Sha, Boehringer Ingelheim Pharmaceuticals, Inc., Ridgefield, USA.

Association of baseline gene expression (ΔCt) and expression change in a 2-fold change transformation from baseline at 4 hours, day 4 and day 8 ($2^{-\Delta\Delta Ct}$) with the SVR, adjusted for different factors (IL28B, HCV genotype and previous treatment) was the primary analysis in this step.

For each ISG, the SVR12 rate was calculated. Average gene expression was calculated with standard deviation. To assess the association between gene expression and SVR12 unadjusted association and adjusted association were performed separately. The association was evaluated using odds ratio, calculated through logistic regression. 95% confidence interval of odds ratio was provided. Statistical significance of association was assessed through test of association. For adjusted association, multivariate logistic regression model was used with adjustment factor included as covariate. The factors include IL28B, HCV genotype, and patient treatment experience, respectively. To see if the results were consistent across treatment groups, these analyses were also performed within each treatment group.

3. RESULTS

3.1. Gene selection by literature review

The literature review was performed based on the predefined criteria (see section 2.3.1). Four publications met inclusion criteria and were considered for the initial gene selection (see Table 7). These publications describe ISGs differently regulated in HCV infected responders as compared to non-responders to PegIFN/RBV, all were based on in-vivo data and had comparably large sample size.

Table 7: Publications and selection criteria for the gene selection by literature review

mRNA=messenger ribonucleic acid; HCV=Hepatitis C Virus; GT=genotype; PegIFN/RBV=pegylated interferon α-2a/ribavirin.

Reference	mRNA Source	Time points	Population	Number of selected genes / Criteria for selection
Chen et al. 2005	Liver	Baseline	**31** HCV infected patients: 15 non-responders and 16 responders	18 genes which expression significantly differed between non-responders and responders ($p < 0.005$)
Brodsky et al. 2007	Peripheral Blood Mononuclear Cells	Day 1, 2, 7, 14, 28	**52** HCV infected patients	36 genes which were linked to a decrease in viral titer during PegIFN/RBV treatment according to the methodology of the analysis
Feld et al. 2007	Liver	Baseline	**30** HCV infected patients	33 genes selected with the fold change >1.5, $p<0.01$ for the rapid responders versus matched pre-treatment group.
Taylor et al. 2007	Peripheral Blood Mononuclear Cells	Day 1, 2, 7, 14, 28	**69** HCV GT1 infected patients (33african americans and 36 caucasian americans)	55 genes selected that were considered to be most prominent in terms of the fold change and/or difference between non-responders and rapid responders (changes in gene expression for rapid versus poor responders with the cutoff 1,5 fold change with p-value <0.001)

Based on the selected publications we developed the initial gene listing. The gene selection represented the majority of the genes well described in the literature. We added MAVS, DDX58 and IRF3 genes based on the biologic function and performed reconciliation considering additional 13 publications (see Table 8). Apart from the review of the biologic function and literature we checked the availability of assays and excluded several genes (e.g. PABPC4 and IL6R) due to technical restrictions.

Table 8: Additional publications and compilation of the final gene listing

Reference	**Comments**
Asahina et al. 2003	PKR and MxA included in our initial selection.
Bièche et al. 2005	CXCL9 added into our selection.
Helbig et al. 2005	All relevant genes reconciled with our initial selection.
Yoneyama et al. 2005	DHX58 added into our selection.
Arimoto et al. 2007	RNF125 added into our selection.
Asselah et al. 2008	All relevant genes were present in our initial selection.
Sarasin-Filipowicz et al. 2008	All relevant genes included in our initial selection. The gene analysis from PBMC was not included, due to the limited sample size and a large number of genes which were up- or down regulated in PBMC.
Honda et al. 2010	All relevant genes were present in our initial selection.
Urban et al. 2010	All relevant genes were present in our selection, except FCN1.
Abe et al. 2011	All relevant genes included in our selection, except Zc3h12a and A20.
Dill et al. 2011	All relevant genes included in our selection, except HTATIP2.
Schoggins et al. 2011	IRF2 added into our selection.
Onomoto et al. 2011	All relevant genes were present in our initial selection.

Reviewed literature data represented extensive knowledge database of the gene expression and their association with the treatment-response and/or host genetic factors in patients with HCV infection. Our final selection comprised of 95 genes (see Table 9).

Table 9: Final selection of the genes based on literature review (N=95)

No	Gene	No	Gene	No	Gene	No	Gene	No	Gene
1	ADAR	20	DUSP1	39	IFIT5	58	LY6E	77	RPLP2
2	APOBEC3A	21	EIF3L	40	IFITM1	59	MAP3K14	78	RPS28
3	ATF5	22	EPHB2	41	IFITM2	60	MAVS	79	RSAD2
4	BLZF1	23	GAPDH	42	IFITM3	61	MB21D1	80	SAMD4A
5	C1S	24	GBP1	43	IFNA4	62	MOV10	81	SERPING1
6	C3AR1	25	HERC5	44	IFNAR2	63	MS4A4A	82	SIGLEC1
7	CCL2	26	HERC6	45	IL18BP	64	MX1	83	SOCS1
8	CCL8	27	HESX1	46	IL1RN	65	MX2	84	SP100
9	CCR1	28	HPSE	47	IL28RA	66	NMI	85	SP110
10	CD38	29	IFI16	48	IRF1	67	NT5C3	86	SSBP3
11	CDK18	30	IFI27	49	IRF2	68	OAS1	87	STAT1
12	CHMP5	31	IFI35	50	IRF3	69	OAS2	88	STXBP5
13	CXCL10	32	IFI44	51	IRF7	70	OAS3	89	TBX3
14	CXCL11	33	IFI44L	52	IRF9	71	OASL	90	TLR7
15	CXCL9	34	IFI6	53	ISG15	72	PKLR	91	TNFSF10
16	DDIT4	35	IFIH1	54	ISG20	73	PLSCR1	92	TRIM5
17	DDX58	36	IFIT1	55	LAMP3	74	PML	93	UBE2L6
18	DDX60	37	IFIT2	56	LAP3	75	RNF125	94	USP13
19	DHX58	38	IFIT3	57	LGALS9	76	RPL22	95	USP18

Dataset Review via Ingenuity® Systems Database

The final gene selection was analyzed via Ingenuity® Systems Database. The dataset of 95 ISGs was reviewed and their canonical pathways analyzed through the use of Ingenuity Pathways Analysis (Ingenuity® Systems, www.ingenuity.com).

Interferon signaling and PRR pathways were the most common canonical pathways for this dataset. Apart from the 'multiple sclerosis pathogenesis' pathway, the majority of most common canonical pathways were associated with the interferon-signaling and included the pathways directly associated to the viral recognition and/or host response to the viral infection (see Figure 4). This outcome supported our assumption of the relevance of these genes for our analysis.

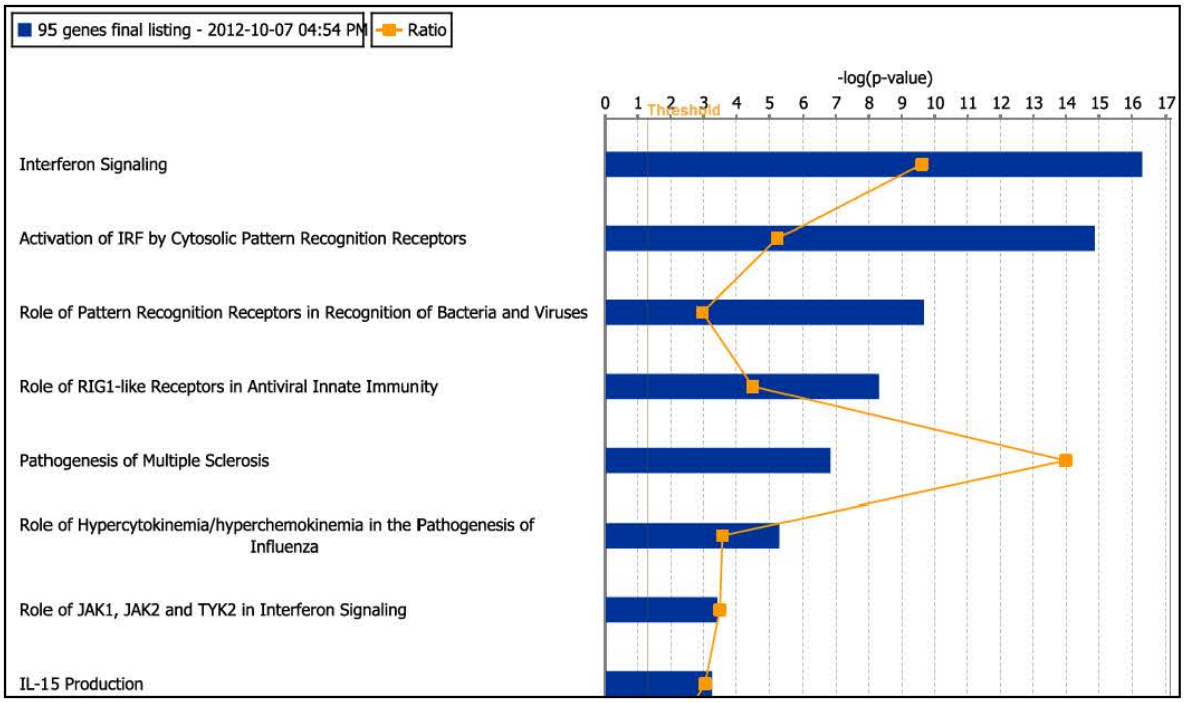

Figure 4: Most common canonical pathways, dataset of 95 selected genes

The significance of the association between the data set and the canonical pathway was measured and presented in the following 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. RIG-1=Retinoid acid inducible gene 1; IRF=Interferon regulatory factor; JAK=Janus kinase; TYK=tyrosine kinase; IL-15=Interleukin 15.

3.2. Groups and demographics of 30 selected patients

For the exploratory screening, the completeness of the data and the quality of the samples were essential. The patients were selected based on the criteria described in the section 2.3.2 and allocated to one of the 6 pre-defined groups (see section 2.3.2). The key selection criterion for the patients was the RNA quality and the availability of the samples at each time point. As a result, each of 6 pre-defined groups consisted of 5 selected patients per group with the total of 30 patients for this analysis (see Table 10).

Table 10: Demographic data and baseline characteristics of 30 selected patients

IL28B=Interleukin-28B, tested on rs12979860 SNP (CC, CT, TT); TN=treatment-naïve patients; TE=treatment-experienced; QD=once daily; PegIFN/RBV=pegylated interferon α-2a/ribavirin; SVR=sustained virological response; F=female; M=male; FDV=faldaprevir; GT=genotype.

Treatment-naïve patients							
Patient No.	**Age (years)**	**Sex**	**Race**	**IL-28B**	**Viral GT**	**Baseline HCV RNA (log_{10})**	**Response**
Group 1 (TN: Placebo + PegIFN/RBV: SVR)							
1553	50	F	Black	CT	1b	7.38	SVR
3265	32	M	White	CT	1b	6.08	SVR
3267	39	F	White	CT	1b	5.37	SVR
3902	46	M	White	CT	1a	6.19	SVR
4009	44	F	Black	CT	1a	5.31	SVR
Group 2 (TN: Placebo + PegIFN/RBV: Non-SVR)							
1701	50	F	White	TT	1b	6.51	Relapse
1952	57	F	White	CT	1a	6.85	Breakthrough
3260	48	F	White	TT	1b	5.92	Non-responder
4305	32	M	White	TT	1a	6.23	Relapse
5402	51	M	White	CT	1a	7.03	Non-responder
Group 3 (TN: FDV 240 mg QD + PegIFN/RBV: SVR)							
1466	49	M	White	CT	1a	5.89	SVR
1468	54	F	White	CT	1a	6.93	SVR
1851	28	F	White	CT	1a	6.38	SVR
1058	45	M	White	Missing	1a	5.94	SVR
3051	37	M	White	TT	1a	6.32	SVR
Group 4 (TN: FDV 240 mg QD + PegIFN/RBV: Non-SVR)							
1009	56	F	White	CT	1b	6.59	Relapse
1460	47	M	White	CT	1b	6.39	Relapse
1610	44	M	White	CT	1a	6.59	Relapse
1801	31	F	White	CT	1b	6.23	Breakthrough
3255	54	F	White	CT	1b	6.24	Relapse
Treatment-experienced patients							
Patient No.	**Age (years)**	**Sex**	**Race**	**IL-28B**	**Viral GT**	**Baseline HCV RNA (log_{10})**	**Response**
Group 5 (TE: FDV 240 mg QD + PegIFN/RBV: SVR)							
1626	59	M	White	TT	1a	6.08	SVR
3556	36	F	White	TT	1a	6.59	SVR
3558	48	M	White	CT	1a	6.41	SVR
4608	37	F	White	CT	1a	7.16	SVR
5304	49	M	White	TT	1a	6.15	SVR
Group 6 (TE: FDV 240 mg QD + PegIFN/RBV: Non-SVR)							
1617	59	F	White	TT	1a	6.48	Breakthrough
1619	43	M	White	CT	1a	6.92	Breakthrough
1471	62	M	White	CT	1a	6.76	Breakthrough
5403	62	M	White	CT	1a	6.89	Breakthrough
1476	42	M	White	CT	1b	6.55	Breakthrough

Table 11: Demographic data and baseline characteristics per group

* - IL28B=Interleukin-28B SNP: Single-nucleotide polymorphism tested on rs12979860 SNP (CC, CT, TT);
VL=viral load; HCV=Hepatitis C Virus.

	Treatment-naïve				Treatment-experienced		Total
	Group 1	**Group 2**	**Group 3**	**Group 4**	**Group 5**	**Group 6**	
Number of patients, N (%)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	30 (100.0)
Gender, N (%)							
Male	2 (40.0)	2 (40.0)	3 (60.0)	2 (40.0)	3 (60.0)	4 (80.0)	16 (53.3)
Female	3 (60.0)	3 (60.0)	2 (40.0)	3 (60.0)	2 (40.0)	1 (20.0)	14 (46.7)
Race, N (%)							
White	3 (60.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	5 (100.0)	28 (93.3)
Black	2 (40.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (6.7)
Age [years]							
Mean (SD)	42 (6.9)	48 (9.3)	42 (10.3)	46 (9.9)	46 (9.5)	54 (10.2)	46 (9.4)
Baseline HCV VL							
Mean [log_{10} IU/mL] (SD)	6.51 (0.45)	6.51 (0.45)	6.29 (0.39)	6.42 (0.17)	6.48 (0.43)	6.68 (0.21)	6.42 (0.44)
HCV genotype, N (%)							
1A	2 (40.0)	3 (60.0)	5 (100.0)	1 (20.0)	5 (100.0)	4 (80.0)	20 (66.7)
1B	3 (60.0)	2 (40.0)	0 (0.0)	4 (80.0)	0 (0.0)	1 (20.0)	10 (33.3)
IL28B SNP*, N (%)							
T_T	0 (0.0)	3 (60.0)	1 (20.0)	0 (0.0)	3 (60.0)	1 (20.0)	8 (26.7)
C_T	5 (100.0)	2 (40.0)	3 (60.0)	5 (100.0)	2 (40.0)	4 (80.0)	21 (70.0)
C_C	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Missing	0 (0.0)	0 (0.0)	1 (20.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (3.3)

The baseline characteristics of the patients were comparable across treatment groups for gender, baseline viral load and IL28B genotype (see Table 11). Mean viral load at baseline ranged from 6.29 to 6.68 log_{10} IU/mL. Mean age was 46 years and was lower in the group 1 (42 years) and the highest in the group 6 (54 years). The majority of patents had IL28B CT genotype (rs12979860 SNP). None of the selected patients had IL28B CC genotype. Viral GT was not balanced across the groups. In the group 1, two of five patients and in the group 2, three of five patients had HCV GT1a. In the groups 3 and 5 all patients had HCV GT1a and the group 6 had four patients with HCV GT1a. Conversely, the group 4 had four of five patients infected with HCV GT1b.

In all SVR groups (group 1, 3, and 5) the patients had HCV RNA undetected 24 weeks after the end of the treatment. In the groups without SVR (group 2, 4, and 6) all patients were non-responders and the majority was categorized as relapse or breakthrough.

3.3. SVR-oriented exploratory screening

3.3.1. Treatment-naïve patients

Results of the analysis of the group interaction effect, main SVR and FDV effects (see section 2.3.3) at different time points (see Tables 12-14) showed the following 12 genes with the highest numerical differences in mean ΔΔCt (threshold >+5.0/<-5.0) which met selection criteria and considered for the inclusion into the confirmatory analysis. Of notice is that there was an overlap for some of the genes which met more than one criterion (e.g. CCL8 and C1S).

Table 12: Genes with the group interaction effect TN

* defined as mean ΔΔCt (G1+G4)-(G2+G3); threshold >+5.0/<-5.0; TN=treatment-naïve.

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
CCL8	7.93	CCL8	7.06	CXCL9	-10.47
		C1S	-6.68	C1S	-7.44
		IFNA4	-5.96	USP13	-5.29
		TBX3	-5.05		

Table 13: Genes with the main SVR effect TN

* defined as mean ΔΔCt (G1+G3)-(G2+G4); threshold >+5.0/<-5.0;
TN=treatment-naïve; SVR=sustained virological response.

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
none		RSAD2	-6.95	CCL8	5.86
		IFI27	-5.90	CXCL11	-7.51
		IFNA4	-5.50	RSAD2	-5.67
		SIGLEC1	-5.08	HESX1	-5.65
				C1S	-5.63
				CXCL9	-5.19

Table 14: Genes with the main FDV effect TN

* defined as mean ΔΔCt (G3+G4)-(G1+G2); threshold >+5.0/<-5.0,
TN=treatment-naïve; FDV=faldaprevir.

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
CXCL11	8.94	CCL8	6.66	C1S	8.76
CCL8	8.32	C1S	6.21	CXCL11	7.24
				CXCL9	5.27
				IFIT1	-5.60

In summary, **C1S, CCL8, HESX1, IFI27, IFIT1, IFNA4, RSAD2, SIGLEC1, TBX3** and **USP13** met inclusion criteria in one or several of the defined categories and were included into the confirmatory analysis. **CXCL11** and **CXCL9** met inclusion criteria but were excluded due to the low quality of the RNA measurement for these genes.

3.3.2. Treatment-experienced patients

The same approach was applied for the treatment-experienced patients (groups 5 and 6). Faldaprevir treatment arms (group 5 and 6) were compared against placebo arm (group 1 and 2) and SVR arms (group 1 and 5) were compared with non-SVR arms (groups 2 and 6). Seven genes met inclusion criteria based on the threshold >+6.0/<-6.0 (see Tables 15-17). We adjusted the threshold in the treatment-experienced population due to the larger number of genes which were up- or down regulated with the difference of near 5.0 mean ΔΔCt. Similarly to the treatment-naïve groups, some genes met inclusion criteria at many time points and/or met several criteria (e.g. PKLR, C1S and CXCL11).

Table 15: Genes with the group interaction effect TE

* defined as mean ΔΔCt (G1+G6)-(G2+G5); (threshold >+6.0/<-6.0); TE=treatment-experienced.

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
PKLR	-12.05	PKLR	-8.65	C1S	-6.50
		C1S	-7.29		
		IFNA4	-7.25		
		RSAD2	-6.71		

Table 16: Genes with the main SVR effect TE

* defined as mean ΔΔCt (G1+G5)-(G2+G6); (threshold >+6.0/<-6.0);
TE=treatment-experienced; SVR=sustained virological response.

