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Strategies for serum chemokine/cytokine assessment as biomarkers of therapeutic response in HCV patients as a prototype to monitor immunotherapy of infectious diseases

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ABSTRACT

In this study, strategies for serum biomarker assessment were developed for therapeutic monitoring of HCV patients. For this purpose, serum chemokine/cytokine levels were measured by cytometric-bead-array in HCV patients, categorized according to immunotherapy response as: non-responder/NR, relapser/REL and sustained-virologic-responder/SVR. The results demonstrated an overall increase of serum chemokine/cytokine levels in HCV patients. In general, therapeutic failure was associated with presence of a predominant baseline proinflammatory pattern with enhanced CCL5/RANTES, IFN-α, IFN-γ along with decreased IL-10 levels in NR and increased IL-6 and TNF in REL. SVR displayed lower baseline proinflammatory status with decreased CXCL8/IL-8, IL-12 and IL-17 levels. The inability to uphold IFN-α levels during immunotherapy was characteristic of NR. Serum chemokine/cytokine signatures further support the deleterious effect of proinflammatory baseline status and the critical role of increased/persistent IFN-α levels to guarantee the sustained virologic response. The prominent baseline proinflammatory milieu observed in NR and REL yielded a restricted biomarker network with small number of neighborhood connections, whereas SVR displayed a network with integrated cytokine connectivity. Noteworthy was that SVR presented a shift towards a proinflammatory pattern upon immunotherapy, assuming a pattern similar to that observed in NR and REL at baseline. Moreover, the immunotherapy guided REL towards a profile similar to SVR at baseline. Analysis of baseline-fold changes during treatment pointed out IFN-α and TNF as high-performance biomarkers to monitor immunotherapy outcome. This knowledge may contribute for novel insights into the treatment and control of the continuous public health threat posed by HCV infection worldwide.

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1. Introduction

Immunotherapy has been proposed as an alternative approach to etiological treatment of parasitic and infectious diseases aiming to assist the natural immune system in achieving control over infection (Vanham and Van Gulck, 2012). Several immunotherapy formats have been evaluated over the years, including non-antigen

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specific strategies, the use of immunomodulators, antibodies that block negative regulatory pathways, as well as cytokines that activate the immune response to control pathogenic agents (D'Elia et al., 2013; Guo et al., 2014; Kong et al., 2010; Qian et al., 2016; Xiang et al., 2015).

Hepatitis C virus (HCV) infection affects approximately 170 million people worldwide (Alavian and Haghbin, 2016; Chassagne et al., 2016; Han and Liu, 2016; King et al., 2016; Muñoz-Gámez et al., 2016; Sharma et al., 2016; Tang et al., 2016; Wirth and Manns, 2016). Efficient therapeutic interventions have been developed in the last two decades to control/minimize the impact of this worldwide health problem. Firstly, type-1 interferon alpha (IFN-α) monotherapy was recommended and, subsequently, combination of IFN-α or pegylated-IFN-α (Peg-IFN-α) with ribavirin (RBV) was extensively used to treat HCV infection. However, the virologic response with IFN-α based therapy depends on the HCV genotype (Dresch et al., 2016; Watanabe et al., 2016). Indeed, patients infected with genotypes 2 and 3 have higher chances of sustained virologic response (SVR), while less impact has been reported for patients infected with genotype 1 (Ogawa et al., 2016; Rizk et al., 2016; Poizot-Martin et al., 2016; Łucejko et al., 2016; Kao et al., 2016; Punzalan et al., 2015), suggesting that the immunological status observed prior to treatment, or the shift of the immune response observed after treatment onset, might be involved in the virologic response. In fact, it has been demonstrated that genetic variations of IFN-λ-related genes are strongly associated with distinct patterns of therapeutic response to Peg-IFN-α/RBV dual therapy (Mangia et al., 2013). Moreover, it has been reported that early change in the baseline cytokine pattern is critical to support the sustained virologic response following Peg-IFN-α/RBV therapy in chronic hepatitis C (de Souza-Cruz et al., 2016; Araujo et al., 2013; Zarife et al., 2011). Several efforts have been made to establish novel biomarkers with relevant predictive values to monitor immunotherapy of HCV infection (Araujo et al., 2013; Rizk et al., 2016; Zarife et al., 2011).

In the last five years, the treatment of HCV infection has evolved from the use of IFN-α (monotherapy or combined with RBV) towards the use of IFN-free protease and polymerase inhibitor combination regimens (Watanabe et al., 2016; Ogawa et al., 2016; Poizot-Martin et al., 2016). However, preclinical studies have shown that several cytokines have considerable clinical potential in treating infectious, parasitic diseases at acute and chronic stages as well as allergic and degenerative conditions. As the field of cytokine-based immunotherapy expands, side effects, development of blocking antibodies and immunotolerance may emerge as idiosyncratic events that limit therapeutic effectiveness.

Effort to reduce or minimize such idiosyncratic effects of immunotherapy may be focused on identifying biomarkers to serve as predictors of therapeutic response. The detailed characterization of biomarker changes triggered by a given cytokine-based therapy may provide relevant insights to subsidize the development of therapeutic monitoring strategies as follow-up to immunotherapy of infectious diseases.

The present study intended to assess the pattern of serum biomarkers in HCV patients upon IFN-α-based immunotherapy, using novel approaches to evaluate the balance, baseline-fold changes, network connectivity and performance as a prototype evaluation to follow-up immunotherapy of infectious diseases.

2. Material and methods

2.1. Study population

A total of 73 subjects were included in the present study. Treatment-naive HCV patients (HCV = 54, 33 males and 21 females, age range from 20 to 70 years old), chronically infected with genotype 1, were enrolled for a longitudinal follow-up investigation in parallel with recruitment of age/gender matching healthy blood donors (NI = 19, 12 males and 7 females, age range from 19 to 58 years old).

The HCV patients under recommendation of antiviral therapy with Peg-IFN-α(2a or 2b)/RBV dual therapy were followed by multidisciplinary medical care at the Outpatient Liver Unit, Instituto Alfa de Gastroenterologia, affiliated to the Universidade Federal de Minas Gerais, Belo Horizonte, Minas Gerais, Brazil. Therapeutic response was assessed by viral load analysis carried

Table 1
Demographic features and laboratorial records of HCV study group.

Parameters	HCV	HCV subgroups[a]		
		NR	REL	SVR
Age	53 (20–70)	53 (20–62)	57 (38–69)	51 (24–70)
Gender (n)				
Males	33	09	11	13
Females	21	07	03	11
ALT (IU)	80.5 (18.0–238.0)	65.0 (26.0–161.0)	103.0 (33.0–191.0)	83.0 (18.0–238.0)
AST (IU)	74.0 (5.0–241.0)	74.0 (5.0–241.0)	76.0 (31.0–169.0)	59.0 (21.0–158.0)
Albumin (g/dL)	4.2 (3.3–5.2)	4.0 (3.3–5.2)	4.4 (4.0–5.0)	4.2 (3.3–5.1)
Platelets (counts/mm^3)	164,500 (50,000–395,000)	123,000 (50,000–246,000)	164,000 (111,000–244,000)	207,000 (97,000–395,000)
PA (%)	95.5 (61.0–117.0)	91.6 (61.0–100.0)	100 (79.0–110.0)	98 (67.0–117.0)
INR	1.03 (0.91–1.29)	1.06 (0.99–1.29)	1.00 (0.91–1.19)	1.01 (0.92–1.23)
Viral Load (IU/mL)	597,000 (135,000–4,914,583)	615,000 (135,000–4,914,583)	634,000 (149,841–1,513,889)	579,000 (180,587–850,000)
METAVIR Score (%)				
A1F2	17.6	6.3	15.4	27.3
A1F4	9.8	12.5	23.1	0.0
A2F1	7.8	6.3	7.7	9.1
A2F2	23.5	18.8	30.8	22.7
A2F3	11.8	12.5	0.0	18.2
A2F4	19.6	37.5	15.4	9.1
A3F2	3.9	0.0	0.0	9.1
A3F4	5.9	6.3	7.7	4.5

