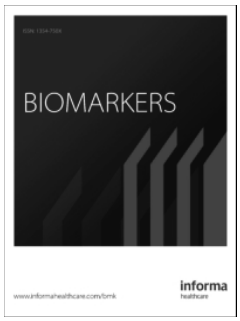

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RESEARCH ARTICLE

Immunological signature of the different clinical stages of the HTLV-1 infection: establishing serum biomarkers for HTLV-1-associated disease morbidity

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Abstract

This study aimed at establishing the immunological signature and an algorithm for clinical management of the different clinical stages of the HTLV-1-infection based on serum biomarkers. A panel of serum biomarkers was evaluated by four sets of innovative/non-conventional data analysis approaches in samples from 87 HTLV-1 patients: asymptomatic carriers (AC), putative HTLV-1 associated myelopathy/tropical spastic paraparesis (pHAM/TSP) and HAM/TSP. The analysis of cumulative curves and molecular signatures pointed out that HAM/TSP presented a pro-inflammatory profile mediated by CXCL10/LTB-4/IL-6/TNF-α/IFN-γ, counterbalanced by IL-4/IL-10. The analysis of biomarker networks showed that AC presented a strongly intertwined pro-inflammatory/regulatory net with IL-4/IL-10 playing a central role, while HAM/TSP exhibited overall immune response toward a predominant pro-inflammatory profile. At last, the classification and regression trees proposed for clinical practice allowed for the construction of an algorithm to discriminate AC, pHAM and HAM/TSP patients with the elected biomarkers: IFN-γ, TNF-α, IL-10, IL-6, IL-4 and CysLT. These findings reveal a complex interaction among chemokine/leukotriene/cytokine in HTLV-1 infection and suggest the use of the selected but combined biomarkers for the follow-up/diagnosis of disease morbidity of HTLV-1-infected individuals.

Keywords

Chemokines, cytokines and morbidity, HAM/TSP, HTLV-1, leukotrienes

History

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Introduction

The human T-lymphotropic virus type 1 (HTLV-1) is a delta-type retrovirus, which infects approximately 10–20 million people worldwide (Carneiro-Proietti et al., 2002; Proietti et al., 2005). In addition to adult T-cell leukemia (ATL) (Poiesz et al., 1980), HTLV-1 is also involved in the etiology of chronic inflammatory diseases, such as the HTLV-1-associated myelopathy/tropical spastic paraparesis (HAM/TSP) (Gessain et al., 1985; Osame et al., 1986) uveitis (Mochizuki et al., 1992) skin disorders (Coelho-dos-Reis et al., 2013) infective dermatitis (La Grenade et al., 1998; Nishimoto et al., 2009; Primo et al., 2005) and may also be associated with arthropathies, polymyositis and Sjögren syndrome (Taylor & Matsuoka, 2005).

While the majority of infected individuals remain lifelong asymptomatic carriers (ACs), the HTLV-associated diseases can occur in the minority, approximately 3% develop ATL (Tajima et al., 1990) and other 4% develop HAM/TSP (Hisada et al., 2004; Nakagawa et al., 1995; Osame M et al., 1986, 1990). Regarding the inflammatory disorders associated with HTLV-1 infection, several immunological factors seem to be involved in the HTLV-1/host interaction influencing the clinical evolution of HTLV-1-infected subjects from asymptomatic status to HAM/TSP (Starling et al., 2013).

HAM/TSP is a neuroinflammatory disease characterized by a chronic progressive myelopathy with infiltrating

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mononuclear cells in the area of demyelination and axonal dystrophy (Cooper et al., 2009). The chronic pro-inflammatory process caused by HTLV-1 is characterized by spontaneous in vitro T cell proliferation (Lunardi-Iskandar et al., 1993; Sibon et al., 2006) and by elevated *ex vivo* production of IFN-γ and TNF-α by the peripheral blood mononuclear cells (Brito-Melo et al., 2001; Carvalho et al., 2001). Moreover, IL-2 and metalloproteinases produced by HTLV-1-infected lymphocytes are potentially inflammatory and may damage the central nervous system (Biddison et al., 1997; Kubota et al., 2002; Muniz et al., 2006). The monocytic cells produce high level of leukotrienes (LTs), mainly Cys type, which also appear to be involved in the progression of the HTLV-neurologic disease (Trindade et al., 2012).