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
CXCL10	8.1	PKLR	-12.89	C1S	-6.56
				CXCL11	-6.43

Table 17: Genes with the main FDV effect TE

* defined as mean ΔΔCt (G5+G6)-(G1+G2); (threshold >+6.0/<-6.0);
TE=treatment-experienced; FDV=faldaprevir

4 hours		Day 4		Day 8	
Gene	**Result***	**Gene**	**Result***	**Gene**	**Result***
CXCL11	-6.52	PKLR	12.46	C1S	9.25
		C1S	8.53		
		USP13	6.79		
		IFNA4	6.76		

In summary, **C1S, CXCL10, IFNA4, PKLR, RSAD2** and **USP13** met inclusion criteria in one or several of the defined categories and were included into the confirmatory analysis. **CXCL11** met inclusion criteria but was excluded due to the low quality of the RNA measurement. **C1S, IFNA4, RSAD2** and **USP13** also met the inclusion criteria in treatment-naïve patient population. **CXCL10** and **PKLR** were new based on the treatment-experienced group analysis.

3.3.3. Baseline gene expression

Apart from the up- or down-regulation at different time points compared to the baseline, we looked at the baseline gene expression and compared absolute mean baseline values (ΔCt) across the groups (see Tables 18-20).

Table 18: Genes with the maximal baseline differences in TN

* defined as baseline mean ΔCt (G1+G3)-(G2+G4); (threshold >+4.0/<-4.0);
** defined as baseline mean ΔCt G1-G2; **** defined as baseline mean ΔCt G3-G4;
TN=treatment-naïve; FDV=faldaprevir; SVR=sustained virological response.

SVR Group Effect TN		SVR Effect Placebo TN		SVR Effect FDV TN	
Gene	**Result***	**Gene**	**Result****	**Gene**	**Result*****
RSAD2	4.96	PKLR	4.91	CXCL11	4.04
		CCL8	-5.43		

Table 19: Genes with the maximal baseline differences in TE

*defined as baseline mean ΔCt (G1+G5)-(G2+G6); (threshold >+4.0/<-4.0);
** defined as baseline mean ΔCt (G5-G6); TE=treatment-experienced;
FDV=faldaprevir; SVR=sustained virological response.

SVR Group Effect TE		SVR Effect FDV TE	
Gene	**Result***	**Gene**	**Result****
PKLR	8.57	none	

Table 20: Group interaction baseline effect TN

* defined as baseline mean ΔCt (G1+G4)-(G2+G3);
(threshold >+4.0/<-4.0), TN=treatment-naïve.

Group Interaction Effect	
Gene	**Result***
CCL8	-8.83
CXCL11	-5.47
HESX1	-5.11

In summary, **RSAD2, PKLR, CCL8, CXCL11** and **HESX1** met inclusion criteria based on this analysis. All these genes also met selection criteria for the group interaction, SVR or FDV effect based on treatment-naïve or treatment-experienced group analysis (see sections 3.3.1 and 3.3.2).

3.3.4. Manual selection

In addition to the selection based on the group, main SVR and FDV effect, we selected 3 genes based on the biologic function. The following genes were added: **IRF3**, **MAVS** and **DDX58** (see section 1.4).

3.3.5. Final gene listing for the confirmatory gene expression analysis

Final selection of the genes for the confirmatory gene expression analysis included 15 genes (see Table 21). The genes with the highest difference in mean ΔΔCt (on-treatment time points as compared to placebo) or ΔCt (baseline) between defined groups were primarily selected. The negative difference between the groups should be interpreted as a positive effect or higher gene expression in the group of interest, as the Ct is inversely proportional to RNA copy number (i.e. low Ct = high copy number of transcripts). The summary of the criteria and the effect (positive or negative) is presented in the Table 21. The majority of genes were selected due to positive SVR effect (higher gene expression in SVR patients) or negative FDV effect (lower gene expression in FDV patients as compared to placebo) during the treatment as compared to baseline.

C1S, CCL8 (TN patients) and **C1S, IFNA4, PKLR** and **USP13** (TE patients) at day 4 (+/- day 8) showed negative FDV effect. **C1S, HESX1, IFI27, RSAD2, SIGLEC1** (TN patients) and **C1S, PKLR** (TE patients) showed positive SVR effect mostly at day 4 and day 8. Genes encoding complement component 1 (**C1S**) and chemokine **CCL8** matched multiple criteria.

Negative SVR effect (expression lower in SVR patients) was seen for **CCL8** (TN patients at day 8) and **CXCL10** (TE patients at 4 hours). Positive FDV effect (expression higher in FDV patients) was observed for **IFIT1** gene (TN patients at day 8). **PKLR** met selection criteria in all categories of TE patients (mostly day 4). The highest difference at baseline in SVR versus non-SVR patients as well as FDV versus placebo patients was seen for **CCL8, HESX1, PKLR** and **RSAD2** genes (see Table 21).

Table 21: Final gene selection summary

FDV=faldaprevir; SVR=sustained virological response;

'1' - effect seen at 4 hrs, '2' - effect seen at day 4, '3' - effect seen at day 8 '*'- manual selection, '+' positive effect; '-' negative effect.

No	Gene	Selection Criteria							
		Treatment-naïve				Treatment-experienced			
		Baseline	Group interaction	SVR Effect	FDV Effect	Baseline	Group interaction	SVR Effect	FDV Effect
1	C1S		Yes(+)/2,3	Yes(+)/3	Yes(-)/2,3		Yes(+)/2,3	Yes(+)/3	Yes(-)/2,3
2	CCL8	Yes	Yes(-)/1,2	Yes(-)/3	Yes(-)/1,2				
3	CXCL10							Yes(-)/1	
4	DDX58*		-	-	-		-	-	-
5	HESX1	Yes		Yes(+)/3					
6	IFI27			Yes(+)/2					
7	IFIT1				Yes(+)/3				
8	IFNA4		Yes(+)/2				Yes(+)/2		Yes(-)/2
9	IRF3*		-	-	-		-	-	-
10	MAVS*		-	-	-		-	-	-
11	PKLR	Yes				Yes	Yes(+)/1,2	Yes(+)/2	Yes(-)/2
12	RSAD2	Yes		Yes(+)/2,3			Yes(+)/2		
13	SIGLEC1			Yes(+)/2					
14	TBX3		Yes(+)/2						
15	USP13		Yes(+)/3						Yes(-)/2

3.3.6. Characteristics of 15 selected genes

Each of 15 selected genes was reviewed for available information about its biological function. All of them were well known and characterized genes which are classified as interferon-stimulated gene. Based on their biological function (see Table 22), we used the following sub-classification of our gene set:

- Complement component 1& chemotaxis chemokines : C1S, CCL8, CXCL10
- Virus Recognition (e.g. via PRR): DDX58, IRF3, MAVS, RSAD2, IFIT1
- Genes induced after HCV & INF activation: IFNA4, CXCL10, IFI27, SIGLEC1
- Nucleus transcription factors: TBX3, HESX1
- Other functions: PKLR (glycolysis), USP13 (proteolysis)

Table 22: Function of selected genes

*Source: Database Ingenuity IPA, Version 1440082, date 01-Dec-2012

Gene	Function*
C1S	Complement component 1, s subcomponent Activates the first component of the classical pathway of the complement system
CCL8	Chemokine (C-C motif) ligand 8 Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils
CXCL10	Chemokine (C-X-C motif) ligand 10 / Synonym: Interferon-inducible protein-10 Chemotactic factor that attracts monocytes, lymphocytes, basophils and eosinophils
DDX58	DDX58 (DEAD box polypeptide 58) / Synonym: Retinoic inducible gene 1 (RIG1) (ds) RNA recognition (RIG1 like receptor family). Activates IRFs and NFkB
HESX1	HESX homeobox 1 Transcription regulator in nucleus (incl. chromatin binding etc.)
IFI27	Interferon alpha-inducible protein 27 Promotes cell death. Mediates IFN-induced apoptosis
IFIT1	Interferon-induced protein with tetratricopeptide repeats 1 Cellular response to exogenous dsRNA & type I interferon
IFNA4	Interferon alpha 4 Response to virus; type I interferon-mediated signaling, interferon-alpha/beta receptor binding
IRF3	Interferon regulatory factor 3 Activated via TLR and RLRs after ds RNA recognition
MAVS	Mitochondrial antiviral signaling protein Synonyms: IPS1 (IFN-ß promoter stimulator-1)/VISA /Cardif RIG1 (activated by dsRNA) makes a complex with MAVS (located on Mitochondria) and activates IRF3
PKLR	Pyruvate kinase, liver and RBC Plays a key role in glycolysis
RSAD2	Radical S-adenosyl methionine domain containing 2 Regulated by IRF3, MAVS, DDX58, TLR IFNs. Binds IRF7
SIGLEC1	Sialic acid binding Ig-like lectin 1, sialoadhesin Macrophage-restricted adhesion molecule that mediates sialic-acid dependent binding to lymphocytes
TBX3	T-box 3 T-box transcription factor Encodes transcription factors in nucleus
USP13	Ubiquitin specific peptidase 13 Proteolysis; ubiquitin (regulatory protein)-dependent protein catabolic process

Dataset Review via Ingenuity® Systems Database

The analysis via Ingenuity® Systems Database showed similar results to those seen in 95 genes set. The most common canonical pathways were RIG1 receptors role in antiviral innate immunity, activation of IRF and role of PRRs in recognition of bacteria and viruses (see Figure 5).

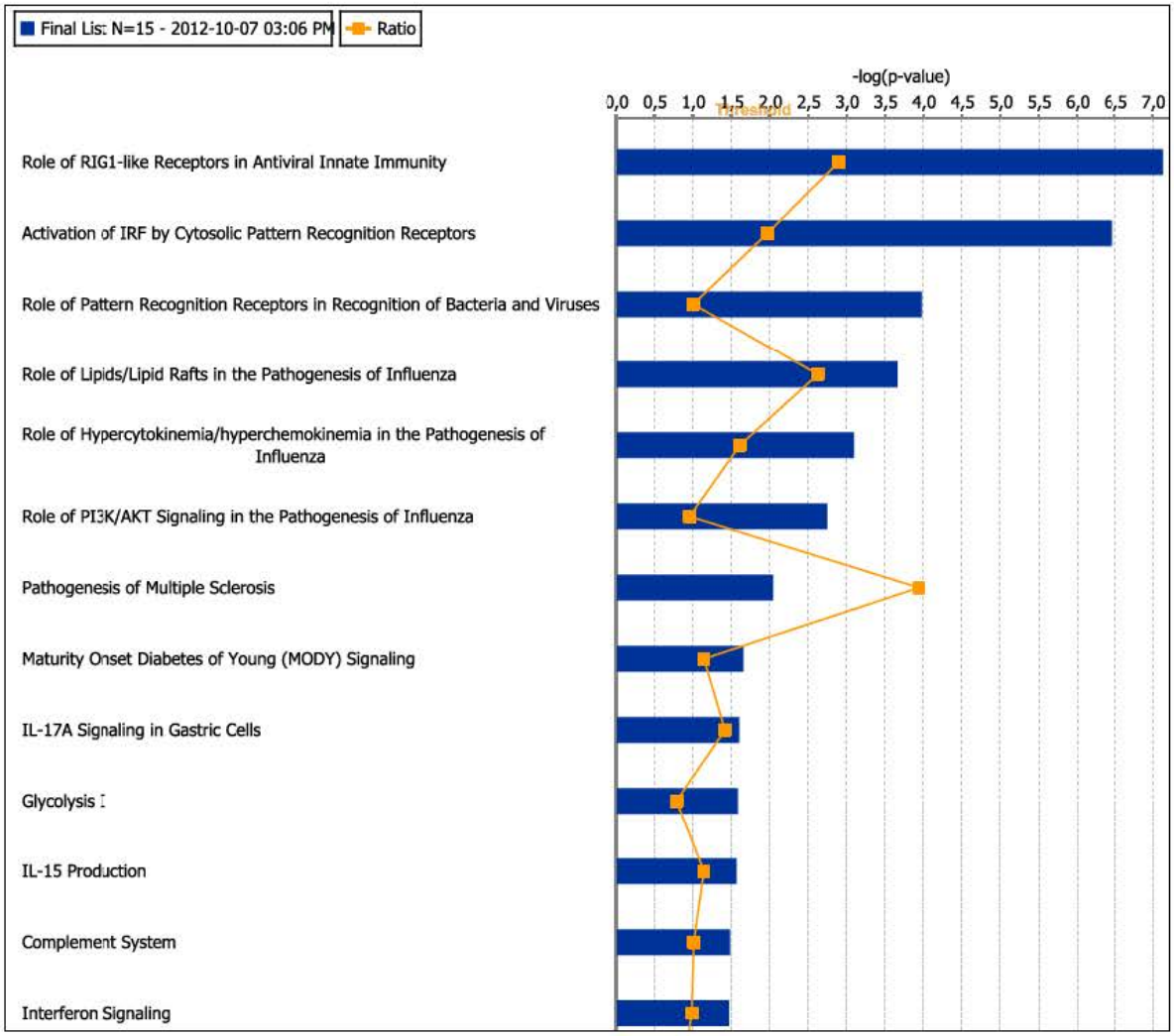

Figure 5: Most common canonical pathways, dataset of 15 selected genes

Canonical pathways analysis identified the pathways from the IPA library of canonical pathways that were most significant to the data set. The significance of the association between the data set and the canonical pathway was measured and presented in the following 2 ways: 1) A ratio of the number of molecules from the data set that map to the pathway divided by the total number of molecules that map to the canonical pathway is displayed. 2) Fisher's exact test was used to calculate a p-value determining the probability that the association between the genes in the dataset and the canonical pathway is explained by chance alone. RIG-1=Retinoid acid inducible gene 1; IRF=Interferon regulatory factor; PI3K=1-phosphatidylinositol 3-kinase; IL=Interleukin.

Further analysis of this dataset by looking into the molecular relationships between genes and other molecules showed a close connection and overlap of the networking of the genes. CXCL 10, DDX58, RSAD2, IRF3, IFIT1, MAVS and others are closely overlapped and show strongly evident biologic connections (see Figure 6). This outcome supported inclusion of all identified genes into the confirmatory gene expression analysis.

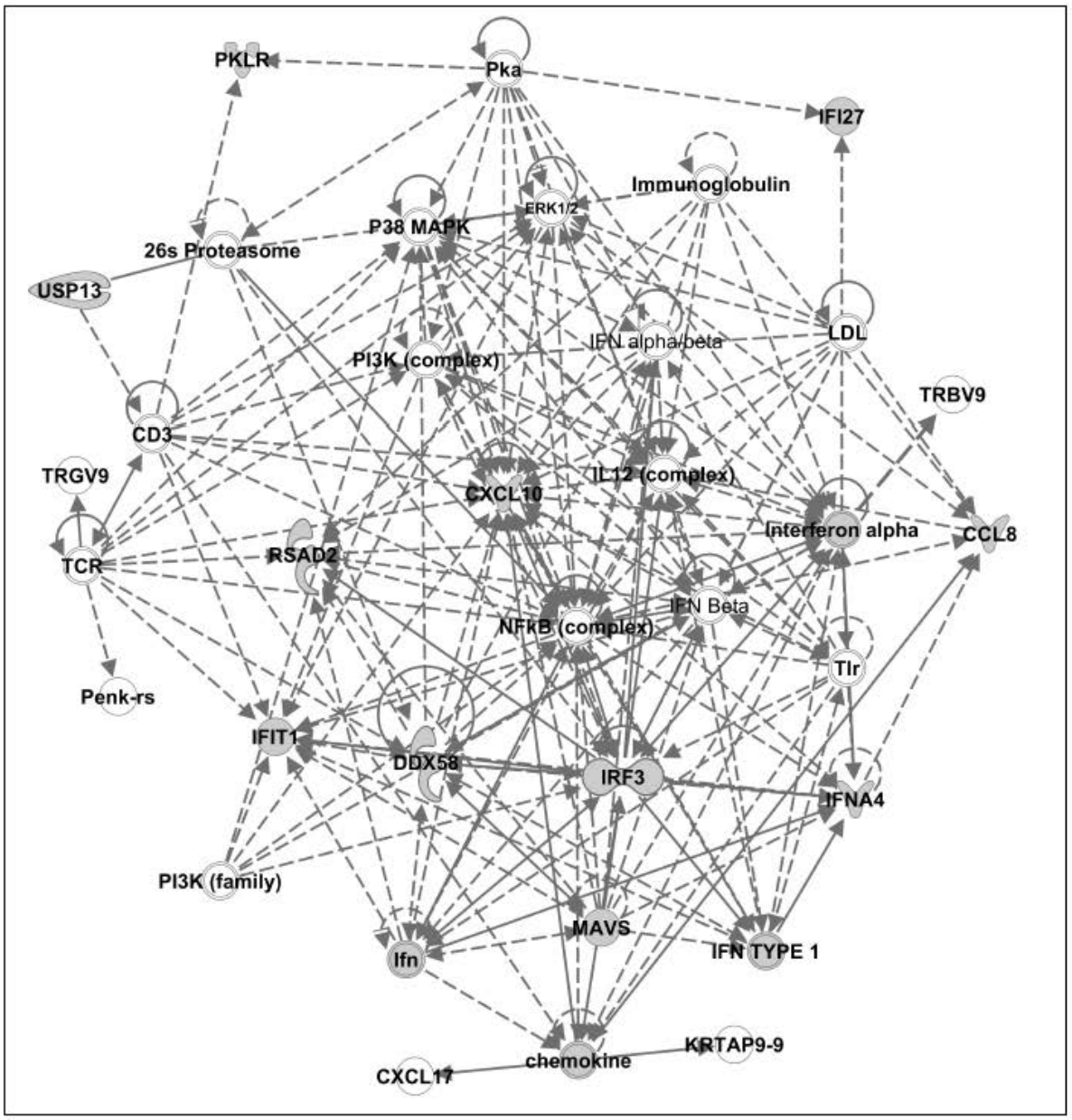

Figure 6: Overlapping network, dataset of 15 selected genes

Overlapping network is a graphical representation of the molecular relationships between molecules. Molecules are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. Human, mouse, and rat orthologs of a gene are stored as separate objects in the Ingenuity Knowledge Base, but are represented as a single node in the network.
PKLR=Pyruvate kinase, liver and RBC; Pka=Protein kinase a; IFI27=Interferon alpha-inducible protein 27; USP13=Ubiquitin specific peptidase 13; MAPK=Mitogen activated protein kinase; ERK=Extracellular signal-regulated protein kinases; LDL=Low density lipoprotein; CD3=Cluster of differentiation 3; IFN=Interferon; CXCL10=Chemokine (C-X-C motif) ligand 10; IL12=Interleukin 12; CCL8=Chemokine (C-C motif) ligand 8; TRBV9=T cell receptor beta variable 9; NF-kB=Nuclear Factor-Kappa B; RSAD2=Radical S-adenosyl methionine domain containing 2; TCR=T-cell receptor; DDX58=DEAD box polypeptide 58; IRF3=Interferon-regulatory factor 3; IFNA4=Interferon alpha 4; MAVS=Mitochondrial antiviral signaling protein; Penk-rs=preproenkephalin, related sequence; PI3K=1-phosphatidylinositol 3-kinase; CXCL17=Chemokine (C-X-C motif) ligand 17; KRTAP9-9=Keratin associated protein 9-9;, **Ifn=** Interferon; TRGV9**=**T cell receptor gamma variable 9; **TLR=**Toll like receptor; IFIT1= Interferon-induced protein with tetratricopeptide repeats 1.

3.4. Confirmatory gene expression analysis

3.4.1. Demographics, baseline characteristics, and HCV related genetic factors

171 treatment-naïve and 92 treatment-experienced patients were included into the confirmatory gene expression analysis. 30 patients tested in the SVR-oriented exploratory screening were not repeatedly included into this confirmatory step.