[a] HCV subgroups were considered according to the virologic response; NR = non-responders; REL = relapsers; SVR = sustained virologic response; PA = prothrombin activity; INR = international normalized ratio; data are reported as median (min-max) values; METAVIR score (A = activity; F = fibrosis score), where F0 = no fibrosis; F1 = portal fibrosis without septa; F2 = portal fibrosis with few septa; F3 = numerous septa without cirrhosis; F4 = cirrhosis and Activity score: A0 = no activity; A1 = mild activity; A2 = moderate activity; A3 = severe activity. No significant differences were observed in demographic features or laboratorial records amongst the HCV subgroups.

out by competitive reverse transcription (RT)-PCR by AMPLICOR v1.0 assay, according to the manufacturer's instructions (Roche Diagnostics Systems, Pleasanton, CA, USA).

Three sub-groups of HCV patients were considered according to the virologic response: i) Non-responders (NR = 16, 09 males and 07 females, age range from 20 to 62 years old), consisting of patients presenting less than 2 Log drop in viral load at T12, or patients without RNA HCV clearance at T48; ii) Relapsers (REL = 14, 11 males and 03 females, age range from 38 to 69 years old), comprising patients with negative RNA HCV results at T48, but showing breakthrough in the first six months after treatment intervention; and iii) Sustained virologic responders (SVR = 24, 13 males and 11 females, age range from 24 to 70 years old), including patients with negative qualitative RNA HCV results at T48 and at six months after treatment regimen. Ten milliliters of peripheral blood were drawn by a trained health care professional, according to standard operational procedures. Samples were collected before treatment (T0; NR = 16, REL = 13 and SVR = 24) and throughout antiviral treatment: at 12 weeks (T12; NR = 15, REL = 11 and SVR = 17) and 48 weeks (T48; NR = 06, REL = 12 and SVR = 18). Detailed information about demographic features and laboratorial records of HCV study group are provided in the Table 1.

Healthy blood donor controls were recruited at the Blood Bank Unit at Felício Rocho Hospital, Belo Horizonte, Minas Gerais, Brazil. Serum samples were obtained, aliquoted and stored at −80 °C until use for chemokine/cytokine analysis by flow cytometry.

Exclusion criteria were: 1) patients infected by HCV genotype other than 1; 2) HBV and/or HIV coinfection; 3) other concomitant liver diseases; 4) use of immunosuppressants; 4) active alcohol consumption (>20 g/day for women and > 40 g/day for men); 5) previous antiviral treatment for chronic hepatitis C; 6) patients submitted to less than 48 weeks' therapy with Peg-IFN-α/RBV.

Informed written consent was obtained from all participants. The protocols were approved by the local Ethics Committee (Federal University of Minas Gerais, protocol number ETIC 226/06).

2.2. Chemokine and cytokine quantitation by cytometric bead array − CBA

Serum levels of chemokines (IL-8/CXCL-8, MCP-1/CCL-2, MIG/CXCL-9, IP-10/CXCL-10 and RANTES/CCL5) and cytokines (INF-α, IL-1β, IL-6, TNF, IL-12, INF-γ, IL-4, IL-10 and IL-17) were measured by enhanced sensitivity CBA kits (BD Biosciences, San Jose, CA, USA), according to manufacturer's protocol. Samples were run in a FACSVerse flow cytometer using the FACSuite™ software (BD Biosciences, San Jose, CA, USA) for acquisition. Data analysis was performed using the FCAP Array™ software version 2.0 (BD Biosciences, San Jose, CA, USA) and the mean fluorescence intensity (MFI) of each bead cluster was determined for each chemokine/cytokine tested. Forth logistic regression analyses were used to generate standard curves for each analyte. Concentrations (pg/mL) were extrapolated according to the standard curves. Chemokine concentrations were expressed as pg/mL. Cytokine levels were expressed in MFI. Additionally, chemokine/cytokine levels were expressed as categorical data (frequency of subjects with high serum levels) and baseline-fold changes (T12/T0) as described in the Data Analysis section.

2.3. Data mining, statistics and systems biology approaches

2.3.1. Conventional statistical analysis

Comparative analysis between NI and HCV groups was carried out by non-parametric Mann-Whitney test. Multiple comparisons amongst HCV subgroups (NR, REL and SVR) and NI were performed by non-parametric Kruskal-Wallis variance analysis followed by Dunn's post-test. In all cases, significance was considered at $p < 0.05$. GraphPad Prism software (version 5.03, San Diego, California, USA) was used for statistical and graphical arts.

2.3.2. Biomarker signature analysis

Chemokine/cytokine ascendant signatures were performed as previously reported by Luiza-Silva et al. (2011), modified as follows: briefly, the global median values were calculated for each serum biomarker, considering the whole data universe (NI + HCV at T0 + HCV at T12 + HCV at T48 = total of 149). The global median was used as the cut-off to categorize each subject as they present "Low" (below or equal the global median) or "High" (above the global median) serum levels. In the next step, data was compiled on black-and-white diagrams to calculate the frequency of subjects with "High" serum levels by column statistics. The scatter plots displaying the cut-off for each biomarker, the diagram compilation and the column statistics are provided in Supplementary Fig. 1. The frequency of subjects with "High" serum levels was assembled to build the overall ascendant biomarker curves. Data mining was carried out considering as relevant biomarkers those with frequency of subjects with "High" serum levels above the 50th percentile. GraphPad Prism software (version 5.03, San Diego, California, USA) was used for bar charts and ascendant curve assembling and graph arts.

2.3.3. Systems biology approaches

2.3.3.1. Biomarker networks. Correlations between chemokines and cytokines for each HCV subgroups (NR, REL and SVR) were calculated by Spearman correlation test to generate a "p" and "r" matrices. Significant correlations at $p < 0.05$ were selected for network assembling using the open source Cytoscape software, version 3.1.1. The biomarker networks were constructed using circular layouts in which each biomarker is represented by a globular node. Connecting edges representing "r" correlation index underscored: strong positive ($r = 0.68$), moderate positive ($0.36 \leq r < 0.68$), strong negative ($r = -0.68$), moderate negative ($-0.68 < r = -0.36$) as proposed by Taylor (1990).

2.3.3.2. Heatmap constructions. The baseline-fold (T12/T0) indices were calculated for each biomarker and the whole data universe (NR + REL + SVR = total of 43) used to define the global median values. The global median was used to categorize each subject as they present "Low" (below or equal the global median) or "High" (above the global median) baseline-fold index. The heatmaps were assembled to display the overall proportion of subjects with "Low" and "High" baseline-fold index (T12/T0) using Microsoft Excel software. The frequency of subjects with "High" baseline-fold index (T12/T0) was compiled by column statistics to generate the equalized number of subjects. Those biomarkers associated with frequencies of "High" baseline-fold index (T12/T0) above 50% were selected as putative tools to discriminate HCV subgroups (NR, REL and SVR). GraphPad Prism software (version 5.03, San Diego, California, USA) was used for bar charts and ascendant curve assembling and graph arts.

