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TITLE: Negative predictive value of *IL28B, SLC28A2* and *CYP27B1* SNPs and low RBV plasma exposure for therapeutic response to PEG/IFN-RBV treatment.

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Short Running Title: SNPs and RBV PK predict failure of therapy.

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List of Abbreviations: HCV, Hepatitis C virus; SVR, sustained virological response; PEG/IFN, pegylated interferon; RBV, ribavirin; Hb, haemoglobin; Pk, pharmacokinetic; ITPA, inosine triphosphatase; SNPs, single nucleotide polymorphisms; TDM, therapeutic drug monitoring; IQR, inter-quartile range; ROC, receiver operating characteristic.

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Abstract

Objectives. The response rate to treatment of chronic HCV-genotype 1 and 4 infection was recently found to be strongly influenced by many polymorphisms. The aim of our study was to carry out a integrated analysis of the effects of polymorphisms and ribavirin plasma exposure on outcome.

Methods. The retrospective analysis included 174 patients. *IL28B*, *CYP27B1*, *SLC29A1*, *SLC28A3* and *SLC28A2* polymorphisms were genotyped and tested for association with sustained virological response. The impact of RBV plasma exposure during the first three months of therapy on outcome was also investigated.

Results. Considering patients infected by HCV-1/4, three polymorphisms (*IL28B rs8099917TT*, *CYP27B1 rs4646536TT* and *CNT2 rs11854484TT*) were associated with sustained virological response. The number of negative variant allele and low ribavirin exposure were correlated to percentage increasing to therapy failure, suggesting some degree of cumulative effect of the four factors. A cut-off of 2.5 μg/mL of RBV was found to be associated with outcome (AUROC=0.64, sensitivity=55.0% and specificity=71.2%, p=0.020). In multivariate logistic regression analyses each variant allele and RBV plasma exposure cut-off were independently associated with outcome.

Conclusions. In this study we found that additional polymorphisms as well as RBV plasma exposure are also able to influence the achievement of response. Regardless the magnitude of RBV pharmacokinetic exposure, the negative predictive value of the polymorphisms here investigated is much stronger than the positive one.

Keywords: HCV; Therapeutic Drug Monitoring; Ribavirin Plasma Exposure; Single Nucleotide Polymorphisms; Sustained Virological Response.

Introduction

Hepatitis C is a major global public health problem. One hundred and eighty million individuals worldwide are chronically infected with hepatitis C virus (HCV) and at risk for related morbidity and mortality from cirrhosis and hepatocellular carcinoma [1-3]. Approximately 10%-20% of the infected people spontaneously eliminate the virus [4]. Different factors may influence the ability to spontaneously clear the virus and to favourably respond to treatment [5].

Although two new antiviral drugs are being introduced into clinical use, the combination of pegylated-interferon-alfa (pegIFN-alfa) and ribavirin (RBV) still remain the backbone of anti-HCV therapy.

The probability of eradicating HCV varies according to genotype; thus, it is recommended that the duration of therapy be chosen according to HCV genotype -48 weeks for difficult-to-cure patients infected with genotypes 1 or 4, and 24 weeks for the less difficult-to-cure patients infected with genotypes 2 or 3 [2, 3].

However, of patients infected with genotype 1 and genotype 4 HCV, only approximately 40% are cured by pegIFN-alfa/RBV therapy [2, 3, 6-9]. Furthermore, therapy may be associated with considerable toxicity. Therefore, the ability to prospectively identify individual patients who are likely to respond to treatment would be clinically valuable.