The demographic and baseline characteristics of the treatment-naive patients were overall comparable between the treatment groups (see Table 23). Nearly half of the patients were male. The majority of patients were white. Mean age was 45 years, ranging from 44 years in the placebo group up to 47 years in the 240 mg QD group. BMI distribution was balanced across the groups. Placebo group had more patients with high baseline viral load (87% in the placebo group, versus 67.9%, 71.1% and 79.6% in the 240 mg QD LI, 120 mg QD LI and 240 mg QD groups, respectively). Distribution of viral genotype subtypes was slightly unbalanced. In the placebo group 47.8% patients had GT1a and 47.8% of patients had GT1b. Conversely, 120 mg QD LI and 240 mg QD groups had more patients with viral GT1b (89.5%, 62.5% and 81.5% of patients had GT1b in the 120 mg QD LI, 240 mg QD LI and 240 mg QD groups, respectively). The placebo group had the highest number of patients with favorable IL28B genotype CC (39.1%). The lowest number of IL28B CC patients was in 120 mg QD LI group (15.8%). SVR rates were 60.9%, 78.4%, 75.5% and 87.7% in the placebo, 120 mg QD LI, 240 mg QD LI and 240 mg QD groups, respectively and were overall comparable with the SILEN-C1 SVR rates (56.3%, 72.5%, 72.3% and 83.8%, respectively).

The demographic data and baseline characteristics were similar to the population of the SILEN-C1 trial. Of notice is only the minor difference in the total number of GT1b patients (72.5% in this study versus 59% in SILEN-C1) and Asian patients (3.5% in this study versus 12.9% in SILEN-C1). Overall, the study population was representative of the HCV infected patients.

Table 23: Demographic data, baseline characteristics, and HCV related genetic factors for treatment-naive patients

HCV=Hepatitis C Virus; QD=once daily; LI=lead-in; IL28B=Interleukin-28B; SVR=sustained virological response.

	Placebo	120 mg QD LI	240 mg QD LI	240 mg QD	Total
Number of patients, N (%)	**23 (100.0)**	**38 (100.0)**	**56 (100.0)**	**54 (100.0)**	**171 (100.0)**
Gender, N (%)					
Male	10 (43.5)	20 (52.6)	24 (42.9)	31 (57.4)	85 (49.7)
Female	13 (56.5)	18 (47.4)	32 (57.1)	23 (42.6)	86 (50.3)
Race, N (%)					
White	20 (87.0)	36 (94.7)	53 (94.6)	51 (94.4)	160 (93.6)
Asian	0 (0.0)	2 (5.3)	3 (5.4)	1 (1.9)	6 (3.5)
Black/African American	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)
American Indian/Alaska Native	2 (8.7)	0 (0.0)	0 (0.0)	2 (3.7)	4 (2.3)
Age [years]					
Mean (SD)	43.6 (11.35)	45.3 (11.22)	44.2 (11.00)	47.2 (12.60)	45.3 (11.60)
Age group, N (%)					
18-40 years	9 (39.1)	11 (28.9)	22 (39.3)	17 (31.5)	59 (34.5)
>40-55 years	10 (43.5)	20 (52.6)	24 (42.9)	20 (37.0)	74 (43.3)
>55 years	4 (17.4)	7 (18.4)	10 (17.9)	17 (31.5)	38 (22.2)
Body Mass Index [kg/m2]					
Mean (SD)	26.1 (4.94)	25.9 (4.29)	25.5 (4.74)	25.7 (3.67)	25.8 (4.32)
Alcohol status, N (%)					
Non-drinker	16 (69.6)	23 (60.5)	40 (71.4)	38 (70.4)	117 (68.4)
Drinker - no interference with trial	7 (30.4)	15 (39.5)	15 (26.8)	16 (29.6)	53 (31.0)
Drinker - possible interference with trial	0 (0.0)	0 (0.0)	1 (1.8)	0 (0.0)	1 (0.6)
Smoking status, N (%)					
Never smoked	10 (43.5)	19 (50.0)	23 (41.1)	26 (48.1)	78 (45.6)
Ex-smoker	3 (13.0)	7 (18.4)	14 (25.0)	12 (22.2)	36 (21.1)
Current smoker	10 (43.5)	12 (31.6)	19 (33.9)	16 (29.6)	57 (33.3)
Baseline HCV viral load [IU/mL]					
Mean (SD)	3412652.2 (4141357.54)	2449131.6 (2586396.48)	4849085.7 (6104360.88)	3890966.0 (3810383.83)	3819576.5 (4592311.24)
Baseline viral load category N (%)					
<800 000 IU/mL	3 (13.0)	11 (28.9)	18 (32.1)	10 (18.5)	42 (24.6)
≥800 000 IU/mL	20 (87.0)	27 (71.1)	38 (67.9)	43 (79.6)	128 (74.9)
Missing	0 (0.0)	0 (0.0)	0 (0.0)	1 (1.9)	1 (0.6)
HCV NS3-4A protease genotype, N (%)					
1	1 (4.3)	0 (0.0)	0 (0.0)	0 (0.0)	1 (0.6)
1A	11 (47.8)	4 (10.5)	20 (35.7)	8 (14.8)	43 (25.1)
1B	11 (47.8)	34 (89.5)	35 (62.5)	44 (81.5)	124 (72.5)
3A	0 (0.0)	0 (0.0)	0 (0.0)	2 (3.7)	2 (1.2)
6E	0 (0.0)	0 (0.0)	1 (1.8)	0 (0.0)	1 (0.6)
IL28B gene polymorphism, N (%)					
T_T	4 (17.4)	7 (18.4)	10 (17.9)	5 (9.3)	26 (15.2)
C_T	10 (43.5)	24 (63.2)	29 (51.8)	25 (46.3)	88 (51.5)
C_C	9 (39.1)	6 (15.8)	15 (26.8)	17 (31.5)	47 (27.5)
Missing	0 (0.0)	1 (2.6)	2 (3.6)	7 (13.0)	10 (5.8)
Response rate					
SVR (%)	60.9	78.4	75.5	87.7	

Treatment-experienced patients demographic and baseline characteristics were overall balanced between the treatment groups (see Table 24). As expected, the patients were older (mean age 50 years), had higher baseline viral load (approximately 90% of all patients had HCV RNA >800.000 IU/mL at baseline), and had less patients with favorable IL28B genotype CC (9% to 14%). The majority of patients were null or partial responders to the previous HCV treatment. Relapsers were excluded in the SILEN-C2 protocol. Viral GT distribution was equal in 240 mg BID LI group (48.3% each 1a and 1b). 63.8% and 43.8% of patients had GT1a in the 240 mg QD LI and 240 mg QD, respectively. SVR rates of the patients included into the confirmatory analysis were 25.5%, 37.5% and 34.5% in 240 mg QD LI, 240 mg QD and 240 mg BID LI groups, respectively and were overall comparable with the SILEN-C2 SVR rates (28.2%, 40.8% and 31.4%, respectively). The demographic data and baseline characteristics of the treatment-experienced patient groups were similar to the population of the SILEN-C2. Overall, the study population was representative of the treatment-experienced chronic HCV infected patients.

Table 24: Demographic data, baseline characteristics, and HCV related genetic factors for treatment-experienced patients

HCV=Hepatitis C Virus; QD=once daily; BID=twice daily; LI=lead-in; IL28B=Interleukin-28B; SVR=sustained virological response; * - confirmed non-responders with insufficient data to categorize them as null- or partial-responders; ** patients who discontinued previous treatment between 12 and 24 weeks of treatment and had detectable HCV RNA at the end of treatment.

	240 mg QD LI	240 mg QD	240 mg BID LI	Total
Number of patients, N (%)	**47 (100.0)**	**16 (100.0)**	**29 (100.0)**	**92 (100.0)**
Gender, N (%)				
Male	36 (76.6)	11 (68.8)	16 (55.2)	63 (68.5)
Female	11 (23.4)	5 (31.3)	13 (44.8)	29 (31.5)
Race, N (%)				
White	47 (100.0)	12 (75.0)	28 (96.6)	87 (94.6)
Asian	0 (0.0)	2 (12.5)	0 (0.0)	2 (2.2)
Black/African American	0 (0.0)	2 (12.5)	1 (3.4)	3 (3.3)
Age [years]				
Mean (SD)	49.7 (8.75)	50.4 (9.41)	50.1 (9.15)	49.9 (8.89)
Age group [years]				
18-40	4 (8.5)	3 (18.8)	5 (17.2)	12 (13.0)
>40-55	30 (63.8)	9 (56.3)	16 (55.2)	55 (59.8)
>55	13 (27.7)	4 (25.0)	8 (27.6)	25 (27.2)
Body Mass Index [kg/m2]				
Mean (SD)	25.4 (3.94)	24.0 (3.45)	25.5 (3.56)	25.2 (3.75)
Alcohol status, N (%)				
Non-drinker	28 (59.6)	12 (75.0)	18 (62.1)	58 (63.0)
Drinker - no interference with trial	19 (40.4)	4 (25.0)	11 (37.9)	34 (37.0)
Drinker - possible interference with trial	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Smoking status, N (%)				
Never smoked	15 (31.9)	6 (37.5)	12 (41.4)	33 (35.9)
Ex-smoker	14 (29.8)	4 (25.0)	7 (24.1)	25 (27.2)
Current smoker	18 (38.3)	6 (37.5)	10 (34.5)	34 (37.0)
Baseline HCV viral load [IU/mL]				
Mean (SD)	6659127.7 (6566154.51)	5414937.5 (4355999.47)	6258642.9 (5519683.79)	6317142.9 (5877551.90)
Baseline viral load category [IU/mL]				
<800 000 IU/mL	5 (10.6)	1 (6.3)	2 (6.9)	8 (8.7)
≥800 000 IU/mL	42 (89.4)	15 (93.8)	26 (89.7)	83 (90.2)
Missing	0 (0.0)	0 (0.0)	1 (3.4)	1 (1.1)
HCV NS3-4A protease genotype, N (%)				
1	0 (0.0)	0 (0.0)	1 (3.4)	1 (1.1)
1A	30 (63.8)	7 (43.8)	14 (48.3)	51 (55.4)
1B	17 (36.2)	8 (50.0)	14 (48.3)	39 (42.4)
1D	0 (0.0)	1 (6.3)	0 (0.0)	1 (1.1)
IL28B gene polymorphism, N (%)				
T_T	13 (27.7)	3 (18.8)	9 (31.0)	25 (27.2)
C_T	30 (63.8)	11 (68.8)	16 (55.2)	57 (62.0)
C_C	4 (8.5)	2 (12.5)	4 (13.8)	10 (10.9)
Missing	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Previous response to treatment				
Null Responder	18 (38.3)	7 (43.8)	18 (62.1)	43 (46.7)
Partial Responder	17 (36.2)	5 (31.3)	9 (31.0)	31 (33.7)
Type of non-response unknown*	3 (6.4)	1 (6.3)	0 (0.0)	4 (4.3)
Efficacy not assessable**	9 (19.1)	3 (18.8)	2 (6.9)	14 (15.2)
Response rate				
SVR (%)	25.5	37.5	34.5	

3.4.2. Analysis of association with SVR

3.4.2.1. Pooled analysis

The pooled analysis of the association of the baseline ISG expression with the achievement of SVR showed that **IFNA4** low baseline expression was significantly associated with SVR (OR 1.128, 95% CI 1.016-1.252, p=0.0238). Low baseline expression level of **DDX58** was the second ISG which was similarly strongly associated with SVR (OR 1.278, 95% CI 1.009-1.617, p=0.0415). The low baseline expression of **IRF3** was nearly significantly associated with the SVR and had the highest odds ratio (OR 1.395, 95% CI 0.999-1.947, p=0.0504). Association of the baseline gene expression of all other tested genes at baseline was not significant (see Table 25).

Table 25: Baseline gene expression relationship to SVR: Pooled Analysis

SVR=sustained virological response; ΔCt=delta cycle threshold; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	ΔCt, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFNA4	239	146 (61.1)	28.6 (2.74)	1.128	1.016-1.252	0.0238
DDX58	251	156 (62.2)	17.5 (1.21)	1.278	1.009-1.617	0.0415
IRF3	255	158 (62.0)	17.1 (0.87)	1.395	0.999-1.947	0.0504
C1S	117	79 (67.5)	26.0 (1.42)	1.391	0.996-1.941	0.0525
CXCL10	212	130 (61.3)	25.1 (1.78)	1.182	0.997-1.401	0.0535
MAVS	254	158 (62.2)	20.0 (1.12)	1.262	0.982-1.622	0.0695
IFIT1	235	147 (62.6)	23.7 (1.81)	1.144	0.974-1.343	0.1011
PKLR	107	71 (66.4)	27.0 (1.47)	1.275	0.923-1.763	0.1408
TBX3	202	127 (62.9)	28.8 (1.34)	1.181	0.936-1.491	0.1609
IFI27	244	152 (62.3)	22.5 (2.16)	1.091	0.955-1.248	0.2007
CCL8	138	88 (63.8)	26.0 (1.99)	1.121	0.931-1.350	0.2283
USP13	242	153 (63.2)	21.9 (1.02)	1.181	0.889-1.570	0.2517
SIGLEC1	247	155 (62.8)	21.5 (1.91)	1.089	0.932-1.271	0.2829
HESX1	161	92 (57.1)	28.0 (1.49)	1.120	0.887-1.415	0.3402
RSAD2	253	156 (61.7)	19.9 (1.84)	1.057	0.906-1.233	0.4805

Pooled analysis of associations of the gene expression change from baseline at 4 hours after the start of the treatment, at day 4 and day 8 across all treatment groups showed that the gene expression change of **IRF3** at 4 hours was associated with SVR (OR 0.749, 95% CI 0.561-0.999, p=0.0491), meaning that the down regulation of this gene was associated with the SVR. For this gene the fold change difference for SVR versus non-SVR patients was very low (mean FC was 1.19 [SD=0.72] versus 1.55 [SD=1.64] in SVR versus non-SVR, respectively), thus this association might be less meaningful. Other remarkable genes were **RSAD2** and **DDX58** at 4 hours (see Table 26). These genes had lowest p-value

across other genes (p>0.05) and high odds ratio (OR) seen at various time points that could evidence their important biological role.

At day 4 and day 8, **IFI27** expression change from baseline was statistically significantly associated with SVR (Day 4: OR 1.003, 95% CI 1.001-1.005, p=0.005; Day 8: OR 1.003, 95% CI 1.001 – 1.005, p=0.0103). **RSAD2** and **DDX58** change from baseline at day 4 was significantly associated with the SVR (OR 1.018, 95% CI 1.004-1.033, p=0.0142 and OR1.160, 95% CI 1.018-1.322, p=0.0259). Due to the high variability of the data and high standard deviation of mean for **IFI27** and **RSAD2** the results for these genes at day 4 and day 8 should be interpreted with caution (see Tables 26-28).

Table 26: Gene expression change from baseline at 4 hours relationship to SVR: Pooled Analysis

SVR=sustained virological response;; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IRF3	232	144 (62.1)	1.27 (1.07)	0.749	0.561-0.999	0.0491
RSAD2	228	141 (61.8)	45.3 (255)	1.009	0.999-1.018	0.0662
DDX58	230	144 (62.6)	8.86 (10.3)	1.031	0.995-1.069	0.0902
USP13	212	137 (64.6)	1.41 (2.05)	0.861	0.703-1.055	0.1491
IFNA4	208	129 (62.0)	159 (1202)	1.000	1.000-1.000	0.1643
PKLR	40	32 (80.0)	2.89 3.26)	1.661	0.771-3.579	0.1949
SIGLEC1	223	142 (63.7)	15.7 (143)	1.028	0.982-1.076	0.2411
MAVS	228	142 (62.3)	1.58 2.21)	0.921	0.799-1.061	0.2526
CXCL10	192	119 (62.0)	275 (494)	1.000	1.000-1.001	0.3319
TBX3	155	99 (63.9)	1.80 (4.73)	0.967	0.902-1.037	0.3449
HESX1	126	72 (57.1)	43.9 (61.6)	1.004	0.996-1.011	0.3451
C1S	57	42 (73.7)	4.19 (15.0)	0.955	0.862-1.058	0.3780
CCL8	120	78 (65.0)	478 (1144)	1.000	1.000-1.001	0.4587
IFIT1	216	136 (63.0)	47.9 80.0)	1.001	0.997-1.006	0.4761
IFI27	221	139 (62.9)	3.77 (21.0)	1.016	0.953-1.082	0.6316

Table 27: Gene expression change from baseline at Day 4 relationship to SVR: Pooled Analysis

SVR=sustained virological response; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFI27	206	129 (62.6)	254 (560)	1.003	1.001-1.005	0.0050
RSAD2	210	129 (61.4)	40.9 (164)	1.018	1.004-1.033	0.0142
DDX58	210	130 (61.9)	3.42 (3.58)	1.160	1.018-1.322	0.0259
SIGLEC1	205	128 (62.4)	66.5 (386)	1.009	0.999-1.018	0.0651
HESX1	119	72 (60.5)	29.3 (37.7)	1.019	0.999-1.039	0.0699
IFIT1	193	121 (62.7)	22.2 (41.3)	1.009	0.997-1.022	0.1531
C1S	47	30 (63.8)	1.94 (2.98)	1.465	0.784-2.736	0.2313
MAVS	211	132 (62.6)	1.19 (0.91)	1.250	0.859-1.819	0.2441
PKLR	45	30 (66.7)	1.78 (2.81)	1.310	0.775-2.212	0.3135
USP13	185	119 (64.3)	1.08 (0.88)	1.214	0.802-1.838	0.3583
CXCL10	175	111 (63.4)	10.8 (19.2)	1.006	0.987-1.026	0.5256
IRF3	213	132 (62.0)	1.19 (0.87)	1.082	0.759-1.540	0.6636
CCL8	105	66 (62.9)	27.6 (41.9)	1.001	0.991-1.012	0.8080
TBX3	154	93 (60.4)	1.78 (3.38)	0.989	0.898-1.089	0.8147
IFNA4	195	119 (61.0)	2.48 (8.07)	1.004	0.952-1.059	0.8914

Table 28: Gene expression change from baseline at Day 8 relationship to SVR: Pooled Analysis

SVR=sustained virological response; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFI27	192	125 (65.1)	240 (657)	1.003	1.001 – 1.005	0.0103
RSAD2	201	128 (63.7)	25.8 (72.7)	1.019	0.999-1.039	0.0662
HESX1	103	63 (61.2)	19.1 (24.3)	1.027	0.997-1.059	0.0811
DDX58	199	127 (63.8)	2.59 (2.96)	1.113	0.959-1.292	0.1587
IFIT1	175	113 (64.6)	12.5 (20.7)	1.018	0.990-1.046	0.2158
SIGLEC1	194	126 (64.9)	51.6 (308)	1.006	0.995-1.018	0.2991
MAVS	201	129 (64.2)	1.20 (0.84)	1.207	0.803-1.813	0.3650
IFNA4	184	116 (63.0)	2.13 (3.58)	0.968	0.891-1.051	0.4371
TBX3	133	84 (63.2)	1.89 (2.91)	1.073	0.898-1.281	0.4377
PKLR	34	21 (61.8)	1.30 (1.67)	1.132	0.657-1.951	0.6555
IRF3	203	130 (64.0)	1.15 (0.82)	1.058	0.724-1.546	0.7706
CXCL10	148	98 (66.2)	3.25 (4.48)	0.990	0.916-1.070	0.8042
CCL8	82	56 (68.3)	11.9 (20.4)	1.003	0.978-1.029	0.8072
C1S	48	34 (70.8)	3.13 (5.11)	1.008	0.879-1.157	0.9086
USP13	173	112 (64.7)	1.15 (0.96)	1.021	0.709-1.471	0.9113

In summary, the pooled analysis showed that gene expression of **5 ISGs** was statistically significantly associated with SVR at baseline or during the treatment (**IFNA4, DDX58, IRF3, RSAD2** and **IFI27**, see Table 29). The strongest association was observed for **IFNA4** and **DDX58** at baseline ($p<0.05$). **RSAD2, DDX58** and **IFI27** showed association at multiple time points that could evidence their significant biological role and relevance in this analysis. **RSAD2**, **IRF3** and **IFI27** had either high data variability or near significant association, therefore the results for these genes should be interpreted with caution.