2.3.3.3. Decision trees. This novel approach is a widely used machine learning algorithm to select the minimal set of attributes that efficiently segregates unbiased clusters. Baseline-fold indices (T12/T0) were also employed in the dataset to create decision trees. The decision trees were built using the Waikato Environment for Knowledge Analysis software (WEKA, version 3.6.11, Waikato, New Zealand). This method searches attributes for group segregation with maximum classification accuracy and elects root and branch attribute nodes.

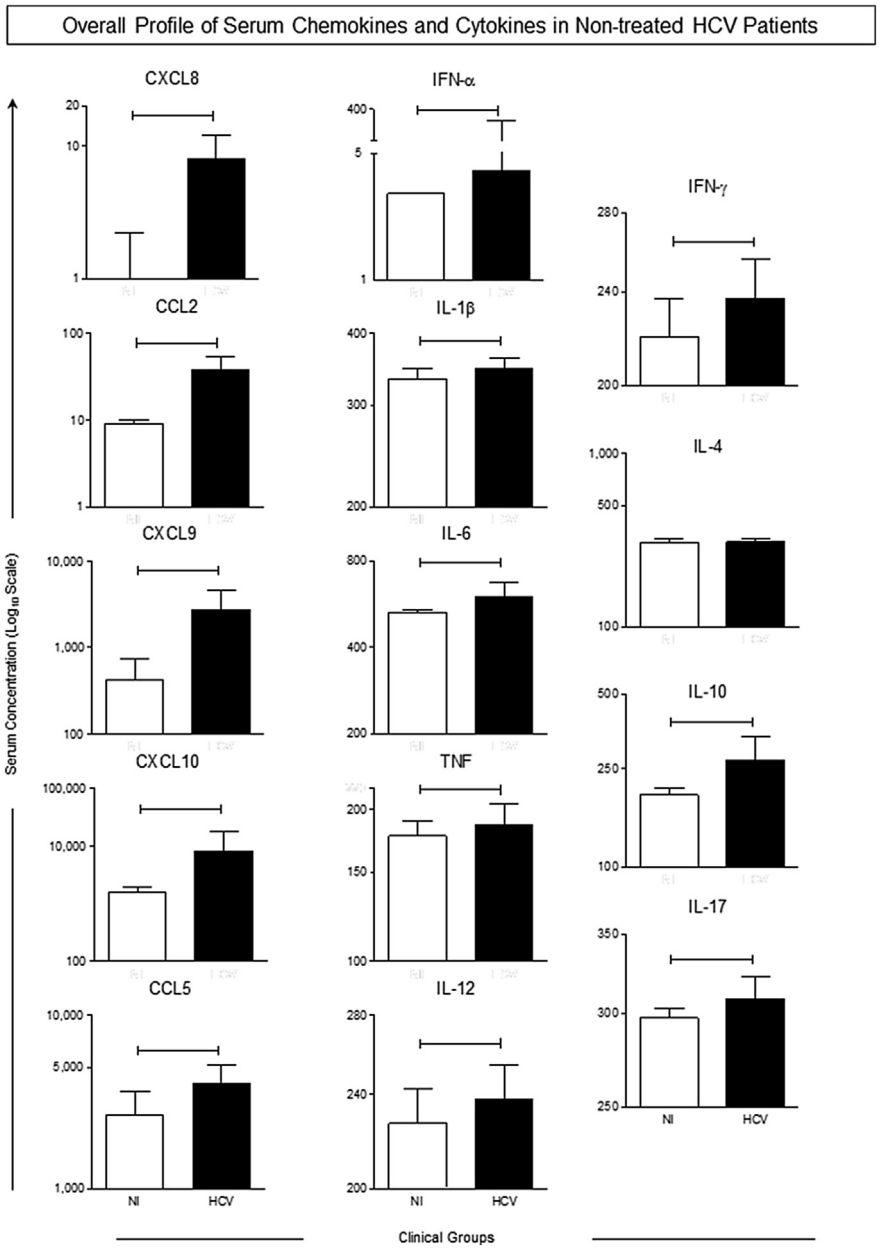

Fig. 1. *Overall profile of serum chemokines and cytokines in non-treated HCV patients.* Chemokine (IL-8/CXCL-8, MCP-1/CCL-2, MIG/CXCL-9, IP-10/CXCL-10 and RANTES/CCL5) and cytokine (IFN-α, IL-1β, IL-6, TNF, IL-12, IFN-γ, IL-4, IL-10 and IL-17) levels were measured in serum samples from Non-treated HCV patients (HCV = ■) and Non-infected Controls (NI = □) by cytometric bead array as described in Methods. The results are presented by bar plots, in Log scale, displaying the median serum concentration and 75% interquartile range. Significant differences at $p < 0.05$ between groups are highlighted by connecting lines.

2.3.3.4. Performance analysis. The performance analysis was comprised of two distinct approaches including the Receiver Operating Characteristic (ROC) curve and Two-Graph ROC (TG-ROC) analysis. The operating-characteristic curves were assembled considering the sensitivity and specificity at distinct cut-off values. The ROC curve analysis confirmed the cut-off edges proposed by the decision tree analysis and also determined the global accuracy (area under the curve - AUC) of selected biomarkers. The statistical indices: (Se) sensitivity = [true positives/(true positives + false negatives)] × 100 and (Sp) specificity = [true negatives/(true negatives + false positives)] × 100. The TG-ROC plots further validated the proposed cut-off thresholds. The MedCalc software package (ver. 7.3) was used for the operating-characteristic curve analysis. GraphPad Prism software (version 5.03, San Diego, California, USA) was utilized for TG-ROC plotting and graphical arts. Principal components discriminant analysis (PCA) was carried out to assess the association between serum biomarker baseline levels and the baseline-fold variability (T12/T0) to identify biomarkers able to clusterize the immunotherapy responders (NR *versus* SVR). The PCA graphs were built as XY plots considering the Log_{10} Baseline Levels (T0) and Log_2 Baseline-fold changes (T12/T0) of all biomarkers, respectively.

3. Results

3.1. Overall profile of serum chemokines/cytokines in non-treated HCV patients

Quantitative analysis of serum chemokines (IL-8/CXCL8, MCP-1/CCL2, MIG/CXCL9, IP-10/CXCL10 and RANTES/CCL5) and cytokines

