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*Bioinformatics e Biostatistics applied to research in
pediatric genetic disease*

**Clinical evidence in IFN λ 4 polymorphisms associated with
HCV infection in patients with beta thalassemia and WGCNA
analysis weighted for IFN λ 4 genotype rs12979860 to detect
RPL9P18 as hub in HCV infected cell.**

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Ad Alessia e Ilaria

**Clinical evidence in IFN λ 4
polymorphisms associated with HCV
infection in patients with beta
thalassemia**

and

**WGCNA analysis weighted for IFN λ 4
genotype (rs12979860) to detect
RPL9P18 as hub in HCV infected cell.**

Abstract

Genome-wide association studies have identified host genetic variation to be critical for spontaneous clearance and treatment response in patients infected with hepatitis C virus (HCV). We demonstrated the same in patients with thalassemia major infected by genotype 1b of HCV.

In the present first part study we retrospectively analyzed 368 anti-HCV positive patients with beta-thalassemia in two Italian major thalassemic centers (Cagliari and Turin).

The strongest IFN λ 4 SNP found associated with HCV was rs12979860 where C/C genotype was related to response to the interferon treatment and, above all, to spontaneous clearance of the virus. However, the positive predictive power was stronger for viral persistence than spontaneous clearance indeed TT allele was more predictive than CC.

Another polymorphism rs4803221 was analyzed because had independent effects respect to rs12979860. The haplotype tagged by SNP rs12979860 and rs4803221 significantly could improve the viral clearance prediction in infected patients.

Neither necrotic-inflammation or bilirubin values in the chronic phase of the hepatitis C were related to IFN λ 4 polymorphisms. No relation among IFN λ 4 polymorphisms and fibrosis stage directly shown by the liver biopsy was found.

Second part of our study was to identify hub genes associated in pathways closely related to IFN λ 4 variants in HCV response. We used gene expression profile data of GSE54648, downloaded from Gene Expression Omnibus (GEO). We focused our attention on expression genes differential between rs12979860 unfavorable TT genotype and favorable CC genotype, using weighted gene expression network analysis (WGCNA - R package). Significant modules were selected using the clustering analysis. At the final the best significant module was “black” module. Its

pathways were involved in translation mechanisms such as translation termination, translation elongation, nuclear-transcribed mRNA catabolic process, cellular protein complex disassembly, therefore biological mechanisms that occur inside ribosome.

We discovered RPL9P18 pseudogene as a hub potentially related in inhibition of spontaneous clearance and furthermore likely involved in drug treatment inhibition. Our result suggests an active role for ribosome pseudogene in innate antiviral response probably during ISG (IFN-stimulated genes) translation. Moreover, through co-expression analysis we demonstrate a new possible role of IFN λ 4 genotype in HCV infection, associate with expression of ribosomal pathways.

Introduction

Background

Some 3% of the World's population is infected with the hepatitis C virus (HCV). In most cases, the virus, if either untreated or treated but not cleared, causes chronic infection and thereby increases the risk of liver fibrosis and liver cancer. HCV infection is also the major cause of liver transplantation. The current standard of care is pegylated-interferon and ribavirin, which clears the virus only after weekly injections for up 48 weeks and in less than half of those infected with the most common form of the virus, genotype 1.

Hepatitis C, as with all viruses, seeks entry into human host cells to evade the dangerous extracellular milieu repleted with cytokines, natural killer cells, macrophages and interferon each seeking to destroy the virus. Once inside the hospitable hepatocyte, the HCV uses its RNA as a template for replication. Into the trillions of new copies every day.

The majority of people infected with hepatitis C virus (HCV) develops chronic infection, which can remain asymptomatic for many years, ultimately leading to the development of liver fibrosis, cirrhosis, and hepatocellular carcinoma (Brown et al., 2005; Alter and Liang et al., 2012). Interestingly, 20%-30% of people infected with HCV are able to clear the infection and do not progress to chronicity. The molecular mechanisms driving this clinical dichotomy remain unknown, in part for the complications to study HCV in its native environment, the human hepatocyte in the liver.

In 2009, four independent groups, using genome wide association studies (GWAS), identified SNPs from IFN λ 4 [RefSeq:NM_172139.2] region that could predict drug response. A further study demonstrated that viral clearance without therapy was also predicted by these SNPs. Previously, prediction of treatment failure was based on

phenotypic features such as viral load, body mass index, ethnicity and liver fibrosis. IFN λ 4 genotype, however, predicts treatment failure with greater sensitivity and specificity.

SNPs at the IFN λ 4 locus (e.g. rs12979860) are strongly associated with HCV clearance and treatment response, but the underlying mechanisms responsible remain unclear (Ge et al., 2009; Naggie et al., 2012; Thomas et al., 2009; Urban et al., 2010). More recently, polymorphisms at an IFN-lambda locus (IFN λ 3, IFN λ 4), were shown to have similar predictive value, thus galvanizing the importance of IFN λ in HCV infection (Prokunina-Olsson et al., 2013). However many SNPs for IFN λ 3 and IFN λ 4 appear in strong linkage disequilibrium (Prokunina-Olsson et al., 2013), therefore rs12979860 could predict same HCV response, finding other SNPs not in linkage disequilibrium is a research challenge.

It is desirable to predict treatment response or spontaneous clearance response for many reasons. For those unlikely to respond, alternative therapies are in late phase clinical trials and have a greatly improved success rate due to the ability to target those that are unlikely to respond to the standard treatment. Patients with non-response genotype could therefore delay treatment, or have preferential use of the more expensive new therapies, which are all designed to be used in combination with the current standard of care. The predictive value of SNPs is better calculated from routine clinical practice, rather than the clinical trial scenario, since there are the conditions in which most patients are treated and where there is usually much lower compliance with drug usage regimens. Consequently, in the first part of this study we used a new cohort (Sardinian population), to compare response rates for just known SNPs and other not well known SNPs. Thus, variants could be included to tag additional haplotype, for a better predictive values than the common haplotype.

Second part of our study is aimed to understand poorly known functional mechanisms in which IFN λ 4 is involved during spontaneous clearance. For this purpose we used co-expression analysis method (WGCNA) from GEO dataset.

Hepatitis C virus relevance in Sardinia

Hepatitis C virus (HCV) is a single-stranded RNA virus belonging to the Flaviviridae family characterized by a high degree of genetic heterogeneity (Lai et al., 2013).

The major routes of transmission are injection drug use, blood transfusion, hemodialysis, organ transplantation and less frequently, sexual intercourse.

Controversial results have been reported about the relation between post-transfusion transmission, aggressive histological activity, and development of cirrhosis. Before the implementation of blood donor screening in 1991, the risk of acquiring post-transfusion hepatitis C in Italy was about 20 cases per 1000 blood units. Although hepatitis C is no longer a major threat in the Italian blood supply, HCV acquired through transfusion before 1990 remains one of the most important problems among patients with thalassemia, given the reported presence of HCV antibodies in 80%-90% of multi-transfused Italian patients and a prevalence of clinically significant fibrosis in 45% of them.

In patients with thalassemia major, post transfusional iron overload and HCV infection seem to be independent risk factors for liver fibrosis progression and their concomitant presence results in a greater risk.

Multiple episodes of acute hepatitis C due to reinfection or reactivation of primary infection have been described.

IFN λ 4 gene description

IFN-lambda is a cytokine that comes in two isoforms, IFN λ 3 and IFN λ 4, and plays a role in immune defense against viruses, including the induction of an "antiviral state". IFN λ 4 belongs to the interferon family of cytokines and is highly similar in amino acid sequence of IFN λ 3. Their classification as interferons is due to their ability to induce an innate antiviral state, while their additional classification as cytokines is due to their (Sheppard et al., 2003) chromosomal location as well as the fact that

they are encoded by multiple exons, as opposed to a single exon, as most type-I IFNs are.

IFN λ 4 was discovered in 2002 by Zymogenetics using a genomic screening process in which the entire human genome was scanned for putative genes, was named IL28B. Once these genes were found, a second scan was performed to look specifically for cytokines.

IFN λ 4 genes are located near IFN λ 3 on chromosome 19 in humans. The two isoforms of are 96% homologous. Differences in function between the two forms remains unclear.

IFN-lambda has also been shown to play a role in the adaptive immune response, as its inclusion as an immune-adjuvant during small animal vaccination lead to augmented antigen-specific Interferon Gamma release as well as an increased cytotoxic potential in CD8⁺ T cells (Morrow et al., 2009).

Studies of IFN λ 4 in non-human primate models of vaccination confirmed the small animal models, leading to an increase in Interferon Gamma production and CD8⁺ T cell activity in the form of cytotoxicity in an HIV vaccine study (Morrow et al., 2010).

A single nucleotide polymorphism (SNP) near the IFN λ 4 gene predicts response to hepatitis C treatment with interferon and ribavirin (Ge et al., 2009a). The SNP was identified in a genome-wide association study (GWAS – table 1) and is to date the best example of a successful GWAS hit that is clinically relevant (Maxmen, 2011).

Author	Region	HCV genotype	RS12979860	RS8099917	RS12980275	RS4803219	RS8103142	RS8105790	RS10853728	RS7248668	RS482221	RS2841683	RS4803217	RS1881222
Ge et al. (7)	USA	1	✓	✓				✓						
Suppiah et al. (10)	Australia		✓											
Tanaka et al. (11)	Japan	1		✓	✓									
Rauch et al. (18)	Switzerland	1, 4		✓										
Abe et al. (19)	Japan			✓										
Mangia et al. (20)	Italy	2, 3	✓											
McCarthy et al. (21)	USA		✓											
Thompson et al. (22)	USA		✓											
Bochud et al. (23)	Switzerland		✓	✓	✓									
Smith et al. (24)	Europe		✓	✓						✓	✓			
Yu.M.Lin et al. (25)	Taiwan		✓											
Chen et al. (26)	Taiwan		✓	✓	✓	✓		✓	✓	✓				✓
Scherzer et al. (27)	Austria		✓	✓										
Ridruejo et al. (28)	Argentina	1	✓	✓										
Yu et al. (25)	Taiwan	2				✓			✓					
Jun-qiang et al. (29)	China		✓											
Pedergnana et al. (30)	Egypt	4	✓					✓						
Shi et al. (31)	China		✓	✓	✓			✓						
de Castellarnau et al. (32)	Spain		✓	✓		✓	✓					✓	✓	
Grandi et al. (33)	Brazil	1	✓											
Prokunina-Olsson et al. (13)	USA		✓											
Stenkvist et al. (34)	Sweden		✓											
Gelinas et al. (35)	France		✓	✓										
Ezzikouri et al. (36)	Morocco		✓	✓										
Jung et al. (37)	Korea		✓	✓										

Table 1: A list of SNPs studied by several group in the recent years

Genetics test

Genetic testing [GWAS] have recently identified host genetic variation to be critical for spontaneous clearance and treatment response in patients infected with hepatitis C virus (HCV). Compared with all other baseline host and viral variables, different polymorphisms of the interferon lambda 4 gene, situated on chromosome 19, have been reported as the strongest predictors of HCV therapy response and spontaneous viral clearance. In this respect, the rs12979860 C and the rs8099917 T variants, located 3 and 8 kb upstream of the IFN λ 4 gene, which have some level of linkage disequilibrium (R^2 : 0.50 in Caucasians), seem to be the two strongest genetic predictors. Beyond their identification, little is known about the mechanisms involved between these genomic variants and viral clearance and it is still uncertain whether these polymorphisms play a causal role. It has been shown that unfavorable IFN λ 4 genetic variations are associated with higher pre-activated levels of ISGs, which could explain the more frequent chronicization and poor response to antiviral treatment in this patients. Data on the influence of the IFN λ 4 gene polymorphisms on

the natural history of untreated chronic HCV infection are scarce. The correlation between IFN λ 4 polymorphisms and progression of liver fibrosis is still controversial. Interestingly, Tillman et al., have reported that jaundice during acute hepatitis C is more common among patients with rs12979860 C/C genotype than non-C/C patients and that women with the C/T or T/T genotype who did not develop jaundice have a lower chance of spontaneous clearance of HCV infection.

