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SERUM ANTI-INTERFERON ALPHA ANTIBODIES IN CHRONIC HEPATITIS C PATIENTS TREATED WITH PEGYLATED INTERFERON ALPHA CONTAINING THERAPY

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1. INTRODUCTION

The primary goal of hepatitis C virus (HCV) treatment is to achieve a sustained virological response, defined as persisting undetectable HCV-RNA after treatment withdrawal likely leading to the resolution of liver disease, at least in patients without cirrhosis (1). The treatment of hepatitis C has dramatically improved over the past decade, so much that a significant proportion of chronic patients can now be cured.

To date, the standard of care for chronic HCV infection (CHC) has been based on the antiviral and immunomodulatory effect of pegylated interferon alpha (PEG-IFNα) and ribavirin (RBV) (2). Current schedules take advantage of direct acting antivirals (DAAs), agents able to interfere with HCV enzymes essential for viral replication. The “new era” of HCV treatment has started with the approval of two drugs against the NS3/4A serine protease (PI) for genotype 1 infections (3-6), and since then, several DAAs with other viral targets have been approved or are in the pipeline (7). These drugs promise, in the future, to lead to viral eradication with simplified regimens, very low toxicity, and without the use of interferon. However, the “first generation” (telaprevir and boceprevir) and the most recent second-wave DAAs (simeprevir, sofosbuvir and daclatasvir) will continue to include PEG-IFNα plus RBV, even if with shorter schedules (1). These therapies have extremely high costs and still require a careful monitoring for side effects despite a great improvement of the overall safety profile. Thus, their final efficacy also depends on an appropriate selection of patients which should include a careful evaluation of a well-described host and of viral factors associated with therapeutic failure (8).

Among these factors, the development of serum anti-IFN alpha antibodies (anti-IFNα-Ab), able to both bind and neutralize the biologic activity of IFNα, has been proposed as a mechanism against non-response in patients treated with recombinant IFNα (9-11), while others did not arrive at the same conclusions (12,13). More recently, the role of anti-IFNα-Ab in combination with the pegylated form of IFN has been evaluated, providing controversial results (14-17).

In addition, the “natural” production of anti-IFNα-Ab has been reported in patients with various autoimmune disorders (18-20), and anti-cytokines autoantibodies have also been detected in healthy donors, with unknown significance (21).

Thus, the phenomenon of production of anti-IFNα-Ab could be more complex than it appears and its clinical impact remains elusive. In particular, its role should be addressed in difficult-to-treat patients, who remain the clinical category less prone to benefit from advancements in HCV therapy, and for whom there is the urgent need of optimizing the cost-effectiveness of new treatment schedules containing both interferon and direct antivirals.

On the basis of these remarks, a retrospective study on stored serum samples from CHC patients was performed with the following purposes:

1. To assess the presence of anti-IFNα-Ab during PEG-IFNα plus RBV treatment in CHC, both treatment-failure and naive patients, and in a group of healthy blood donors as control;

2. To assess the impact of anti-IFNα-Ab on serum levels of IFNα and the virological response to treatment.

2. PATIENTS AND METHODS

2.1 Patients

The serum levels of anti-IFNα-Ab and IFNα were retrospectively measured on stored serum samples collected from 90 consecutive CHC patients who had received antiviral treatment with PEG-IFNα2a (180 μg/weekly subcutaneously) and RBV (1000-1200 mg/daily, according to body weight, <75 or ≥75 kg, respectively) in the period from 2008 to 2010 at the Outpatient Clinic for Liver Disease, Azienda Ospedaliero-Universitaria di Bologna, Policlinico Sant'Orsola-Malpighi, Bologna, Italy. In particular:

- 76 patients were previously non-responders to one or more course of IFNα/PEG-IFNα plus RBV.
- 14 patients were treatment-naive.

For the control group, serum samples from 57 healthy donors, recruited at the Hospital Blood Transfusion Service, were also frozen and stored until analysis. The inclusion and exclusion criteria were those applied for the dual antiviral treatment eligibility (22). More specifically, the major inclusion criteria were the following: adult age (>18), CHC confirmed by detectable serum HCV-RNA levels and histologically documented. The major exclusion criteria were the following: decompensated cirrhosis, evidence of hepatocellular carcinoma, other concomitant causes of hepatic disease, pregnancy or breastfeeding, co-infection with HIV or HBV, severe concomitant diseases and haematological values not compatible with treatment.

Virological definitions used in the text and graphics are the following:

- Early Virological Response (EVR): HCV-RNA decrease ≥2 log10 IU mL−1 at week 12 of treatment;
- End of treatment response: HCV-RNA undetectable at the end of treatment;
- Sustained Virological Response (SVR): HCV-RNA undetectable at 24 week after the end of therapy;
- Null response: HCV-RNA decrease <2 log10 IU mL−1 at week 12 of treatment;
- Partial response: HCV-RNA decrease ≥2 log10 IU mL−1 at week 12 but still detectable at week 24 of treatment;
- Relapse: HCV-RNA undetectable at the end of treatment, with viral rebound after the end of treatment.

The anti-IFNα-Ab and IFNα concentration measurement was performed at baseline and at week 12 of treatment. The local Ethical Committee approved the study protocol and written informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the "World Medical Association (WMA) Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects" adopted by the 18th WMA General Assembly, Helsinki, Finland, June 1964 and amended by the 59th WMA General Assembly, Seoul, South Korea, October 2008.