The response rate to treatment of chronic HCV-genotype 1 and 4 infection was recently found to be strongly influenced by polymorphisms (SNPs) in the genetic region coding for *IL28B* [5, 10-18], possibly through modulation of the IFN-λ system [19-21]. Specifically, the *rs12979860* polymorphism was strongly associated with the viral clearance, and *rs8099917* with the sustained virological response (SVR) [10, 13, 14, 22]. According to the analyses of several clinical cohorts, the response rate to standard PEG/IFN-RBV therapy in carriers of favourable genes may be as high as 80%, while the current expectations in a non-IL28B-stratified population are around the value of 45% [23]. While a series of other individual features (e.g. degree of liver fibrosis, HCV viral load at baseline, age, gender, etc) were also found to have a predictive role on treatment response [9], the only so far

identified modifiable variable that has been consistently associated to the therapeutic outcome is RBV dose and pharmacokinetic exposure [24-27]. The RBV pharmacokinetic determinants associated to the response rate have however yet to be firmly defined, mainly due to differences in study design and sampling time amongst the clinical series so far investigated. RBV tends to accumulate in plasma over time, and as a consequence, the pharmacokinetic parameters measured at different times since treatment initiation may show significantly different values [24, 25, 27-31]. Nevertheless, in most clinical studies, RBV concentration was found to correlate with the therapeutic outcome, as also historically testified by the better treatment response seen with increased RBV doses [24, 25, 27, 28, 30-36]. Additionally, considering the importance of ribavirin concentrations [27], there are limited data as the ribavirin transporters polymorphisms may affect RBV concentrations and the clinical outcome [37-42].

In this setting, the association between low therapeutic responsiveness and vitamin D deficiency has been recently reported by several authors, particularly for HCV difficult-to-treat infections, likewise *IL28B* (*rs12979860* and *rs8099917*) SNPs and low ribavirin plasmatic concentrations [22, 43, 44].

With the introduction of IL28B screening in the therapeutic procedures for chronic HCV infection [45-48] (and also considering the forthcoming introduction of a third drug), the question arises whether RBV pharmacokinetic exposure still retains any value in predicting the response to anti-HCV treatment. Should the therapeutic response becoming no longer dependent upon RBV pharmacokinetic exposure, potential advantages are foreseeable in terms of side effects reduction.

Few data are, however, yet available on the interplay between RBV exposure and these newly introduced genetic factors. We describe here the results of a retrospective study of HCV patients with chronic hepatitis who underwent standard PEG/IFN-RBV treatment. The treatment outcome has been analysed according to individual polymorphisms (*IL28B*, *CYP27B1*, *SLC29A1*, *SLC28A3* and *SLC28A2* SNPs) and measurement of RBV pharmacokinetic exposure.

Materials and Methods.

Patients

In this retrospective study, 174 patients with chronic HCV infection, who were treated at two university hospitals (Amedeo di Savoia and S. Giovanni Battista) of the city of Turin, Italy, between March 2005 and November 2008 were enrolled (table 1).

Patients were treated with PEG/IFN-α-2b (1.5 μg/kg s.c. once a week; sub dermal injection) or PEG/IFN-α-2a (180 μg once a week; sub dermal injection) plus RBV (600–1400 mg daily depending on bodyweight; orally). Sampling was performed after obtaining written informed consent in accordance with local ethics committee indications. Since patients' authorization for routine sampling at the time of treatment did not include genetic analyses for polymorphisms (while therapeutic drug monitoring -TDM- of ribavirin is customarily done and authorized on a regular basis since early 2005), patients were asked an additional authorization for the genetic screening of their stored samples. Main inclusion criteria were: no concomitant interacting drugs and self-reported adherence > 95%. Patients with other forms of liver disease, active hepatitis A, hepatitis B infection, HIV infection, decompensated liver disease, hepatocellular carcinoma, severe anaemia, severe depression or other psychiatric diseases, significant cardiac or renal disease, seizure disorders or pregnancy were excluded from this study. Data collected included: age, gender, weight, previous IFN therapy, concomitant drugs, baseline biochemical parameters, such as white blood cells, Hb, alanine aminotransferase (ALT) catalytic activity, serum HCV RNA level (logIU/mL).