It is unknown whether rs12979860 and rs8099917 exert direct biological effects or are in linkage disequilibrium with other functional polymorphisms. Several investigators have performed gene mapping failing to detect new single nucleotide polymorphisms (SNPs) with a stronger genetic effect or with a clear functional mechanism. Other studies, however, have demonstrated that the polymorphisms are associated with a change of methylation (loss or a gain of methylation), located within the 5' region upstream from the transcription start of the IFN λ 4. Among them, rs4803221 is a C to G (C/G) polymorphisms resulting in a loss of methylation, located -500 bp upstream rs12979860 which has been associated with reduced clearance and response to treatment.

Recently the role of the IFN λ 4 polymorphisms in influencing the spontaneous clearance of HCV, the response to interferon and the progression of liver fibrosis, was also associated in patients with thalassemia major infected by genotype 1b.

Although hepatitis C is no longer a major threat in the European blood supply, these findings are relevant because HCV acquired through transfusion before the implementation of blood donor screening, remaining one of the most important problems among patients with thalassemia, given the reported high presence of HCV antibodies in multi-transfused patients and a noteworthy prevalence of clinically significant fibrosis.

Moreover, hepatocarcinoma is becoming frequent with the aging population of patients with thalassemia.

HCV replication cycle

General replication cycle for HCV infection is well known as well as the IFN λ 4 entry in one or more of those. General replication in figure 1 starts by the interaction between HCV-associated lipoprotein receptors: LDL-R and/or SR-BI and/or cell surface heparan sulphate proteoglycans (GAGs). HCV subsequently interacts with CD81, a “post-entry” receptor, which forms a complex with SB-BI. CD81 plays fundamental role in HCV infectivity as it triggers signaling cascades essential for virus entry and further downstream events (Brazzoli et al, 2008). The virus is then transferred to the tight junction proteins Claudin-1 (or Claudin-6 or Claudin-9, (Zheng et al., 2007)), and Occludin (Ploss et al., 2009) which provides “the final entry key” for HCV cellular uptake. E1 and E2 envelope glycoproteins mediate pH-dependent fusion (Lavillette et al., 2007) with limiting membranes of early endosome, which trigger nucleocapsid release into the cell cytoplasm and permits the virus to escape the lipoprotein degradation pathway.

After virus cell entry, HCV-RNA replication takes place in membrane-associated replication complexes designated as membranous webs (Gosert et al., 2003). HCV replication complexes are subjected to intracellular transport, and their formation is closely linked to the dynamic organization of the endoplasmic reticulum, actin filaments and the microtubule network (Jones et al., 2007, Lai et al., 2008). However, how the virus is trafficked from the virion attachment at the cell surface to the delivery of the viral genome to its replication site remains unclear. Moreover microtubules play a key role in the early steps of the virus cycle, leading to the establishment of productive HCV infection (Budkowska et al., 2009).

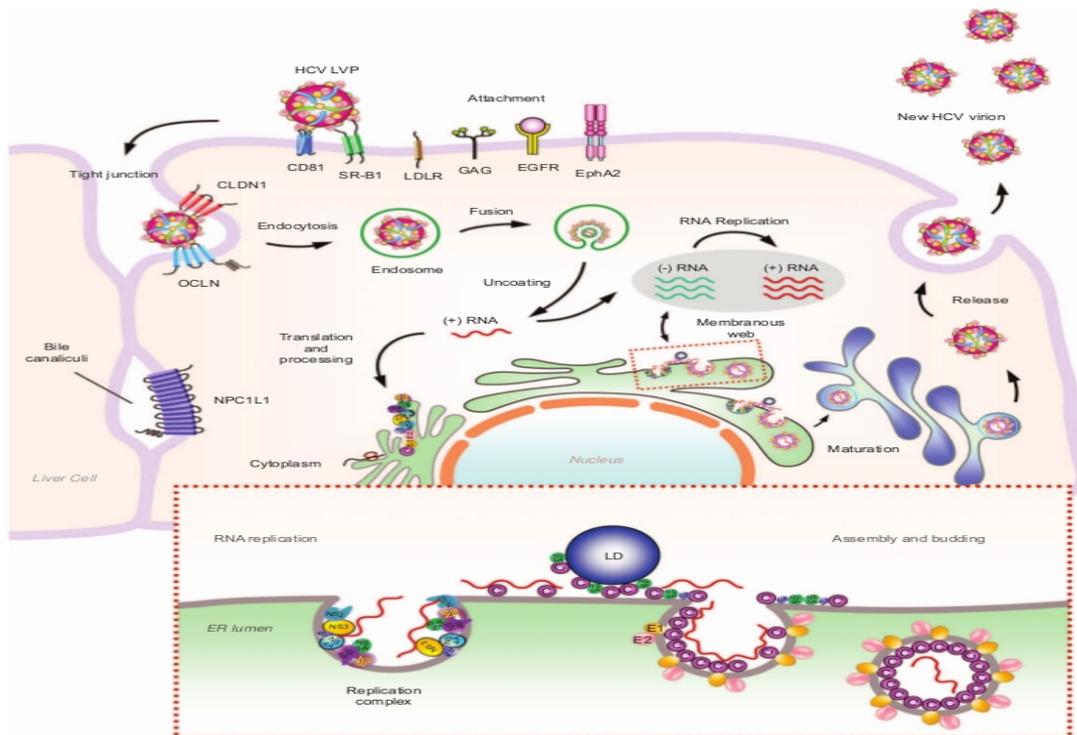


Figure 1: Canonical HCV replication cycle. (Wong et al., 2014).

Innate antiviral response

First defense of hepatocyte against HCV is innate antiviral response.

The innate immune response, also known as the nonspecific immune system (Grasso et al., 2002), is an important subsystem of the overall immune system that comprises the cells and mechanisms that defend the host from infection by other organisms. The cells of the innate system recognize and respond to pathogens in a generic way, but, unlike the adaptive immune system, it does not confer long-lasting or protective immunity to the host.

Recently has emerged a key role of IFN λ 4 genotype in the induction of the innate antiviral immunity.

Rs12979860 (at the IFN λ 4 locus) is strongly associated with HCV spontaneous clearance and treatment response, but the underlying mechanisms responsible in innate antiviral response remain unclear (Ge et al., 2009; Naggie et al., 2012; Thomas

et al., 2009; Urban et al., 2010). IFN λ 4 variants proteins correlated with unfavorable IFN λ 4 genotype had less antiviral activity against HCV replicons and were capable to determine a variation of ISG (IFN-stimulated genes) expression (Terczyńska-Dyla E. et al., 2014). Sheahan T. et al.(2014), confirms the association between IFN λ 4 polymorphism rs12979860 and the innate antiviral response though expression studies. While examining genotype-specific host responses, they observed that the numbers of significantly regulated genes increased stepwise from homozygous major to homozygous minor alleles HCV-infected cells. People with unfavorable genotype chronically infected with HCV typically have higher baseline levels of ISGs. In order to understand poorly known mechanisms of spontaneous clearance in which IFN λ 4 is involved, we used co-expression analysis method (WGCNA) from GEO dataset. To our knowledge, no weighted correlation network analysis is available in literature to describe interaction between IFN λ 4 and HCV infection.

Potential role of the IFN λ 4 in the HCV response

The molecular pathways that link the IFN λ 4 genotype with antiviral effector systems of the innate and adaptive immune system are not known. However, substantial progress has been made in basic understanding of the induction of interferons through toll-like receptor and RIG-I/MDA5 pathways, and of interferon-induced signaling pathways and antiviral effector systems.

HCV was able to interfere not only with IFN signaling through the Jak-STAT pathway (figure 2), but also with the translation of ISG mRNAs to proteins at the ribosomes. Elegant evidence for such a translational block has been obtained in Huh7.5 cells infected with HCV and treated with IFN α . In this system HCV infection did not block the transcriptional induction of ISGs by IFN α . However, HCV infection triggered phosphorylation and activation of the RNA-dependent protein kinase PKR, which inhibits eukaryotic translation initiation factor eIF2 alpha, and thereby cap-dependent translation of cellular mRNAs, but not the IRES-dependent translation of HCV RNA (Horner et al., 2014).

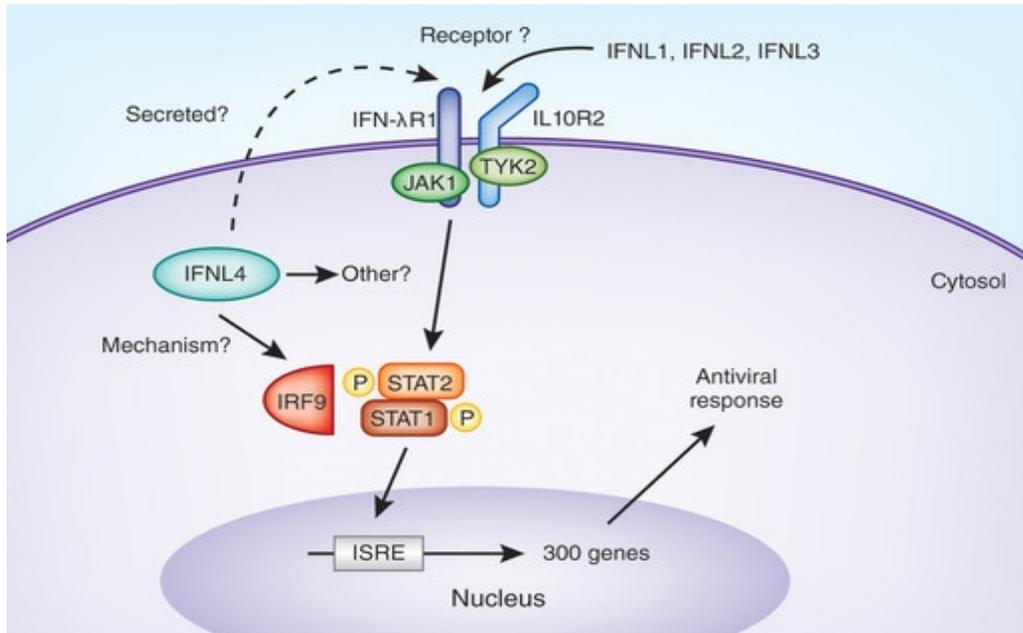


Figure 2: IFNλ4 role in HCV antiviral response.

Materials and Methods

Clinical study patients

In the present study we retrospectively analyzed 511 patients with beta-thalassemia at two Italian major centers in Cagliari and Turin transfused before 1990 and 122 transfused after 1990 in which only four patients were infected with hepatitis C virus. In totally we analyzed 418 patients that were infected. Two hundred and fifteen were anti-HCV negative and 168 anti-HCV positive.

Of 418 patients infected we could extract DNA only of 368 patients, 364 before 1990 and 4 after the implementation of blood donor screening in 1991. We have lost some patients due to several reasons like denaturation of DNA of old patients that we had available.