While the pro-inflammatory immunological environment in HAM/TSP was well-scrutinized over the years, little is known about the predictive value of the inflammatory biomarkers to distinguish the asymptomatic stage from the early phase of HAM/TSP. Considering such lack of information on the diagnostic value of these immunological molecules and their importance on HTLV-1 infection, this study aimed at establishing the immunological signature of different clinical status of the HTLV-1 infection as well as the interaction between these molecules in the different clinical statuses evaluated with the ultimate goal of understanding the power of these molecules as biomarkers of disease morbidity.

Materials and methods

Study population

This was a transversal study comprised of 108 participants that belong to a cohort study of former blood donors infected by HTLV, all living in Minas Gerais, Brazil. This cohort has been followed by the HTLV Interdisciplinary Research Group (Grupo Interdisciplinar de Pesquisa em HTLV – GIPH) since 1997 concerning the clinical and immunological aspects of this infection.

Different spectrums of the HTLV-1-associated outcome other than HAM/TSP were included in this study in order to identify possible biomarkers that may present prognostic value. In this respect, HTLV-1 carriers with no symptoms (AC) and patients with initial symptoms, but without full clinical diagnosis of HAM/TSP (putative HAM/TSP), were included in this study.

Participants were diagnosed as infected with HTLV-1, if they presented positive serology for HTLV-1, both by the enzyme-linked immunosorbent assay (ELISA) test and western blot (WB) test. As described in Box 1, 87 HTLV-1 infected patients were categorized according to their clinical status as ACs (AC = 27), patients with putative HTLV-1 associated myelopathy/tropical spastic paraparesis (pHAM/TSP = 32) and patients with defined HAM/TSP (HAM/TSP = 28). In addition, a group of 21 healthy blood donors with negative HTLV-1 serologic tests (ELISA and WB tests) were included as an uninfected control group.

All the participants were submitted to serologic screening for blood-borne pathogens and showed negative serology for *Trypanosoma cruzi*, *Treponema pallidum*, HBV, HCV and HIV infections.

This study was approved by the Research Ethics Committee of the Federal University of Minas Gerais, protocol ETIC 090/07 and the Research Ethics Committee of Hemominas – protocol n 83/2007. Free and informed consent forms were obtained from all participants.

Flow cytometry quantitative analysis of serum chemokines and cytokines

The serum levels of chemokines (CXCL8/IL-8, CCL5/RANTES, CXCL9/MIG, CCL2/MCP-1, CXCL10/IP-10) and cytokines (IL-2, IL-4, IL-6, IL-10, TNF-α and IFN-γ) were measured in serum samples from all patients. The samples were centrifuged at 4000*g* for 15 min at 18 °C, aliquoted and stored at −20 °C until analysis. The serum chemokines and cytokines were quantified using the Cytometric Bead Array (CBA) System, Becton Dickinson (BD) according to Peruhype-Magalhaes et al. (2006). Data acquisition was achieved by flow cytometry using a FACSCalibur flow cytometer (Becton Dickinson, La Jolla, CA).

Leukotrienes immunoassay

A specific enzyme-linked immunoassay (Cayman Chemical Company, Ann Arbor, MI) was used to quantify LTB_4 and CysLT in serum samples as recommended by the manufacturer. Serum samples stored at 27 °C were purified on Waters C18 Sep-Pak cartridges (Waters Associates, Milford, MA), spun dried in a vacuum centrifuge, and reconstituted in assay buffer prior to performing the assay.

Analysis of biomarker signatures

The biomarker signature was assembled as previously reported by Costa-Silva et al. (2014). The frequency of ''high'' and ''low'' producers was defined according to the global median values. The median values of all data set were calculated for each biomarker in a blind manner prior to assigning the groups. The median value for each biomarker was utilized as a cut-off in such a way that patients with results for a particular biomarker above the global median value were attributed as high producers, whereas a patient with a result lower than the global median value was assigned as a low producer. In is important to consider that the concept of ''high'' and ''low''

Box 1. Study population.

		Gender		Median age
		Male	Female	(Min and max)
Healthy controls	(*n*=21)	12 (57.1%)	9 (42.9%)	42 (28–60)
Asymtomatic carriers (AC)	(*n*=27)	13 (48.1%)	14 (51.9%)	52 (19–69)
putative HAM/TSP (pHAM)	(*n*=32)	8 (25.0%)	24 (75.0%)	51 (31–72)
HAM/TSP	(*n*=28)	3 (10.7%)	25 (89.3%)	58 (34–72)

producer only applies to the present data set and the global median values should only be taken as a threshold for this specific study population. Following, the ''ascendant biomarker signature'' for each group was then assembled and compared. Additionally, the ''ascendant biomarker signature'' for each clinical group was also assembled and overlaid to identify changes in the overall biomarker profile.