Genotyping

Patients for whom DNA samples were available and agreed to undergo genetic analyses were genotyped at polymorphic sites showed in table 2 using the ABI TaqMan allelic discrimination kit by real time PCR using standard methodology. All primers, probes, and PCR conditions are available on request.

Measurement of plasma ribavirin concentration

At week 4, 8 and 16, plasma samples were collected ~12 hours after the dose administration of ribavirin, just before the subsequent administration (trough value). Samples were centrifuged at 1400 *g* for 10 minutes to separate plasma and then stored at -80°C until analysis. Ribavirin concentrations were measured by a previously validated HPLC method [49]. Calibration curve ranged from 0.078 up to 10 µg/mL. Accuracy and precision standard errors were below 10%.

Definition of Clinical End Points

Alike in most therapeutic studies on HCV chronic hepatitis treatment, efficacy analysis was performed on patients with a definitive virological outcome of SVR (negative qualitative HCV PCR test 24 weeks after cessation of the treatment), relapse (positive qualitative HCV PCR test during the first 24 weeks after the cessation of a successful therapy) and therapeutic failure (positive qualitative HCV PCR test at 24 weeks of therapy, or <2 log decrease in HCV-RNA from baseline to week 12).

Statistical Analysis

For descriptive statistics, continuous variables were summarized as median (25th to 75th percentiles). Categorical variables were described as frequency and percentage. All data were assessed for normality using a Shapiro-Wilk test and categorical data were compared using a Mann Whitney or Kruskal-Wallis statistical test. To investigate continuous data, a Spearman Rank correlation was utilized.

Mean ribavirin Ctrough was calculated as the mean of all available Ctrough determinations for each subject between the first and the third month of therapy. The association between individual SNP and plasmatic concentrations was tested by a basic allelic test and calculated using the χ^2-test.

Receiver operating characteristic (ROC) curve was used to calculate cut-off values. Multivariate logistic regression analysis with stepwise forward selection was performed with *P*-values of less than 0.05 as the criteria for model inclusion. Furthermore, for the univariate analysis, it was also evaluated the correction of the p-value according to Benjamini-Hochberg rules [50, 51]. Statistical analyses were conducted by using SPSS software package ver. 18.0 (Chicago, IL, USA).

Results

The main characteristics of the patients were summarized in Table 1. Specifically, of the 174 patients included in the analysis, 77 had HCV genotype 2-3 and 97 had HCV genotype 1-4, 95 (55%) were male, median age was 48 (IQR, 41-61) and median body weight 69 (IQR, 62-75). Patients were almost totally (n=168; 96.6%) of Caucasian ethnicity, and only 6 (3.4%) were African. Most of the patients received 800 mg (n=52) or 1000 mg (n=107) of ribavirin, one patient was treated with 400 mg, one with 1400 mg, two patients were treated with 600 mg, while 11 patients received 1200 mg. Median dose of RBV was 13.88 mg/kg (IQR 12.7–15.38). 90 patients were treated with PEG IFN-a-2b (1.5 mg/kg s.c. once a week) and 84 patients were treated with PEG IFN-a-2a (180 mg/kg once a week). Sustained virological response was achieved in 38 patients (39%) with HCV-1/4 and 58 patients (75.3%) with HCV-2/3. 13 HCV-1/4 patients (13.5%) and 8 patients (10.4%) HCV-2/3 did not complete the therapy for side effect and were classified as non responder.

The allele frequencies for all polymorphisms observed in our population were listed in Table 2.

All SNPs were in Hardy-Weinburg equilibrium. Since the SNPs *rs12979860 and rs12980275* (R^2=0.90, as compared to R^2=0.54 for *rs12979860* and *rs8099917* as well as R^2=0.60 for *rs12980275* and *rs8099917*) for *IL28B,* and *rs4646536* and *rs10877012* (R^2=0.91) for *CYP27B1*, were respectively in strong *linkage disequilibrium* only SNP variants for *rs12979860, rs8099917 and rs4646536* were included in the following analyses.