HCV status in thalassemia major patients by starting of transfusion
(Pediatric and Adult Thalassemia Units – Cagliari and Torino)

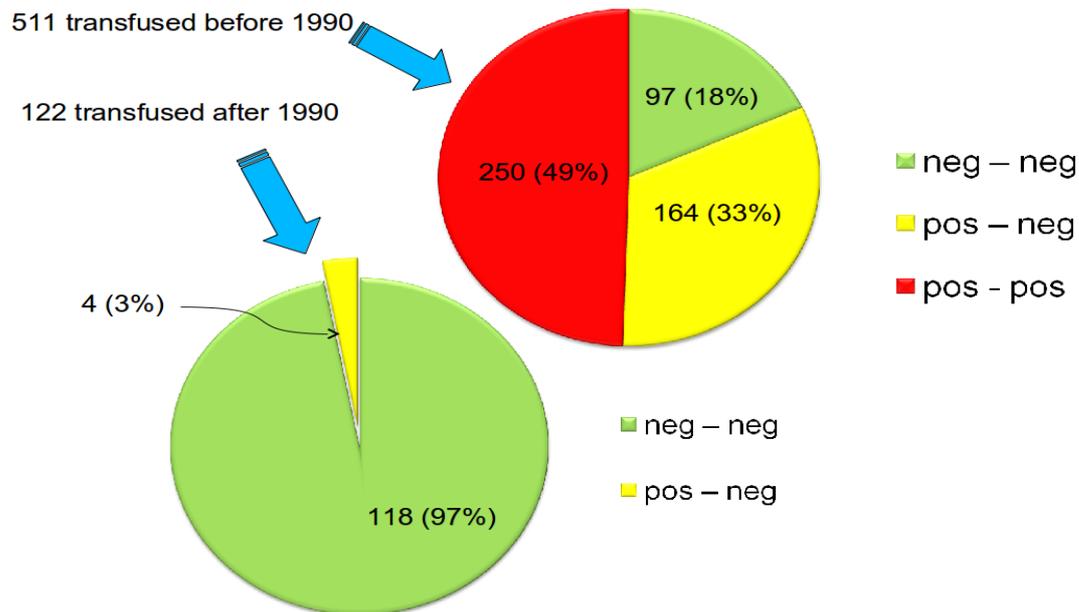


Figure 3: HCV status in Beta-Thalassemia Mayor in the cohort.

Neg-neg = Anti-hcv neg - RNA neg, *pos-neg* = Anti-hcv pos - RNA neg, *pos-pos* = Anti-hcv pos - RNA pos

Viral genotype was known in 215 patients. One hundred thirty-three subject with chronic infection had been infected by severe genotypes (1 or 4) and 83 by side genotypes (2,3 or 5).

Clinical data

The 368 anti-HCV positive patients were genotyped at the polymorphic sites on chromosome 19. The relationship between IFNλ4 variants, liver necrotic-inflammation, and fibrosis was evaluated. Although the bilirubin values during the acute phase of hepatitis C were not available, four values per year until 2010 for the last 20 years per patient (or until the antiviral treatment) were recorded by mean of Webthal, a Web-Based Multicentric DataBase, as well as ALT and serum ferritin values measured at the same time. The use of Webthal for the clinical follow-up of the patients and for scientific purposes was approved by the Ethics Committees of the

two hospitals. All patients registered in WebThal signed informed consent to the use of their clinical data for research studies and objectives. Moreover, each patient, and their parents if minor, signed a written consent for DNA testing.

Among 368 anti-HCV positive patients 149 (40,5%) had spontaneously cleared the virus (HCV+/RNA-) and 219 (59.5%) were chronically infected (HCV+/RNA+), at least at a biannual determination by real time PCR.

	Spontaneous Clearance			tot
	Cagliari Pediatria	Cagliari Adulti	Torino	
HCV+/RNA+	94	57	69	219
HCV+/RNA-	75	23	47	149
	164	80	116	368

Table 2: Records description

244 patients came from Sardinia, Microcitemico of Cagliari divided in pediatric patients (164) and adult patients (80), while 116 patients came from pediatric center of Turin.

Of our cohort of beta-thalassemic major, we have evaluated the frequency of the rs12979860 genotypes (figure 4).

Recessive genotype (TT) have a frequency in whole cohort equal to 12,5%, heterozygous (CT) has 47%, and dominant homozygous (CC) has 40,5%.

Even without statistical tests it is straightforward the difference in the TT genotype behavior, as those that maintain virus persistence have 18% of frequencies and vice-versa the population in clearance has only 3% of this genotype. Also CC change in frequency when observing cleared patients (52%) or persisted patients (31%).

Rs1279860 genotype frequency in Spontaneous HCV clearance and Persistence HCV ()

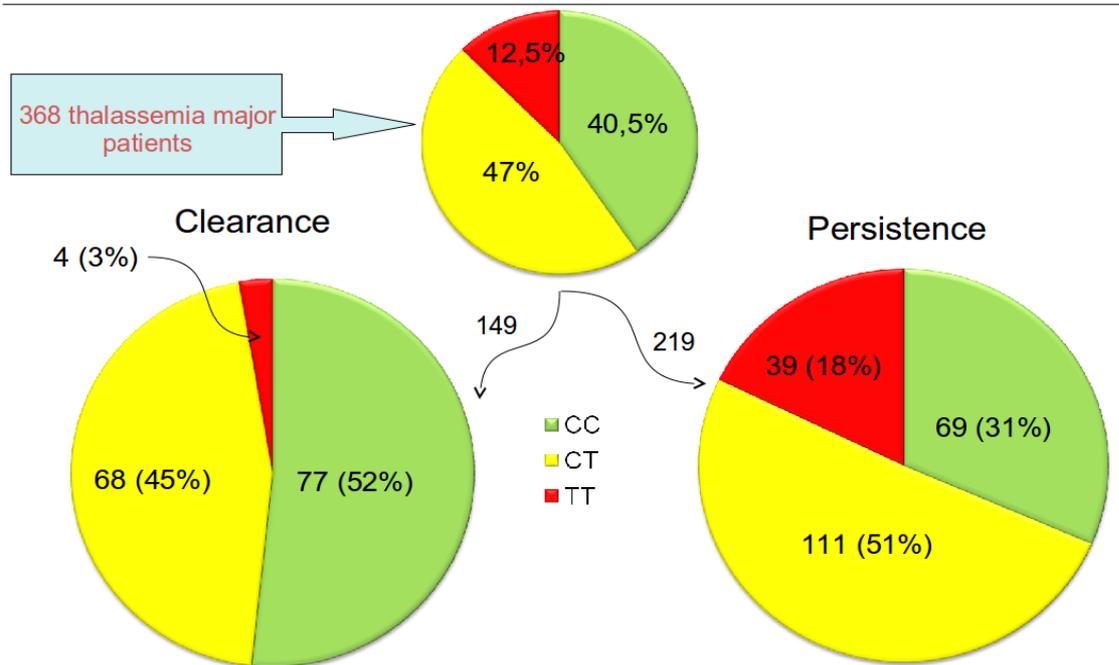


Figure 4: Rs1279860 frequency

IFNλ4 genotype

Three SNPs, rs1279860, rs8099917, and rs4803221 were genotyped. DNA was extracted from venous peripheral blood with standard methods while SNPs were directly genotyped using ABI TaqMan assay (Applied Biosystem Warrington, UK). SNP details as well as genotype frequency measures are reported on figure 4 for clearance virus.

Ninety four HCV-RNA positive patients had undergone liver biopsy and none of them had received antiviral therapy before. Mean age at the time of liver biopsy was 20+/-5 years.

Fifty patients had been treated with Interferon in the '90s of whom 20 presented sustained virological response (SVR) and 30 presented non sustained virological response (NSVR).

Since 2010, most patients with chronic persistent HCV infection received Peg-

interferon and ribavirin, but, having only 27 of them completed the treatment since at least 6 month, the correlation between IFN λ 4 polymorphisms and the response to this kind of treatment could not be evaluated due to the low number.

Haplotypes

We constructed haplotype from the two best SNPs of IFN λ 4 in patients chronically infected and in patients who cleared virus.

SNPs data were phased using PHASE software (Stephens M. et al., 2003) and kept when phasing probability was higher than 0.9.

Differences between groups were tested using genotype test as implemented in p-Link software and Mann-Whitney U-test upon data characteristics.

Furthermore, to check whether SNPs or haplotypes frequencies were associated with spontaneous viral response while controlling for confounders, a binary logistic regression model was developed. Goodness of fit of the model was assessed through Hosmer & Lemeshow test while Nagelkerke R² was used to measure how useful explanatory variables were in predicting the outcome.

Statistical methods

The Mann-Whitney, U-test or Student's test were used to compare continuous variables, and the chi-square were used to compare categorical variables. Univariate logistic regression was used to assess odd ratios of factors associated with sustained virological response. Multivariate analysis included all parameters from univariate analysis with a p-value <0.2. Receiver operator curves (ROC) were constructed to evaluate the areas under curve regarding spontaneous viral clearance associated with the SNPs and Haplotype.

All genetic analysis were performed using the PLINK software, version 1.07 (Purcell S. et al. 2007) while the SPSS statistical software package, version 18 (SPSS, IBM, Somers, NY, USA), was used for subsequent analysis, using a critical alpha of 0.05. Patients characteristics were described as relative frequencies or median with 5th and

95th percentile values.

Linkage disequilibrium between SNPs was assessed by Haploview version 4.2, which provided D' and r^2 values.

Statistical analysis

- 1) χ^2 and fisher and LogReg for SNPs/Haplotype/confounders
- 2) ROC for prediction ability
- 3) Decisional Tree description
- 4) Statistical package and significance

SNPs and Haplotypes frequencies were compared between groups using Chi-square and fisher exact test when appropriate, and also we performed Logistic Regression to account for confounders.

ROC curve analysis was performed to compare single SNPs and Haplotypes predictive ability, evaluating the area under the curve.

All p-value presented are two tailed and a value of $p > 0.05$ was considered statistically significant, and PLINK v.1.07, PHASE v.2.1 was used to generate haplotype data.

Mann-Whitney test was conducted to compare medians. The significance of associations between genotype/alleles variants and susceptibility to infection or spontaneous clearance were assessed using Chi-square and Fisher's tests with an estimation of the risk by computing OR (odd ratio).

A p-value < 0.05 was considered statistically significant. All tests were two-sided.

Haplotype frequencies and pairwise differences bet groups analyzes were performed using PHASE.

GEO dataset

We have downloaded a public expression dataset in order to develop co-expression network study. Expression data were already normalized to a housekeeping gene, beta actin, and donor and time-matched mock-infected samples using the comparative Ct method developed by Schmittgen and Livak (2008).

That dataset is downloadable at NCBI GEO DATASET, can be found at

<http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE54648>.

Dataset GSE54648 samples (Shehara et al , 2014) derived from culture of PHHs (primary human hepatocyte), offering an attractive alternative to human environmental. Like native hepatocytes. PHHs are polarized, largely terminally differentiated, and can robustly upregulate the innate immune response upon infection. The hepatocytes were infected with HCV genotype 2a. Association between IFN λ 4 and effective antiviral response was determined by comparing IFN λ 4 genotype with high and low infection frequencies in HCV infected cells (named high, medium, low “donors”).

Shehara's study revealed that hepatocytes from infection frequencies with clinically less favorable IFN λ 4 genotype (rs12979860, minor allele T/T) were more permissive for HCV infection (high or medium donors) compared to the cells with favorable alleles (rs12979860, major allele C/C), that were low donors (table 1).

We selected dataset from 20 cDNA libraries amplified from whole-genome microarray, hybridized to Illumina Whole-Genome Expression BeadChips (Illumina, HumanHT-12 V4 0 R2), from a total of 188 amplified cDNA libraries generated from LCM (Laser Capture Microdissection) samples of mock, HCV-infected, and adjacent hepatocytes.

The cDNA amplified selected for our aim were HCV infected cells. Moreover, we selected cells captured on 1dpi (day post infection) and 3dpi, excluding cells on 7 dpi, because during 7dpi occurred a reduction of infection frequencies for all rs12979860 genotype.