Table 29: Genes associated with SVR ($p\leq0.05$): Summary

SVR=sustained virological response; n.s.=not significant.

Gene/Time point	Baseline	4 hours	Day 4	Day 8
IFNA4	p<0.05	n.s.	n.s.	n.s.
IRF3	p=0.05	p<0.05	n.s.	n.s.
DDX58	p<0.05	n.s.	p<0.05	n.s.
RSAD2	n.s.	n.s.	p<0.05	n.s.
IFI27	n.s.	n.s.	p<0.05	p<0.05

3.4.2.2. Adjusted analysis

Adjustment by IL28B (CC versus non-CC)

The analysis adjusted by IL28B showed that the baseline expression of **IFNA4** was associated with SVR (OR 1.116, 95% CI 1.003-1.242, p=0.0442, see Table 30). This association was weaker as compared to the unadjusted analysis, but still meaningful (pooled unadjusted analysis: OR 1.128, 95% CI 1.016-1.252, p=0.0238, see Table 25). This provides the evidence that **IFNA4** gene expression is predictive of SVR independently from IL28B genotype. **DDX58** was not found to be significantly associated with SVR at baseline in the adjusted analysis by IL28B that provides the evidence of its dependence to IL28B genotype. However, **DDX58** was significantly associated with SVR at day 4 in this adjusted analysis (OR 1.157, 95% CI 1.012-1.322, p=0.0325, see Table 32). The results for other genes were similar to the unadjusted analysis (see Tables 30-34). At 4 hours after the treatment start the expression change of **IRF3** was associated with SVR (OR 0.737, 95% CI 0.550-0.989, p=0.0419, see Table 31) similarly to the result of unadjusted analysis (OR 0.749, 95% CI 0.561-0.999, p=0.0491, see Table 26). **RSAD2** was associated with SVR at day 4 (OR 1.018, 95% CI 1.003-1.033, p=0.0170, see Table 32). At day 4 and day 8 the results for **IFI27** were similar to those seen in the unadjusted analysis (day 4: OR 1.003, 95% CI 1.001-1.005, p=0.0062; Day 8: OR 1.003, 95% CI 1.001-1.006, p=0.0139, see Tables 32-33).

Table 30: Baseline gene expression relationship to SVR: Adjusted by IL28B

SVR=sustained virological response; ΔCt=delta cycle threshold , IL28B=Interleukin-28B; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	ΔCt,, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFNA4	239	146 (61.1)	28.6 (2.74)	1.116	1.003-1.242	0.0442
PKLR	107	71 (66.4)	27.0 (1.47)	1.366	0.958-1.948	0.0851
IRF3	255	158 (62.0)	17.1 (0.87)	1.342	0.959-1.877	0.0859
DDX58	251	156 (62.2)	17.5 (1.21)	1.233	0.966-1.574	0.0920
CXCL10	212	130 (61.3)	25.1 (1.78)	1.155	0.972-1.373	0.1017
C1S	117	79 (67.5)	26.0 (1.42)	1.318	0.939-1.850	0.1102
MAVS	254	158 (62.2)	20.0 (1.12)	1.219	0.940-1.581	0.1353
IFIT1	235	147 (62.6)	23.7 (1.81)	1.119	0.947-1.322	0.1863
IFI27	244	152 (62.3)	22.5 (2.16)	1.092	0.950-1.255	0.2158
CCL8	138	88 (63.8)	26.0 (1.99)	1.106	0.917-1.335	0.2931
TBX3	202	127 (62.9)	28.8 (1.34)	1.122	0.884-1.424	0.3436
USP13	242	153 (63.2)	21.9 (1.02)	1.140	0.852-1.525	0.3788
SIGLEC1	247	155 (62.8)	21.5 (1.91)	1.065	0.907-1.250	0.4441
HESX1	161	92 (57.1)	28.0 (1.49)	1.084	0.853-1.377	0.5090
RSAD2	253	156 (61.7)	19.9 (1.84)	1.042	0.888-1.222	0.6164

Table 31: Gene expression change from baseline at 4 hours relationship to SVR: Adjusted by IL28B

SVR=sustained virological response; IL28B=Interleukin-28B; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IRF3	232	144 (62.1)	1.27 (1.07)	0.737	0.550-0.989	0.0419
RSAD2	228	141 (61.8)	45.3 (255)	1.009	0.999-1.019	0.0740
DDX58	230	144 (62.6)	8.86 (10.3)	1.033	0.995-1.073	0.0927
USP13	212	137 (64.6)	1.41 (2.05)	0.855	0.709-1.030	0.1000
MAVS	228	142 (62.3)	1.58 (2.21)	0.896	0.777-1.033	0.1289
IFNA4	208	129 (62.0)	159 (1202)	1.000	1.000-1.000	0.1522
PKLR	40	32 (80.0)	2.89 (3.26)	1.740	0.735-4.121	0.2080
SIGLEC1	223	142 (63.7)	15.7 (143)	1.028	0.980-1.078	0.2567
TBX3	155	99 (63.9)	1.80 (4.73)	0.956	0.884-1.035	0.2659
CXCL10	192	119 (62.0)	275 (494)	1.000	1.000-1.001	0.2729
C1S	57	42 (73.7)	4.19 (15.0)	0.945	0.852-1.049	0.2867
HESX1	126	72 (57.1)	43.9 (61.6)	1.004	0.996-1.011	0.3829
CCL8	120	78 (65.0)	478 (1144)	1.000	1.000-1.001	0.3889
IFIT1	216	136 (63.0)	47.9 (80.0)	1.001	0.997-1.006	0.4900
IFI27	221	139 (62.9)	3.77 (21.0)	1.016	0.957-1.078	0.6034

Table 32: Gene expression change from baseline at Day 4 relationship to SVR: Adjusted by IL28B

SVR=sustained virological response; IL28B=Interleukin-28B; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFI27	206	129 (62.6)	254 (560)	1.003	1.001-1.005	0.0062
RSAD2	210	129 (61.4)	40.9 (164)	1.018	1.003-1.033	0.0170
DDX58	210	130 (61.9)	3.42 (3.58)	1.157	1.012-1.322	0.0325
HESX1	119	72 (60.5)	29.3 (37.7)	1.019	0.998-1.040	0.0743
SIGLEC1	205	128 (62.4)	66.5 (386)	1.008	0.998-1.018	0.1022
IFIT1	193	121 (62.7)	22.2 (41.3)	1.009	0.996-1.021	0.1713
C1S	47	30 (63.8)	1.94 (2.98)	1.457	0.798-2.657	0.2202
PKLR	45	30 (66.7)	1.78 (2.81)	1.325	0.770-2.279	0.3100
USP13	185	119 (64.3)	1.08 (0.88)	1.214	0.806-1.827	0.3532
MAVS	211	132 (62.6)	1.19 (0.91)	1.184	0.811-1.728	0.3818
CXCL10	175	111 (63.4)	10.8 (19.2)	1.008	0.989-1.028	0.3948
CCL8	105	66 (62.9)	27.6 (41.9)	1.002	0.991-1.012	0.7395
IRF3	213	132 (62.0)	1.19 (0.87)	1.061	0.746-1.509	0.7422
TBX3	154	93 (60.4)	1.78 (3.38)	0.987	0.894-1.088	0.7865
IFNA4	195	119 (61.0)	2.48 (8.07)	1.006	0.954-1.061	0.8121

Table 33: Gene expression change from baseline at Day 8 relationship to SVR: Adjusted by IL28B

SVR=sustained virological response; IL28B=Interleukin-28B; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFI27	192	125 (65.1)	240 (657)	1.003	1.001-1.006	0.0139
HESX1	103	63 (61.2)	19.1 (24.3)	1.030	0.998-1.062	0.0668
RSAD2	201	128 (63.7)	25.8 (72.7)	1.017	0.998-1.037	0.0831
DDX58	199	127 (63.8)	2.59 (2.96)	1.100	0.951-1.274	0.1999
SIGLEC1	194	126 (64.9)	51.6 (308)	1.006	0.994-1.018	0.3082
IFIT1	175	113 (64.6)	12.5 (20.7)	1.013	0.984-1.043	0.3725
MAVS	201	129 (64.2)	1.20 (0.84)	1.198	0.793-1.809	0.3901
TBX3	133	84 (63.2)	1.89 (2.91)	1.067	0.900-1.266	0.4526
PKLR	34	21 (61.8)	1.30 (1.67)	1.179	0.694-2.001	0.5427
IFNA4	184	116 (63.0)	2.13 (3.58)	0.976	0.898-1.061	0.5679
IRF3	203	130 (64.0)	1.15 (0.82)	1.104	0.758-1.608	0.6067
CCL8	82	56 (68.3)	11.9 (20.4)	1.003	0.979-1.029	0.7940
CXCL10	148	98 (66.2)	3.25 (4.48)	0.990	0.915-1.072	0.8110
USP13	173	112 (64.7)	1.15 (0.96)	1.037	0.722-1.490	0.8443
C1S	48	34 (70.8)	3.13 (5.11)	1.012	0.881-1.161	0.8707

Table 34: Genes associated with SVR, adjusted by IL28B ($p \leq 0.05$): Summary

SVR=sustained virological response; n.s.=not significant; IL28B=Interleukin-28B.

Gene/Time point	Baseline	4 hours	Day 4	Day 8
IFNA4	p<0.05	n.s.	n.s.	n.s.
IRF3	n.s.	p<0.05	n.s.	n.s.
DDX58	n.s.	n.s.	p<0.05	n.s.
RSAD2	n.s.	n.s.	p<0.05	n.s.
IFI27	n.s.	n.s.	p<0.05	p<0.05

Adjustment by viral GT (1a versus 1b)

The following 7 ISGs were statistically significantly associated with SVR ($p \leq 0.05$) in the pooled analysis adjusted by viral genotype at various time points: **IFNA4, DDX58, RSAD2**, **IFI27, IFIT1, CXCL10** and **HESX1** (see Tables 35-39). All genes which were identified to be associated with SVR in the pooled unadjusted analysis (**IFNA4, DDX58, RSAD2** and **IFI27**) except **IRF3** were also significantly associated in the adjusted analysis by viral GT. This provides the evidence of their independence from the viral genotype category.

Table 35: Baseline gene expression relationship to SVR: Adjusted by Viral Genotype

SVR=sustained virological response; SD=standard deviation; CI=confidence interval, ΔCt=delta cycle threshold.

Gene	N	Achieved SVR, n (%)	ΔCt,, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFNA4	239	146 (61.1)	28.6 (2.74)	1.130	1.013-1.262	0.0284
CXCL10	212	130 (61.3)	25.1 (1.78)	1.220	1.019-1.460	0.0300
DDX58	251	156 (62.2)	17.5 (1.21)	1.310	1.019-1.684	0.0351
IFIT1	235	147 (62.6)	23.7 (1.81)	1.197	1.006-1.425	0.0425
C1S	117	79 (67.5)	26.0 (1.42)	1.367	0.971-1.926	0.0736
CCL8	138	88 (63.8)	26.0 (1.99)	1.168	0.953-1.430	0.1339
IFI27	244	152 (62.3)	22.5 (2.16)	1.113	0.961-1.290	0.1527
IRF3	255	158 (62.0)	17.1 (0.87)	1.275	0.906-1.793	0.1637
MAVS	254	158 (62.2)	20.0 (1.12)	1.203	0.924-1.567	0.1694
TBX3	202	127 (62.9)	28.8 (1.34)	1.180	0.916-1.521	0.1990
PKLR	107	71 (66.4)	27.0 (1.47)	1.238	0.878-1.746	0.2224
SIGLEC1	247	155 (62.8)	21.5 (1.91)	1.105	0.935-1.306	0.2404
HESX1	161	92 (57.1)	28.0 (1.49)	1.160	0.899-1.498	0.2544
RSAD2	253	156 (61.7)	19.9 (1.84)	1.104	0.929-1.310	0.2610
USP13	242	153 (63.2)	21.9 (1.02)	1.152	0.855-1.551	0.3524

Table 36: Gene expression change from baseline at 4 hours relationship to SVR: Adjusted by Viral Genotype

SVR=sustained virological response; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
DDX58	230	144 (62.6)	8.86 (10.3)	1.048	1.003-1.094	0.0344
RSAD2	228	141 (61.8)	45.3 (255)	1.012	1.001-1.023	0.0345
HESX1	126	72 (57.1)	43.9 (61.6)	1.014	1.000-1.028	0.0524
IRF3	232	144 (62.1)	1.27 (1.07)	0.744	0.533-1.038	0.0818
CXCL10	192	119 (62.0)	275 (494)	1.001	1.000-1.002	0.1321
SIGLEC1	223	142 (63.7)	15.7 (143)	1.038	0.986-1.092	0.1578
IFIT1	216	136 (63.0)	47.9 (80.0)	1.003	0.999-1.008	0.1643
PKLR	40	32 (80.0)	2.89 (3.26)	1.675	0.768-3.652	0.1944
USP13	212	137 (64.6)	1.41 (2.05)	0.867	0.689-1.092	0.2262
IFNA4	208	129 (62.0)	159 (1202)	1.000	1.000-1.000	0.2717
TBX3	155	99 (63.9)	1.80 (4.73)	0.965	0.899-1.036	0.3302
MAVS	228	142 (62.3)	1.58 (2.21)	0.931	0.791-1.096	0.3883
CCL8	120	78 (65.0)	478 (1144)	1.000	0.999-1.001	0.4031
C1S	57	42 (73.7)	4.19 (15.0)	0.955	0.839-1.086	0.4791
IFI27	221	139 (62.9)	3.77 (21.0)	1.022	0.928-1.126	0.6582

Table 37: Gene expression change from baseline at Day 4 relationship to SVR: Adjusted by Viral Genotype

SVR=sustained virological response; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
RSAD2	210	129 (61.4)	40.9 (164)	1.024	1.007-1.042	0.0064
IFI27	206	129 (62.6)	254 (560)	1.003	1.001-1.005	0.0068
DDX58	210	130 (61.9)	3.42 (3.58)	1.214	1.048-1.407	0.0096
SIGLEC1	205	128 (62.4)	66.5 (386)	1.009	1.000-1.019	0.0577
HESX1	119	72 (60.5)	29.3 (37.7)	1.020	0.999-1.042	0.0600
IFIT1	193	121 (62.7)	22.2 (41.3)	1.013	0.998-1.028	0.0934
MAVS	211	132 (62.6)	1.19 (0.91)	1.361	0.930-1.993	0.1130
C1S	47	30 (63.8)	1.94 (2.98)	1.515	0.800-2.868	0.2023
USP13	185	119 (64.3)	1.08 (0.88)	1.260	0.823-1.930	0.2876
CXCL10	175	111 (63.4)	10.8 (19.2)	1.010	0.990-1.030	0.3478
IRF3	213	132 (62.0)	1.19 (0.87)	1.138	0.802-1.615	0.4691
PKLR	45	30 (66.7)	1.78 (2.81)	1.175	0.709-1.949	0.5314
IFNA4	195	119 (61.0)	2.48 (8.07)	1.016	0.962-1.072	0.5781
CCL8	105	66 (62.9)	27.6 (41.9)	1.003	0.992-1.014	0.5962
TBX3	154	93 (60.4)	1.78 (3.38)	0.992	0.896-1.099	0.8800

Table 38: Gene expression change from baseline at Day 8 relationship to SVR: Adjusted by Viral Genotype

SVR=sustained virological response; SD=standard deviation; CI=confidence interval.

Gene	N	Achieved SVR, n (%)	Fold change, mean (SD)	Odds Ratio	95% CI, lower – upper limit	p-value
IFI27	192	125 (65.1)	240 (657)	1.003	1.000-1.005	0.0442
RSAD2	201	128 (63.7)	25.8 (72.7)	1.020	0.998-1.043	0.0749
HESX1	103	63 (61.2)	19.1 (24.3)	1.029	0.996-1.064	0.0893
DDX58	199	127 (63.8)	2.59 (2.96)	1.102	0.948-1.280	0.2045
IFIT1	175	113 (64.6)	12.5 (20.7)	1.019	0.987-1.052	0.2526
TBX3	133	84 (63.2)	1.89 (2.91)	1.146	0.883-1.487	0.3057
SIGLEC1	194	126 (64.9)	51.6 (308)	1.004	0.992-1.016	0.5190
PKLR	34	21 (61.8)	1.30 (1.67)	1.209	0.650-2.247	0.5493
MAVS	201	129 (64.2)	1.20 (0.84)	1.129	0.732-1.741	0.5821
IFNA4	184	116 (63.0)	2.13 (3.58)	0.979	0.892-1.074	0.6486
USP13	173	112 (64.7)	1.15 (0.96)	0.928	0.632-1.360	0.7003
CXCL10	148	98 (66.2)	3.25 (4.48)	0.990	0.912-1.074	0.8087
C1S	48	34 (70.8)	3.13 (5.11)	1.009	0.876-1.162	0.9038
CCL8	82	56 (68.3)	11.9 (20.4)	1.001	0.973-1.030	0.9412
IRF3	203	130 (64.0)	1.15 (0.82)	1.012	0.676-1.515	0.9557

Table 39: Genes associated with SVR, adjusted by viral GT ($p \leq 0.05$): Summary

SVR=sustained virological response; n.s.=not significant; GT=genotype.

Gene/Time point	**Baseline**	**4 hours**	**Day 4**	**Day 8**
IFNA4	p<0.05	n.s.	n.s.	n.s.
IFIT1	p<0.05	n.s.	n.s.	n.s.
CXCL10	p<0.05	n.s.	n.s.	n.s.
DDX58	p<0.05	p<0.05	p<0.05	n.s.
HESX1	n.s.	p=0.05	n.s.	n.s.
RSAD2	n.s.	p<0.05	p<0.05	n.s.
IFI27	n.s.	n.s.	p<0.05	p<0.05

Adjustment by previous treatment (TN versus TE)

The association analysis adjusted by treatment-naïve and treatment-experienced groups did not show any of the tested gene to be statistically significantly associated with SVR.

In summary, the adjusted analysis by IL28B, viral genotype and previous treatment showed similar results to those seen in the pooled unadjusted analysis for **IFNA4, DDX58, IRF3, RSAD2** and **IFI27.** These genes were associated with SVR also in the adjusted analysis by IL28B and/or viral genotype. All but **IRF3** were associated with SVR in both unadjusted analysis and the adjusted analysis by IL28B and viral genotype. **IR3F** was associated with SVR in the unadjusted analysis and in the adjustment by IL28B only. Additionally, **IFIT1, CXCL10** and **HESX1** showed association with SVR in the analysis adjusted by viral genotype at baseline (**CXCL10** and **IFIT1**) and at 4 hours time point (**HESX1**), but these genes did not show statistically significant association with SVR in the pooled unadjusted analysis. **IFNA4** showed statistically significant association with SVR in all adjusted and not adjusted pooled analyses at baseline only. **DDX58** showed consistent data at day 4 in all analyses, but it was not statistically significantly associated with SVR at baseline in the analysis adjusted by IL28B. **IRF3** showed association with SVR at baseline and 4 hours in the pooled unadjusted analysis but this association was seen in the analysis adjusted by IL28B only at 4 hours. **RSAD2** showed association with SVR consistently in all analyses at day 4 and **IFI27** association was observed at day 4 and day 8 in the pooled unadjusted and all adjusted analyses.