No differences between demographic, physical characteristics and biochemical parameters, (Hb, platelet count, alanine aminotransferase (ALT) catalytic activity, serum HCV RNA level) was observed among genetic groups.

Considering patients infected by HCV-1/4, *rs8099917* correlated with SVR, as 49.1% patients with *rs8099917TT* (n=55) had SVR compared to 26.2% of patients with *rs8099917TG/GG* (n=42), p=0.035. Similarly, *rs4646536TT* (n=62) was associated with higher frequency of SVR (50%)

compared *to rs4646536CC/CT* (20%), p=0.004. Moreover *rs11854484* correlated with SVR; 56.3% patients with *rs11854484TT* (n=32) had SVR compared to 30.8% of patients with *rs11854484CC/CT* (n=65), p=0.016. Considering patients infected by HCV 2-3, *rs11854484* correlated with SVR; 89.3% patients with *rs11854484TT* (n=28) had SVR compared to 67.3% of patients with *rs11854484CC/CT* (n=65), p=0.032. The other SNPs investigated showed no influence on outcome.

Median ribavirin plasma concentrations in the first three months of treatments were 2.27 μg/mL (1.89 to 2.95) for patients with HCV-2/3 and 2.02 μg/mL (1.56 to 2.46) for patients with HCV-1/4. No correlation between weight at baseline, dose/kg of ribavirin, age and mean RBV concentration in the first three months of treatment could be identified. Similarly, RBV concentrations were not influenced by the three SNPs, except for *rs11854484* only in patients infected by HCV-1/4. In this group, patients with *rs11854484CC/CT* genotype had a median ribavirin plasma concentrations in the first three months of treatments of 2.32 μg/mL (1.98 to 3.1) compared to patients with *rs11854484TT*, 2.07 μg/mL (1.6 to 2.56), p=0.034. But considering all HCV genotype RBV concentrations were not influenced by SNP *rs11854484.*

As shown in Figure 1, RBV concentrations were higher in patients infected by HCV-1/4 who achieved SVR (n=38): 2.56 μg/mL (2.10 to 3.12) vs 2.1 μg/mL (1.68 to 2.57), p=0.02. This difference of concentration was not observed (p=0.30) for HCV-2/3 patients. In HCV-1/4 patients, by using ROC curve analysis, a cut-off of 2.50 μg/mL of RBV was found to be associated with SVR (AUROC=0.64, sensitivity=55.0% and specificity=71.2%, p=0.020). Using this cut-off, 21 out of 38 (55%) patients with a RBV concentration above 2.50 μg/mL achieved SVR, while this occurred in only 17/59 (28.8%) patients with RBV concentrations below 2.50 μg/mL ($\chi 2$=6.7, p=0.009).

To further confirm the effect of pharmacokinetic, genetic and demographic factors on SVR a multivariate logistic regression analysis was conducted.

As shown in Table 3, for patients infected with HCV-1/4, the results of multivariate logistic regression analysis were: *IL28B rs8099917TT* genotype (OR = 3.05, p=0.018), RBV concentrations above 2.50 μg/mL (OR=4.03, p=0.004), *rs4646536TT* (OR=4.5, p=0.006) and *rs11854484TT* (OR=3.7, p=0.014). By means of this logistic regression analysis sensitivity was 50% (19 out of 38) and specificity 91.5% (54 out of 59). *rs4646536TT*, *rs11854484TT*, IL28 *rs8099917TT* and RBV concentrations above 2.5 μg/mL were classified as favourable factors and could explain 11.9%, 8.2%, 6.6% and 6.9% of the total interpatient variability, respectively.

As summarised in Figure 2, with a different perspective, patients with increasing number of unfavourable factors had lower chances of achieving SVR. All five patients with four unfavourable factors (100%), 24 out 29 (83%) patients carrying three factors, 25 out of 39 (64%) patients carrying two factors, 4 out of 16 (25%) patients carrying one factors and 1 out of 8 (12%) patients without unfavourable factors have not achieved SVR, respectively.