Biomarker network analysis

Biomarker networks were assembled to assess the association between the serum chemokines, leukotrienes and cytokines for each clinical group. Spearman's correlation test was performed to assess the association between serum biomarker levels (pg/mL). The positive and negative correlations were significant when p value <0.05. Cytoscape (version 2.8, San Diego, CA), an open source software, was used for composing networks of interactions among biomolecules in order to better represent the interactivity among the molecules tested (Shannon et al., 2003). The biomarker networks were constructed using three layouts, one for each biomarker category (chemokine, leukotrienes and cytokine) represented by globular nodes to underscore the biomarkers with ''High'' levels (●) as compared to those with ''Low'' levels (○). Connecting edges display underscore negative (_ _), moderate (___) and strong (▬) as proposed by Taylor (1990).

Data analysis

Three sets of innovative/non-conventional data analysis approaches for observational investigation of the immune response related to the HTLV-1 infection were applied: (1) Gehan–Breslow–Wilcoxon test, (2) biomarker signature analysis, (3) serum biomarker networking and (4) logistic regression and classification tree. These approaches have been shown to be relevant to detect, with high sensitivity, putative changes in the cytokine signatures that are not detectable by conventional statistical approaches.

Gehan–Breslow–Wilcoxon test

This cumulative frequency analysis is usually employed to compare survival curves. The method can be applied to identify relevant differences in the cumulative frequency of subjects with a given biomarker serum level among groups. The dashed line underscore when the number of patients achieved 80% of the sample size and at the significant differenced at $p<0.05$ highlighted on graphs by brackets between groups as shown in Figure 1. The Graphpad Prism 5.0 software (San Diego, CA) was used data analysis and graph arts.

Biomarker signature analysis

The use of this approach to identify relevant differences in the chemokine/cytokine/LT signatures between the groups has been adapted from a pioneering study by Costa-Silva et al. (2014). Initially, serum levels of cytokines/chemokines/leukotrienes were initially classified as ''low'' or ''high'' based on the global median (as the cut-off) as proposed by Costa-Silva et al. (2014). Each data set was assembled in gray-scale diagrams in order to calculate the frequency ''high producers'' within each clinical group. Relevant frequencies were considered when above 50% of the study group. Following, ascendant biomarkers signatures for AC, pHAM and HAM/TSP were assembled and overlaid to point out for each clinical groups the biomarkers with relevant ''high'' levels ($\geq$50%) as highlighted by black rectangles. The Graphpad Prism 5.00 software was used for graph arts.

Serum biomarker network

To evaluate the association between chemokine, leukotrienes and cytokines, the Spearman's correlation test and significance were calculated and statistically significant differences were considered if p value <0.05. The correlation index (r) was used to categorize the correlation strength as negative ($r<0$), moderate ($0.36>r<0.67$) and strong ($r>0.68$) as proposed by Taylor (1990) (Figure 4). The Graphpad Prism 5.0 software was used for data analysis and the Cytoscape (version 2.8) used for assembling networks.

Logistic regression and classification tree

To establish a relationship between HTLV-1-associated disease and changes in serum biomarker, we fitted the stereotype ordinal logistic regression model, $\log[\pi_j/\pi_{AC}] = \alpha_j + \emptyset_j\beta_i x_i$, where $j=2,3$ represents the pHAM and HAM/TSP classes, respectively, and i represents the biomarker expression, and estimate the value of β as proposed by Anderson (1984). We used the distributions of variables in the control group for categorization of the biomarkers and log-likelihood method for to test the coefficient significance. In order to classify the elements of the sample and to apply the data set in clinical practice we used discriminant analysis and classification and regression tree (CART), respectively. A result was considered statistically significant when the $p<0.05$. We performed the statistical analyses with the VGAM, Rpart, Rattle, Rpart.plot, RColorBrewer, Party, Partykit Caret packages of the R statistical software (Version 2.15.3; http://www.r-project.org).