3.4.3. Group differences

Pooled Analysis: SVR

Overall pooled analysis of the group difference between SVR and non-SVR patients showed that the baseline expression of **C1S, CXCL10, DDX58** and **IFNA4** genes was lower in SVR patients ($p<0.05$). On-treatment induction of **DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2** was higher in SVR patients (see Table 40). For the interpretation of the data presented in the tables it should be noted that the Ct is inversely proportional to RNA copy number (i.e. low Ct = high copy number of transcripts). The higher baseline ΔCt means the lower gene expression at baseline. The fold change transformation means that the higher fold change translates as higher expression of the gene in the respective category.

Table 40: Genes with statistically significant difference between SVR versus non-SVR patients (p≤0.05): pooled data

SVR=sustained virological response; ΔCt=delta cycle threshold; SD=standard deviation;
FC=fold change; ↓ - lower expression in SVR patients; ↑ - higher expression in SVR patients.

Gene		**Baseline, ΔCt**	**4 hours, FC**	**Day 4, FC**	**Day 8, FC**
C1S	Trend (p-value)	SVR↓ (0.04)	-	-	-
	SVR: mean (SD)	26.2 (1.43)			
	No SVR: mean (SD)	25.6 (1.28)			
CXCL10	Trend (p-value)	SVR↓ (0.049)	-	-	-
	SVR: mean (SD)	25.3 (1.78)			
	No SVR: mean (SD)	24.7 (1.73)			
DDX58	Trend (p-value)	SVR↓ (0.039)	SVR↑ (0.035)	SVR↑ (0.005)	-
	SVR: mean (SD)	17.6 (1.20)	9.97 (11.9)	3.81 (4.04)	
	No SVR: mean (SD)	17.2 (1.19)	7.25 (6.60)	2.53 (2.16)	
HESX1	Trend (p-value)	-	-	SVR↑ (0.02)	SVR↑ (0.037)
	SVR: mean (SD)			34.3 (44.3)	22.5 (28.3)
	No SVR: mean (SD)			19.4 (20.7)	13.5 (13.3)
IFI27	Trend (p-value)	-	-	SVR↑(0.007)	SVR↑(0.018)
	SVR: mean (SD)			315 (697)	298 (807)
	No SVR: mean (SD)			141 (121)	122 (115)
IFIT1	Trend (p-value)	-	-	SVR↑ (0.05)	-
	SVR: mean (SD)			25.6 (48.8)	
	No SVR: mean (SD)			14.9 (22.9)	
IFNA4	Trend (p-value)	SVR↓ (0.046)	-	-	-
	SVR: mean (SD)	28.9 (2.09)			
	No SVR: mean (SD)	28.0 (3.66)			
PKLR	Trend (p-value)	-	SVR↑ (0.003)	-	-
	SVR: mean (SD)		3.32 (3.51)		
	No SVR: mean (SD)		1.18 (0.74)		
RSAD2	Trend (p-value)	-	-	-	SVR↑ (0.025)
	SVR: mean (SD)				30.9 (89.1)
	No SVR: mean (SD)				12.5 (15.0)

For the majority of these genes the difference was also seen in the median, min/max values as shown in the boxplots (see Figure 7 and 8).

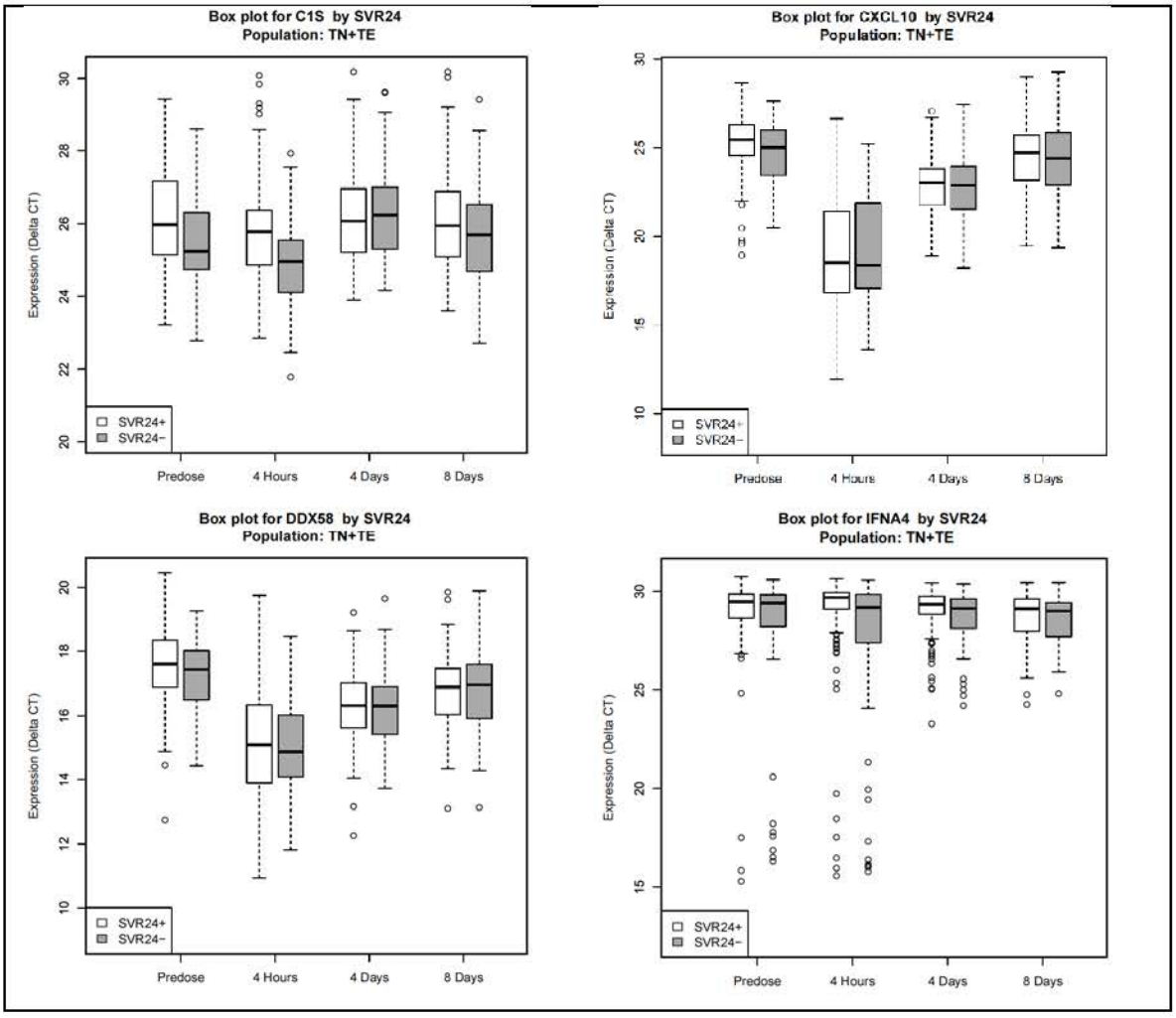

Figure 7: Boxplot ΔCt Gene Expression for the selected ISGs ($p<0.05$ for the difference at baseline): SVR versus non-SVR patients. Pooled data

SVR24=sustained virological response; ISG=interferon-stimulated gene; CT=cycle threshold; TN=treatment-naïve; TE=treatment-experienced.

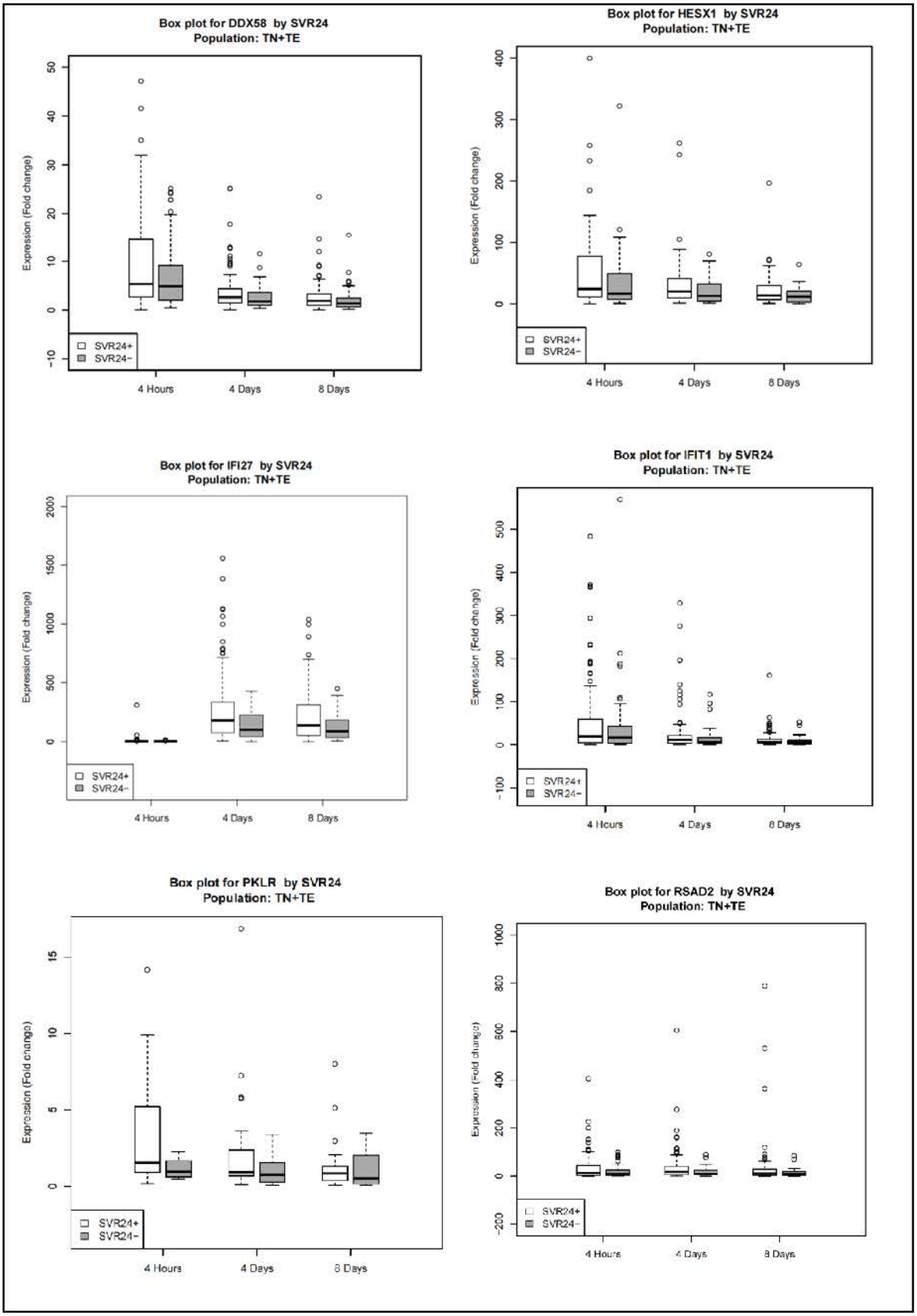

Figure 8: Boxplot ΔΔCt gene expression change for the selected ISGs ($p<0.05$ for the fold change difference at 4 hours, day 4 or day 8): SVR versus non-SVR patients. Pooled data

SVR24=sustained virological response; ISG=interferon-stimulated gene; ΔΔCt=delta delta cycle threshold; TN=treatment-naive; TE=treatment-experienced.

Pooled Analysis: IL28B

No differences were reported in the pooled analysis of the IL28B CC versus non-CC patients.

Pooled Analysis: TN versus TE

The majority of the genes (N=14) had lower baseline expression in the treatment-naive patients and many of them (**C1S, DDX58, HESX1, IFI27, IFIT1, IRF3, MAVS, RSAD2** and **USP13**) had stronger induction during the treatment in treatment-naive as compared to treatment-experienced patients (see Table 41).

Table 41: Genes with statistically significant difference between TN versus TE patients ($p<0.05$): pooled data

ΔCt=delta cycle threshold; TN=treatment-naïve; TE=treatment-experienced; SD=standard deviation; FC=fold change; ↓ - lower expression in TN patients; ↑ higher expression in TN patients.

Gene		Baseline, ΔCt	4 hours, FC	Day 4, FC	Day 8, FC
C1S	Trend (p-value)	TN↓ (<0.01)	-	TN↑ (0.015)	-
	TN, mean (SD)	26.3 (1.49)		2.37 (3.40)	
	TE, mean (SD)	25.4 (1.05)		0.80 (0.72)	
CXCL10	Trend (p-value)	TN↓ (<0.01)			
	TN, mean (SD)	25.5 (1.65)			
	TE, mean (SD)	24.4 (1.75)			
DDX58	Trend (p-value)	TN↓ (<0.01)	TN↑ (0.032)	TN↑ (0.007)	TN↑ (0.001)
	TN, mean (SD)	17.8 (1.16)	9.74 (11.4)	3.95 (3.60)	3.06 (3.27)
	TE, mean (SD)	17.0 (1.15)	7.02 (7.49)	2.59 (3.41)	1.76 (2.08)
HESX1	Trend (p-value)	TN↓ (0.001)	-	-	TN↑ (0.017)
	TN, mean (SD)	28.3 (1.41)			22.9 (27.5)
	TE, mean (SD)	27.5 (1.5)			12.6 (15.5)
IFI27	Trend (p-value)	TN↓ (<0.01)	-	TN ↑ (0.006)	TN ↑ (0.014)
	TN, mean (SD)	22.9 (2.08)		324 (694)	304 (805)
	TE, mean (SD)	21.7 (2.09)		141 (191)	120 (128)
IFIT1	Trend (p-value)	TN↓ (<0.01)	-	-	TN↑ (0.02)
	TN, mean (SD)	24.1 (1.68)			14.8 (24.1)
	TE, mean (SD)	23.0 (1.85)			8.36 (12.0)
IFNA4	Trend (p-value)	TN↓ (<0.01)	-	-	-
	TN, mean (SD)	29.2 (1.04)			
	TE, mean (SD)	27.5 (4.23)			

Table 41: Genes with statistically significant difference between TN versus TE patients ($p<0.05$): pooled data (continued)

ΔCt=delta cycle threshold; TN=treatment-naïve; TE=treatment-experienced; SD=standard deviation; FC=fold change; ↓ - lower expression in TN patients; ↑ higher expression in TN patients.

Gene		Baseline, ΔCt	4 hours, FC	Day 4, FC	Day 8, FC
IRF3	Trend (p-value)	TN↓ (<0.01)	-	TN↑(0.029)	TN↑(0.015)
	TN, mean (SD)	17.2 (0.8)		1.29 (0.79)	1.25 (0.82)
	TE, mean (SD)	16.7 (0.91)		1.01 (0.97)	0.96 (0.79)
MAVS	Trend (p-value)	TN↓ (<0.01)	-	TN↑(0.009)	TN↑(0.007)
	TN, mean (SD)	20.2 (1.10)		1.32 (0.94)	1.31 (0.9)
	TE, mean (SD)	19.6 (1.07)		0.98 (0.85)	1.0 (0.66)
PKLR	Trend (p-value)	TN↓ (0.042)	-	-	-
	TN, mean (SD)	27.2 (1.34)			
	TE, mean (SD)	26.6 (1.63)			
RSAD2	Trend (p-value)	TN↓ (0.001)	-	-	TN↑ (0.016)
	TN, mean (SD)	20.2 (1.88)			32.7 (88.6)
	TE, mean (SD)	19.4 (1.66)			13.1 (17.0)
SIGLEC	Trend (p-value)	TN↓ (<0.01)	-	-	-
	TN, mean (SD)	21.8 (1.96)			
	TE, mean (SD)	21.0 (1.68)			
TBX3	Trend (p-value)	TN↓ (0.021)	-	-	-
	TN, mean (SD)	29.0 (1.2)			
	TE, mean (SD)	28.5 (1.51)			
USP13	Trend (p-value)	TN↓ (<0.01)	-	TN↑(0.006)	TN↑(0.009)
	TN, mean (SD)	22.0 (1.01)		1.21 (0.92)	1.26 (1.04)
	TE, mean (SD)	21.5 (0.96)		0.86 (0.77)	0.91 (0.7)

Treatment-naïve patients: SVR

The analysis of the group differences in treatment-naïve patients showed that none of the gene had statistically significant difference of the expression at baseline between SVR versus non-SVR patients, however **C1S, IFI27, IFIT1** and **RSAD2** had stronger induction in SVR patients during the treatment (see Table 42).

Table 42: Genes with statistically significant difference between SVR versus non-SVR patients ($p<0.05$): TN patients

SVR=sustained virological response; ΔCt=delta cycle threshold; TN=treatment-naïve;
FC=fold change; ↓ - lower expression in SVR patients; ↑ higher expression in SVR patients.

Gene		**Baseline, ΔCt**	**4 hours, FC**	**Day 4, FC**	**Day 8, FC**
C1S	Trend (p-value)	-	SVR↑(0.009)	-	-
	SVR: mean (SD)		2.37 (2.6)		
	No SVR: mean (SD)		0.89 (0.45)		
IFI27	Trend (p-value)	-	-	-	SVR↑(0.038)
	SVR: mean (SD)				335 (887)
	No SVR: mean (SD)				142 (104)
IFIT1	Trend (p-value)	-	SVR↑(0.016)	SVR↑(0.035)	-
	SVR: mean (SD)		53.8 (87.0)	24.5 (43.7)	
	No SVR: mean (SD)		27.7 (28.6)	12.9 (10.4)	
RSAD2	Trend (p-value)	-	-	-	SVR↑(0.035)
	SVR: mean (SD)				35.1 (97.7)
	No SVR: mean (SD)				14.1 (9.53)

For the majority of these genes the tendency was also seen in the median, min/max values as shown in the boxplots (see Figure 9).