Our restricted selection from original dataset was composed by 12 cDNA libraries from cells with TT genotype (8 high donors and 4 medium donors) and 8 cDNA libraries from cells with CC genotype (all low donors) in order to discover the greatest difference between favorable and unfavorable genotype (Table 3)

Cell ID	rs12979860	Infect freq. Classification	Average infect Freq			SLC45A2
			1dpi	3dpi	7dpi	
472B	C/T	HIGH	28	24	19	Af/Eur
<i>HFTR</i>	<i>T/T</i>	<i>HIGH</i>	<i>21</i>	<i>28</i>	<i>8</i>	<i>Af</i>
5737	C/T	HIGH	23	12	5	Af
7185	C/T	MEDIUM	8	22	4	Af
<i>3868</i>	<i>T/T</i>	<i>MEDIUM</i>	<i>7</i>	<i>14</i>	<i>7</i>	<i>Eur</i>
5123	C/T	LOW	9	12	4	Eur
<i>5780</i>	<i>C/C</i>	<i>LOW</i>	<i>9</i>	<i>12</i>	<i>7</i>	<i>Af</i>
<i>5763</i>	<i>C/C</i>	<i>LOW</i>	<i>4</i>	<i>6</i>	<i>4</i>	<i>Af/Eur</i>

Table3: (A) Description of the major and minor alleles for IFN λ 4. IFN λ 4 and SLC45A2 genotype data for all cell type and their relation to infection frequency. Infection frequency classification is based on the infection frequency for each cell at 1 day (dpi) and 3 dpi. SLC45A2 can predict European or African ethnicity In red are highlighted cell chosen for our analysis. (Sheahan T. et al., 2014).

WGCNA – R package

WGCNA begins with the understanding that information captured by microarray experiments is far richer than a list of differentially expressed genes. Rather, microarray data are more completely represented by considering the relationships between measured transcripts, which can be assessed by pair-wise correlations between gene expression profiles. In most microarray analyses, however, these relationships go essentially unexplored. WGCNA starts from the level of thousands of genes, identifies clinically interesting gene modules and finally uses intramodular connectivity, gene significance (based on the correlation of a gene expression profile with a sample trait) to identify key genes in the disease pathways for further validation (Langfelder P. et al., 2008). WGCNA alleviates the multiple testing problem inherent in microarray data analysis. Instead of relating thousands of genes to a microarray sample trait, it focuses on the relationship between a few modules and

the sample trait. We excluded outliers through observing cluster graph, all samples that cross red abline, will be deleted to avoid artifacts. Toward this end, it calculates the eigengene significance (correlation between sample trait and eigengene) and the corresponding p-value for each module.

The module definition does not make use of a priori defined gene sets. Instead, modules are constructed from the expression data by using hierarchical clustering. Although it is advisable to relate the resulting modules to gene ontology information to assess their biological plausibility, it is not required. Because the modules may correspond to biological pathways, focusing the analysis on intramodular hub genes amounts to a biologically motivated data reduction scheme. Because the expression profiles of intramodular hub genes are highly correlated, typically dozen of candidate biomarkers result. Although these candidates are statistically equivalent they may differ in terms of biological plausibility or clinical utility. Gene ontology information can be useful for further prioritizing intramodular hub genes.

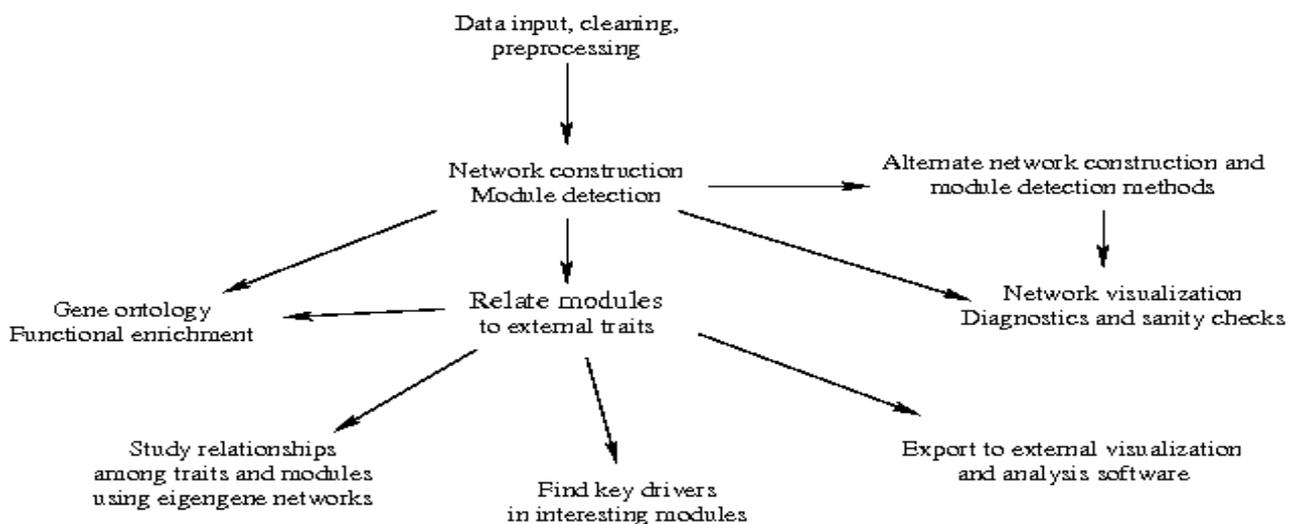


Figure 5: WGCNA standard proof-of-concept analysis

Co-expression network is defined as undirected, weighted gene networks. The nodes of such network correspond to gene expression profiles and edges between genes are

determined by the pairwise correlations between gene expression. Finally, Cytoscape version 3.2 and its plug-in Cluego software were used to visualize networks.

Weighted Gene Co-expression Network Analysis

WGCNA is based on topological overlap measurements derived from pairwise correlation-based adjacency values to estimate the neighborhood similarity among genes, followed by hierarchical clustering to identify gene (Figure 5) co-expression modules. Instead of focusing on individual genes WGCNA is highly effective for characterizing the feature of co-expression gene modules each of which is represented by a color classifier (modules). Here the correlation power is raised by a power of 10 to satisfy scale-free criteria. The minimum module size was set to 20 genes and the height for merging modules was set to 0.25, which required at least 25% dissimilarity among modules in expression, and network type was “signed”.

We identified several modules, each summarized by its eigengene (ME, defined as the first principal component of the standardized expression values). The significant of module eigengene-phenotype association (rs129798860 genotype, Donors, days or SLC45A2) was evaluated by linear regression model using WGCNA function.

Association less than 0,05 (Bonferroni Correction) was considered as significant. The top kME connection or the top gene was selected in order to facilitate visualization.

Functional enrichment analysis

Functional enrichment analysis was assessed using gene ontology categories. The background was set to the total list of genes expressed in this data set.

Gene are selected on the basis of MM (module membership) value color >0.6 or <-0.6 , always if cross the p-value threshold, and then selected the best genes with highest absolute MM values. Subsequently all gene selected were used as input of a functional enrichment analysis in KEGG using Cluego.

Result

Polymorphisms significance

				Spontaneous virus Clearance			
<i>rs8099917</i>	<i>genemo build 37</i>	1000 genome	tot freq	AntiHCV pos RNA neg	AntiHCV pos RNA pos	<i>p</i>	AUROC
TT	<i>chr19:39738787</i>	43	40,5	109 (48,9)	114 (51,1)	6,2E ⁻⁶	0,614
GT		44	47	40 (30,5)	91 (69,5)		
GG		11,5	12,5	0 (0)	14 (100)		
<i>rs4803221</i>				AntiHCV pos RNA neg	AntiHCV pos RNA pos	<i>p</i>	AUROC
GG	<i>chr19:39743165</i>	74	57,6	111 (50)	111 (50)	4,0E ⁻⁷	0,628
CG		24	36,4	38 (29,2)	92 (70,8)		
CC		2	6	0 (0)	16 (100)		
<i>rs12979860</i>				AntiHCV pos RNA neg	AntiHCV pos RNA pos	<i>p</i>	AUROC
CC	<i>chr19:39739129</i>	69	57,4	77 (52,7)	69 (47,3)	3,8E ⁻⁷	0,635
CT		28	36,6	68 (38)	111 (62)		
TT		3	5,9	4 (9,3)	39 (90,7)		

Table 4: Genotype frequencies of IFN λ 4 polymorphisms in 368 subjects with beta thalassemia and anti-HCV antibodies. The frequencies were calculated in the whole group and separately, in HCV RNA positive and HCV RNA negative patients. AUC= area under the curve.

As shown in table 4, rs12979860 and rs4803221 were the polymorphisms more associated with viral clearance and persistence ($p=3.8E-7$ and $p=4.0E-7$ respectively). The positive predictive power (PPP) of rs 12979860 was stronger for viral persistence than for spontaneous clearance (PPP=91%) and in such respect it corresponded to a recessive model for the T allele ($p=3.8E-4$ for TT versus CT genotype while $p=0.08$ for CT versus CC genotype). On the other hand, a dominant model for the C allele of rs12979860 was not alike predictive of viral clearance (PPP=0.52) (figure 6).

49 patients had been treated with Interferon in the '90s, of whom 19 presented sustained virological response (SVR) and 30 presented non sustained virological response (NSVR). Since 2010, most patients with chronic persistent HCV infection received Peg-interferon and ribavirin, but, having only 27 of them completed the treatment since at least 6 months, the correlation between IFNλ4 polymorphisms and response to this kind of treatment could not be evaluated due to the low number.

Spontaneous HCV clearance and IL28B genotype
(368 thalassemia major patients)

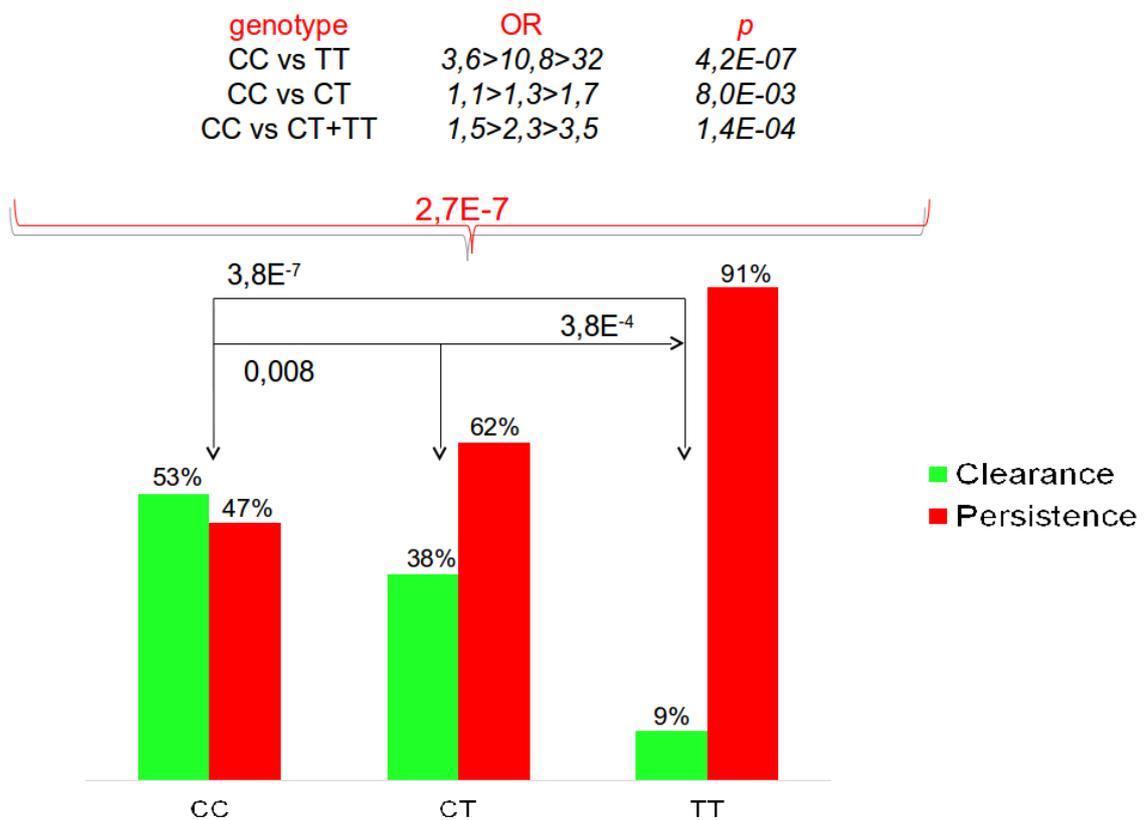


Figure 6: Positive predictive power of rs12979860 polymorphisms in respect of viral persistence and of spontaneous clearance. The positive predictive power (PPP) of rs12979860 was stronger for viral persistence than for spontaneous clearance and a recessive model for the T allele was the most predictive of viral persistence.