Results

The concentration of chemokines, leukotrienes and cytokines is altered in patients with different clinical status of HTLV-1 infection

Figure 1 shows the results of concentration of chemokines, leukotrienes and cytokines in the sera of asymptomatic (AC), putative HAM/TSP (pHAM) and HAM/TSP (HAM/TSP) patients. The gray rectangles express the concentration range in healthy blood donors (first and third tertiles). It was possible to observe that the HAM/TSP group presented statistically higher levels of CXCL9, CXCL10, CysLT, IL-6, IFN-γ, TNF-α when compared to pHAM. In parallel, the levels of CysLT, IL-6, IFN-γ, TNF-α and IL-4 in HAM/TSP were significantly higher when compared to AC.

Biomarker cumulative curves analysis demonstrated a predominant pro-inflammatory profile in HAM/TSP patients

Figure 2 shows the cumulative frequency (%) of subjects according to their chemokine, leukotriene and cytokine

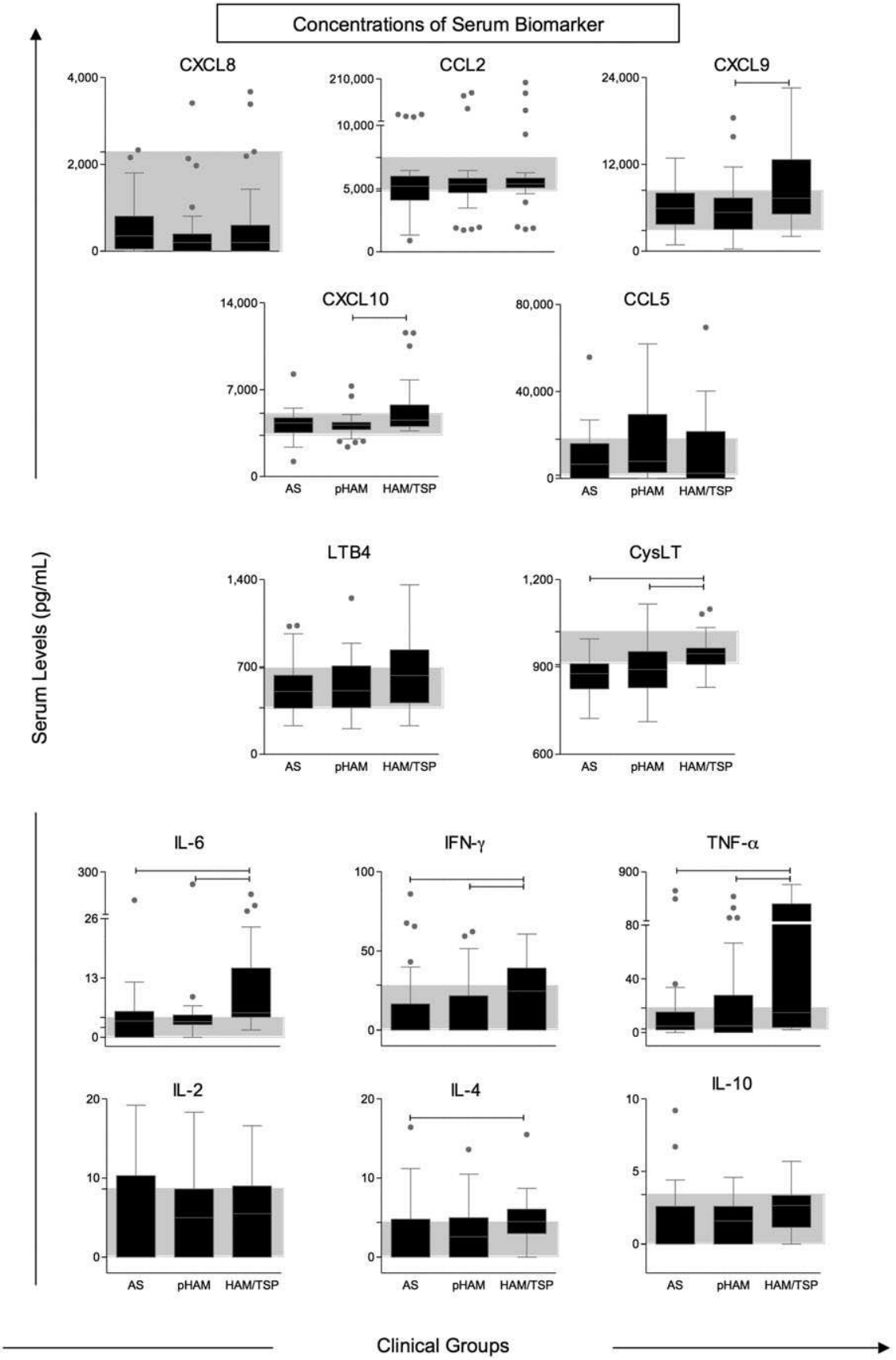

Figure 1. Quantitative biomarker measurements were performed to identify the serum levels of IFN-γ, TNF-α, IL-10, IL-6, IL-4, IL-2, CXCL10, CCL2, CXCL9, CCL5, CXCL8, LTB_4 and CysLT as described in the ''Materials and methods'' section. Results are expressed as box-and-whiskers plot. Gray rectangles demonstrate the range of concentrations observed in healthy blood donors (first and third quartiles). Statistical differences at $p < 0.05$ are displayed as connecting lines.