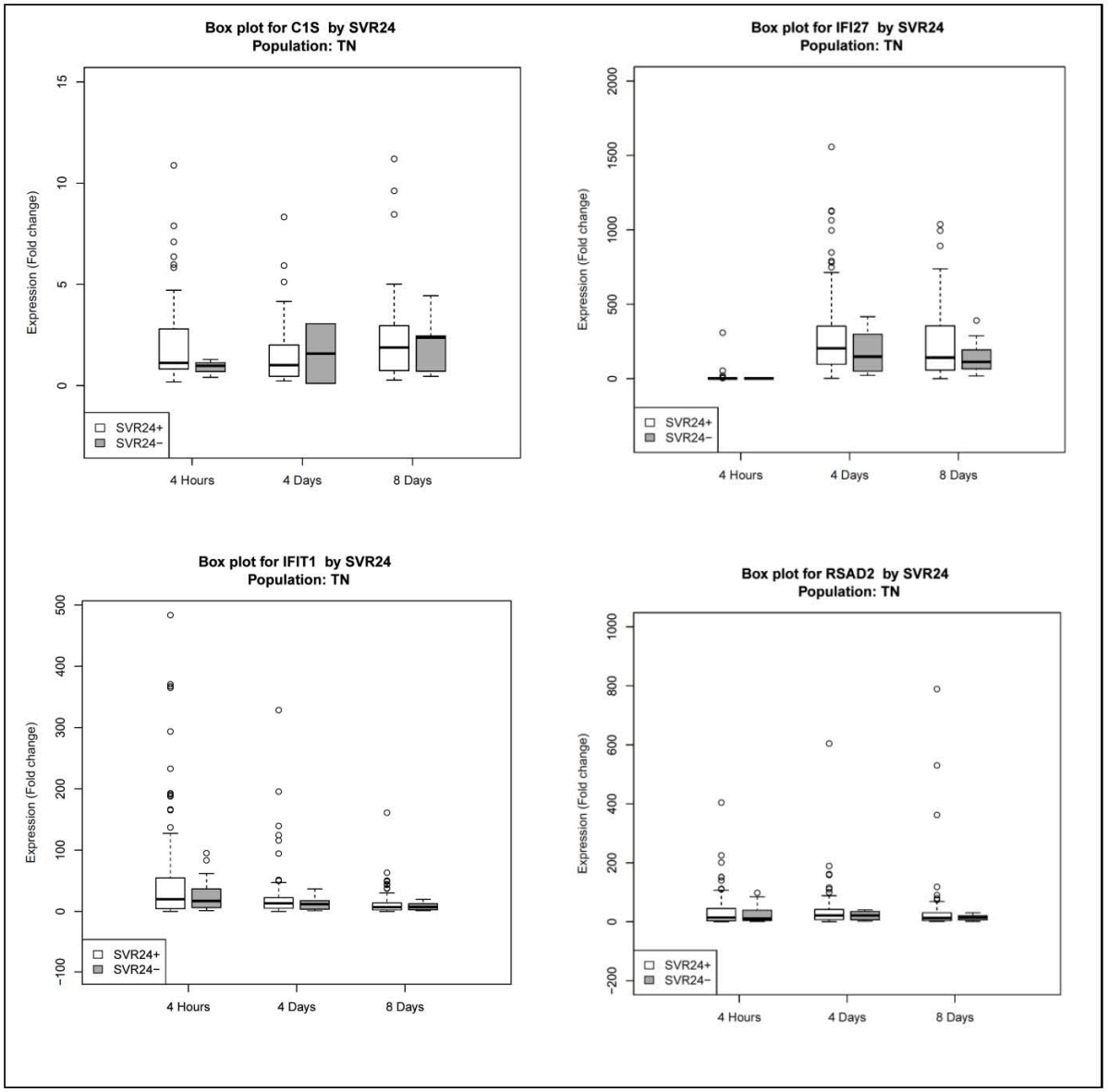

Figure 9: Boxplot ΔΔCt gene expression change for the selected ISGs ($p<0.05$ for the fold change difference at 4 hours, day 4 or day 8): SVR versus non-SVR patients. TN Patients

SVR24=sustained virological response; ISG=interferon-stimulated gene, ΔΔCt=delta delta cycle threshold; TN=treatment-naïve.

Treatment-experienced patients: IL28B

In the analysis of the treatment-experienced patients by IL28B, no statistically significant differences were reported in IL28B CC versus IL28B non-CC patients at baseline. In this group, no differences in IL28B CC versus non-CC patients were reported at baseline, however the induction of **CXCL10, HESX1, IFI27**, **IFIT1, RSAD2** and **SIGLEC** was lower in IL28B CC patients (see Table 43 and Figure 10).

Table 43: Genes with statistically significant difference between IL28B CC versus non-CC patients ($p<0.05$): TE patients

IL28B=Interleukin-28B; ΔCt=delta cycle threshold; TE=treatment-experienced; SD=standard deviation; FC=fold change; ↓ - lower expression in CC patients; ↑ - higher expression in CC patients.

Gene		**Baseline, ΔCt**	**4 hours, FC**	**Day 4, FC**	**Day 8, FC**
CXCL10	Trend (p-value)	-	-	CC↓(0.004)	CC↓(0.007)
	CC, mean (SD)			2.37 (2.32)	0.9 (0.66)
	CT/TT, mean (SD)			13.6 (28.1)	3.57 (6.05)
HESX1	Trend (p-value)	-	-	-	CC↓(0.004)
	CC, mean (SD)				3.15 (3.78)
	CT/TT, mean (SD)				13.8 (16.0)
IFI27	Trend (p-value)	-	-	CC↓(0.037)	-
	CC, mean (SD)			75.5 (78.0)	
	CT/TT, mean (SD)			151 (201)	
IFIT1	Trend (p-value)	-	-	CC↓ (0.005)	CC↓ (0.039)
	CC, mean (SD)			5.32 (5.5)	3.94 (4.08)
	CT/TT, mean (SD)			22.1 (45.4)	8.91 (12.6)
RSAD2	Trend (p-value)	-	CC↓(0.031)	CC↓ (0.034)	CC↓ (0.022)
	CC, mean (SD)		10.5 (7.95)	8.84 (14.1)	5.62 (7.91)
	CT/TT, mean (SD)		19.3 (23.3)	23.2 (40.2)	14.2 (17.8)
SIGLEC	Trend (p-value)	-	-	-	CC↓ (0.016)
	CC, mean (SD)				10.3 (10.6)
	CT/TT, mean (SD)				23.3 (24.6)

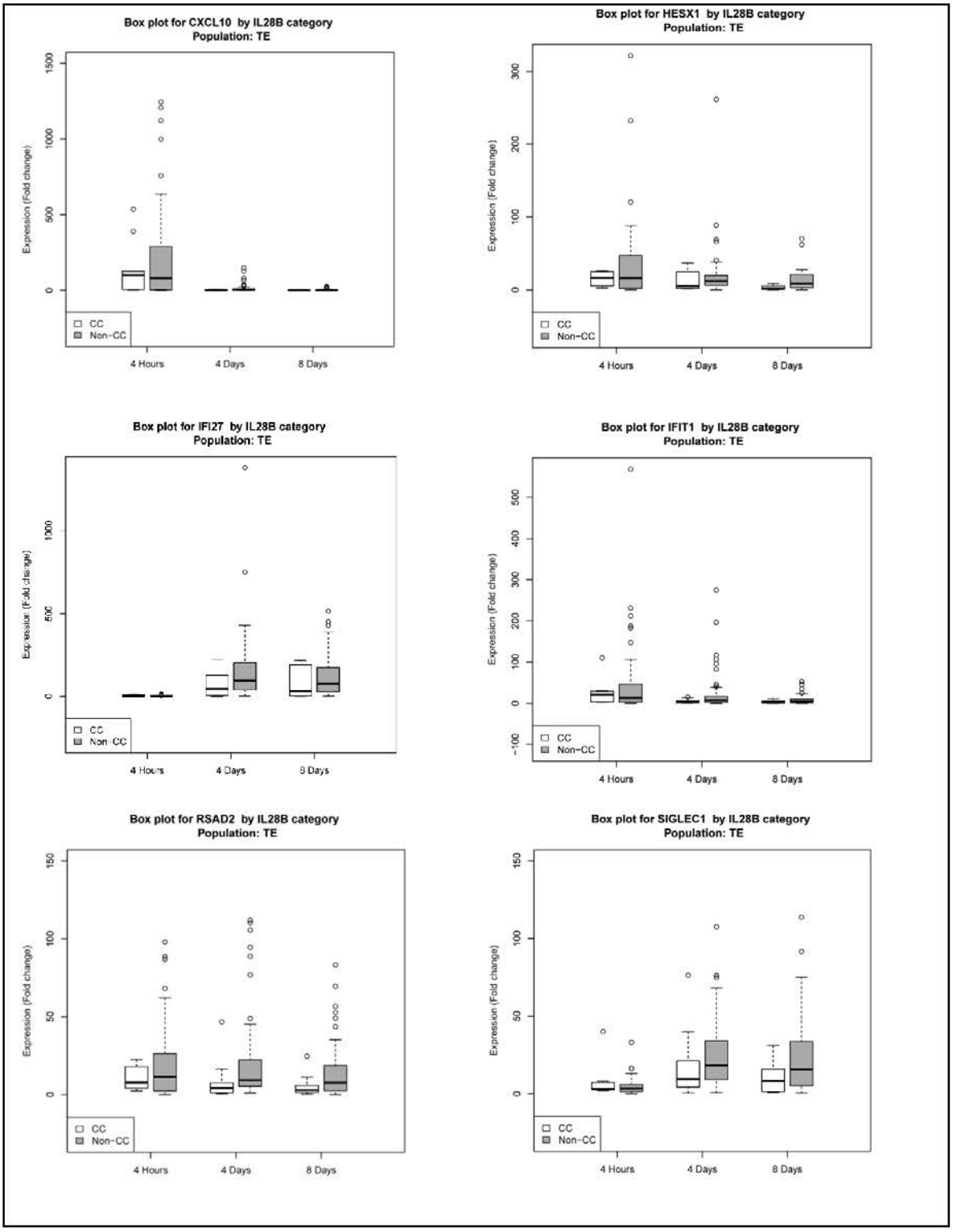

Figure 10: Boxplot ΔΔCt gene expression change for the selected ISGs ($p<0.05$ for the fold change difference at 4 hours, day 4 or day 8): IL28B CC versus non-CC patients. TE Patients

IL28B=Interleukin-28B; ΔΔCt=delta delta cycle threshold; TE=treatment-experienced.

In summary, treatment-naïve patients had lower baseline expression of the majority of the tested genes at baseline as compared to treatment-experienced patients (14 of 15 tested genes) and many of them had higher induction during the treatment (**C1S, DDX58, HESX1, IFI27, IFIT1, IRF3, MAVS, RSAD2** and **USP13**). SVR patients had higher induction of 6 genes (**DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2**) during the treatment as compared to non-SVR patients ($p<0.05$). **C1S, CXCL10, DDX58** and **IFNA4** had lower baseline expression in SVR patients as compared to non-SVR patients ($p<0.05$). Pooled analysis of the IL28B CC versus non-CC patient did not show any significant differences, however differences were reported in the treatment-experienced sub-population.

3.4.4. Faldaprevir/Placebo and lead-in effect

3.4.4.1. Faldaprevir vs. placebo effect

Pooled analysis in the treatment-naïve patients showed no statistically significant differences between placebo and faldaprevir lead-in and no lead-in groups and no faldaprevir effect was observed in the majority of 15 tested genes. Sub-analysis of these groups in SVR patients did not show any relevant differences between these groups as well.

3.4.4.2. Lead-in effect

Overall, the placebo group had the tendency of stronger regulation of the ISGs as compared to FDV lead-in (LI) and FDV no lead-in (non-LI) groups, whenever the differences across the LI versus non-LI were not remarkable. For many of the genes this difference was already seen at 4 hours and day 4 when both placebo and lead-in patients received the same treatment (PegIFN/RBV without FDV), and the difference in the induction of these genes between the placebo and FDV LI group cannot be explained by adding FDV to the treatment, but rather stronger induction in the placebo group and other factors (see Figure 11).

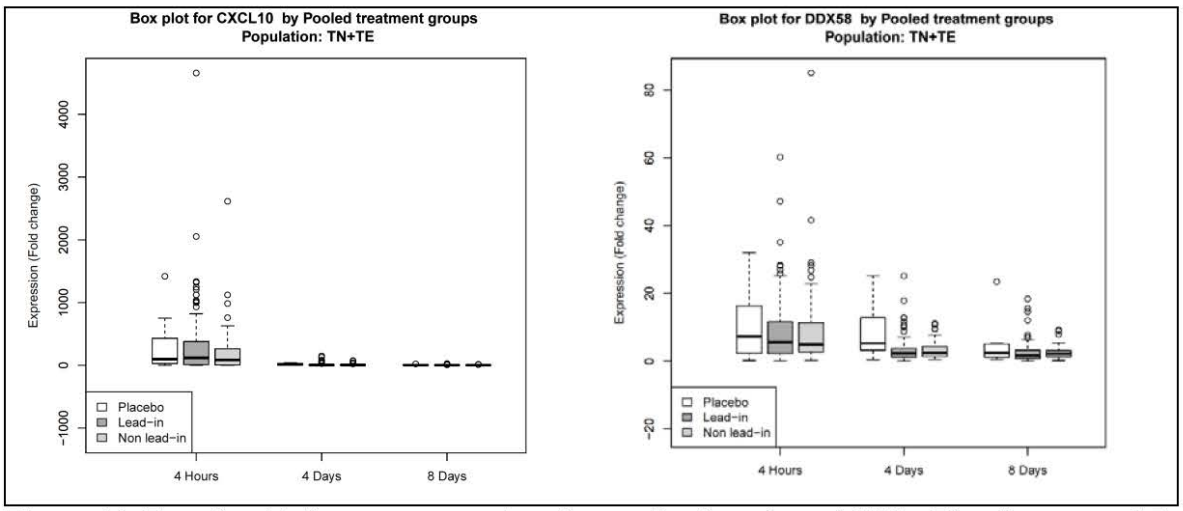

Figure 11: Boxplot ΔΔCt gene expression change for the selected ISGs: Placebo versus LI and no LI groups

IL28B=Interleukin-28B; ISG=interferon-stimulated gene; TN=treatment-naïve; TE=treatment-experienced; LI=lead-in; ΔCt=delta cycle threshold.

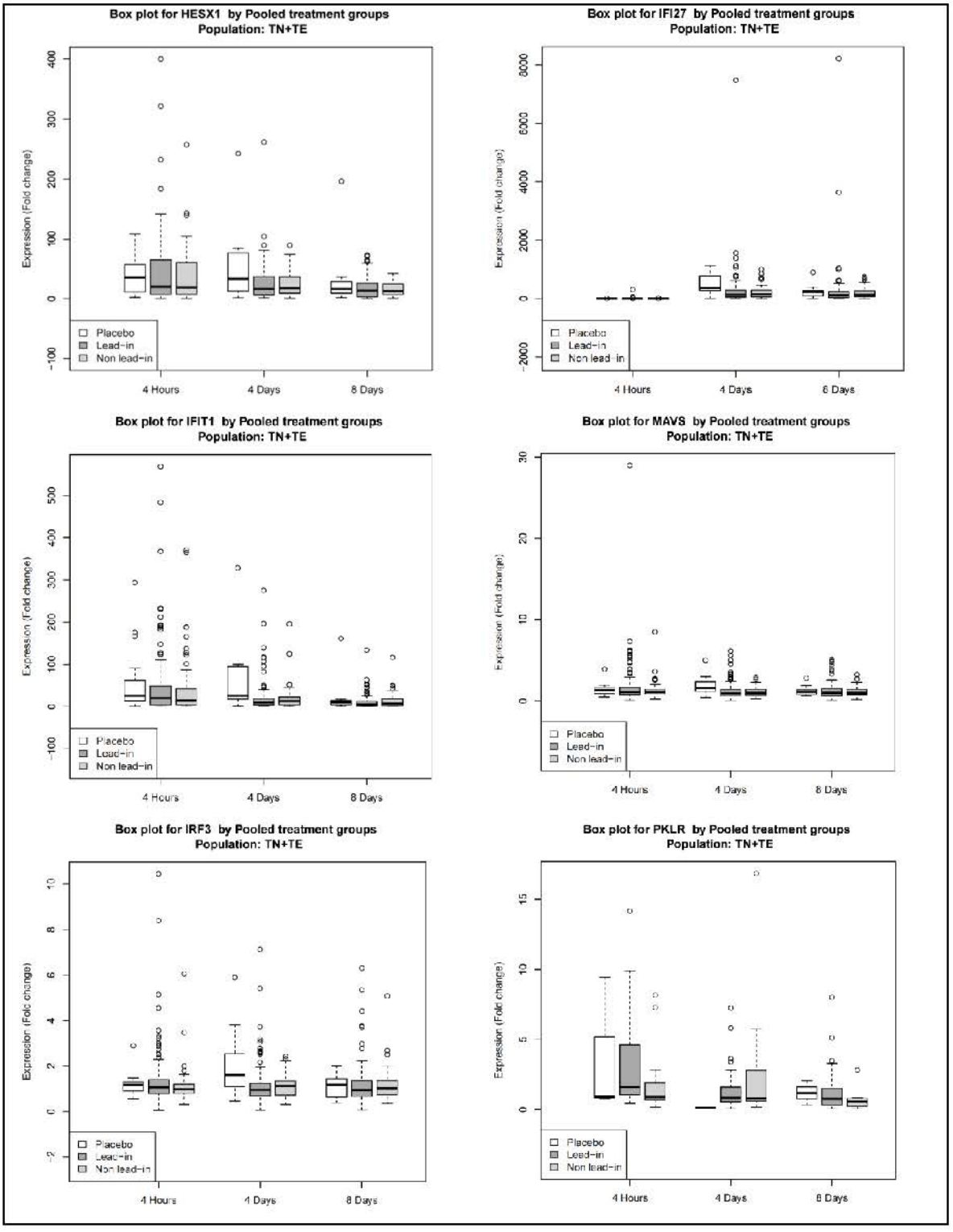

Figure 11: Boxplot ΔΔCt gene expression change for the selected ISGs: Placebo versus LI and no LI groups (continued)

IL28B=Interleukin-28B; ISG=interferon-stimulated gene; TN=treatment-naïve; TE=treatment-experienced; LI=lead-in; ΔΔCt=delta delta cycle threshold.

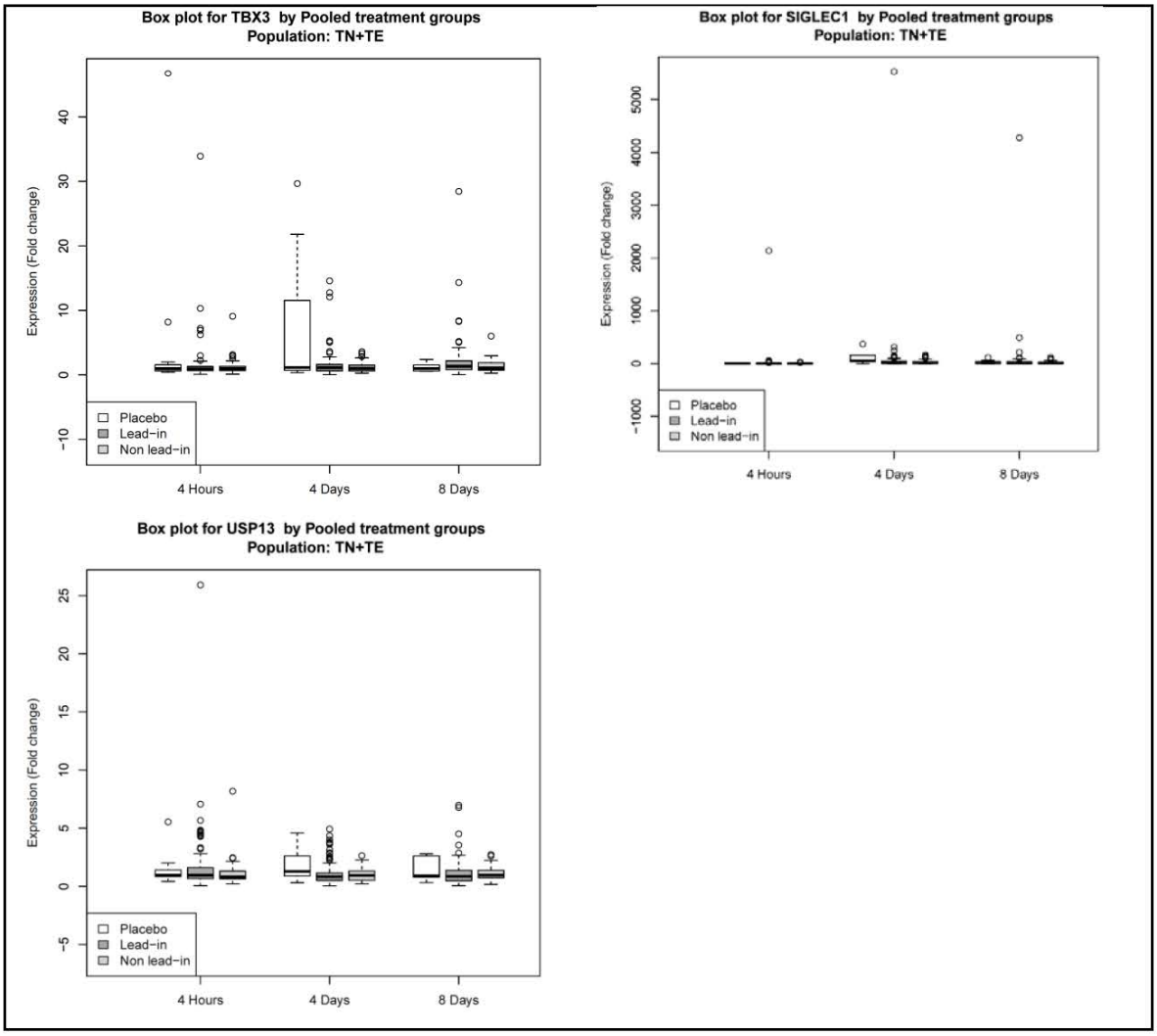

Figure11: Boxplot ΔΔCt gene expression change for the selected ISGs: Placebo versus LI and no LI groups (continued)

IL28B=Interleukin-28B; ISG=interferon-stimulated gene; TN=treatment-naïve; TE=treatment-experienced; LI=lead-in; ΔΔCt=delta delta cycle threshold.