IFN Virological Response			
<i>rs8099917</i>	Responder	Non responder	<i>P-value</i>
TT	13 (50)	13 (50)	0,075
GT	6 (35,3)	11 (64,7)	
GG	0 (0)	6 (100)	
<i>rs4803221</i>	Responder	Non responder	<i>P-value</i>
GG	13 (52)	12 (48)	0,063
CG	6 (33,3)	12 (66,7)	
CC	0 (0)	6 (100)	
<i>rs12979860</i>	Responder	Non responder	<i>P-value</i>
CC	12 (54,5)	10 (45,5)	0,028
CT	7 (35)	13 (65)	
TT	0 (0)	7 (100)	

Table 5: Genotype frequencies of IFN λ 4 polymorphisms in 368 subjects with beta thalassemia infected with Hepatitis C virus and treatment with interferon-alpha. The frequencies were calculated in the whole group and separately, in responder and non responder

Rs12979860 and rs4803221 were also associated with SVR and NSVR ($p=0.028$ and $p=0.063$ respectively). Among 49 patients treated with interferon 19 achieved a SVR. None of them carried the T/T genotype in the rs12979860 while 12 were C/C. Vice-versa, among 30 patients with persistent HCV viremia (non responder) 7 patients carrying recessive genotype (T/T) versus no one in SVR (responder), and with a frequency of 50% in dominant genotype with 13 out of 19 patients who achieved a SVR (table 5)

Due to low records number unfortunately for this test we don't have enough statistical power.

Clinical data Associations

Binary logistic regression analysis, including rs12979860, rs4803221, gender and

UGT1A1, showed that not categorized rs4803221 as well as genotype TT of rs12979860 were associated with viral persistence (OR= 2.36 p=0.007 and OR=6.04 p=0.02 respectively). Rs8099917 was not included in the model for its almost complete linkage disequilibrium with rs4803221.

		Clearance	Persistence
FERRITINA	Media / Ds	1995 / 1293	2021 / 1446
BILIRUBINA		1,7 / 1,6	1,8 / 1,7
ALT		47,8 / 30,6	91,3 / 57,3
LIC		6,1 / 5,9	5,2 / 5,1
p (Ferritina)	0,862		
p (Bilirubina)	0,435		
p (LIC)	0,376		
p (ALT)	6.2E-19		

Table 6: Clinical evidence – In red is reported the p-value for each test

As expected, patients with chronic persistent HCV infection had mean ALT values of the last 22 years significantly higher than patients who had cleared the virus (47,8+/-30,6 vs 91.3+/-57,3 , p=6.2E-19).

However, among HCV-RNA positive patients, mean ALT values were not related to the rs12979860 and rs4803221 polymorphisms (p=0.76 as for rs12979860 and p=0.66 as for rs4803221). On the contrary, mean ALT were significantly related to mean serum ferritin values both in patients with chronic infection and in those who cleared the virus (p=1.25E-09 and 2.58E-03, respectively) and serum ferritin was not statistically different in the two groups of patients (1990 +/- 1290 ng/mL vs 2020 +/- 1440 mg/mL, p=0,984). Mean total bilirubin values were not statistically different

between HCV-RNA positive and negative patients (1,7 vs 1,8 , $p=0,435$), and among HCV-RNA positive, with respect to the rs12979860 and rs4803221 polymorphisms ($p=0.14$ and 0.09 , respectively).

By the Desmet score, 16 patients (17%), had no fibrosis, 35 (37.2%) had mild fibrosis (F1), 24 (25.5%), moderate fibrosis (F2), 17 (18.1%) severe fibrosis (F3) and 2 (F4) had cirrhosis.

Rs12979860 C/C genotype was present in 24 patients with F0-F2 and 9 patients with F3-F4 C/T in 46 patients with F0-F2 and 8 patients with F3-F4 and T/T in 5 patients with F3-F4 and C/T in 2 patients with F3-F4 and C/C ($p=0.26$). As for rs4803221, G/G genotype was present in 37 patients with F0-F2 and 11 patients with F3-F4, C/G in 34 patients with F0-F2 and 8 patients with F3-F4 and T/T in 4 patients with F3/F4 and no patients with F0-F2 ($p=0.75$). However, the present study is underpowered to observe clinically relevant differences in proportion, indeed we only have 20% power to observe a 10% difference between group F0-F2 and F3-F4 with the present sample size (table 7).

FIBROSIS	rs12979860			p-value
	CC	CT	TT	
	%			
F0-F2	32,0	61,3	6,7	1,49E-03
F3-F4	47,4	42,1	10,5	
FIBROSIS	rs4803221			p-value
	GG	CG	CC	
	%			
F0-F2	49,3	45,3	5,3	1,28E-03
F3-F4	57,9	42,1	0,0	

Table 7: Statistical Test to evaluate significance between Desmet score and IFN λ 4 variants.

Haplotype analysis

Four SNPs were genotyped: rs7248668, rs4803221, rs12979860, rs8099917 (Ge et al., 2009b), after GWAS study of all chromosome 19 (figure 9) imputed with 1000

genomes database using hg19 (GRCh37). We used as cases HCV infected and cleared to the infection and as controls HCV infected and in persistence.

GWAs confirmed the two just known SNPs rs12979860, rs8099917 and two less described SNPs (rs7248668, rs4803221,) (Smith et al., 2011).

These SNPs are in complete Linkage Disequilibrium (figure 8) in couple in the other hand rs12979860 is in complete LD with rs7248668 and rs8099917 is in complete LD with rs4803221. The final haplotype was built with only rs12979860 and rs4803221 that are the best predictors for each couple of LD.

The polymorphism rs8099917, was observed to have a high linkage disequilibrium with rs4803221 ($R^2=0.9$), while rs12979860 showed a low linkage disequilibrium with rs8099917 or rs4803221 ($R^2=0.43$ and 0.47 respectively).

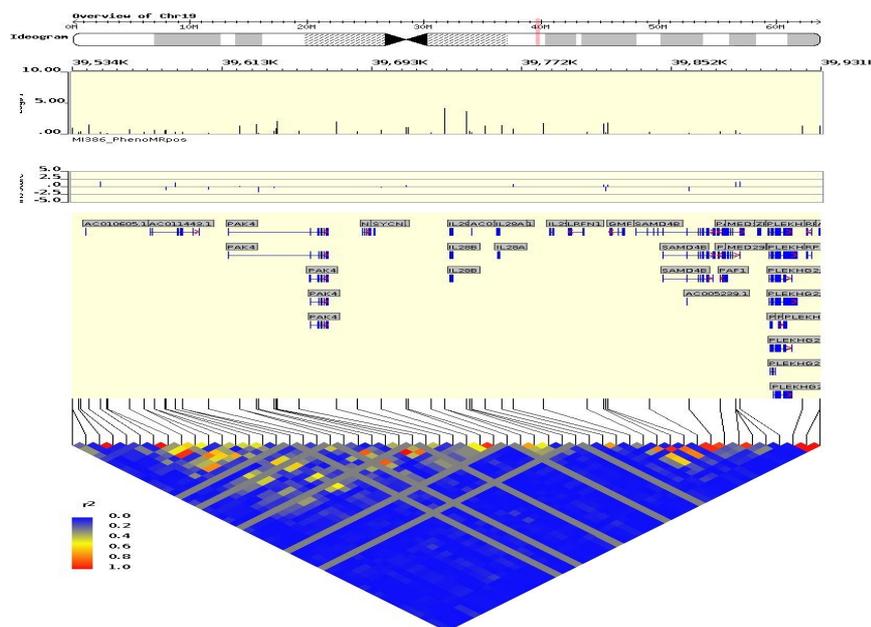


Figure 7: LD block of all IFNλ4 region.

GWAS result confirmed rs4803221 responds better than the current best rs8099917 also in our beta-thalassemic population replaying the result of Smith.

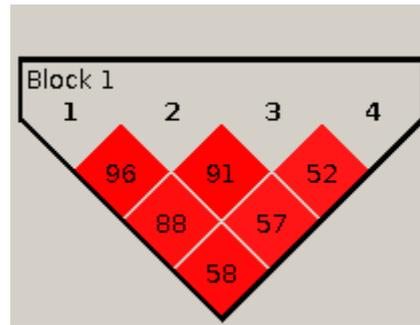


Figure 8: LD block where the numbers above the graph correspond respectively to: 1: rs7248669, 2: rs8099917, 3: rs4803221, 4: rs12979860

Moreover, in our population, while rs8099917 has an almost complete LD with rs4803221 the methylation associated polymorphism rs4803221 has independent effect with respect to rs12979860. This finding led us to develop an algorithm that, considering rs4803221, significantly may improve the viral clearance prediction in patients presenting the T allele at rs12979860. Recently, the same rs4803221 was found to predict failure to respond to antiviral therapy better than rs8099917 and rs12979860 in 199 treated patients infected by C genotype 1.

Haplotype Study, decisional Tree, and ROC curve

Predictive ability of haplotypes formed by rs4803221 and rs129798860 (table 10), as well as haplotype formed by all three SNPs, compared to predictive ability of the best predictor single SNP (rs12979860) using ROC curve analysis (figure 9), showed that the haplotype including rs4803221 and rs12979860 was only slightly less predictive than haplotype including all the three SNPs (AUC=0.656 and AUC=657 respectively). On the other hand the haplotype including rs4803221 and rs12979860 outperformed rs12979860 in predicting viral clearance (AUC=0.635).

	Rs...860	Rs...221	% spontaneous clearance
consensus	C	G	
Protective Haplotypes	·	·	53%
	·	·	47%
	T	·	32%
Non protective Haplotypes	·	C	17%
	T	C	7%
	T	C	7%
p=1,1E-8 R2=0,47			

Table 10 :IFNλ4 haplotype study for two best predictors SNP in spontaneous clearance

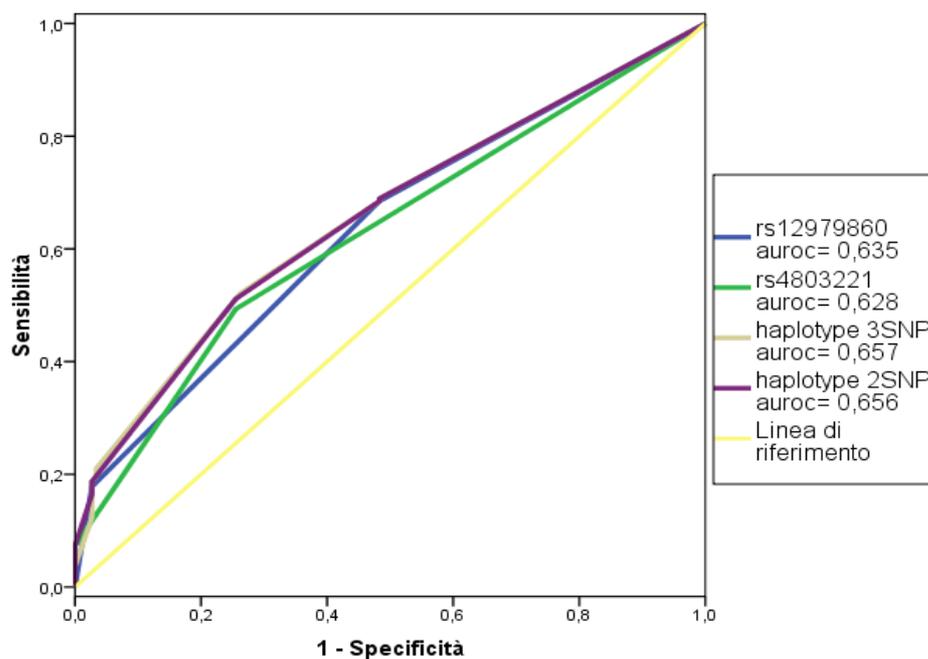


Figure 9: Predictive ability of viral clearance of single SNPs and haplotypes using ROC curve analysis.