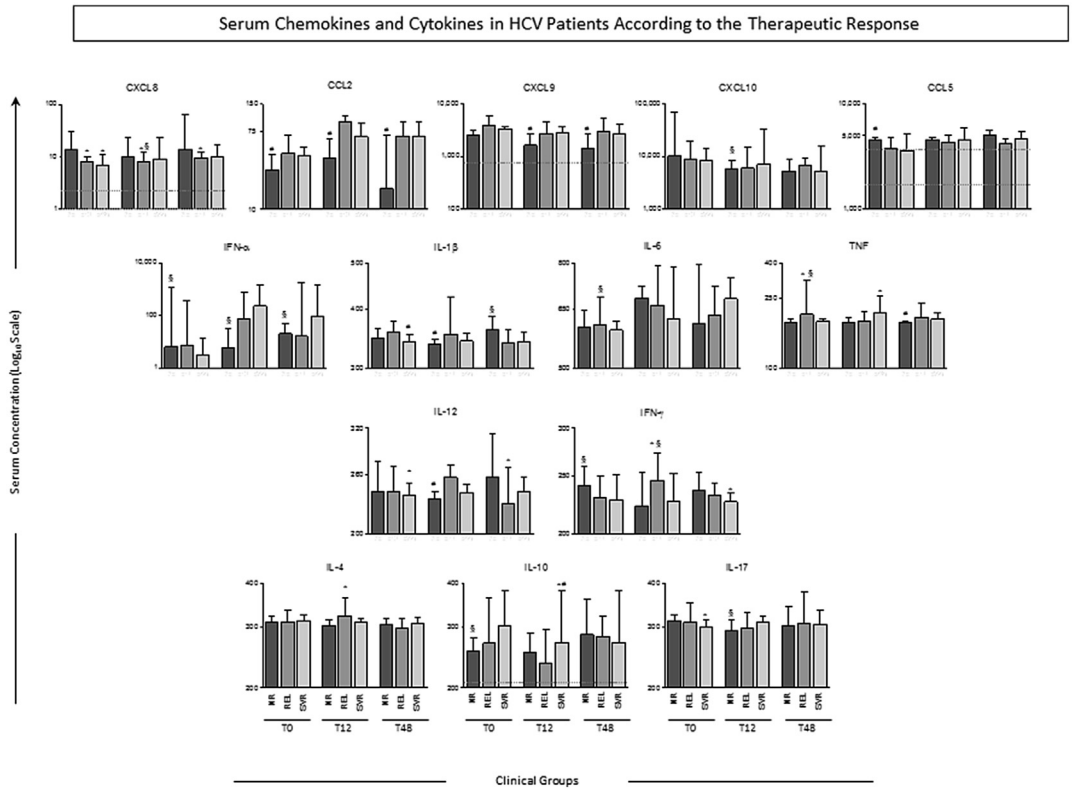

Fig. 2. *Serum chemokine/cytokine levels in HCV patients according to the therapeutic response.* Chemokine (IL-8/CXCL-8, MCP-1/CCL-2, MIG/CXCL-9, IP-10/CXCL-10 and RANTES/CCL5) and cytokine (IFN-α, IL-1β, IL-6, TNF, IL-12, IFN-γ, IL-4, IL-10 and IL-17) levels were quantified by cytometric bead array in serum samples from HCV patients, sub-grouped according to the therapeutic response (NR = ●; REL = ■ and SVR = □), as described in Methods. The chemokine/cytokine level assessment was performed before (T0) and throughout the immunotherapy intervention (T12 and T48). The reference ranges of values for non-infected controls are provided in the figure as indicated by dashed lines. The results are presented by bar plots, in Log scale, displaying the median serum concentration and 75% interquartile range. Significant differences at $p < 0.05$ between groups are underscored by *, # and § for comparisons with NR, REL and SVR, respectively.

(IFN-α, IL-1β, IL-6, TNF, IL-12, IFN-γ, IL-4, IL-10 and IL-17) were performed to characterize the overall profile of non-treated HCV patients as compared to healthy non-infected blood donors (NI). The median values of serum concentrations are shown in Fig. 1. Data analysis demonstrated that HCV patients presented an overall storm of serum biomarkers except for IL-4 when compared to NI reference values (Fig. 1).

3.2. Serum chemokines/cytokines in HCV patients according to the virologic status after immunotherapy

Follow-up analysis of serum chemokines and cytokines were carried out at baseline (T0), 12 weeks (T12) and 48 weeks (T48) after immunotherapy onset. The biomarker levels were compared amongst HCV patients categorized according to the therapeutic response in three subgroups named non-responders (NR), relapsers (REL) and sustained virologic responders (SVR). The median values of serum concentrations are provided in Fig. 2. In general, therapeutic failure profiles (NR and REL) were associated with a prominent proinflammatory pattern at baseline, composed of increased levels of CCL5/RANTES, IFN-α and IFN-γ. While NR displayed decreased levels of IL-10, REL showed increased baseline levels of IL-6 and TNF as compared to SVR. Nonetheless, SVR displayed an evident and significant decrease of proinflammatory status as compared to NR and REL, with decreased levels of CXCL8/IL-8, IL-12 and IL-17 (Fig. 2). Noteworthy was that NR patients lacked the ability to uphold increased IFN-α serum levels as compared to SVR, throughout the immunotherapy follow-up (T12 and T48).

3.3. Ascendant biomarker signatures in treated HCV patients are consistent with distinct patterns of immunotherapy response

The chemokine/cytokine signatures were assembled in order to further characterize the reference groups (NI and untreated HCV) as well as HCV patients undertaking immunotherapy at baseline, 12 and 48 weeks follow-up (T0, T12 and T48). Biomarker signatures were constructed by converging continuous values of chemokine/cytokine serum concentration into categorical data (low and high levels), taking the global median as the cut-off edge (Supplementary Fig. 1). The ascendant frequency of subjects with high serum levels were compared by overlaid curves for NI *vs* HCV as well as for NR *vs* REL *vs* SVR at each time-point of immunotherapy follow-up (Fig. 3).

The results showed that serum chemokine/cytokine signatures further corroborate that non-treated HCV patients display a massive serum biomarker storm as compared to NI, except for IL-4 (Fig. 3). Moreover, the overlaid ascendant chemokine/cytokine curves further support the deleterious effect of prominent proinflammatory status at baseline (T0) for achieving successful results upon immunotherapy. In fact, at baseline (T0), an increased frequency of subjects with high levels of proinflammatory biomarkers was observed in NR and REL as compared to SVR. The ability of mounting a robust balanced proinflammatory/regulatory response at T12 seems to be critical to support the therapeutic success of SVR. This balanced profile could also be observed in SVR at T48 (Fig. 3).

The relevance of persistent elevated IFN-α levels throughout immunotherapy in generating the sustained virologic response was also highlighted by the ascendant biomarker signature analysis.