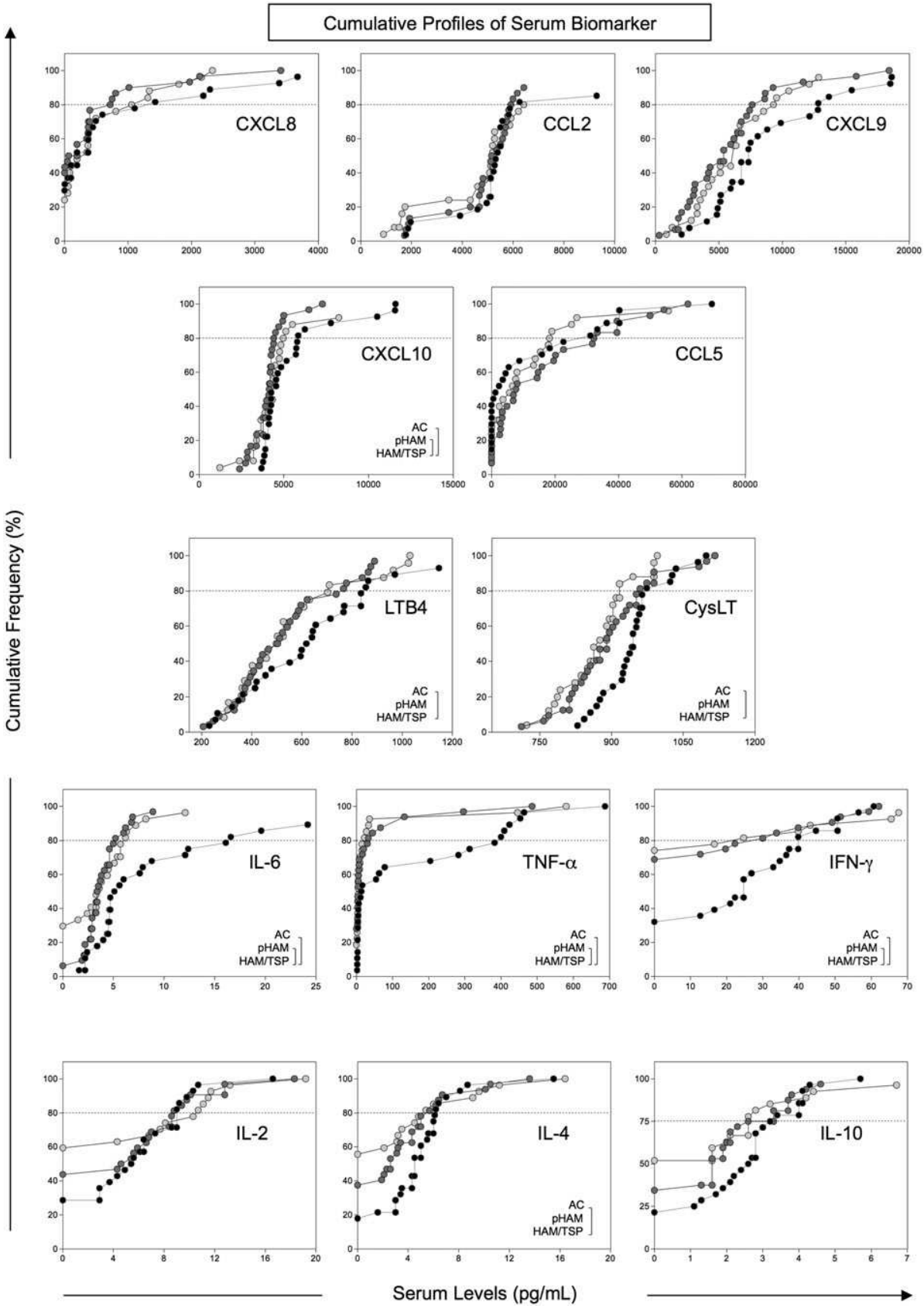

Figure 2. Cumulative frequency (%) of serum chemokines, leukotrienes and cytokines in HTLV-1 infected patients and healthy controls. HTLV-1 patients were categorized according to their clinical records as asyptomatic carrier (AC=◯), patients with putative HTLV-1 associated mielopathy (pHAM=◉) and patients with HTLV-1 associated mielopathy/tropical spastic paraparesis (HAM/TSP=●). The results are expressed as cumulative percentages of subjects with a given serum level of each biomarker expressed as pg/mL. The statistical analysis was carried out by Gehan–Breslow–Wilcoxon Test and significant differences among groups at $p < 0.05$ highlighted by brackets on the graphs. The dashed line underscore when the number of patients achieved 80% of the sample size.

sera levels. Data analysis was performed using the Gehan–Breslow–Wilcoxon test to identify differences between sets of cumulative curves. Dashed lines were used to illustrate the maximum sera levels of each biomarker produced by 80% of individuals in each clinical group. Data analysis demonstrated that HAM/TSP group has a CXCL10 cumulative curve that is shifted toward higher values as compared to AC and pHAM groups. No difference was observed to CXCL8, CCL2, CXCL9 and CCL5 cumulative curves.

HAM/TSP groups also demonstrated distinct LTB_4 cumulative curve shifted to higher values as compared to AC groups. However, AC clinical group showed CysLT cumulative curve deviated toward reduced values as compared to HAM/TSP groups. CysLT cumulative curves for pHAM were also with frequency peak in lower levels of this molecule as compared to healthy controls.

The analysis of cytokine demonstrated that the inflammatory cytokines IL-6, TNF-α and IFN-γ cumulative curves were significantly shifted toward higher serum levels in HAM/TSP group as compared to AC and pHAM groups. Moreover, HAM/TSP also displayed cumulative curve peaks in higher levels for IL-4 as compared to AC groups. No difference was observed for IL-2 and IL-10 cumulative curves.