2.2 Detection of anti-IFNα-Ab and IFNα

Serum concentrations of anti-IFNα-Ab and IFNα were measured by commercially available enzyme-linked immunosorbent assays (Bender Medsystems, eBiosciences, Vienna, Austria), according to manufacturer's instruction. The level of sensitivity for the anti-IFNα-Ab test was 1.4 ng mL−1. The level of sensitivity for the IFNα test was 3.2 pg mL−1.

2.3 Virological assays

HCV–RNA was measured by PCR ([Cobas–Roche, Hoffmann-La Roche Ltd, Basel, Switzerland]; Amplicor HCV qualitative, version 2.0, LOD 50 IU mL−1; Amplicor HCV quantitative, HCV RNA Monitor, version 2.0, LOD 500–600 IU mL−1). HCV genotype was determined using a commercially available line-probe assay (INNOLiPA ®, Innogenetics, Antwerp, Belgium). HCV-RNA quantification was performed at baseline, at weeks 12, 24, and 48 during treatment, and at 24 weeks after the end of treatment.

2.4 IL28B genotyping

IL28B genotyping was performed in patients who consented to the analysis and for whom mononuclear cell samples were available.

Genomic DNA was extracted from whole blood samples collected in EDTA-tubes or total PBMCs by paramagnetic particles using an automated platform (Maxwell 16, Promega, Milan, Italy), according to the manufacturer's protocol.

The single nucleotide polymorphisms (SNPs) rs 12979860 in the gene for IL28B were determined by real time PCR using a commercially available kit (Experteam, Venice, Italy).

2.5 Statistical analysis

Parametric and non-parametric tests were used, as appropriate. In particular, quantitative variables were expressed as a mean ± standard deviation (SD), and categorical variables as absolute and relative frequencies. Groups of quantitative and qualitative variables were compared using the Mann-Whitney and the Fisher-exact tests, respectively. Correlations were performed using Spearman rank correlation. A p-value <0.05 was considered to be statistically significant. Data handling and analysis were performed with GraphPad Prism software, version 5.

3. RESULTS

3.1. Anti-IFNα-Ab prevalence

The levels of serum anti-IFNα-Ab were assessed in 90 consecutive CHC patients at baseline of treatment with PEG-IFNα plus RBV, and in a group of 57 healthy donors. The baseline characteristics of CHC patients are listed in Table 1.

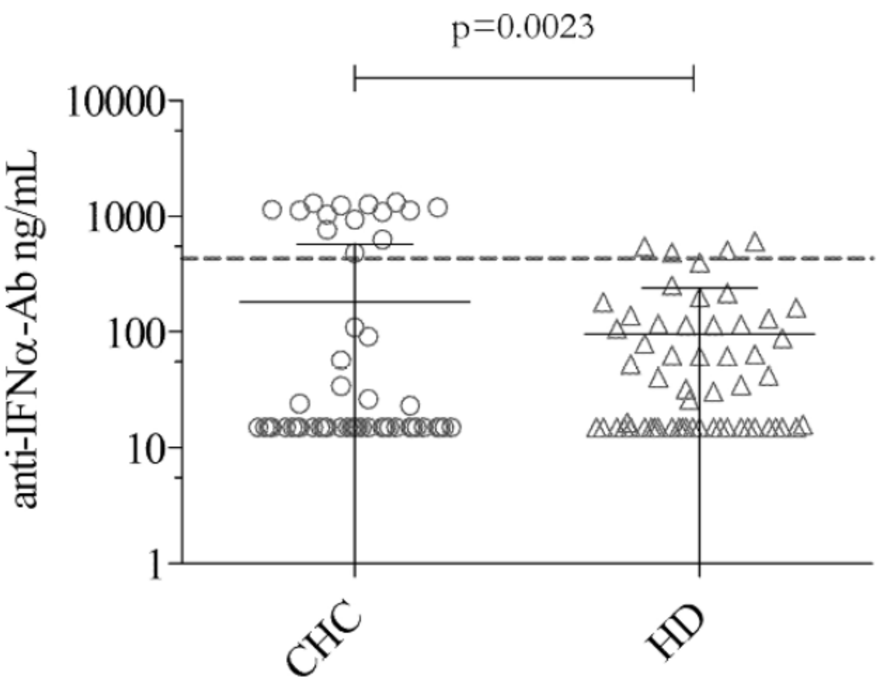
Table 1. Baseline Characteristics of CHC Patients

Age, median [range]	55 [28-71]
Sex, male [%]	51 [56.6]
HCV-RNA IU/mL, median [range]	1,2x10^6 [4.4x10^4-2.7x10^6]
High viral load (≥8x10^5 IU/mL), no. [%]	56 [62]
HCV genotype 1, no. [%]	87 [96.6]
IL28B (rs12979860) genotype*, no. [%]	
CC	7 [10.8]
CT	41 [63]
TT	17 [26.2]
Metavir Score, no. [%]	
F0-F1	36 [40]
F2,	14 [15.4]
F3	25 [28]
F4	15 [16.6]
Body Mass Index, median [range]	26 [20-41]
Number of previous treatment in experienced patients (N=76) no. [%]	
1	40 [52]
2	18 [23]
3	6 [8]
4	6 [8]
5	5 [6]
6	1 [3]