3.4.4.3. Correlation of gene expression with the viral load

Supplementary to the lead-in effect analysis, we performed the correlation analysis of the gene expression change from baseline with the HCV RNA viral load (VL) decline.

The majority of the ISGs across the groups had a weak or no correlation of the gene expression change with the HCV RNA VL decline. Strong correlation was reported only for **C1S** gene at day 4 in the placebo group (r=-0.79), but no correlation was observed in both the lead-in (r=-0.07) and no lead-in groups (r=0.15).

Similar pattern was reported for other genes, for example **TBX3.** In the placebo group **TBX3** had the weak inverse correlation (r=0.66), no correlation was observed in both the lead-in (r=-0.15) and no lead-in groups (r=0.16) at day 4 (see Figure 12).

In summary, the analysis of the correlation of the gene expression change with the HCV RNA VL decline in placebo, non-LI and LI patient groups did not show any pattern of the differences in the LI versus non-LI patients and does not explain the dependence of the gene expression change to the HCV RNA VL decline.

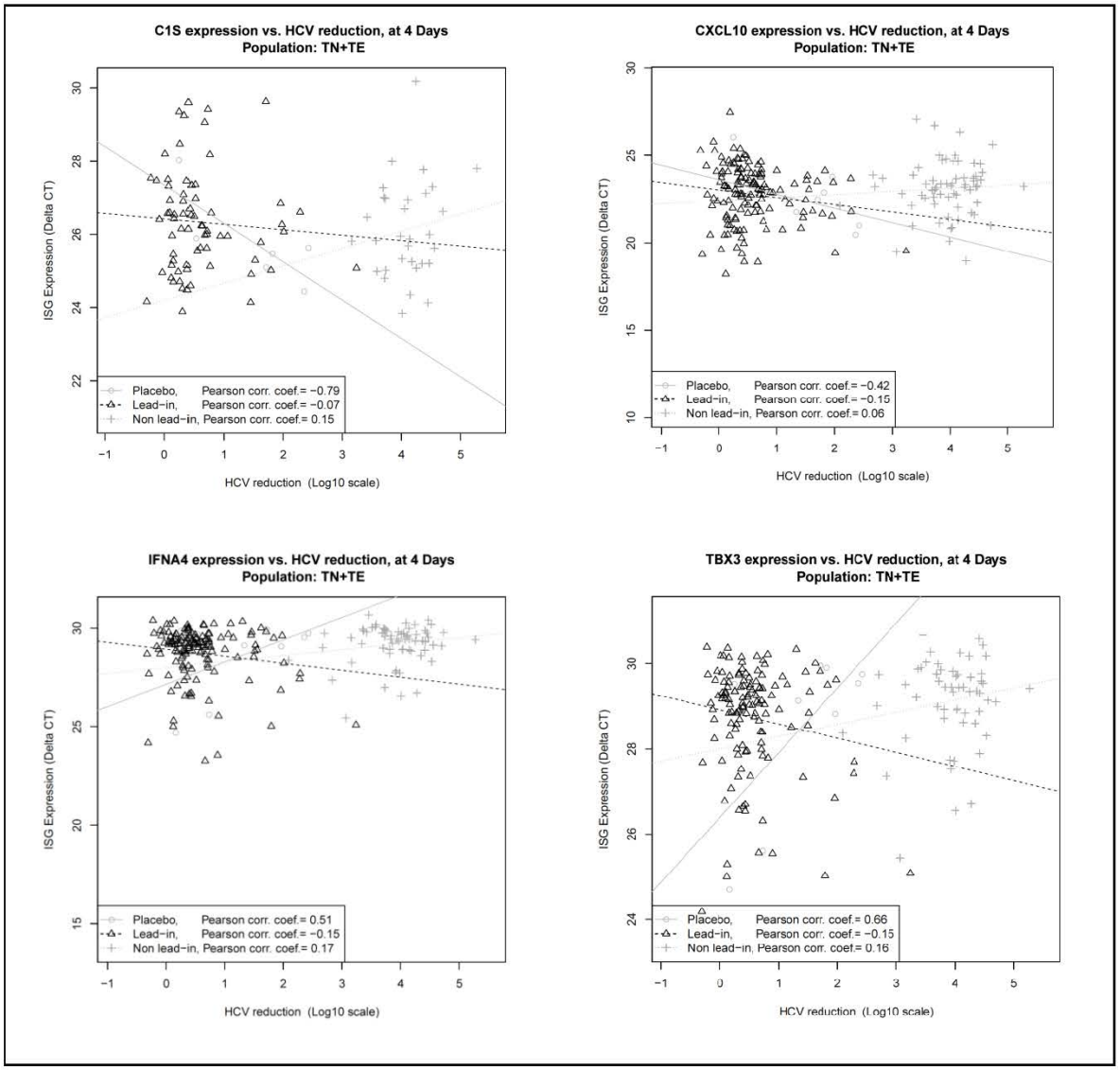

Figure 12: Correlation of the gene expression change with the HCV RNA VL decline at day 4 and day 8: pooled analysis (r=>0.4/<-0.4)

HCV=Hepatitis C Virus; RNA=ribonucleic acid; TN=treatment-naïve; TE=treatment-experienced; CT=cycle threshold; r=pearson correlation coefficient.

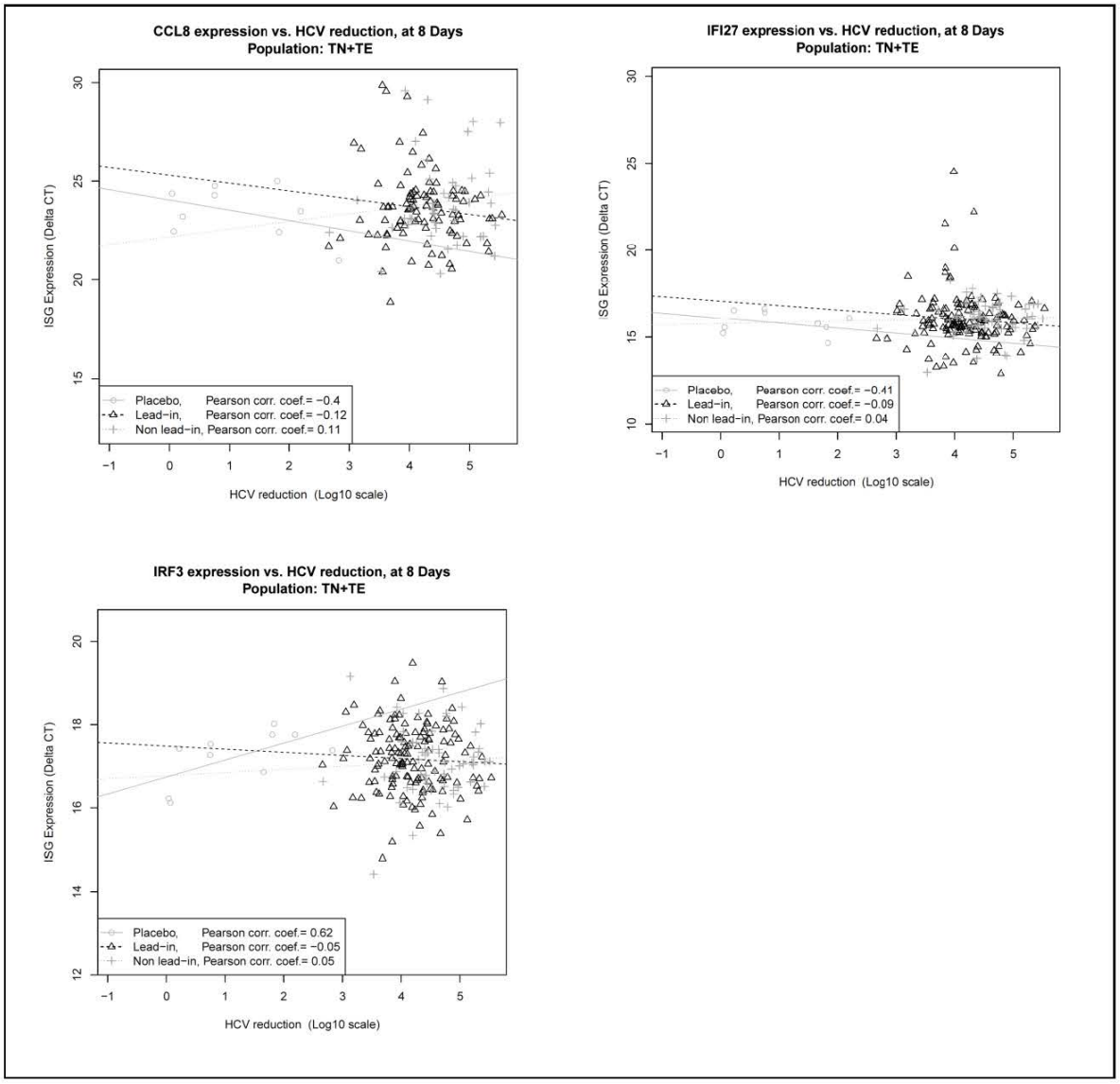

Figure 12: Correlation of the gene expression change with the HCV RNA VL decline at day 4 and day 8: pooled analysis (r=>0.4/<-0.4) (continued)

HCV=Hepatitis C Virus; RNA=ribonucleic acid; TN=treatment-naïve; TE=treatment-experienced; CT=cycle threshold; r=pearson correlation coefficient.

3.4.5. Summary of results

The SVR-oriented exploratory screening of 95 selected genes in 30 patients resulted in the identification of 12 genes with the highest difference of the baseline gene expression across the groups, highest SVR (highest difference between SVR and non-SVR patients) or highest faldaprevir effect (highest difference between faldaprevir and placebo patients): **C1S, CCL8, CXCL10, HESX1, IFI27, IFIT1, IFNA4, PKLR, RSAD2, SIGLEC1, TBX3** and **USP13**. Three genes were added manually based on their biological function: **DDX58, IRF3** and **MAVS**.

The primary analysis of the confirmatory step (association with SVR) showed that the expression of **5 ISGs** was associated with SVR at various time points (**IFNA4, DDX58, IRF3, IFI27 and RSAD2)** based on the analysis of 15 selected genes in 263 patients. The strongest statistically significant association was observed for the baseline expression of **IFNA4** and **DDX58** genes (OR 1.128, p=0.0238 and OR 1.278, p=0.0415, respectively).

Treatment-naïve patients had statistically significant ($p<0.05$) lower baseline expression of the majority of the tested genes at baseline as compared to treatment-experienced patients (14 of 15 tested genes) and many of these genes had higher induction during the treatment (**C1S, DDX58, HESX1, IFI27, IFIT1, IRF3, MAVS, RSAD2** and **USP13**).

SVR patients had higher induction of 6 genes (**DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2**) during the treatment as compared to non-SVR patients ($p\leq0.05$). **C1S, CXCL10, DDX58** and **IFNA4** had lower baseline expression in SVR patients ($p<0.05$). Pooled analysis of the IL28B CC versus non-CC patient did not show any significant differences.

Placebo group had a tendency of the stronger ISG induction as compared to faldaprevir LI and no-LI groups. No significant differences were reported for the LI versus non-LI patients. Overall, no relevant effect of protease inhibitor faldaprevir and no significant differences between faldaprevir and placebo groups were observed.

The majority of the ISGs across the groups had a weak or no correlation of the gene expression change with the HCV RNA VL decline. No pattern was observed across the placebo, LI and non-LI groups.

4. DISCUSSION

Chronic hepatitis C infection still represents a major healthcare problem worldwide. Twenty four years after the discovery of the hepatitis C virus by Choo et al. [18], the novel direct-acting antiviral agents (DAAs) in combination with PegIFN/RBV have substantially improved the cure rates in HCV GT1 infected patients to up to 80% [41, 71]. DAA containing interferon-free treatment regimens are currently under the clinical development that should reduce the burden of the side effects of interferon treatment [31, 32, 70]. In this situation it is of great importance to optimize the individualized treatment approach for HCV infected patients in order to further improve the response rates by investigation of the influence of genetic host factors, like IL28B genotype or interferon-stimulated gene expression in patients treated with the novel interferon-based and interferon-free treatment regimens.

Large genomic studies allowed identification of hundreds of interferon-stimulated genes which are differently regulated in patients with HCV infection and differ in responders to the treatment with PegIFN/RBV [36, 39, 43, 65, 76, 85, 86]. The majority of the studies were conducted in vitro and/or in HCV infected patients treated with PegIFN/RBV alone, but the data for the patients treated with direct-acting antiviral agents like protease inhibitor faldaprevir plus PegIFN/RBV are limited. In our study we provide the in-vivo results from a large cohort of HCV GT1 infected patients treated with the protease inhibitor faldaprevir plus PegIFN/RBV.

364 patients from SILEN-C1 and SILEN-C2 trials have signed the specially designated informed consent for the optional substudy to investigate the expression and induction of interferon-stimulated genes peripheral blood mononuclear cells at different time points and were considered for this thesis. This thesis was performed in three consecutive steps. First, 95 most representative ISGs were selected based on the literature. Secondly, we selected 30 patients for the SVR-oriented exploratory screening and tested all 95 genes in these patients. Finally, we tested 15 genes identified during the SVR-oriented exploratory screening in all patients eligible for this study (N=263). In total, the interferon-stimulated gene expression analysis was performed in 293 treatment-naïve and treatment experienced HCV GT1 infected patients. 240 patients, for whom 15 selected ISGs were analyzed, were treated with the protease inhibitor faldaprevir plus PegIFN/RBV. In terms of the sample

size, this is one of the largest studies for ISG expression performed in-vivo in HCV infected patients during an interferon-based treatment regimen.

The demographic data and baseline characteristics of the patients included into this investigation were overall representative of HCV patients in western countries, but limited to White ethnic group with a very low number of Asian and Black patients due to the predominant location of the study sites in this sub-study in Europe and North America. Due to the randomization in the SILEN-C1&2 trials, the demographic data and baseline characteristics were overall balanced across all groups in this study as well, as all patients enrolled into SILEN-C1&2 trial were offered the participation in this substudy. The response rates of the patients included into this thesis were similar to those reported in the parent trial SILEN-C1&2 and also similar to the first generation protease inhibitors phase II trial population [38, 47, 59].

All patients who signed the informed consent were considered for the investigation, but a number of patients (N=71) were excluded due to the missing blood samples at baseline or during the treatment or withdrawn informed consent. The influence of this positive selection factor did not unbalance the patient selection as this subset of patients had the similar demographic data and response rates as compared to SILEN-C1&2 trial.

The exploratory screening was performed in a limited number of patients (n=30) who were selected primarily based on the availability of the samples at each time point and quality of RNA. This selection was at pseudo random basis because it was highly influenced by these factors and performed manually by the author. As a result, less balanced selection across the groups in terms of IL28B genotype and viral genotype was achieved for the SVR-oriented exploratory screening (see Table 11) and this limited number of patients may be not representative of the larger group of patients. This could have influenced the results of the SVR-oriented exploratory screening where we could have missed some important genes due to the limitations of the patient selection and/or a sample size.
The patients who were included into the SVR-oriented exploratory screening were not repeatedly included into the confirmatory analysis step. This measure should have avoided the positive influence of these patients (N=30) onto the entire study population (N=263).

Our selection of 95 most representative genes was performed based on the literature. We believe to have selected the large pool of most representative interferon-stimulated genes, however we may have not included all important interferon-stimulated genes which could play predictive role for the treatment success and not yet described in literature. Due to the budget limitations, the investigation of larger number of genes was not feasible.
During the SVR-oriented exploratory screening, the assumption was to identify the genes with the highest difference across FDV and SVR groups as compared to placebo and non-SVR patients. In order to get the genes with the most prominent difference between the groups, we analyzed the absolute gene expression data without data transformation (fold change). During the main step (confirmatory analysis) we analyzed the data in fold change transformation and used a well described comparative delta delta Ct method (see Section 2.3.3.4). The $\log_{10}$ data transformation was not reasonable. This methodological approach could have had limitations and influenced our selection of 15 genes. Among the genes which were selected based on their biological function (**MAVS**, **IRF3** and **DDX58**), two genes appeared to have association with SVR (**DDX58** and **IRF3**). This may evidence a weaker point of the selection of the genes for the confirmatory analysis where we may have missed other relevant genes across 95 candidates tested in 30 patients.

Additionally to the baseline expression time point, we had 3 time points of the gene expression testing after the start of the treatment (4 hours, day 4 and day 8). It is expected that the gene expression change may be detected during the first hours after the first injection of interferon, but various ISGs show different timely expression pattern with some ISG having an early induction (within the first 4 hours) and some ISGs at later (>16 hours) or very late time points in more than 2 days [23]. With our 4 hours on-treatment time point we should have detected the early changes in the gene expression, whenever the later time points at day 4 and day 8 should be used for the detection of the later changes in the gene expression. The results obtained in our study did not show the same pattern of the gene expression change at all 3 time points, suggesting that the tested genes had dynamic expression changes which differed at various points in time. The association with SVR was stronger and more consistent at baseline as compared to the on-treatment time points and differed at various time points as well. This may be due to less specificity of the gene expression change in PBMC and also due to the possible impact of other external factors which were not considered in this analysis. Other authors used similar time points for the investigation of the on-treatment gene expression. Taylor et al. [85] and Brodsky et al. [14]

investigated the gene expression in PBMC at days 1, 2, 7 and 14. Sarasin-Filipowicz et al. [76] investigated the gene expression change at 4 hours after the first injection of PegIFN when comparing the biopsy and PBMC gene expression.

The gene selection datasets review via Ingenuity Pathway Analysis (Ingenuity® Systems) database based on the most current knowledge available on genes and protein families helped us to ensure that the selection of the genes represents the relevant ones which play a role in the host response to HCV infection. Apart from the interferon-signaling and PRR common canonical pathways, the pathogenesis of multiple sclerosis was among eight most common canonical pathways of 95 genes dataset. It was based on the p-value and had the highest ratio (calculated as the number of molecules in this dataset in this pathway divided by the total number of molecules that map to this pathway). This relatively small pathway (e.g. the total number of molecules in this pathway is low) and the overlap with the interferon pathway could create such a high ratio. Overall only 4 genes encoding chemokines **CCR1**, **CXCL9**, **CXCL10** and **CXCL11** were included in our selection from the pathogenesis of multiple sclerosis pathway. This observation together with the prominent role of interferon signaling in the pathogenesis of multiple sclerosis explains the significant association of this pathway. All other most common canonical pathways were classical for HCV infection. The same pattern was seen for the dataset of 15 selected genes for the confirmatory analysis.