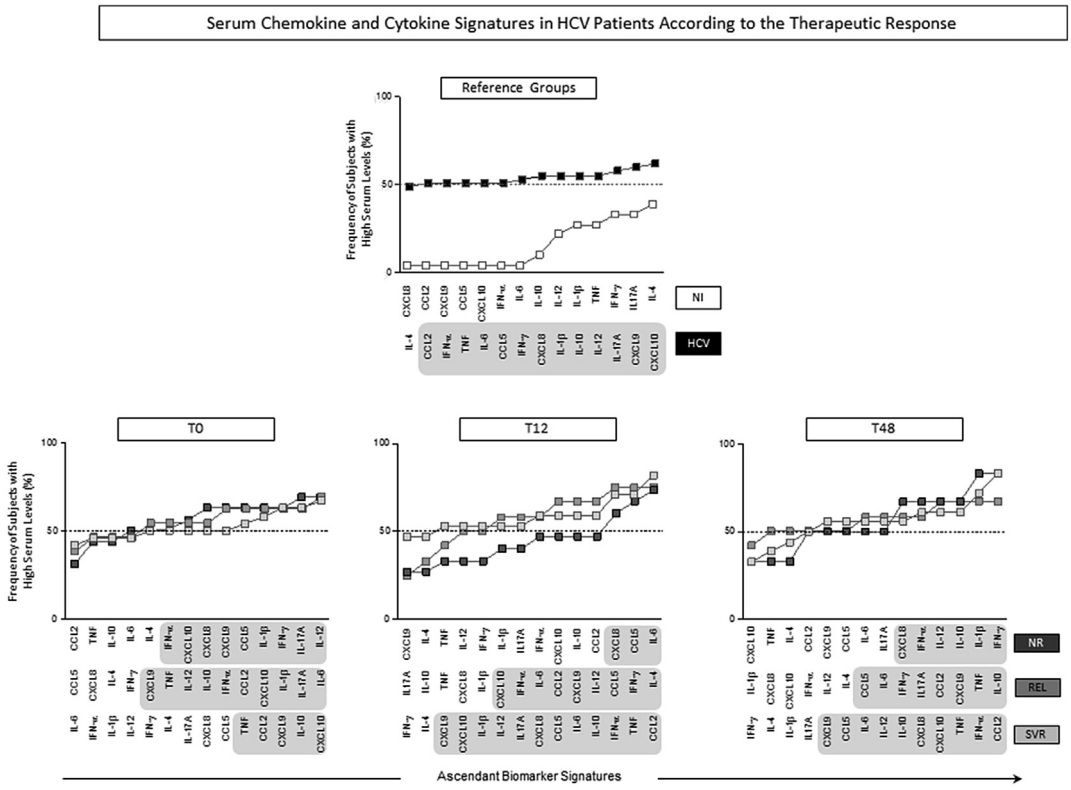

Fig. 3. *Serum chemokine and cytokine signatures in HCV patients according to the therapeutic response.* Ascendant chemokine/cytokine signatures were constructed for reference groups (HCV = ■ and NI = □) along with HCV sub-groups (NR = ■; REL = ▒ and SVR = □), assessed before (T0) and throughout the immunotherapy intervention (T12 and T48). Data are expressed as ascendant frequency of subjects with high serum biomarker levels as described in Methods. Biomarkers for which the frequency of subjects with "High" serum levels was above the 50th percentile were used for data mining and are highlighted with gray rectangle backgrounds.

Indeed, an increased frequency of subjects with high serum levels of IFN-α was observed selectively in SVR throughout the immunotherapy follow-up (T12 and T48), but not in NR (Fig. 3).

3.4. Restricted biomarker network connectivity at baseline reflects therapeutic failure, whereas robust cytokine linkage predicts sustained virologic response

Systems biology approaches applied to identify biomarker connectivity as predictive parameters for virologic response following immunotherapy are presented in Fig. 4.

Data mining demonstrated that the prominent proinflammatory milieu observed in NR and REL at baseline, yielded a restricted biomarker network with a small number of neighborhood connections. Conversely, SVR displayed integrated cytokine connectivity, characterized by a large number of inter-node edges.

Noteworthy was that SVR presented, at T12, a shift towards a proinflammatory pattern upon immunotherapy, assuming a pattern similar to that observed in NR and REL at baseline.

The immunotherapy intervention guided REL forward into developing, at T12 through T48, a profile similar to that observed for SVR at baseline (Fig. 4).

3.5. Baseline-fold changes during immunotherapy (T12/T0) allowed for the selection of putative predictive hallmarks of therapeutic failure as well as sustained virologic response

Baseline-fold changes are a reliable strategy to evaluate at the individual level changes in biomarker profiles, avoiding bias due to idiosyncratic features observed before immunotherapy onset. Fig. 5 shows changes in serum chemokine and cytokine levels observed at T12 relative to autologous levels at T0 (T12/T0). The analysis of individual baseline-fold values showed that changes towards higher levels of CXCL8, CCL2 and IFN-α are observed for SVR, as displayed in scatter plot distribution (Fig. 5 – top panels). Categorical analysis of subjects with high baseline-fold index revealed that amongst a range of biomarkers able to discriminate NR, REL and SVR, only CCL2 and IFN-α were selective for SVR (Fig. 5 – middle panels in gray background).

Decision tree analysis further demonstrated that CCL2 and IFN-α are root biomarkers for categorizing immunotherapy outcome (Fig. 5 – decision trees). In fact, whereas the baseline-fold change in CCL2 is the root attribute followed by CCL5 and CXCL10 nodes for identifying therapeutic failure with moderate performance (REL – 55% and NR – 75%), IFN-α root followed by TNF as first level node yields a decision tree able to segregate NR (92%) from SVR (72%) with superior performance.

3.6. Performance indices supported the baseline-fold changes of IFN-α as a useful biomarker for monitoring sustained virologic response after immunotherapy

Intending to determine the value of IFN-α and TNF for monitoring post-therapeutic outcome (NR *versus* SVR), scatter plot analysis was carried out using the cut-offs elected by the decision tree analysis, further confirmed by ROC and TG-ROC plots (Fig. 6). Data indicated that IFN-α baseline-fold change above 14 and a drop of TNF below 0.8 are hallmarks of SVR, with high-performance operating-characteristics (Sensitivity = 71%; 82% and Specificity = 92%; 100%, respectively). Data mining by principal

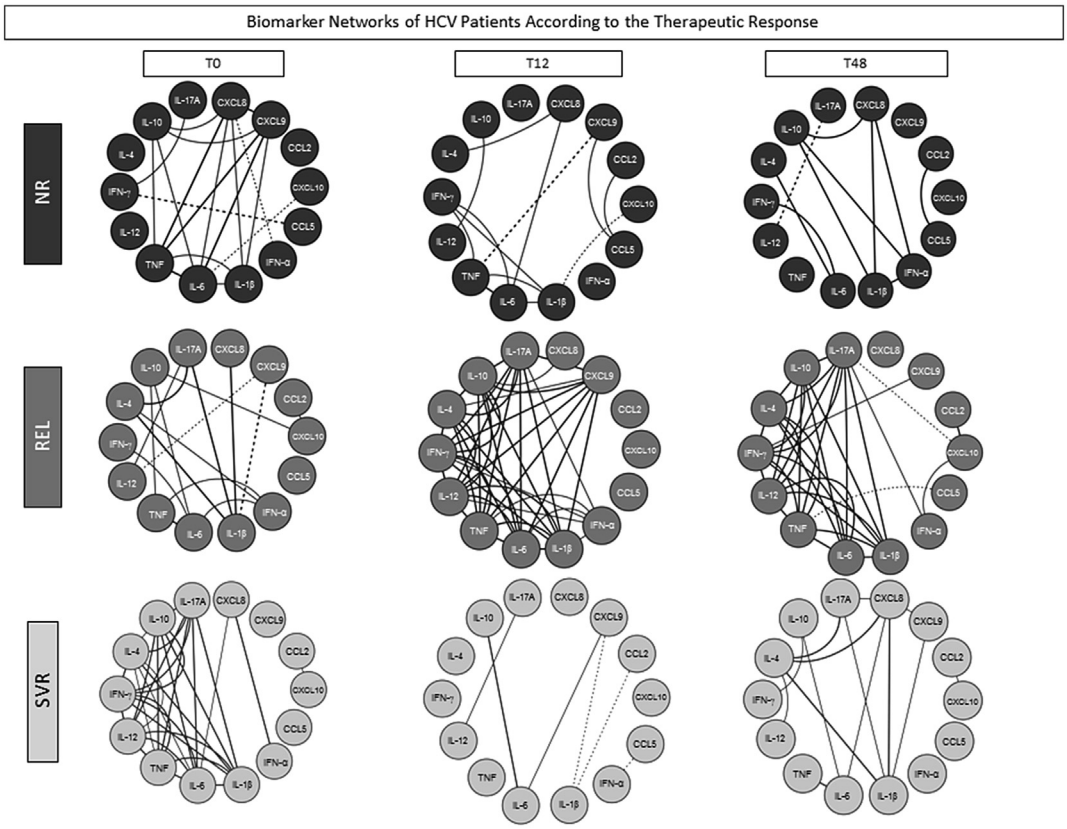

Fig. 4. *Biomarker networks of HCV patients according to the therapeutic response.* Networks of Chemokine/cytokine were assembled for HCV sub-groups (NR = ●; REL = ● and SVR = ◍), assessed before (T0) and throughout the immunotherapy intervention (T12 and T48). Correlation matrices were built with significant indices and circular layouts with globular nodes representing each biomarker connected by edges to identify significant ($p < 0.05$) correlation, as described in Methods. Connecting edges representing "r" correlation index underscored: strong positive ($r = 0.68$) and moderate positive ($0.36 \leq r < 0.68$) represented by solid lines; strong negative ($r = -0.68$) and moderate negative ($-0.68 < r = -0.36$) as dashed lines as proposed by Taylor (1990)[27].

component analysis revealed that the cross-matching pairs (baseline-levels/baseline-fold change) of IFN-α individual values stand out amongst all biomarkers evaluated. In fact, a cluster of patients with high baseline-fold changes of IFN-α (T12/T0) can be clearly identified selectively in SVR (Fig. 6 – bottom panels).