** Data available on 65/90 patients; No: absolute number*

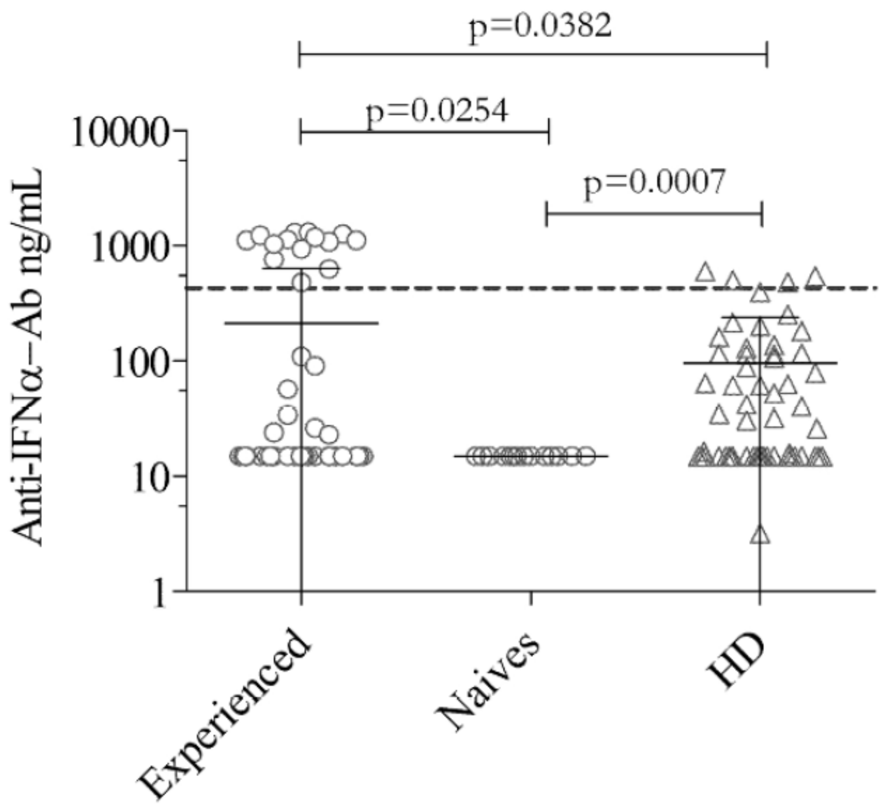
Among the 90 treated patients, 76 were treatment-failure and 14 were treatment-naive. The baseline serum levels of anti-IFNα-Ab were significantly higher in CHC patients than in healthy donors (mean±SD: 181.5±389.9 *vs* 95.9±143.0 ng mL−1, respectively, p=0.0023, Figure 1A).

Figure 1A: Baseline anti-IFNα-Ab (ng/mL) in chronic HCV patients (CHC) and healthy donors (HD)



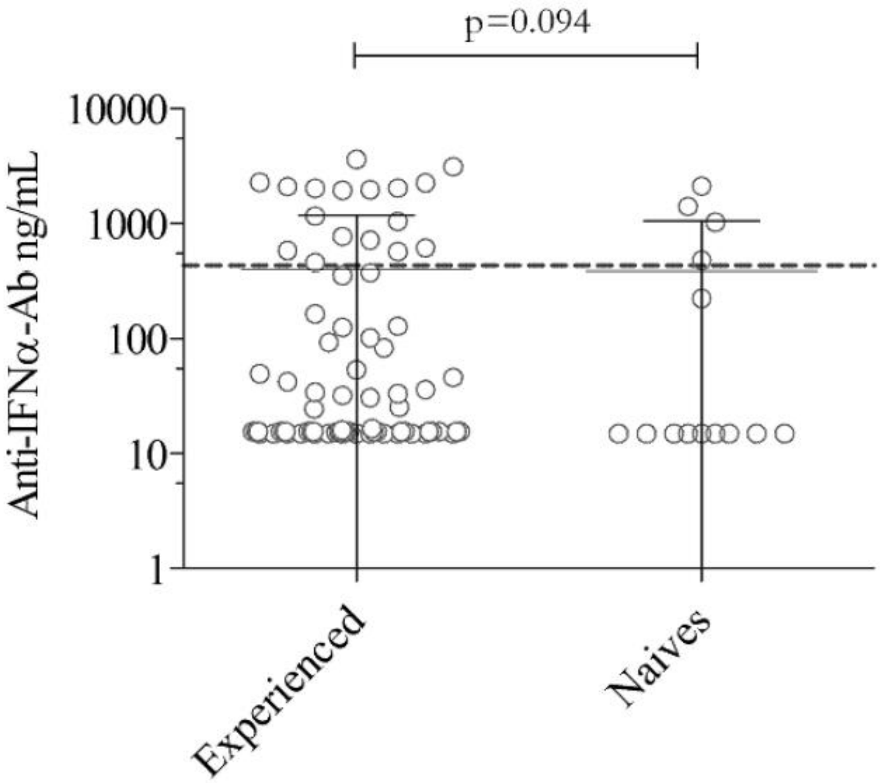
On the basis of values obtained in the control group, a conservative value of 430 ng mL−1, assessed as 3 times the mean plus SD (23), was assumed as the threshold for positivity, so that CHC patients and healthy donors (HD) were categorized into anti-IFNα-Ab positive or anti- IFNα-Ab negative groups. Using this threshold, 14/90 (15.5%) in the entire CHC group were anti-IFNα-Ab positive, compared to 4/57 (7.0%) in the HD group (p=0.196). When the CHC patients were stratified into experienced and naive, the highest levels of anti-IFNα-Ab were measured in experienced patients, while the levels of anti-IFNα-Ab were undetectable in naive patients. Thus, anti-IFNα-Ab levels of treatment-naive patients were significantly lower when compared to those of the other two groups (Figure 1B).