Enhanced frequency of cytokine and leukotrienes ''high-producers'' was observed in HAM/TSP patients

Serum levels of cytokines/chemokines/leukotrienes were initially classified as ''low'' or ''high'' based on the global median (Figure 3). The number of subjects for each biomarker varied depending on the total number of tested samples included in each group (Figure 3A). Each data set was assembled in black and white diagrams in order to calculate the frequency of ''high producers'' within each clinical group (Figure 3A). After establishing the frequency of high producers for each biomarker, these frequencies were organized in an ascendant fashion and a curve of biomarkers was built for each clinical group. In order to select the relevant biomarkers for each group, it was established a 50% cut-off corresponding to the frequency of high producers among groups (Figure 3B). Data analysis demonstrated that the majority of serum biomarkers tested in HAM/TSP remained above the 50% threshold (CXCL10, IL-2, CCL2, TNF-α, IL-10, CXCL9, LTB_4, IFN-γ, IL-4, IL-6 and CysLT), while only few of them were above this threshold in AC (CXCL8 and CXCL10) and pHAM (IL-2, CCL2 and CCL5) curves. Below the ascendant curve, it is highlighted in black the biomarkers which were above 50% (Figure 3B).

A strong connection of the pro-inflammatory/ regulatory network is observed in AC, whereas CysLT seems to interfere in the IL-4/IL-10 regulatory axis in HAM/TSP patients

Aiming at accessing the dynamics of interaction between chemokines, leukotrienes and cytokines in HTLV-1 infected patients; we have assembled the biomarkers network based on the significant Spearman correlation indices. Black circles refer to elevated frequency of high producers of this