4. Discussion

Immunotherapy is a rational approach to empower the natural immune system with effector mechanisms in order to achieve clearance of etiological agents causing parasitic/infectious diseases (Vanham and Van Gulck, 2012). The understanding of chemokine/cytokine changes induced by immunotherapy could provide insights to support the development of novel therapeutic strategies and minimize idiosyncratic effects.

The treatment of HCV infection has evolved over the years and the protease/polymerase/NS5 inhibitors era has started worldwide (Ogawa et al., 2016; Poizot-Martin et al., 2016; Watanabe et al., 2016). In Brazil, these protocols based on third-generation antiviral drugs are still restricted to patients with moderate/severe fibrosis. Although the IFN-α-based treatment is no longer indicated for chronic HCV infection in several countries, the lessons learned from this immunotherapy strategy has clearly demonstrated the role of the immunological status on therapeutic outcome.

The present study assessed the pattern of serum chemokine/cytokine levels in HCV patients over treatment with IFN-α-based immunotherapy as a prototype evaluation following immunotherapy of infectious diseases. The results demonstrated that, in general, therapeutic failure was associated with presence of a predominant proinflammatory pattern at baseline composed of enhanced CCL5/RANTES, IFN-α and IFN-γ along with decreased IL-10 levels in NR and increased IL-6 and TNF in REL. In addition, SVR displayed lower proinflammatory status with decreased levels of CXCL8/IL-8, IL-12 and IL-17. The prominent proinflammatory milieu observed in NR and REL at baseline built a restricted biomarker network with a small number of neighborhood connections. Noteworthy was that NR fails to uphold IFN-α levels during immunotherapy. It is possible that NR patients may have developed a "sink effect" or a clearance mechanism mediated by the generation of anti-IFN-α antibodies. Moreover, repeated IFN-α administration resulted in "tolerance", and overdosing could even result in paradoxical immune suppression. Interestingly, the immunotherapy guided REL forward into developing a profile similar to SVR at baseline. These data may suggest that monitoring the immunological status at baseline in order to prescribe an anti-inflammatory therapy prior to treatment onset may contribute to successful response in NR and REL.

System biology analysis pointed out that SVR showed a biomarker network with integrated cytokine connectivity at baseline and shifted towards a proinflammatory pattern upon immunotherapy, assuming a pattern similar to that observed in NR and REL at baseline. In fact, a critical role of increased and persistent IFN-α levels throughout immunotherapy was imperative to guarantee the SVR. The serum IFN-α, a pivotal asset of the innate antiviral response, has been shown as essential for controlling viral replication. The ability of IFN-α therapy in eliciting early changes in

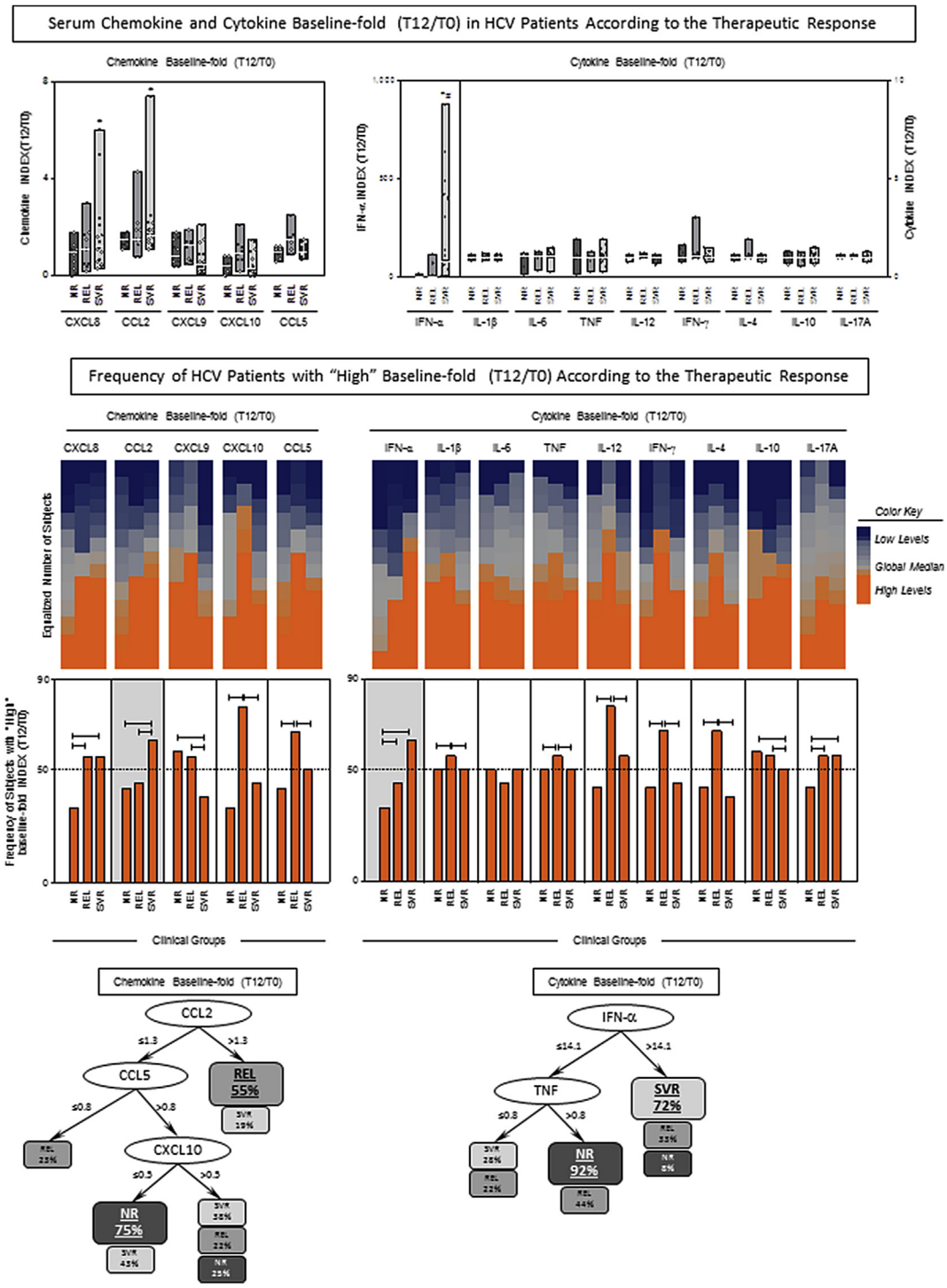

Fig. 5. *Serum chemokine and cytokine baseline-fold (T12/T0) in HCV patients according to the therapeutic response.* Baseline-fold of chemokine/cytokine index were calculated for HCV sub-groups (NR = ■; REL = ▒ and SVR = □) as the ratio T12/T0, as described in Methods. The baseline-fold (T12/T0) indices were displayed as floating bars of minimum and maximum values overlayed by scatter plots of individual values for each biomarker. The heatmaps of equalized number of subjects, shown in the middle panels, were assembled to display the overall proportion of subjects with "High" baseline-fold index (T12/T0) according to the therapeutic response. Those biomarkers associated with frequencies of "High" baseline-fold index (T12/T0) above 50% were selected as relevant tools to differentiate HCV subgroups (NR, REL and SVR). Decision trees with putative biomarkers were assembled by machine learning algorithm in order to select unbiased clusters. Significant differences at $p < 0.05$ between groups are either underscored by connecting lines (lower panels) as well as symbols (*, # and §) for comparisons with NR, REL and SVR, respectively.

the baseline immune response towards a proinflammatory microenvironment has been pointed out as a critical event that leads to effective SVR (Araujo et al., 2013). Analysis of chemokine/cytokine baseline-fold changes during treatment pointed out IFN-α and TNF as high-performance biomarkers to monitor immunotherapy outcome. These findings were further validated by data mining of principal components, which shows that patients with baseline-fold changes of IFN-α (T12/T0) higher than 14 can be clearly identified as SVR.