Figure 1B: Baseline anti-IFN α -Ab in CHC subdivided into experienced, naïve and HD



When the CHC patients were evaluated at week 12 of treatment, the prevalence rates of anti-IFNα-Ab positivity increased in both experienced and naive patients (17/76, 22.3% *vs* 4/14, 28.5%, respectively), without significant differences between the two groups (p=0.732). Similarly, the anti-IFNα-Ab concentrations were not different between experienced and naïve patients (391±792.3 *vs* 384.7±662.6 ng mL−1, respectively, p=0.094, Figure 1C).

Figure 1C: anti-IFN α -Ab in experienced and naives at week 12 of treatment. The Y-axis is a logarithmic scale. The grey dashed line represents the threshold of positivity (430 ng/mL). Line and bars: Mean $\pm$ SD.

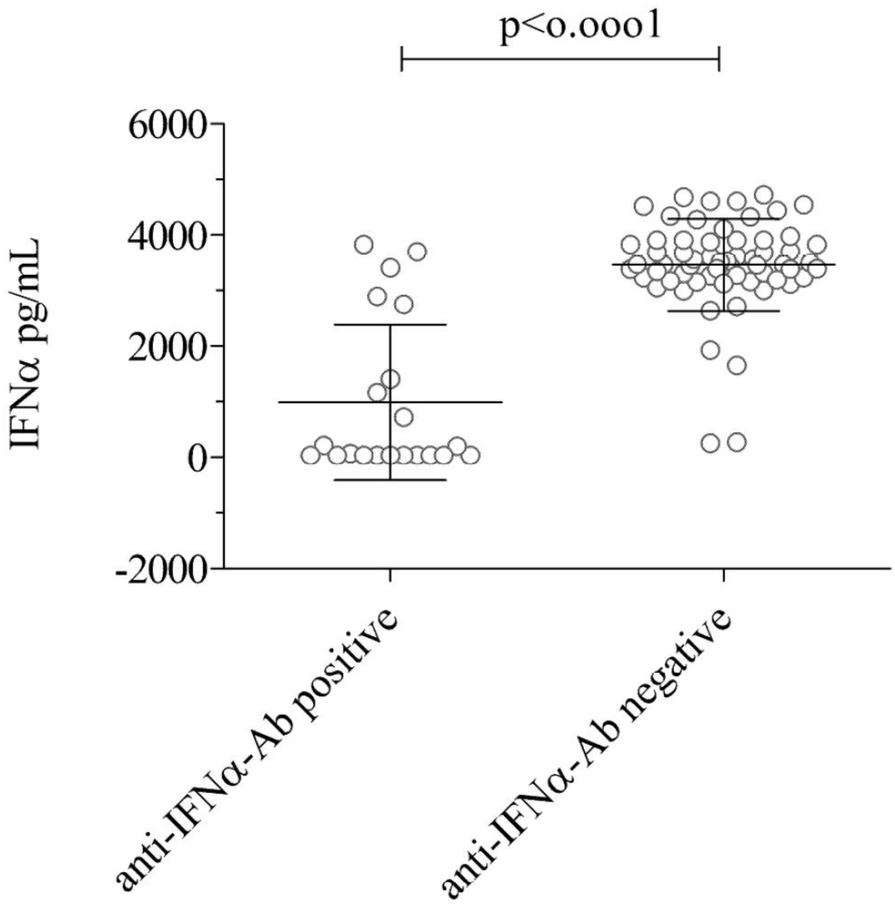


Overall, 21 patients out of 90 CHC patients (23.3%) tested anti-IFNα Ab positive at week 12. Among the 14 patients positive at baseline, two tested negative at week 12, while the remaining maintained the positive status. Nine patients developed antibodies positivity during treatment. In general, there was a trend of anti-IFNα-Ab increase from baseline to week 12, while a decrease was observed in only two patients. The increase was highly variable, ranging from 1.5 to 140-fold the basal value. Interestingly, the positivity of anti-IFNα-Ab at baseline was independent from the wash-out time from the previous treatment course, which ranged from 6 to 106 months.