In the same way decisional tree (figure 11) described what happened in the haplotype with rs12979860 T (minor allele) genotype and rs4803221 C genotype (minor allele). In figure 11 is shown the increase of prediction, as decisional tree, in the persistence infection of hepatitis C virus, this could lead a better diagnosis in clinical management.

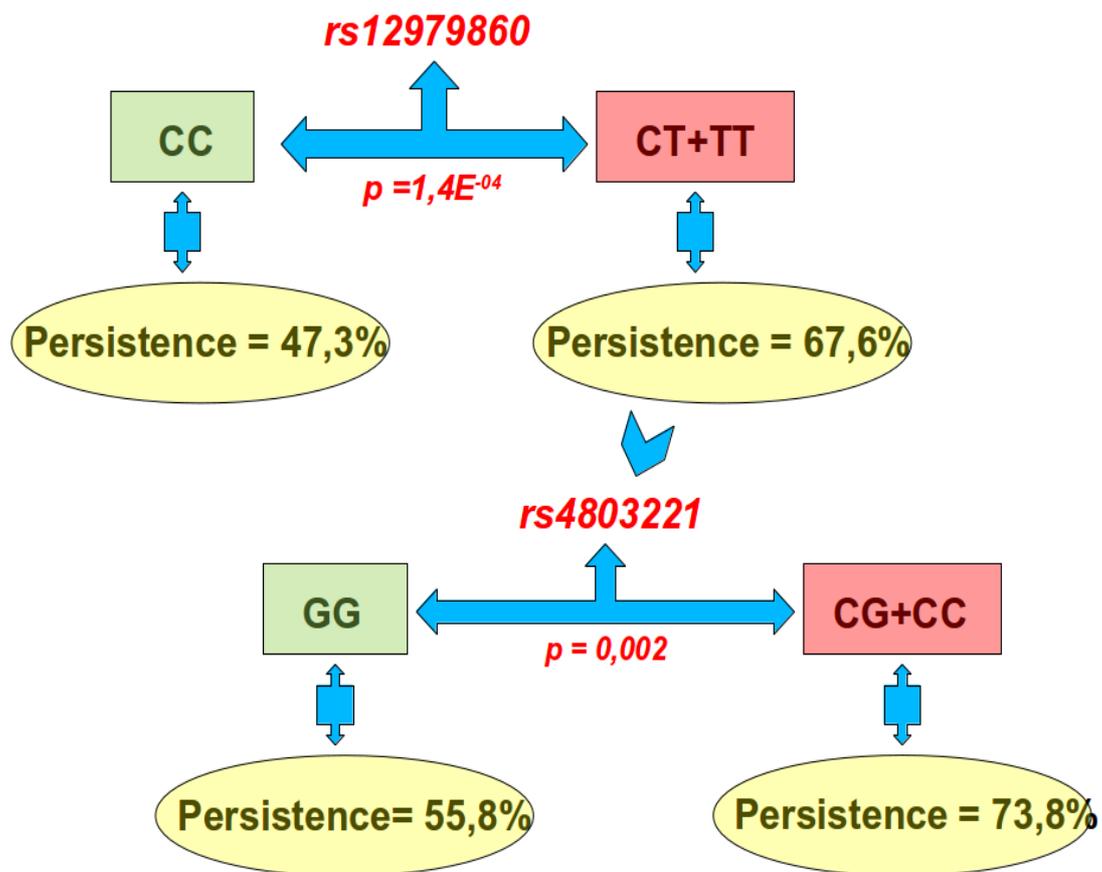


Figure 11: Decisional Tree to see the impact of the rs4803221 polymorphism analysis on viral clearance prediction.

Co-expression enrichment analysis

Using co-expression analysis in public expression data previously described, we have weighed array data for “donors” and for rs12979860. On this study we expected to find a correlation between rs12979860 unfavorable genotype TT and favorable genotype CC in HCV infected cell.

Following standard WGCNA workflow we obtained, after clusterization and module generation, the enrichment table depending on module colors, for the 20 samples selected.

Enrichment analysis revealed as the most significant modules “purple” and “white” (table 8). “Purple” module belongs to pathways related to retinoic acid receptor. It’s known that RIG-I, is a retinoic acid-inducible gene 1 and it belongs to one of the main biological process involved in PRR (pattern recognition receptor). At the “white” modules belong pathways related to translation and transcription, protein localization and protein transport, mechanisms already known to be activated in HCV infected cell.

rs12979860 CC vs TT – HCV weighed rs12979860 (47324 genes)					
module	size	p-val	Bonf	ontology	term name
purple	704	3.30E-9	4.70E-5	BP	negative regulation of retinoic acid receptor
purple	704	1.50E-8	2.20E-4	BP	regulation of retinoic acid receptor signal
purple	704	8.90E-8	1.30E-3	MF	retinoic acid receptor binding
white	127	1.50E-7	2.10E-3	BP	translational initiation
purple	704	1.70E-7	2.40E-3	BP	retinoic acid receptor signaling pathway
white	127	1.80E-7	2.50E-3	BP	SRP-dependent cotranslational protein
white	127	2.00E-7	2.90E-3	BP	cotranslational protein targeting
white	127	2.00E-7	2.90E-3	BP	protein targeting to ER
white	127	2.20E-7	3.00E-3	BP	establishment of protein localization
darkorange	148	2.40E-7	3.30E-3	CC	extracellular vesicular exosome
white	127	3.90E-7	5.50E-3	BP	nuclear-transcribed mRNA catabolic process
white	127	5.20E-7	7.30E-3	BP	protein localization to endoplasmic reticulum
white	127	1.40E-6	1.90E-2	CC	cytosolic ribosome
white	127	1.40E-6	2.00E-2	BP	translational termination
white	127	2.70E-6	3.80E-2	BP	viral transcription

Table 8. Color Pathways depend on module colors. All are close related to ribosome process (translation and transcription). Enrichment analysis of the CC vs TT rs12979860 WGCNA result (had also pathways relate to retinoic acid where the most known for HCV interaction is RIG-I).

At a next step, we kept first 9830 best genes of first analyses (selected for p-value < 0.05), in order to resolve computational problem in “eigengene” analysis due to work with more than 47324 genes: we have made a large selection of genes inside the best module found. Obviously new selected genes test compared with all genes test got similar results, but since we have to change “soft-thresholding power”, modules color were different from first co-expression analysis (module color depends number of genes), we had as best modules “yellow” and “black” (table 9).

rs12979860 CC vs TT – HCV weighed rs12979860 (9830 genes)					
module	size	p-val	Bonf	ont	term name
yellow	751	2.6e-8	3.7e-4	BP	negative regulation of retinoic acid receptor
yellow	751	5.9e-8	8.4e-4	BP	regulation of retinoic acid receptor signal
yellow	751	5.9e-8	8.4e-4	MF	retinoic acid receptor binding
yellow	751	4.8e-7	6.8e-3	BP	retinoic acid receptor signaling pathway
yellow	751	9.8e-6	1.4e-1	MF	nuclear hormone receptor binding
black	72	1.2e-5	1.8e-1	BP	nuclear-transcribed mRNA catabolic process
brown	1036	2.3e-5	3.3e-1	BP	dephosphorylation
pink	29	2.4e-5	3.4e-1	BP	cobalamin biosynthetic process
red	231	4.1e-5	5.8e-1	BP	neurological system process
black	72	5.3e-5	7.5e-1	BP	nuclear-transcribed mRNA catabolic process
black	72	6.3e-5	8.9e-1	BP	mRNA catabolic process
black	72	6.8e-5	9.6e-1	CC	cytosolic ribosome
black	72	7.4e-5	1.0e+0	BP	translational termination
black	72	1.0e-4	1.0e+0	BP	RNA catabolic process
black	72	1.3e-4	1.0e+0	CC	cytosolic small ribosomal subunit
black	72	1.5e-4	1.0e+0	BP	cotranslational protein targeting
black	72	1.5e-4	1.0e+0	BP	SRP-dependent cotranslational protein
black	72	1.6e-4	1.0e+0	BP	translational elongation

Table 9: WGCNA analysis performed with 9830 best genes found in CC vs TT analysis made with 47324 genes.

Relationship between module color and rs12979860 showed an high correlation between “black” module and trait, vice-versa even if we had significant p-value in “yellow” module (table 9) doesn't exist a direct correlation with rs12979860, as possible as to see in the “MEyellow” row (figure 12).

Eigengene cluster graphs highlighted the correlation between genes and modules for CC vs TT and weighted for donors (figure 13 (a)), or for the rs12979860 genotype (figure 13 (b)). Also the dendrogram indicates that “black” module is highly related in weights chosen, indeed the “black” module had stronger correlation than other modules.

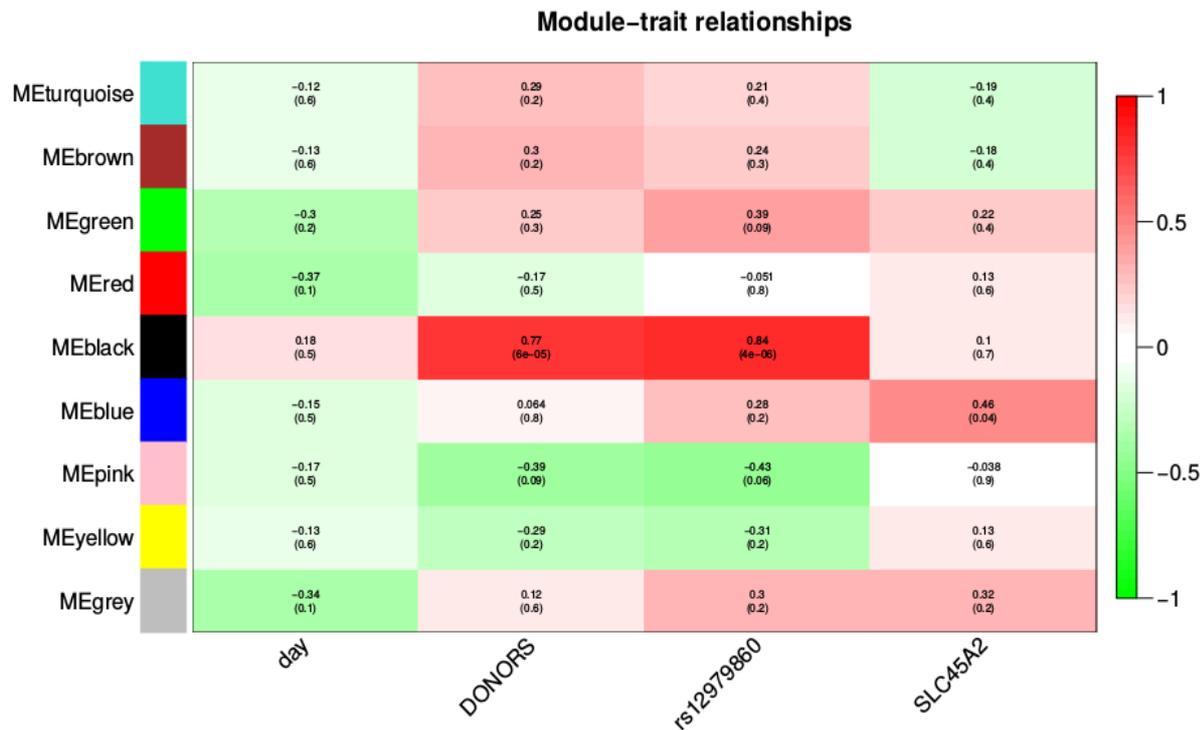


Figure 12: Relationship between module color and traits. Dark red represent positive correlation and dark green represent negative correlation. Black module is the most correlated.