The results clearly support that an exacerbated

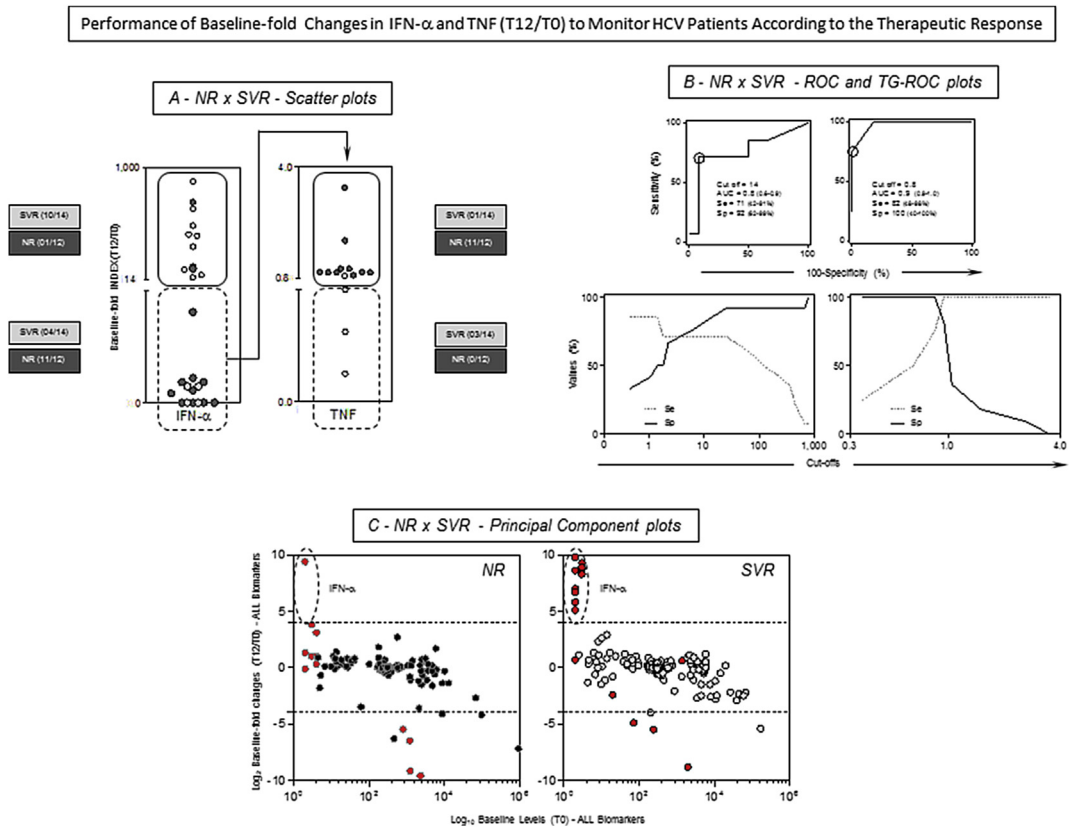

Fig. 6. *Performance of baseline-fold changes in IFN-a and TNF (T12/T0) to monitor HCV patients according to the therapeutic response.* Baseline-fold indices (T12/T0) of IFN-α followed by TNF were assembled for HCV sub-groups (NR = ● and SVR = ◍), assessed by cytometric bead array as described in Methods. Proportions of subjects from NR and SVR subgroups confined below (dashed rectangles) and above (continuous line rectangles) the cut-off value are provided in panel A. Receiver Operating Characteristics (ROC) as well as Two-Graph ROC curves were constructed for the performance analysis of IFN-α followed by TNF as the biomarker algorithm for segregating NR versus SVR. Performance of operating characteristics such as the sensitivity (Se), specificity (Sp) and area under the curve (AUC) are displayed as insets in panel B. The principal component analysis displayed in panel C were built as XY plots considering the Log_{10} Baseline Levels (T0) and Log_2 Baseline-fold changes (T12/T0) of all biomarkers. Amongst the total of components considered, the IFN-α (●) results were able to clusterize (dashed-line ellipsis) most SVR subjects above the cut-off edge (baseline-fold = ±14; dashed lines).

proinflammatory status at baseline has a deleterious effect during HCV immunotherapy, leading to therapeutic failure. Our findings indicated that while the immune response was boosted by IFN-α therapy in SVR, the IFN-α sink effect was clearly detected in NR. Previous reports have demonstrated that a pro-inflammatory state at treatment onset is risky to support the SVR following Peg-IFN-α/RBV therapy in chronic hepatitis C (Araujo et al., 2013; Sharafi et al., 2012; Yoneda et al., 2011). This inappropriate immune activation was partly due to exacerbated IFN-α level at baseline in NR. Consistently, increased type-1 IFN activity has been related to a bad prognosis of viral infection (Craxi et al., 1995).

Efforts to reduce or minimize such idiosyncratic effects of immunotherapy based on identifying biomarkers predictors of therapeutic response are a plausible approach to generate scientific support for future immunotherapeutic protocols. It has been demonstrated that modifying or targeting nonspecific immune responses is an important aspect of intervention of ongoing viral infections. The understanding provided by IFN-α-based approaches has paved the way into orchestrating the immune system favoring viral clearance. This knowledge may contribute for novel insights into the treatment and control of the continuous public health threat posed by HCV infection worldwide.

5. Conclusions

Analysis of baseline-fold changes during treatment pointed out IFN-α and TNF as high-performance biomarkers to monitor immunotherapy outcome. This knowledge may contribute for novel insights into the treatment and control of the continuous public health threat posed by HCV infection worldwide.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.antiviral.2017.02.001.

References

Alavian, S.M., Haghbin, H., 2016. Relative importance of hepatitis B and C Viruses in hepatocellular carcinoma in EMRO countries and the Middle East: a systematic review. Hepat. Mon. 16 (3), e35106.

Araújo, A.R., Peruhype-Magalhães, V., Coelho-dos-Reis, J.G., Chaves, L.P., de Lima, T.A., Pimentel, J.P., et al., 2013. Dual role of IL-12 in the therapeutic efficacy or failure during combined PEG-Interferon-α2A and ribavirin therapy in patients with chronic hepatitis C. Immunol. Lett. 154 (1–2), 61–69.

Chassagne, F., Rojas Rojas, T., Bertani, S., Bourdy, G., Eav, S., Ruiz, E., et al., 2016. A 13-year retrospective study on primary liver cancer in Cambodia: a strikingly high hepatitis C occurrence among hepatocellular carcinoma cases. Oncology 91 (2), 106–116.

Craxì, A., Magrin, S., Fabiano, C., Linea, C., Almasio, P., 1995. Host and viral features in chronic HCV infection: relevance to interferon responsiveness. Res. Virol. 146 (4), 273–278.