3.2 Anti-IFNα-Ab and IFNα concentrations

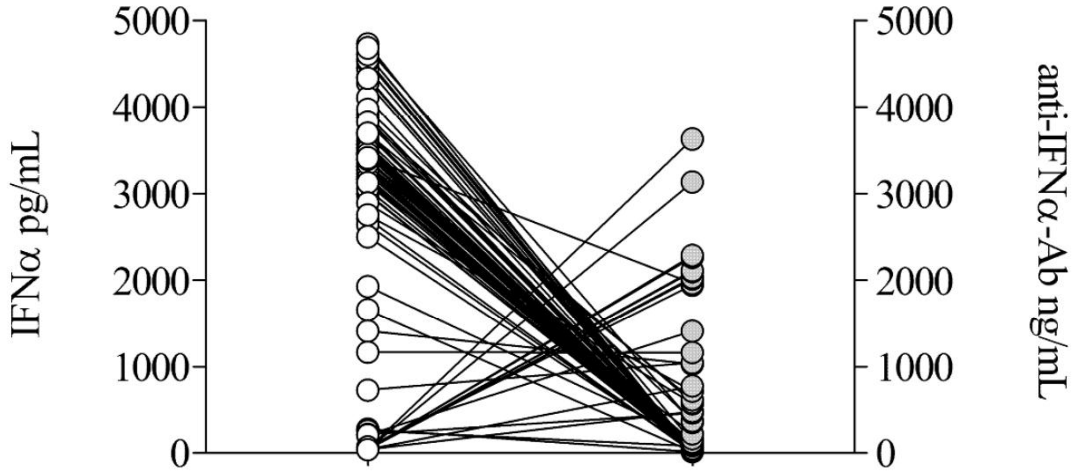
To address the therapeutic impact of the anti-IFNα-Ab, we measured the serum levels of IFNα at week 12, that resulted significantly lower in the anti-IFNα-Ab positive group compared to the negative group (mean±SD: 988.2±1402 *vs* 3462±830.8 pg mL^{-1}, respectively, $p<0.0001$, Figure 2A). More specifically, in the anti-IFNα-Ab negative group, just two out of 69 patients showed remarkably lower serum concentrations of IFNα (Figure 2A).

Figure 2A: IFNα levels (pg/mL) in anti-IFNα-Ab positive and anti-IFNα-Ab negative patients at week 12 of treatment. Line and bars: Mean ± SD.



The reason for this inconsistency is difficult to ascertain; however, an incomplete adherence to treatment or a defect in drug absorption cannot be excluded. Instead, in the anti-IFNα-Ab positive population, two different patterns can be observed. In the first, despite the presence of antibodies, the concentration of IFNα was comparable to that of patients without antibodies. In the second, the presence of anti-IFNα-Ab abrogates, to a different extent, the concentration of IFNα, resulting in extremely low or even under the test detection limits in twelve patients. Despite these two different conditions, the concentrations of anti-IFNα-Ab and IFNα were inversely correlated, considering both the anti-IFNα-Ab positive patients (n=21; r= -0.6233, p= 0.0025) and the entire group (n=90; r= -0.405, p=0.0001, Figure 2B).

Figure 2B: IFNα levels (left Y-axis and white dot plots) and anti-IFNα-Ab levels (right Y-axis and grey dot plots) in CHC patients (n=90) at week 12



In particular, the patients with the highest concentrations of anti-IFNα-Ab (>2000 ng mL−1, n=8) displayed IFNα levels under the test detection limit.

3.3 Anti-IFNα-Ab and response to treatment

19/21 (90.5%) patients anti-IFNα-Ab positive at week 12 did not achieve SVR at the present treatment course. Overall, the SVR was achieved only in 13/90 (14.4%) patients as it should be considered that this study was performed in a prevalent population of “very” difficult-to-treat patients. Although the SVR rates were not significantly different between anti-IFNα-Ab positive and negative patients, nevertheless these were slightly lower in anti-IFNα-Ab positive than in anti-IFNα-Ab negative patients (9 *vs* 16%, respectively, p=0.19). The presence of anti-IFNα-Ab at week 12 was associated with the failure to achieve EVR, as 14/21 (66.7%) positive patients did not experience a ≥2log decrease in HCV-RNA from baseline, compared to 23/69 negative patients (33.3%, p=0.0105). Moreover, considering the whole study population, the levels of anti-IFNα-Ab were higher in the group of null responders compared to the other groups (Figure 3A).

Figure 3A: Serum levels of anti-IFN α antibodies (ng/mL) according to virological responses. Line and bars: Mean ± SD