Discussion

In the last two years, several relevant advances have been made in the direction of a more favourable outcome of anti-HCV therapy, particularly for HCV-genotype 1. The identification of genetic polymorphisms around the genes coding for *IL28B* makes it today possible to distinguish those patients who are more likely to achieve a SVR, possibly allowing a shorter treatment duration in a proportion of patients around 50%. At the same time, the development of drugs acting on HCV replication has led to increases of the SVR rate to values approaching 80%, as recently seen with the two HCV protease inhibitors telaprevir and boceprevir recently introduced in the clinical practise [52].

The recently acquired *IL28B*-driven knowledge that a sizeable proportion of patients are intrinsically more responsive to standard PEG/IFN therapy leads to the question whether and how other factors may drive to a best response and/or identify the patients as not responder.

In this study we observe that, within *IL28B* SNPs, the *rs8099917* polymorphism has a greater capacity to predict the SVR than *rs12979860*. Moreover, we found that additional polymorphisms as well as RBV plasma exposure are also able to influence the achievement of SVR (Table 3).

Following the discovery of the role of *IL28B*-related genetic polymorphisms and also considering the established role of RBV pharmacokinetic exposure in driving the patients' response to pegIFN-alfa/RBV therapy, it is becoming clear that further identifiable human genetic variations may also contribute to refine the individual chance to have a favorable response to anti-HCV treatment. The analysis carried out in this patients' series shows however that the overall predictive power of the variables here considered works better on the negative side (Table 4 and Figure 2 and Figure 3). It is noteworthy that, regardless the magnitude of RBV pharmacokinetic exposure, the negative predictive value of the polymorphisms here investigated is much stronger than the positive one. Considering the genetic factors and exposure RBV within the first three months of therapy

separately (Figure 3), it is also worth noting how RBV pharmacokinetics still retain its independency in predicting the therapeutic outcome.

Moreover, the difference between patients with favourable and unfavourable genotypes lies in the concentration intervals within which comparable response rates are recorded. That is to say that patients with favourable SNPs may respond with significantly lower concentrations as compared to those with unfavourable genotypes.

It is a point of great interest the possibility of avoiding the use of RBV with the new therapies. Looking at the available evidence, however, it seems that the additional therapeutic action provided by directly acting antiviral agents (DAAs) does not replace completely the role of RBV. Thus, as far as the novel HCV protease inhibitors are concerned, RBV is likely to remain a component of anti-HCV therapy. In this setting a further point to be investigated is whether with the new DAAs the dose of RBV can be safely reduced without the risk of a lower rate of SVR or the recently achieved tri-therapeutic performances can only be maintained by keeping RBV pharmacokinetic exposure at the same level so far required to ensure the best outcome achievable by PEG/IFN-RBV.

The results of our study address the reasonable hope that, at least in patients carrying the favourable genotypes, once a DAA is also added to the regimen, the pharmacokinetic exposure of RBV will become of lesser importance. Should this be the case, we can also envisage how the risk of RBV-associated anaemia, which is also associated to RBV pharmacokinetics [24, 36, 42, 53], will be greatly reduced. Since the latter is even more frequent when RBV is co-administered with the new HCV protease inhibitors, such an issue appears to be of critical importance for the new tri-therapeutic regimens. This working hypothesis deserves to be investigated in order to possibly get eventually rid of the still considerable issue of individual RBV concentration.

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Figure 1. Mean 3 months of RBV plasma concentrations with SVR and No SVR in HCV-1/4 infected patients.