D'Elia, R.V., Harrison, K., Oyston, P.C., Lukaszewski, R.A., Clark, G.C., 2013. Targeting the "cytokine storm" for therapeutic benefit. Clin. Vaccine Immunol. 20 (3), 319–327.

de Souza-Cruz, S., Victória, M.B., Tarragô, A.M., da Costa, A.G., Pimentel, J.P., Pires, E.F., et al., 2016. Liver and blood cytokine microenvironment in HCV patients is associated to liver fibrosis score: a proinflammatory cytokine ensemble orchestrated by TNF and tuned by IL-10. BMC Microbiol. 16, 3.

Dresch, K.F., de Mattos, A.A., Tovo, C.V., de Onofrio, F.Q., Casagrande, L., Feltrin, A.A., et al., 2016. Impact of the pegylated-interferon and ribavirin therapy on the treatment-related mortality of patients with cirrhosis due to hepatitis C virus. Rev. Inst. Med. Trop. Sao Paulo 58, 37.

Guo, F., Zhao, X., Gill, T., Zhou, Y., Campagna, M., Wang, L., et al., 2014. An interferon-beta promoter reporter assay for high throughput identification of compounds against multiple RNA viruses. Antivir. Res. 107, 56–65.

Han, Q.Y., Liu, Z.W., 2016. Current treatment of chronic hepatitis C in China: dilemma and potential problems. World J. Gastroenterol. 22 (19), 4615–4618.

Kao, J.H., Lee, Y.J., Heo, J., Ahn, S.H., Lim, Y.S., Peng, C.Y., et al., 2016. All-oral daclatasvir plus asunaprevir for chronic HCV genotype 1b infection: a sub-analysis in Asian patients from the Hallmark Dual study. Liver Int. 36 (10), 1433–1441.

King, A., Bornschlegel, K., Johnson, N., Rude, E., Laraque, F., 2016. Barriers to treatment among New York City residents with chronic hepatitis C virus Infection, 2014. Public Health Rep. 131 (3), 430–437.

Kong, Y.C., Wei, W.Z., Tomer, Y., 2010. Opportunistic autoimmune disorders: from immunotherapy to immune dysregulation. Ann. N. Y. Acad. Sci. 1183, 222–236.

Luiza-Silva, M., Campi-Azevedo, A.C., Batista, M.A., Martins, M.A., Avelar, R.S., da Silveira Lemos, D., et al., 2011. Cytokine signatures of innate and adaptive immunity in 17DD yellow fever vaccinated children and its association with the level of neutralizing antibody. J. Infect. Dis. 204 (6), 873–883.

Mangia, A., Santoro, R., Copetti, M., Massari, M., Piazzolla, V., Spada, E., et al., 2013. Treatment optimization of and prediction of HCV clearance in patients with acute HCV infection. J. Hepatol. 59 (2), 221–228.

Muñoz-Gámez, J.A., Salmerón, J., Ruiz-Extremera, Á., 2016. Hepatitis C during pregnancy, vertical transmission and new treatment possibilities. Med. Clin. Barc. 147 (11), 499–505.

Ogawa, E., Furusyo, N., Yamashita, N., Kawano, A., Takahashi, K., Dohmen, K., et al., 2016. Effectiveness and safety of daclatasvir plus asunaprevir for HCV genotype 1b patients aged 75 and over with or without cirrhosis. Hepatol. Res. http://dx.doi.org/10.1111/hepr.12738.

Poizot-Martin, I., Bellissant, E., Garraffo, R., Colson, P., Piroth, L., Solas, C., et al., 2016. Addition of boceprevir to PEG-interferon/ribavirin in HIV-HCV-Genotype-1-coinfected, treatment-experienced patients: efficacy, safety, and pharmacokinetics data from the ANRS HC27 study. HIV Clin. Trials 17 (2), 63–71.

Punzalan, C.S., Barry, C., Zacharias, I., Rodrigues, J., Mehta, S., Bozorgzadeh, A., et al., 2015. Sofosbuvir plus simeprevir treatment of recurrent genotype 1 hepatitis C after liver transplant. Clin. Transpl. 29 (12), 1105–1111.

Qian, X.J., Zhang, X.L., Zhao, P., Jin, Y.S., Chen, H.S., Xu, Q.Q., et al., 2016. A Schisandra-derived compound schizandronic acid inhibits entry of pan-HCV genotypes into human hepatocytes. Sci. Rep. 6, 27268.

Rizk, H.H., Hamdy, N.M., Al-Ansari, N.L., El-Mesallamy, H.O., 2016. Pretreatment predictors of response to PegIFN-RBV therapy in Egyptian patients with HCV genotype 4. PLoS One 11, 4.

Sharafi, H., Pouryasin, A., Alavian, S.M., Behnava, B., Keshvari, M., Mehrnoush, L., et al., 2012. Development and Validation of a simple, rapid and inexpensive PCR-RFLP method for genotyping of common IL28B polymorphisms: a useful pharmacogenetic tool for prediction of hepatitis C treatment response. Hepat. Mon. 12, 190–195.

Sharma, A., Halim, J., Jaggi, T., Mishra, B., Thakur, A., Dogra, V., et al., 2016. Time trends of seroepidemiology of hepatitis C virus and hepatitis B virus coinfection in human immunodeficiency virus-infected patients in a Super Specialty Hospital in New Delhi, India: 2012-2014. Indian J. Sex. Transm. Dis. 37 (1), 33–37.

Tang, L., Marcell, L., Kottilil, S., 2016. Systemic manifestations of hepatitis C infection. Infect. Agent Cancer 11, 29.

Taylor, R., 1990. Interpretation of the correlation coefficient: a basic review. J. Diagnostic Med. Sonogr. 6 (1), 35–39.

Vanham, G., Van Gulck, E., 2012. Can immunotherapy be useful as a "functional cure" for infection with Human Immunodeficiency Virus-1? Retrovirology 9, 72.

Watanabe, T., Joko, K., Seike, H., Michitaka, K., Horiike, N., Kisaka, Y., et al., 2016. Simeprevir with peginterferon/ribavirin for patients with hepatitis C virus genotype 1: high frequency of viral relapse in elderly patients. Springerplus 5, 518.

Wirth, T.C., Manns, M.P., 2016. The impact of the revolution in hepatitis C treatment on hepatocellular carcinoma. Ann. Oncol. 27 (8), 1467–1474.

Xiang, Y., Tang, J.J., Tao, W., Cao, X., Song, B.L., Zhong, J., 2015. Identification of cholesterol 25-hydroxylase as a novel host restriction factor and a part of the primary innate immune responses against hepatitis C virus infection. J. Virol. 89 (13), 6805–6816.

Yoneda, S., Umemura, T., Katsuyama, Y., Kamijo, A., Joshita, S., Komatsu, M., et al., 2011. Association of serum cytokine levels with treatment response to pegylated interferon and ribavirin therapy in genotype 1 chronic hepatitis C patients. J. Infect. Dis. 203 (8), 1087–1095.

Zarife, M.A., Reis, E.A., Meira, G.C., Carmo, T.M., Lopes, G.B., Malafaia, E.C., et al., 2011. IL-8 is associated with non-viremic state and IFN-γ with biochemical activity in HCV-seropositive blood donors. Intervirology 54 (2), 87–96.

Łucejko, M., Parfieniuk-Kowerda, A., Flisiak, R., 2016. Ombitasvir/paritaprevir/ritonavir plus dasabuvir combination in the treatment of chronic HCV infection. Expert Opin. Pharmacother. 17 (8), 1153–1164.