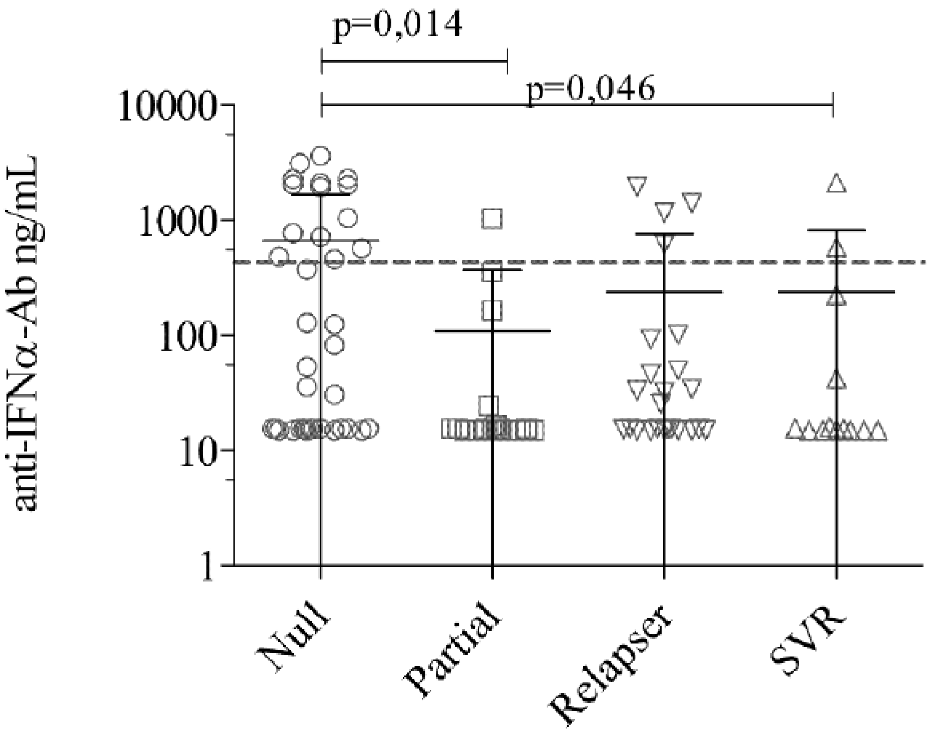
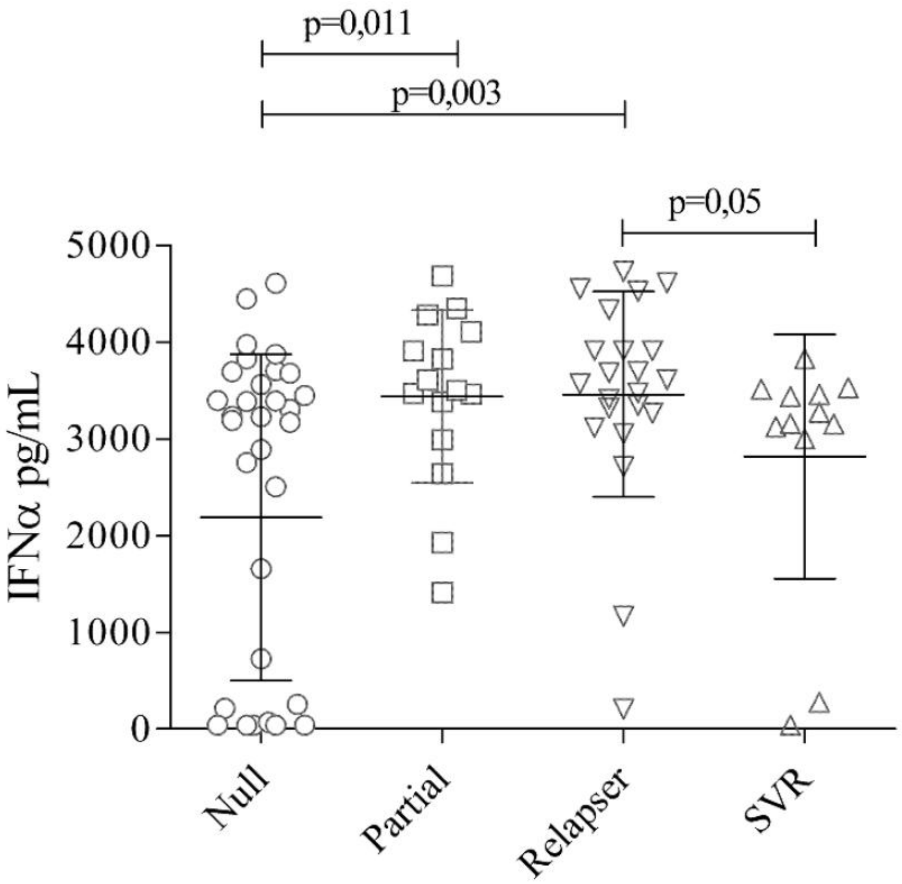


Figure 3B: Serum levels of IFN α (pg/mL) according to virological responses. Line and bars: Mean ± SD