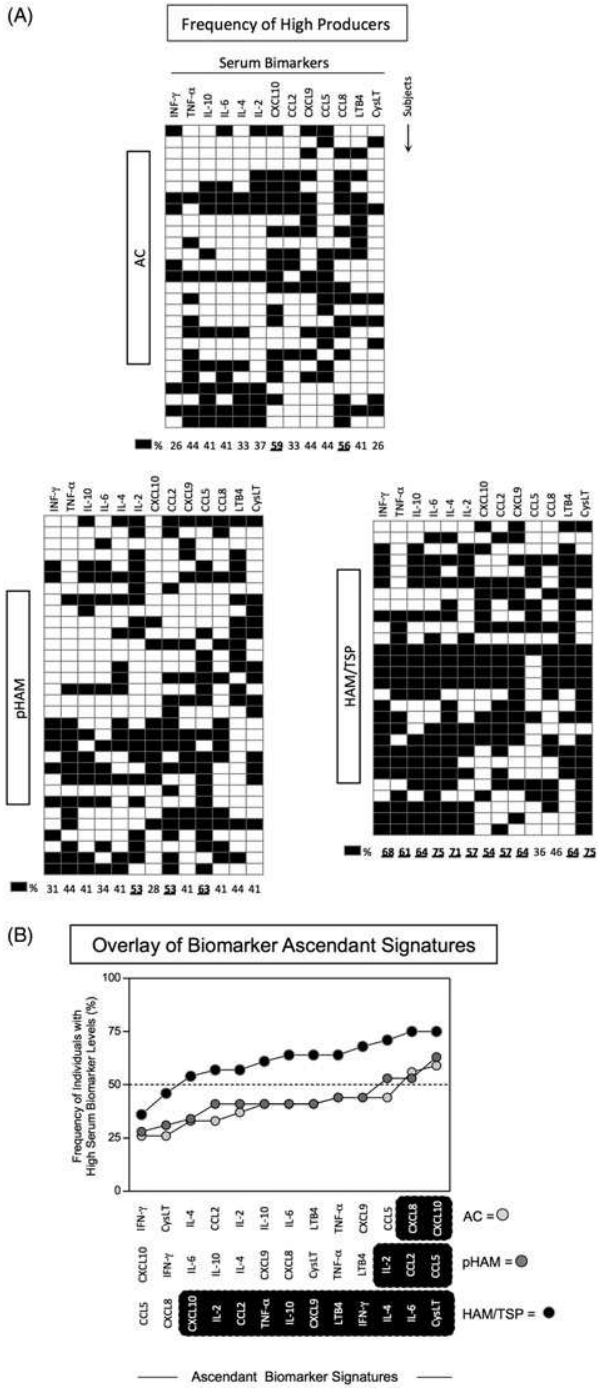

Figure 3. Establishment of serum biomarkers ascendant curve of the frequency of ''high producers'' in HTLV-1 infected patients. Quantitative biomarker measurements were performed to identify the serum levels of IFN-γ, TNF-α, IL-10, IL-6, IL-4, IL-2, CXCL10, CCL2, CXCL9, CCL5, CXCL8, LTB_4 and CysLT. (A) Black-and-white diagrams were used to assemble to calculate the final frequency of ''high producers'' for each clinical group. Relevant frequencies of ''higher producers'' (≥50%) are highlighted by underline format. Subjects were categorized as low (white) or high (black) producers based on their serum biomarker level, according the global data median distribution. The HTLV-1 patients were categorized according to their clinical records as asyptomatic carrier (AC), patients with putative HTLV-1 Associated Mielopathy (pHAM) and patients with HTLV-1 Associated Mielopathy/Tropical Spastic Paraparesis (HAM/TSP). ND, not determined levels. (B) Overlay of ascendant biomarkers signatures of AC(◯), pHAM(◉), HAM (●) was assembled and the biomarkers with frequency higher or equal to the 50th percentile are highlighted by black rectangles in the panel below the curve.