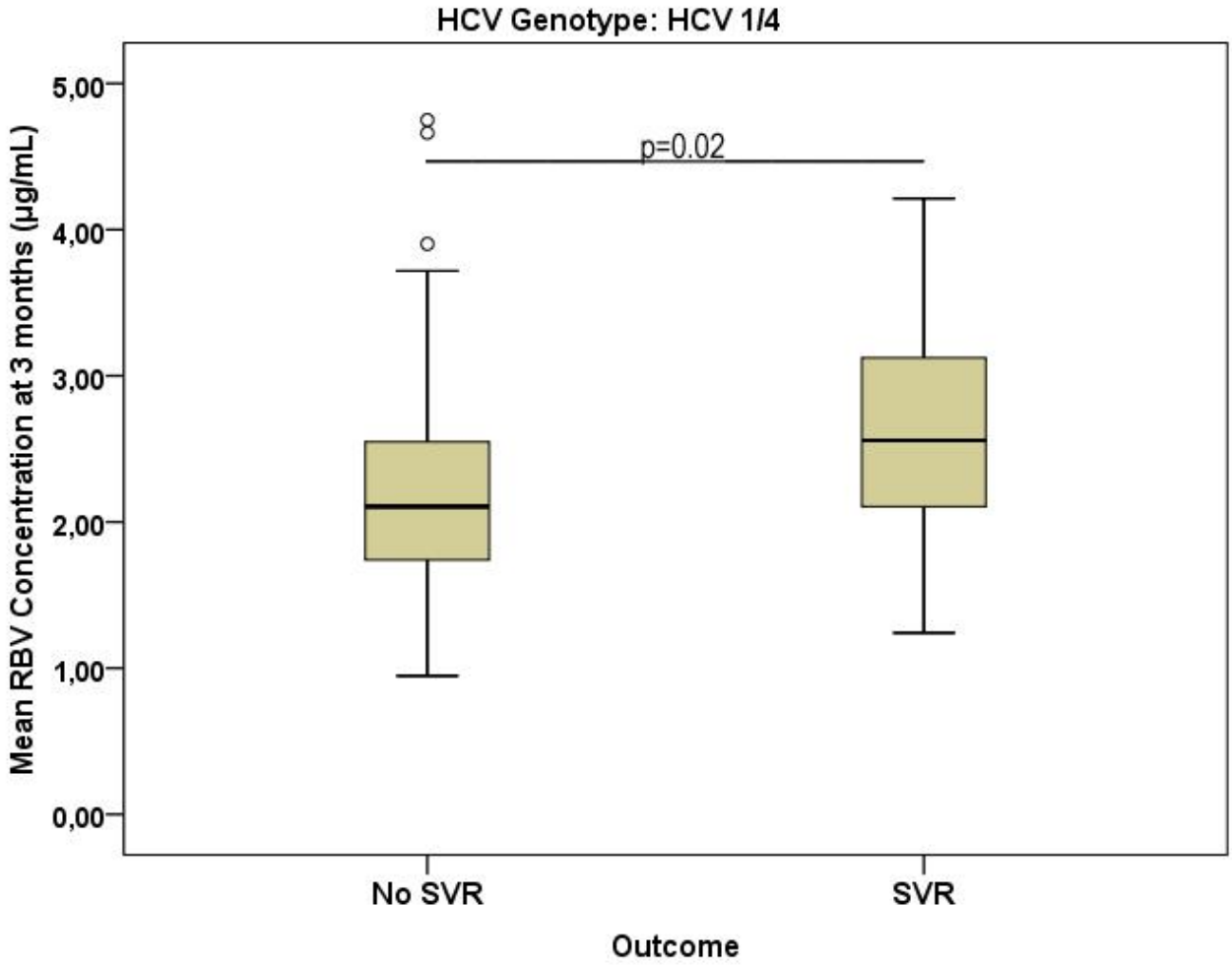

Figure 2. Percentage of SVR and No-SVR HCV-1/4 infected patients with increasing number of unfavourable factors (p=0.0001).