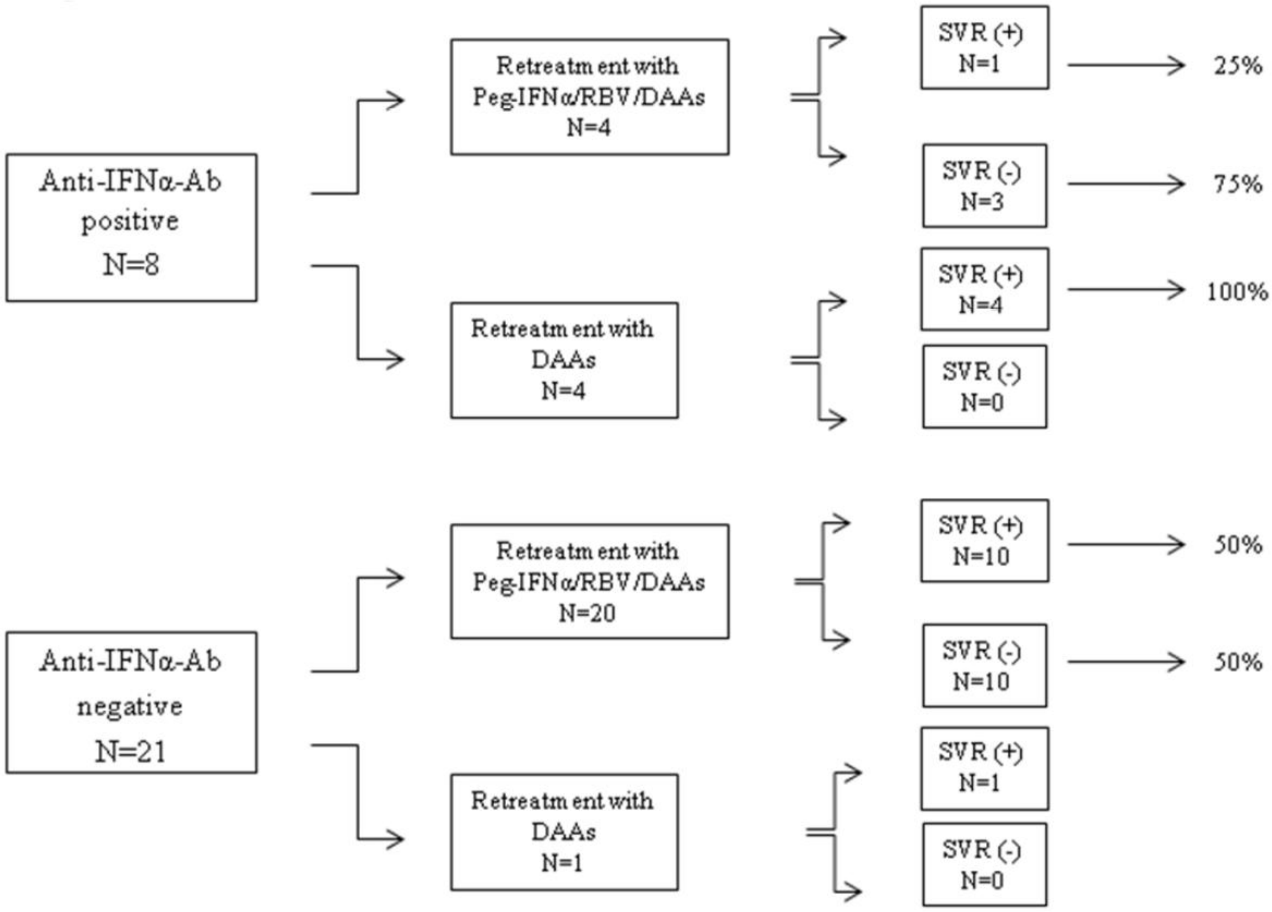


Conversely, the levels of IFNα were significantly lower in null responders (Figure 3B). In particular, all 8 patients, in whom high concentrations of anti-IFNα-Ab were associated with undetectable levels of IFNα, clustered in null responders.

3.4 Additional clinical features of antibodies-positive patients

Some of clinical and genetic parameters characterizing the anti-IFNα-Ab positive patients were explored. As for genetic factors, the IL28B genotype polymorphism was evaluated in 65/90 patients. All variants (CC, CT and TT) were homogenously present in the naive population while among experienced patients only three carried CC allele. Thus, the majority of patients carried the non-favorable allele (CT or TT), so that an association between the presence of antibodies and the IL28B genotype cannot be addressed. Moreover, when the anti-IFNα-Ab positive and negative patients were subdivided based on fibrosis stage, the percentage of patients with F0-F2 or F3-F4 METAVIR score in antibody-positive and in antibody-negative groups (F0-F2: 42.8 *vs* 59.4; F3-F4: 57.2 *vs* 40.6, respectively) indicated a slightly higher prevalence of anti-IFNα-Ab positive patients with more advanced liver disease, but the difference was not statistically significant (p=0.215). Finally, the majority of patients enrolled in this study continued to be followed at our centre, and 29 of them were retreated afterwards with a regimen containing DAAs. Among the anti-IFNα-Ab positive patients, 8 underwent a subsequent course of antiviral treatment with IFN-based (n=4) or IFN-free regimens (n=4). Interestingly, all patients treated with an IFN-free regimen achieved SVR while, among those retreated with a triple regimen containing PEGIFNα, only one reached SVR (Figure 4). Of note, despite the presence of anti-IFNα-Ab, the patient who reached SVR maintained high serum levels of IFNα, while in the remaining three patients the levels were undetectable. Among the anti-IFNα-Ab negative patients, 21 were retreated with schedules containing PEG-IFNα (except for 1 patient) and half of them achieved SVR (Figure 4).

Figure 4. Subsequent antiviral treatments and relative outcomes in anti-IFN α-Ab positive and anti-IFN α-Ab negative patients.



4. DISCUSSION

The results of this study demonstrate that the development of anti-IFNα-Ab in CHC patients undergoing antiviral treatments, including PEG-IFNα, is an occurrence to be considered especially, but not only, in those patients who were previously exposed to IFNα. The study is focused on examining this phenomenon in difficult-to-treat patients since these often represent the most prevalent population in the real clinical practice, are at greater risk of disease progression, are the main candidates to receive new treatment regimens and have the highest probability of treatment failure. Furthermore, although IFN-free regimens are rapidly oncoming, the most recent European recommendations for CHC treatment with DAAs still include treatment options containing PEG-IFNα (24). In addition, while more recent DAAs promise to cure the infection in a large proportion of patients, and to radically change viral epidemiology, the daily clinical practice is already facing the problem of high drug costs, thereby limiting their access not only in developing countries. Thus, treatments including PEG-IFNα will likely continue to represent a consistent part of CHC therapy, at least in the near future (1, 24).