Based on the SVR-exploratory screening performed in 30 patients we identified 12 of 95 selected genes which may play a role in the treatment response to a protease inhibitor faldaprevir plus PegIFN/RBV in treatment-naïve and treatment-experienced chronic HCV GT1a and 1b infected patients and were differently regulated in SVR and/or FDV subgroups. Twelve genes were selected primarily based on the highest difference in the gene expression change in SVR and FDV patients and 3 genes were added in addition based on the biologic function, resulting in the selection of 15 genes for the confirmatory analysis in a large cohort of the HCV infected patients (N=263).
The key finding in our study is that 5 of 15 selected genes (**IFNA4, DDX58, IRF3, IFI27** and **RSAD2)** showed association with SVR at various time points and two genes (**IFNA4** and **DDX58**) were strongly associated with SVR at baseline ($p<0.05$).

IFNA4 (Interferon alpha 4) gene which is primarily activated by IRF7 encodes interferon alpha-4 protein that plays a role in the response to virus and type I interferon-mediated signaling [67]. Low baseline expression of **IFNA4** may represent the readiness of the immune system to establish stronger and more effective induction of interferon signaling and immune response as compared to those who have higher expression of this gene at baseline. Our results did not show any significant association of this gene expression during the treatment, however strong association was seen at baseline and also at day 4 for **DDX58**.

DDX58 (DEAD box polypeptide 58) gene which is also called RIG1 (Retinoic inducible gene 1) represents one of the major pathways of the host defence triggering by HCV infection, it activates **IRF3** and NFkB [29, 90]. Both **DDX58** and **IRF3** showed association with SVR at baseline, however **IRF3** had only near significant association ($p=0.0504$) at baseline and due to data variability was considered to be less meaningful as compared to **IFNA4** and **DDX58**. Both **DDX58** and **IRF3** showed statistically significant association with SVR at some time points after the start of the treatment. Higher induction of **DDX58** at day 4 was associated with SVR. At 4 hours, **IRF3** was inversely associated with SVR, but due to data variability, this observation was not considered meaningful.

IFI27 and **RSAD2** genes were among other tested genes which showed association with SVR during the treatment (at day 4 and/or day 8).

IFI27 encodes interferon alpha-inducible protein 27 which is activated by interferon alpha and mediates interferon-induced apoptosis [35, 73]. **RSAD2** (Radical S-adenosyl methionine domain containing 2) plays an important role in host defense response to virus infection by promoting TLR7- and TLR9-mediated production of type I interferon [17, 75]. Both genes are located in the common canonical pathway of the treatment response to the HCV infection. Induced interferon-mediated signaling mirrored by the expression of these genes in peripheral blood mononuclear cells (PBMC) may evidence their important biological role in the host response to the HCV infection and their predictive role for the treatment response. Due to the high variability of the data and high standard deviation of mean for these genes seen in our study, their predictive role was considered to be less meaningful based on our data analysis. This may be explained by the source of mRNA in our study. Gene expression and induction in peripheral blood mononuclear cells could have been less pronounced and associated with higher variability as compared to the liver tissue.

Recent meta-analysis performed on 20 relevant studies showed significantly positive association of IL28B genotype with SVR in chronic HCV genotype 1 infected patients treated with PegINF/RBV therapy. IL-28B SNPs rs12979860 genotype CC had the OR=4.473, 95% CI=3.814–5.246 and rs8099917 genotype TT had OR=5.171, 95% CI=4.372–6.117) respectively [56]. Currently, the IL28B polymorphism represents a well established genetic biomarker for the reliable prediction of the treatment response in chronic HCV GT1 infected patients. However there is a need to search other methods and genetic factors for the better prediction of the treatment response using a complex approach for the individualized patient treatment. The comparability and complementary value of interferon-stimulated gene expression data in combination with IL28B gene polymorphisms and other baseline factors (e.g. baseline viral load, viral genotype, race, GGT) should therefore be a subject of further investigations.

Overall, the results obtained in our study from the peripheral blood mononuclear cells (PBMC) in HCV infected patients treated with the protease inhibitor faldaprevir plus PegIFN/RBV are consistent with the observations reported in HCV infected patients treated with PegIFN/RBV alone. Asselah and colleagues showed that the treatment response to PegIFN/RBV could be predicted by two genes (**IFI27** and **CXCL9**) in liver tissue [11]. In our study, **IFI27** was also identified as one of the candidates, but due to the data variability was considered as less predictive and not conclusive as compared to **IFNA4** and **DDX58**.

Asahina et al. showed that the baseline levels of two ISGs (**PKR** and **MxA**) in PBMC were significantly correlated with the liver tissue ISGs [9], however this was refuted by other investigations comparing PBMC with liver tissue [76] and suggesting that the PBMC and liver expression of ISGs are differently regulated.

The primary replication site of the HCV is the liver, but one of the reservoirs of the HCV is the blood cells [54]. Apart from the feasibility of the liver biopsy (pain, bleeding, infections, rare but potentially fatal complications, need to take a second biopsy in case of insufficient material, non-invasive alternatives like fibroscan available), the practicality and convenience for the patients as well as the possibility to test the gene expression at different time points in blood were the reasons we selected and investigated the gene expression in PBMC. Although there is a significant overlap of the gene expression in liver and PBMC, ISG expression in PBMC may be less specific and the differences between

responders and non-responders should be less pronounced as compared to the liver tissue [76]. This was taken into account during the analysis and we assumed that the differences in the gene expression and induction across the groups in our study could have been less pronounced with higher variability of the data as it could be seen in the liver tissue. Less meaningful results for **IRF3**, **IFI27** and **RSAD2** could be due to less specific changes in PBMC, higher variation with low specificity in blood as compared the liver tissue which may have influenced this selection. However, given the previous findings by Asselah and colleagues [11] who showed the predictive role of **IFI27** in the liver tissue, the overlap of the gene expression in PBMC and liver may be still present. High variability of the on-treatment gene expression data may be due to other influenced factors which were not considered in this investigation, for example the concomitant acute viral infections or exposure to viruses.

The primary results presented and discussed in this thesis are based on the pooled data analysis combined for all analyzed patients from this study (N=263). In addition to the pooled analysis, we performed the association analysis in the TN and TE subgroups separately, GT1a and GT1b subgroups, placebo group as well as all treatment groups including both unadjusted and adjusted by IL28B and viral genotype analysis. The results of the subgroup analysis were less specific with almost no statistically significant association with SVR found across the subgroups. This could be due to the reduced sample size in the subgroups and our source of mRNA (PBMC) which have more pronounced and less specific changes in PBMC as compared to the liver tissue. Nevertheless, the pooled data represented a large cohort of the HCV infected patients treated with the interferon-based treatment regimen with the baseline gene expression associated with SVR for at least two genes in PBMC which should be representative for the entire population.

In order to rule out the influence of IL28B and viral genotype factors, the adjusted analysis IL28B and viral genotype was performed. Similar results were obtained for the adjusted analysis providing the evidence that the association of the genes was not dependent on these factors.

The results of the exploratory screening showed that more genes were selected due to higher up-regulation of the genes in SVR patients or down-regulation in FDV patients during the treatment. Many of the selected genes met multiple criteria and therefore were

not conclusive in the exploratory screening phase. SVR-oriented exploratory screening selection was performed in treatment-naïve and treatment-experienced patients separately. We adjusted the cutoff for the selection in the treatment-experienced patients because more genes were up or down-regulated in this population (with the cutoff >+5/<-5), thus the treatment-experienced population had less specific gene expression change as compared to those seen in the treatment-naïve patient groups. With the adjusted cutoff (>+6/<-6) we achieved more specific selection resulting in the identification of 7 genes with the highest differences in SVR and/or faldaprevir patients, all but one gene had an overlap with the selection from the treatment-naïve patient groups (cutoff >+5/<-5). The positive SVR effect was also more frequently seen in the confirmatory analysis of 15 ISGs. Six genes (**DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2**) were higher expressed in SVR patients during the treatment as compared to non-responders.

Based on the investigations reported to date in HCV infected patients treated with PegIFN/RBV, the baseline expression of the ISGs has a tendency to be higher in non-responders as compared to good treatment responders. The induction of ISGs during the treatment seems to be stronger in patients who achieve SVR, suggesting that high baseline expression prevents from establishment of effective induction of interferon-signaling and immune response [9, 11, 15, 26, 39, 76]. Taylor et al. showed in 69 HCV infected patients that global gene expression was greater in responders as compared to non-responders [85]. He et al. showed the substantially greater induction of ISGs in PBMC (58 patients) in responders to the PegIFN/RBV treatment [36]. These observations were also seen in our study. SVR patients had a higher induction of 6 genes (**DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2**) during the treatment as compared to non-SVR patients ($p \leq 0.05$). **C1S, CXCL10, DDX58** and **IFNA4** had lower baseline expression in SVR patients. Similar observations were reported for IP-10 protein (encoded by CXCL10) with the lower expression and protein level in peripheral blood at baseline to be predictive of the treatment response [10, 20, 31, 48].

The comparison of the treatment-naïve and treatment-experienced patients showed that treatment-naïve patients had lower baseline expression of the majority of the tested genes at baseline as compared to the treatment-experienced patients (13 of 15 tested genes) and many of them had higher induction of the genes during the treatment (**C1S, DDX58, HESX1, IFIT1, IRF3, MAVS, RSAD2** and **USP13**). This observation shows that the comparably low or relatively intact interferon-signaling in treatment-naïve patients can be more effectively and strongly activated during the treatment in this population as compared to the treatment-experienced patients and this strong activation is consistent with the better response of these patients to the treatment with the protease inhibitor faldaprevir plus PegIFN/RBV as compared to those previously treated with PegIFN/RBV.

Our data showed no relevant differences in the gene expression across IL28B CC versus non-CC patients based on 15 investigated genes. Both pooled analysis and the treatment-naïve sub-group analysis did not show any statistically significant differences between CC versus non-CC patients. This seems to be also consistent with the previous observations [1, 22], but only limited in the treatment-native population and pooled analysis in our study. The results obtained for the treatment-experienced sub-group showed the down regulation of 6 genes in IL28B CC patients as compared to non-CC patients. It should be noted that the number of IL28B CC treatment-experienced patients was very low in our study and this result which is limited for the treatment-experienced patients should be interpreted with caution. In a large study of HCV (N=44) mono-infected and HCV-HIV co-infected patients (N=44), ISG expression in PBMC at baseline and during the treatment with PegIFN/RBV was investigated by Naggie et al. [63]. No difference at baseline was found between IL28B CC versus non-CC patients. The change in ISG expression from baseline was independent of IL28B. Responders had higher deltas as compared to non-responders (lower expression) [63]. IL28B and ISGs appeared to be independent in that study and IL28B did not predict ISG expression or induction of selected genes. These findings were similar to those reported by other authors [1, 22] who suggested that IL28B did not determine ISG expression and also consistent with our observations.

One of the ways HCV escapes the immune response is the cleavage the toll like receptor 3 (TLR3) adaptor (TRIF) and mitochondrial antiviral signalling protein (**MAVS**) [16, 50, 60]. Cheng et al. showed in vitro that the first NS3/NS4A protease inhibitor BILN2061 was able to restore MAVS dependant IFN- β promoter activity [16]. Apart from the

predictive role of the gene expression to the treatment success, one of hypotheses of this thesis was to confirm this observation in-vivo using another NS3/NS4A protease inhibitor (faldaprevir) in the large patient population and investigate the differences in the gene expression between faldaprevir plus PegIFN/RBV as compared to PegIFN/RBV alone.

In terms of our hypothesis that faldaprevir can restore the interferon signaling, the data obtained in our study did not show the expected results. No statistically significant differences were observed between faldaprevir plus PegIFN/RBV as compared to PegIFN/RBV alone (both lead-in and no lead-in) based on the expression data of 15 selected genes. This can evidence that the direct antiviral activity of the protease inhibitor faldaprevir prevails its effect on interferon signaling or that the selected genes were not representative to confirm our hypothesis. Apart from the limited number of genes, it should be noted that the placebo group comprised only 23 of 263 patients. Therefore these results and conclusions should be interpreted with caution.
The investigation of the 3 day lead-in of PegIFN/RBV effect did not show any relevant and statistically significant differences in the gene expression of 15 selected genes. Overall no pattern allowing the distinction of patients treated with the 3 day lead-in of PegIFN/RBV versus non- lead-in patients as compared to placebo was observed. At 4 hours time point and at day 4 the gene expression strongly varied in the groups with the same treatment regiments (placebo and lead-in group). At these time points the placebo and lead-in patient groups received identical treatment (PegIFN/RBV only). In opposite, the non-lead-in group was treated with faldaprevir plus PegIFN/RBV from the first day and the difference was expected already at 4 hours or at least at day 4 between placebo and lead-in group as compared to the non-lead-in group. However, the differences were observed for some genes between placebo and lead-in groups and not in the non-lead-in group as it was expected. Therefore the hypothesis about the differentiation of the treatment with faldaprevir plus PegIFN/RBV compared to placebo plus PegIFN/RBV as well as the lead-in effect on the interferon-stimulated genes could not be confirmed using 15 genes selected for the confirmatory analysis.

Similar observation was reported for the other NS3 protease inhibitor (boceprevir) in PBMC of the HCV infected patients. 1500 patients who participated in the boceprevir phase II trials were included in the gene expression analysis. mRNA in PBMC (89 genes) was measured at baseline, week 4 and week 8 during the treatment [61]. No statistically

significant differences were observed between boceprevir plus PegIFN/RBV versus placebo plus PegIFN/RBV at week 8. Furthermore the SVR status could not be predicted by the gene expression tested in this analysis at day 8. However the sample size in our study is smaller and the list of genes was not identical to those tested in boceprevir study. In our study we used earlier on-treatment time points which may not be compared with the gene expression change at week 4 and week 8.

The majority of the ISGs across the groups had a weak or no correlation of the gene expression change with the HCV RNA. We were aiming to check, if the gene expression change can be attributed to the VL decline. The groups were divided into placebo, LI (lead-in) and non-LI (no lead-in). At the day 4, both placebo and LI patients received identical treatment (PegIFN/RBV alone) and the correlation was expected to be similar as compared to non-LI patients who received the triple therapy with faldaprevir plus PegIFN/RBV from the day 1 (for these patients the viral load decline was stronger and the difference in the gene expression could be more pronounced due to more rapid viral load decline). However the strong correlation was seen only for two genes and only in one group (placebo) whenever the lead-in and no-lead in groups had either inverse correlation or no correlation which does not explain the dependence of the gene expression change to the HCV RNA VL decline and do not provide any distinction of the lead-in versus non-lead-in or placebo groups.

In conclusion, our data showed that the baseline expression of two genes (**IFNA4** and **DDX58)** might be predictive of SVR in the treatment-naïve and treatment-experienced HCV GT1a and 1b infected patients treated with NS3/NS4A protease inhibitor faldaprevir plus PegIFN/RBV.
Treatment-naïve patients had lower baseline expression of ISGs and more pronounced gene induction during the treatment as compared to the treatment-experienced patients. SVR patients had lower baseline expression and higher induction of ISGs during the treatment.
IFNA4 and **DDX58** should be considered for further investigations in prospective studies to confirm their predictive role for the treatment success of interferon-containing treatment regimens combined with protease inhibitors.

An additional predictive role of **IFNA4** and **DDX58** in combination with IL28B and other biomarkers may be recommended for the investigation in prospective studies. Their combination and a multivariate approach may further increase the predictive value of IL28B and/or ISGs (like **IFNA4** and **DDX58)** and additionally contribute to the optimized treatment options and individualized strategies for the HCV infected patients treated with a protease inhibitor plus PegIFN/RBV.

Furthermore it would be important to investigate interferon-stimulated gene expression, its role and predictive value for the treatment success and individualized patient management strategies also for the interferon-free treatment regimens which may become available in future. For all HCV infected patients the endogenous production of interferon alpha and its critical role as a host response factor in the elimination of the virus (not only from the liver, but also from all reservoirs which may be inaccessible for the protease or polymerase inhibitors) will remain to be essential. Therefore, ISG expression in combination with other biomarkers, like IL28B, may become an important component of the individualized approach for HCV infected patients treated with the novel interferon-free DAA regimens.

5. SUMMARY

The objective of this thesis was to investigate the expression of selected interferon-stimulated genes (ISGs) in PBMC (peripheral blood mononuclear cell) messenger ribonucleic acid (mRNA) prior and during the treatment with NS3/NS4A protease inhibitor faldaprevir plus pegylated interferon α-2a/ribavirin (PegIFN/RBV) as compared to PegIFN/RBV alone in different sub-groups of treatment-naïve and treatment-experienced chronic hepatitis C virus genotype (GT) 1a and 1b infected patients. We hypothesized that the baseline and on-treatment expression of various ISGs may have predictive role for the treatment success (SVR) and differentiate the treatment response to PegIFN/RBV plus faldaprevir.

364 patients from SILEN-C1 and SILEN-C2 trials have signed the specially designated informed consent for the optional substudy to investigate the expression and induction of interferon-stimulated genes and were considered for this thesis. 293 patients met eligibility criteria and were included into the analysis. We performed this thesis in three consecutive steps. First, we selected 95 most representative ISGs based on the literature. Secondly, we selected 30 patients for the SVR-oriented exploratory screening and tested all 95 genes in these patients. Finally, we tested 15 genes identified during the SVR-oriented exploratory screening in all patients eligible for this study (N=263).

As a result, we identified 15 genes which may play a role in the treatment response to faldaprevir plus PegIFN/RBV. Five ISGs (**IFNA4, DDX58, IRF3, IFI27** and **RSAD2)** showed association with SVR at various time points. Baseline expression of two ISGs (**IFNA4** and **DDX58**) was strongly associated with SVR.

SVR patients had a higher induction of 6 genes (**DDX58, HESX1, IFI27, IFIT1, PKLR** and **RSAD2**) during the treatment as compared to non-SVR patients ($p=<0.05$). **C1S, CXCL10, DDX58** and **IFNA4** had lower baseline expression in SVR patients compared to non-SVR patients($p<0.05$). Treatment-naïve patients had lower baseline expression of the majority of the tested genes at baseline as compared to the treatment-experienced patients (14 of 15 tested genes) and many of them had higher induction of the genes during the treatment. These findings are consistent to those previously reported in HCV infected patients treated with PegIFN/RBV alone.

The majority of the ISGs across the groups had a weak or no correlation of the gene expression change with the HCV RNA viral load. No patterns were seen to differentiate the lead-in versus non-lead-in groups as compared to placebo and no faldaprevir effect was observed.

In conclusion, our data showed that the baseline expression of two genes (**IFNA4** and **DDX58)** might be predictive of SVR in the treatment-naïve and treatment-experienced HCV GT1a and 1b infected patients treated with NS3/NS4A protease inhibitor faldaprevir plus PegIFN/RBV.
Treatment-naïve patients had lower baseline expression of ISGs and more pronounced gene induction during the treatment as compared to the treatment-experienced patients. SVR patients had lower baseline expression and higher induction of ISGs during the treatment.
IFNA4 and **DDX58** should be considered for further investigations in prospective studies to confirm their predictive role for the treatment success of interferon-containing treatment regimens combined with protease inhibitors.

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APPENDIX

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