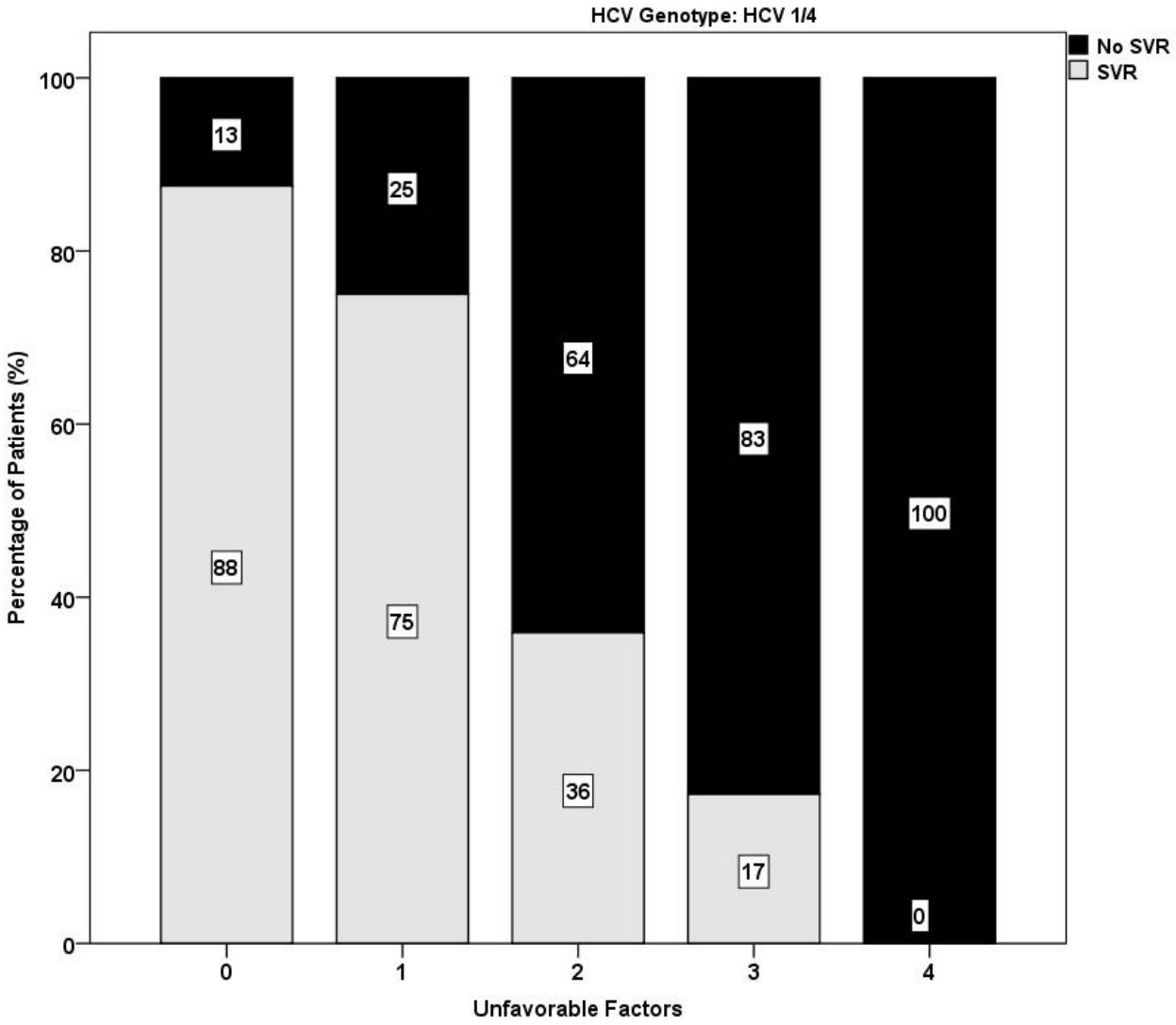
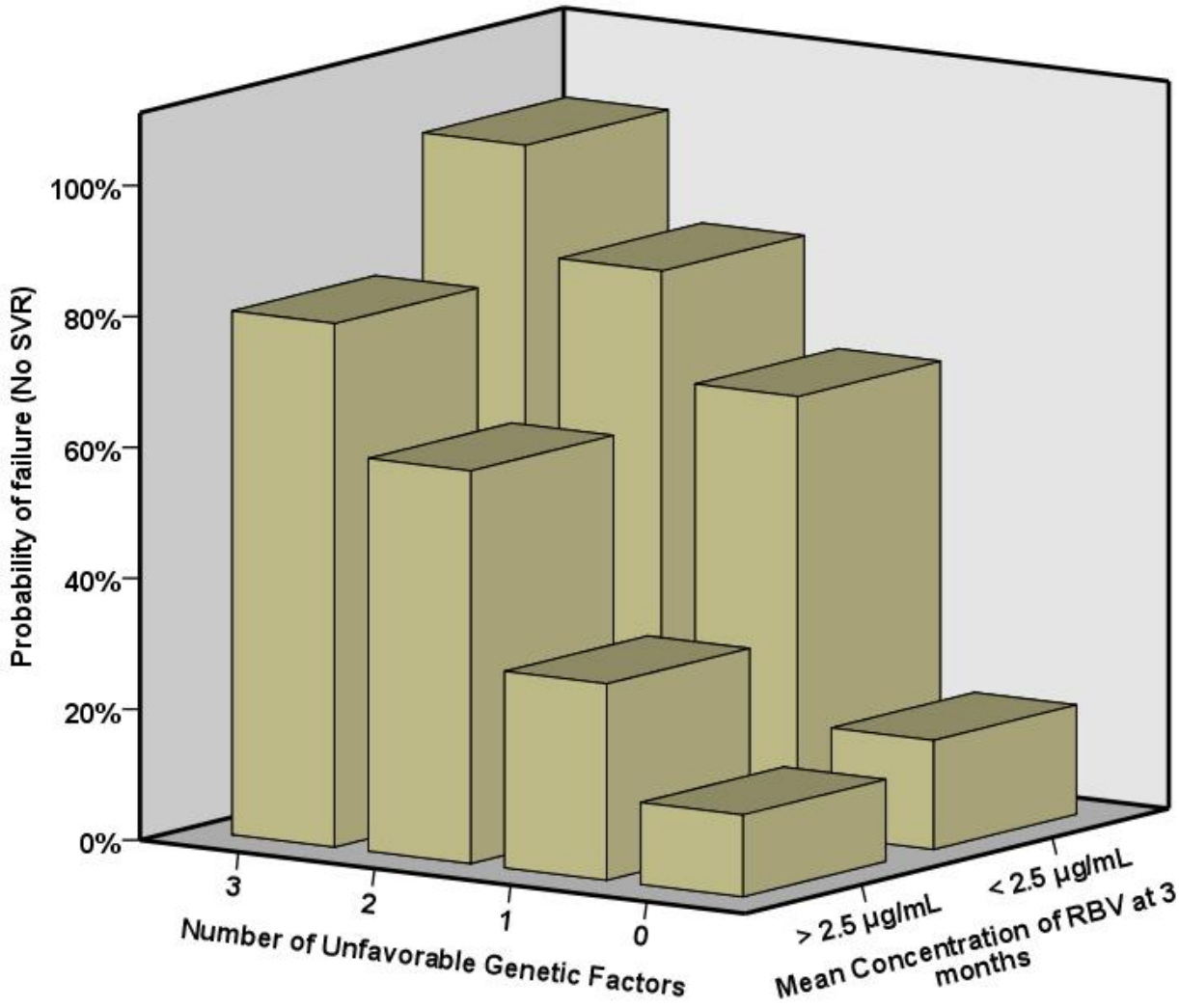
Figure 3. The failure rate percentage in HCV-1/4 patients considering genetic factors and 2.5 µg/mL cut-off, as mean 3 month RBV exposure.