The development of anti-IFNα-Ab is a widespread phenomenon that has been already described in other settings, such as the treatment of some neurological diseases (25). In this setting, the problem has received great attention, so that the European Federation of Neurological Societies Task Force recommended the measurement of anti-IFNβ antibodies during treatment in patients with multiple sclerosis and suggested the modifications of the treatment strategy tailored on the anti-IFNβ antibodies levels (26). The data available in the current literature reported that pegylated preparations have lower immunogenicity compared to standard preparations of IFNα (14, 15). Accordingly with this observation, the prevalence of anti-IFNα-Ab positivity in the present study is almost consistent with that reported in a previous study performed on PEG-IFNα non-responder subjects (16), but is higher on respect to other recent studies (14, 15). The different experimental approaches to detect and to quantify the anti-IFNα-Ab used in these studies may explain the reciprocal discrepancies, as other antiviral neutralization tests, instead of the binding ELISA assay performed in our study, were used. It is known that ELISA allows the detection of different antibodies specificities, some of them with binding but not neutralizing ability. This difference could have a negligible impact on the antiviral mechanism of interferon. However, the biological role of non-neutralizing antibodies is still unclear, but indirect mechanisms of interference with the target could be significant (27, 28). In addition, the ELISA method has the advantage of being very simple, standardized and non-demanding particular equipment, so it is more likely to be widely introduced in a real-life clinical diagnostic setting. On the other hand, testing the IFNα concentrations together with the anti-IFNα-Ab may help in providing an indirect measure of the neutralizing activity of antibodies. By choosing a conservative threshold to discriminate between anti-IFNα-Ab positive and negative patients, in this study it is shown that the immunogenicity of IFNα develops early after exposure, as naive patients resulted positive at the same rate of multiple-experienced ones, and with similar anti-IFNα-Ab levels. In addition, an unexpectedly high prevalence of antibodies also in uninfected healthy donors was found, although at significantly lower levels. Detection of antibodies against several cytokines has been repeatedly reported in healthy donors (21, 29), and a regulatory role in modulating a potential cytokine-derived pathology has been proposed. However, their clinical relevance has not been yet defined and requires additional studies. From this observation, it is more difficult to explain the absence of anti-IFNα-Ab in naive patients at baseline, even if the low patient number in this group likely might have influenced this finding.

In the present study, two different profiles of antibodies positivity have been observed. In some patients, despite high levels of anti-IFNα-Ab, the serum levels of IFNα were not abrogated, while in the others the presence of antibodies is associated with low or absent sera concentrations. In particular, the amount of antibodies appears to be important, as the patients with the highest levels of antibodies have undetectable levels of IFNα. The reasons for these two conditions are difficult to ascertain, but it is possible to speculate that this most likely reflects different capabilities of mounting the antibodies response, in terms of specificities, neutralizing/non neutralizing proportions, affinity and avidity.

Interestingly, the development of antibodies seems to be a particularly stable phenomenon, as the positivity of anti-IFNα-Ab at baseline was independent from the time of wash-out from previous course of therapy. This point should be kept in mind, particularly when patients already exposed to IFNα need to be retreated. As for the treatment response, the overall response rate of this population was particularly low, compared to the standard 40 - 50% of SVR reported for genotype 1 naive patients treated with PEG-IFNα and RBV (2). In addition, it is of note that the majority of anti-IFNα-Ab positive patients clustered in the null responders group. Thus, these data demonstrate that the development of antibodies is most likely responsible for at least part of the non-response rates in a population of difficult-to-treat patients, being a non-necessary but sufficient condition of treatment inefficacy. The development of anti-IFNα-Ab *per se* does not constitute a non-response motif, but it becomes an essential parameter when associated with low or undetectable IFN levels.

Finally, these data suggest that anti-IFNα-Ab positive patients retreated with an adequate IFN-free regimen have the chance to clear the infection, while if they undergo an additional course of therapy containing PEG-IFNα, these chances may be significantly reduced. Although this evaluation was possible in very few patients, this may be a very useful clinical remark, taking into account that the majority of the CHC patients who will need an effective antiviral treatment in the near future will be those who were non-responders to previous treatment courses. In particular, the triple therapy with PEG-IFNα/RBV and sofosbuvir (or simeprevir or daclatasvir) schedule should be prescribed after excluding the presence of anti-IFNα- Ab or determining the IFNα concentrations after a 4-week lead-in phase because the anti-IFNα-Ab positivity presence would hamper the final outcome of treatment remarkably overbalancing the cost-effectiveness of the therapy. In these patients, an alternative IFN-free regimen should be carefully set up, also considering that the data in genotype 1 experienced patients treated with only sofosbuvir/RBV are currently lacking and the available data are not encouraging (30).

In conclusion, the present study indicates that the development of anti-IFNα-Ab is a phenomenon that should be considered when patients undergo treatment with IFN-containing regimens, also if associated with a potent DAA. To measure anti-IFNα-Ab and IFNα levels after a lead-in phase with PEG-IFNα/RBV is a very fast and simple test that might turn out in a valid tool for tailoring and optimizing the treatment for CHC.

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