Both dendrograms had the same clusterization, validating the thesis for which IFN λ 4 variants and HCV infection are associated. Even if the highest enriched terms for “yellow” module was 2.6e-8 whereas the highest enriched terms for “black” module was 1.2e-5, “yellow” module is not part of the same meta-module. On the other hand, even if the “yellow” module had better p-values significance (table 9), it’s not directly associated to rs12979860.

Then, with genes inside “black” module we constructed genes networks using Cytoscape in order to detect hubs (genes with driver positions and strong connection each others) related to SNP genotype (figure 14(a)).

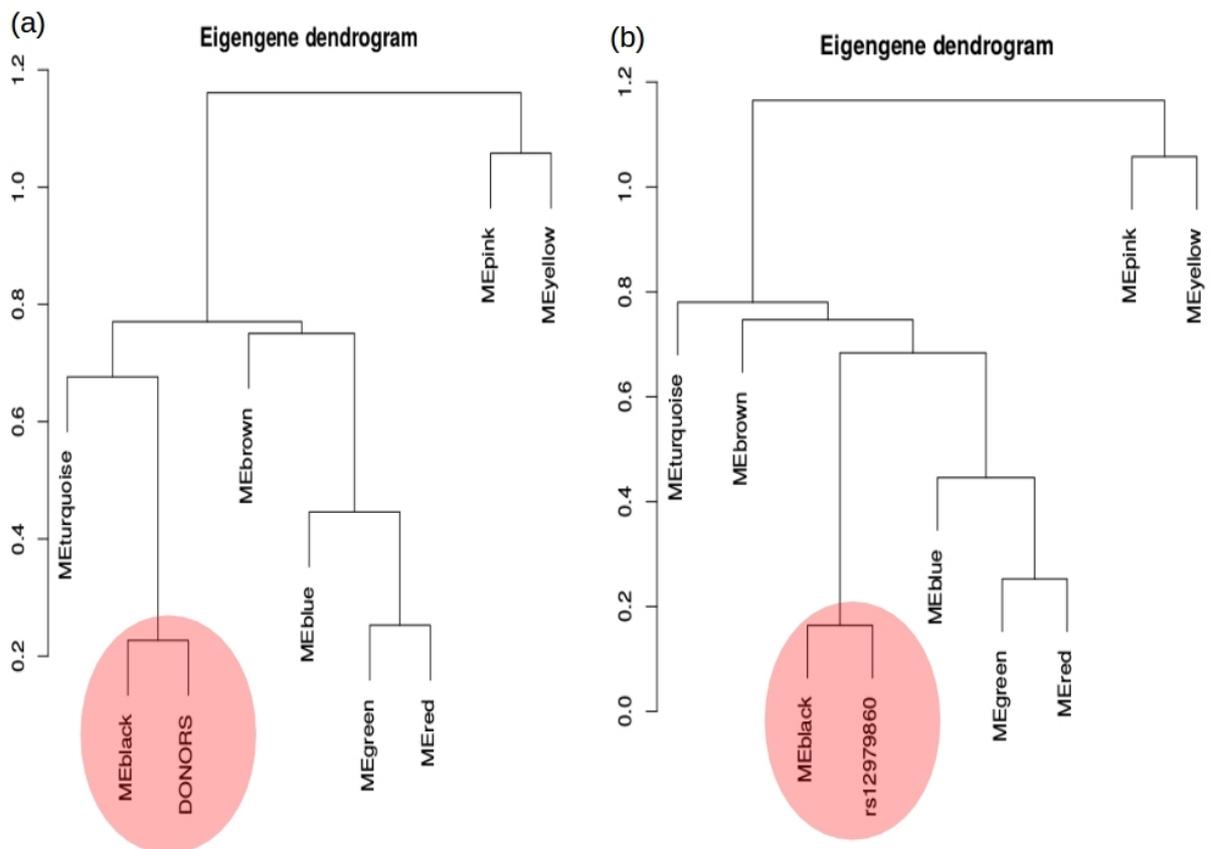


Figure 13: Eigengene dendrogram. (a) weighed for “donors”, (b) weighed for rs12979860 genotype. Red highlighted represent the closest meta-module for each dendrogram. “Black” module is confirmed to be the most related.

Genes with the higher number of correlation were LOC389404 (RPL9P18), LOC727865 (RPL9P32) and LOC651436 (similar RPL9) that we can consider as hub genes of black module. Furthermore this three hub were undirectly linked by LOC441763 with RNA28S5 (LOC100008589) that was the best rank gene in module (the gene with the best p-value associated to rs12979860), shown in table 10.

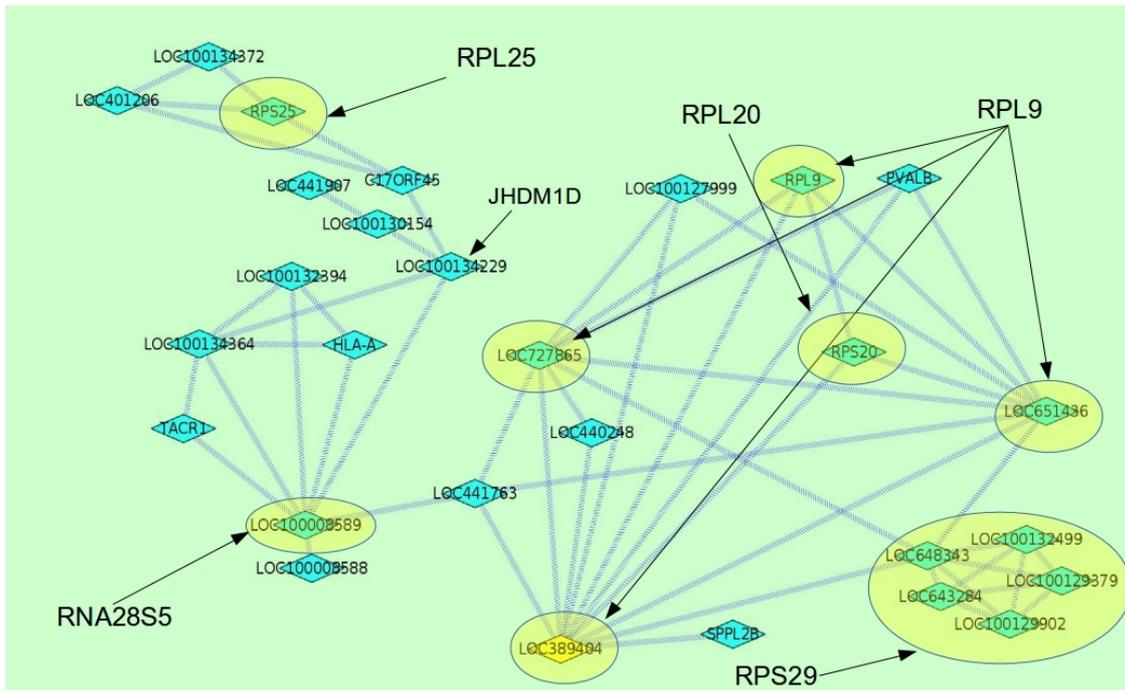
BLACK MODULE GENES LIST					
<i>Probe_Id</i>	<i>geneSymbol</i>	<i>GS.rs12979860</i>	<i>p.GS.rs12979860</i>	<i>MM.black</i>	<i>p.MM.black</i>
ILMN_1733559	LOC100008589	0.88	3.0E-7	0.92	6.3E-9
ILMN_3249578	LOC100132394	0.87	6.4E-7	0.88	2.4E-7
ILMN_1680330	TACR1	-0.86	1.2E-6	-0.87	7.3E-7
ILMN_3246805	LOC100134364	0.85	1.7E-6	0.91	1.6E-8
ILMN_3246279	LOC100134229	-0.84	3.3E-6	-0.90	8.1E-8
ILMN_3243593	LOC100008588	0.83	5.1E-6	0.89	1.4E-7
ILMN_1769277	LOC651436	0.83	6.3E-6	0.94	5.1E-10
ILMN_1759008	ZNF689	0.83	7.1E-6	0.80	2.3E-5
ILMN_3276209	LOC727865	0.83	7.6E-6	0.95	9.7E-11
ILMN_2115669	SEMA4C	-0.82	1.0E-5	-0.81	1.7E-5
ILMN_2203950	HLA-A	0.81	1.3E-5	0.85	1.9E-6
ILMN_3200661	LOC389404	0.81	1.6E-5	0.95	2.5E-10
ILMN_1690295	LOC648343	0.80	2.0E-5	0.91	1.9E-8
ILMN_3261938	LOC100130154	0.79	3.5E-5	0.89	1.5E-7
ILMN_1814812	LOC650546	0.79	3.6E-5	0.87	8.2E-7
ILMN_1672320	LOC441907	0.79	3.9E-5	0.85	2.3E-6
ILMN_1750507	RPL9	0.79	4.0E-5	0.83	4.8E-6
ILMN_1792528	LOC401206	0.78	4.4E-5	0.85	1.8E-6
ILMN_1739263	LOC643284	0.77	7.3E-5	0.83	5.4E-6
ILMN_1746516	RPS25	0.77	8.3E-5	0.83	4.8E-6
ILMN_3252771	LOC100127999	0.76	9.6E-5	0.89	1.4E-7
ILMN_2069224	PVALB	0.76	9.7E-5	0.84	4.1E-6
ILMN_3201600	LOC100132804	0.75	1.4E-4	0.91	2.1E-8
ILMN_1701596	RPS20	0.74	1.7E-4	0.82	1.1E-5
ILMN_1719224	C17ORF45	0.74	1.8E-4	0.82	1.2E-5
ILMN_3283664	LOC100132037	0.74	1.8E-4	0.93	2.6E-9
ILMN_3211079	LOC148430	0.74	2.2E-4	0.90	5.9E-8
ILMN_1735185	CSTF1	0.73	2.3E-4	0.81	1.3E-5
ILMN_1703337	LOC441763	0.73	2.4E-4	0.89	1.2E-7
ILMN_3308921	MIR34B	0.73	2.5E-4	0.80	1.9E-5
ILMN_2165985	RPAP3	-0.73	2.6E-4	-0.76	9.4E-5
ILMN_3242900	HIST2H2AA4	0.73	2.9E-4	0.81	1.5E-5
ILMN_3238018	LOC100134372	0.72	3.0E-4	0.81	1.6E-5
ILMN_3256926	LOC100130764	-0.72	3.9E-4	-0.89	1.0E-7
ILMN_3288268	LOC100132499	0.71	4.0E-4	0.82	1.2E-5
ILMN_1743992	DCP1B	0.71	4.3E-4	0.90	9.8E-8
ILMN_3301372	LOC730167	0.71	4.9E-4	0.84	3.5E-6
ILMN_1803712	LOC653057	0.70	5.3E-4	0.80	2.5E-5
ILMN_3178252	LOC100129379	0.70	5.6E-4	0.78	4.3E-5
ILMN_1667229	HCG22	0.70	6.2E-4	0.82	9.6E-6
ILMN_3266128	LOC100129553	0.70	6.5E-4	0.83	6.0E-6
ILMN_1685985	LOC649150	0.69	6.7E-4	0.86	1.5E-6
ILMN_3200384	LOC441013	0.69	8.3E-4	0.90	7.4E-8
ILMN_2094630	TRIM60	0.67	1.2E-3	0.69	8.3E-4
ILMN_3256024	LOC100128035	-0.67	1.3E-3	-0.81	1.4E-5
ILMN_1770290	CNN2	0.66	1.6E-3	0.78	5.2E-5
ILMN_1691410	BAMBI	-0.65	1.8E-3	-0.83	4.8E-6
ILMN_3256742	LOC100129902	0.65	1.8E-3	0.79	3.8E-5
ILMN_3239629	LOC729679	0.65	1.9E-3	0.88	4.3E-7
ILMN_1715574	ZC3H11A	0.65	1.9E-3	0.79	4.0E-5
ILMN_3228508	LOC731642	0.65	2.0E-3	0.84	3.3E-6
ILMN_1680614	LOC440248	0.65	2.1E-3	0.84	4.4E-6
ILMN_3228294	LOC729279	0.65	2.1E-3	0.85	2.0E-6
ILMN_1698117	DSCR8	-0.64	2.3E-3	-0.89	1.8E-7
ILMN_1697115	FLJ12355	0.64	2.3E-3	0.81	1.3E-5
ILMN_3276409	LOC100133222	0.64	2.6E-3	0.80	2.2E-5
ILMN_1710026	RNF133	0.63	3.1E-3	0.79	3.8E-5
ILMN_3202432	LOC644464	0.63	3.2E-3	0.85	2.5E-6
ILMN_3310745	SNORD116-27	0.62	3.8E-3	0.78	5.4E-5
ILMN_2331890	RPL41	0.61	4.0E-3	0.85	2.0E-6
ILMN_3281988	LOC728157	-0.61	4.1E-3	-0.83	7.6E-6
ILMN_3294335	LOC646294	0.61	4.3E-3	0.86	1.1E-6
ILMN_1755749	PGK1	-0.61	4.6E-3	-0.75	1.3E-4
ILMN_1672461	SPPL2B	0.60	5.0E-3	0.79	3.7E-5
ILMN_2339192	HMGCLL1	0.60	5.0E-3	0.73	2.7E-4
ILMN_3288726	LOC650901	-0.60	5.2E-3	-0.86	8.9E-7
ILMN_1733535	ZNF366	-0.60	5.4E-3	-0.77	8.0E-5
ILMN_1705025	A26C3	0.59	5.8E-3	0.77	7.4E-5
ILMN_2094718	TPT1	0.55	1.2E-2	0.83	5.8E-6
ILMN_2160819	RPS27	0.54	1.3E-2	0.76	9.3E-5
ILMN_3211132	LOC645173	0.54	1.5E-2	0.82	9.3E-6
ILMN_1652955	LOC648622	0.53	1.6E-2	0.78	4.6E-5

Table 10: All 72 gene in black module. Green highlighted are genes present in Cytoscape network. And in red are highlighted the best genes. LOC389404 is RPL9P18 the hub genes in network and LOC100008589 is RNA28S5 the best rank in gene associated to rs12979860.

Enrichment analysis made with Cluego showed in figure 14(b) had two main networks that links in same genes. We just expected this kind of result, due to intrinsic mechanism in HCV infection. This result suggests that innate antiviral response may be hub regulated by pseudogenes (Yan-ZI Wen et al. 2012) indeed unfavorable genotype rs12979860 TT had an overexpression of RPL9P18, RPL9P32, LOC651436 (RPL9 like) and RNA28S5.

The network built in Cluego reveal pathways related to mRNA catabolic process, cytosolic ribosome, translational termination, cytosolic small ribosomal subunit, cotranslational protein targeting to membrane, SRP-dependent cotranslational protein targeting to membrane,translational elongation.

(a)



(b)

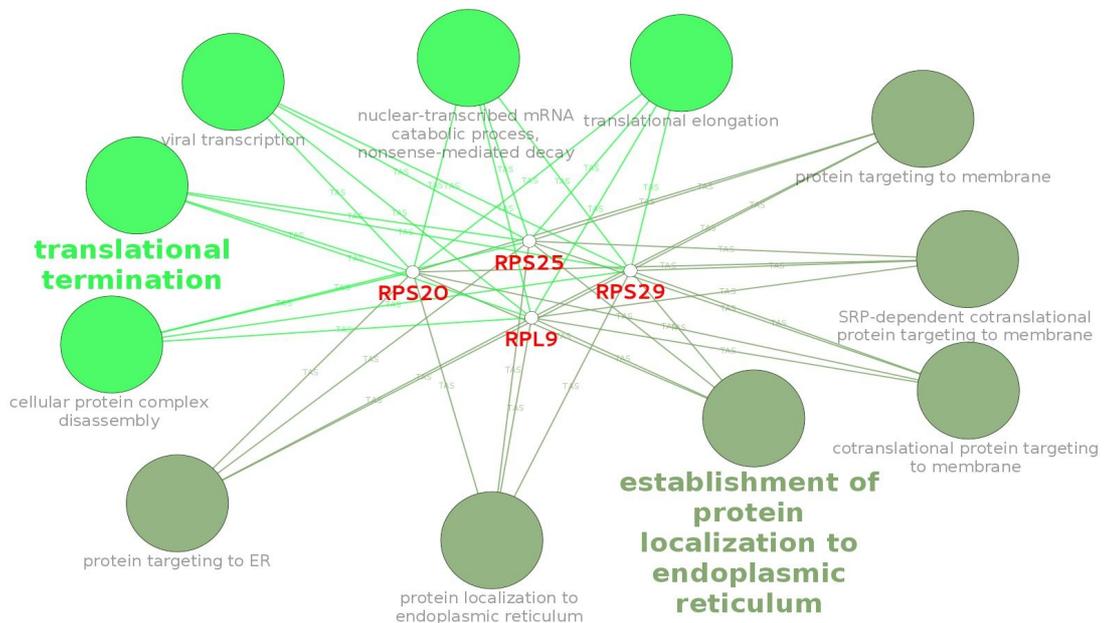


Figure 14: (a) Network displayed genes with higher correlation with rs12979860. RPL9 and RPL9 pseudogenes are the higher correlated, however other ribosome subunits have a central role. (b) Enrichment analysis made with Cluego shows closest pertinent pathways of the main ribosome subunits genes (RPS20, RPS25, RPS29, RPL9).

Discussion

Clinical evidence

The aim of first part of our study was to examine the prevalence and clinical significance of SNPs within the IFN λ 3/IFN λ 4 alleles in a population of HCV-infected patients in Sardinia. We also evaluated the ability to predict response to anti-HCV treatments in this population. We found that rs12979860 and rs4803221 were the most informative markers of treatment response and spontaneous clearance in Sardinia.

Finally we show that combined polymorphisms may show increased predictive value in term of spontaneous clearance, with a significant improvements in the associations.

Clinical Data Association

We have demonstrated that in thalassemia patients the rs12979860 as the rs4803221 polymorphisms are not associated with necrotic-inflammation, differently from that reported by Agundez et al. and Thompson et al. who have found in general population that rs12979860 C/C genotype is associated with higher serum ALT than the remaining genotype and by Abe et al. who found that ALT levels were lower in carrier of the rs8099917 T/T genotype. This difference could be due to the confounding effect of liver iron, which as a primary role in inducing liver damage in subject with thalassemia, as demonstrated by the highly significant relation between mean ALT and mean serum ferritin values in our patients. Tillman reported that the jaundice during acute hepatitis C is more common among patients with rs12979860 C/C genotype than with other variants and that in non-C/C patients, jaundice is associated with a higher likelihood of spontaneous clearance compared with those without jaundice. Although we could not confirm or deny these findings, no correlation was found between UGT1A1 polymorphisms and persistence of the virus.

In addition, the total bilirubin long follow-up allowed us to demonstrate for the first time that, at least in patients with beta-thalassemia, there is no correlation between rs12979860 polymorphisms and bilirubin values, which are confirmed to be strictly dependent on the UGT1A1 polymorphisms.

The relation between IFN λ 4 polymorphisms and the stage of fibrosis is controversial. Di Marco et al. (2012) reported that the carrier state of the minor alleles at rs12979860 and rs8099917 sites were associated with more severe liver fibrosis in a group of 131 patients with thalassemia major and chronic HCV infection. In general population, several authors found higher fibrosis among carriers of the rs8099917 T/T genotype. Nevertheless Thompson et al. reported that the rs12979860 polymorphism was not associated with advanced hepatitis fibrosis in patients with virus C chronic hepatitis, and Agundez et al. did not find any relation among the IFN λ 4 polymorphisms and fibrosis stage directly shown by the liver biopsy. These findings are in agreement with our results. Even though the number of patients undergone liver biopsy in our study is smaller than those examined by Di Marco et al. the lack of association between necrotic-inflammation and IFN λ 4 polymorphisms and the previously demonstrated significant correlation between rate of fibrosis progression and hepatitis iron concentration in HCV-positive patients with thalassemia could explain and support these observations.

Frequency analysis

This study confirms that, in thalassemia patients as in general population, the SNPs on chromosome 19q13 closely associate with innate natural course and treatment response of chronic hepatitis C. As on a recent paper, indeed, also in our population, C/C variant of polymorphism rs12979860 was related to response to interferon treatment and, above all, to spontaneous clearance of the virus. However, it seems noteworthy to underline that, as for rs12979860, the positive predictive power was stronger for viral persistence than spontaneous clearance and in such respect the TT allele was more predictive than CC.

Further studies are needed to demonstrate that the haplotype tagged by SNP rs12979860 and rs4803221 is the major causative.

WGCNA analysis

In second part of our study, based on differentially expressed genes, we employed WGCNA analysis to construct co-expression network analysis for 47324 genes in HCV infection in PHH.

Our study demonstrated that WGCNA is useful for exploring transcriptional changes and identifying difference between HCV-infected cell frequency associated to IFN λ 4 genotype rs12979860. Our results may help to explain biological process occurred in early HCV infection and in particular the deeper role of unfavorable rs12979860 genotype. We identified 72 genes (table 10) in “black” modules which had as mainly hub (RPL9P18).

We observed an overexpression of ribosomal genes when rs12979860 has TT genotype. This could mean that TT genotype encourage overexpression of specific ribosomal pseudogenes (RPL9P18, RPL9P32) probably decreasing innate antiviral response either at the level of ISG regulation or at the level of ISRE mechanism regulation (Prokunina-Olsson et al., 2013).

RPL9P18, has a known transcript (ENST00000439124) of 535 bp, located on Chromosome 6: 63,615,827-63,616,361 reverse strand, to date is classified as processed pseudogene.

RPL9P32 has a known transcript (ENST00000495117) of 578 bp, located on Chromosome 18: 58,631,259-58,632,120 forward strand, to date is classified as processed pseudogene.

This evidence could likely suggest the function of pseudogene transcripts as decoys of miRNAs in which pseudogene competes for miRNA with normal transcript gene. (Muro et al., 2011), on the other hand, an increase in the pseudogene transcription implies that less miRNA will target the parental gene. Therefore the pseudogenes indirectly regulate the corresponding parental gene by competing for binding to the

miRNA.

Our analysis establish a prior event in which HCV response is highly associated with RPL9P18 (as hub) and RNA28S5 (as best p-value single gene) and it is conditioned by IFN λ 4 genotype (figure 14(a)).

From those relevant results appear straightforward two aspects: (1) IFN λ 4 unfavorable genotype is correlated to the ribosomal function; (2) specific pseudogenes could be an active role in innate antiviral response gene regulation. Nevertheless we don't know if pseudogenes overexpression change function, gain function, lose function or none of this, interacting in other way, but we can suppose an active role in regulation of ribosomal genes.

Conclusion

In conclusion, also in thalassemia the SNPs on chromosome 19q13 closely associates with spontaneous clearance and drug treated, induced HCV clearance. The haplotype tagged by SNP rs12979860 and rs4803221 significantly could improve the viral clearance prediction in infected patients. Neither necrotic-inflammation, bilirubin values in the chronic phase of the hepatitis C or fibrosis stage are related to IFN λ 4 polymorphisms.

The network was constructed using WGCNA, R package, in two different steps based on screening best modules after a primary detection, and further analyzed by Cytoscape network. Network showed genes with high module membership value and neighborhood connectivity involved in HCV infection which have different interaction depending on rs12979860 genotype.

We found the most likely hub RPL9P18 could be valuable as candidate genes in further functional analysis.

However, HCV host cell array co-expressed for rs12979860 genotype confirms several mechanisms of innate immune regulation and evasion could provide a molecular basis for viral persistence.

Further analysis could be necessary in order to validate this result, in particular knockdown study essentially to see if that ribosome subunits change expression or change viral response in significant way.

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