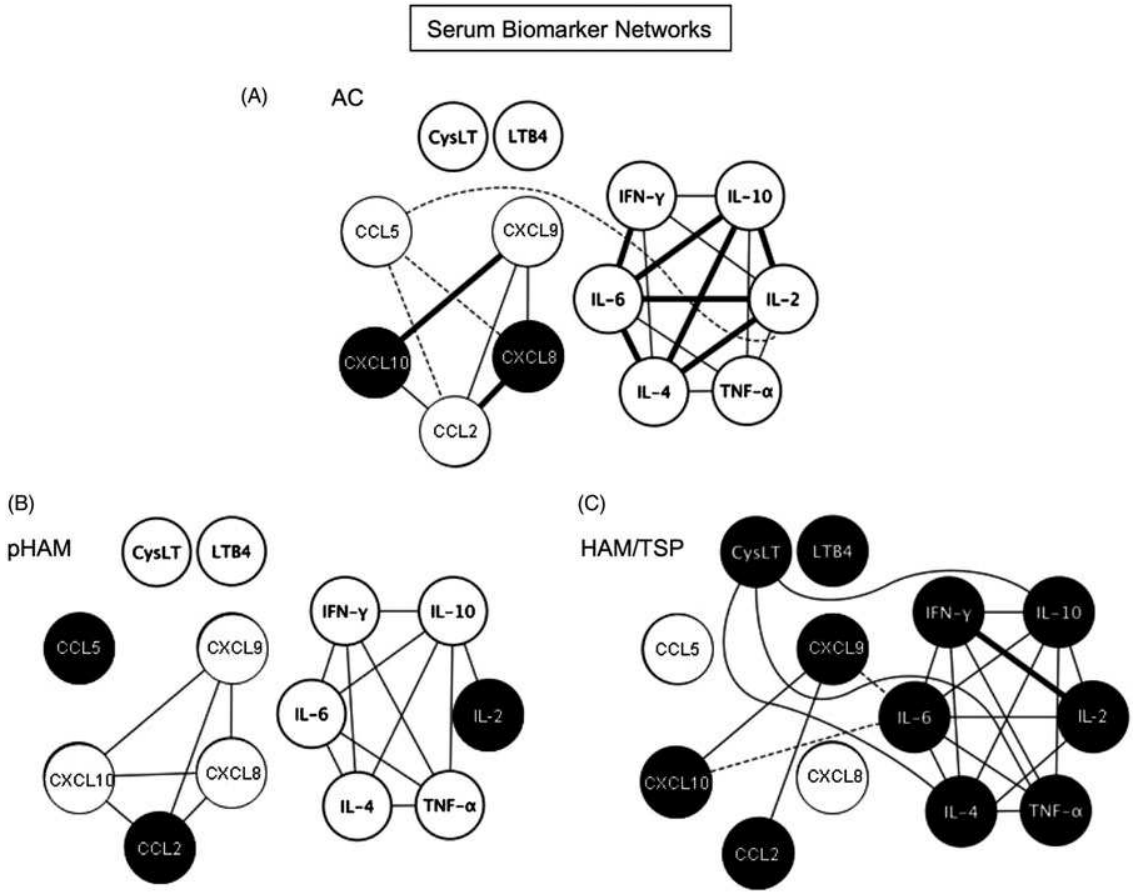

Figure 4. Biomarker networks in AC, pHAM and HAM/TSP. (A) Chemokine, leukotriene and cytokine nodes were assembled to point out significant differences in the serum levels (low=○ and high=● producers) as well as the biomarkers correlation indexes among groups (negative _ _; moderate ____ and strong ▬ positive correlation).

biomarker (Figure 4). AC presented a strongly connected pro-inflammatory/regulatory cytokine network with IL-4 and IL-10 representing central hinges, as demonstrated by the axes IL-6/IL-4/IL-2 and IL-6/IL-10/IL-2 (Figure 4A). No participation of leukotrienes CysLT and LTB_4 was observed in the AC or pHAM networks (Figure 4B).

In HAM/TSP patients, there was a clear up-regulation of pro-inflammatory cytokines, including CysLT, LTB_4, IL-6, TNF-α and IFN-γ with a relevant bridge composed of a strong correlation between two essential mediators such as IFN-γ and IL-2. Although there was a parallel increase in the frequency of high serum levels of IL-4 and IL-10, a clear arm of CysLT counterbalanced the putative regulatory activity of IL-4 and IL-10. Moreover, a complementary arm between CysLT and TNF-α further favored a shift of the immune response toward a predominant pro-inflammatory response in HAM/TSP patients (Figure 4C).

An additional analysis of biomarker connections revealed that despite the differences in the magnitude and intensity of the correlations, some preserved axes of pro-inflammatory/regulatory cytokines such as IL-6/IL-4/IL-10/IL-2/IFN-γ and chemokines CXCL10/CXCL9/CCL2/CXCL8 could be observed in all groups (Figure 4). This analysis showed that, independently of the HTLV-clinical status, there was a general pro-inflammatory/regulatory axis composed of IL-6/TNF-α counterbalanced by IL-10 and IL-4 with contributions of IFN-γ and IL-2 (Figure 4). The hallmark of HAM/TSP-immunological profile was the inclusion on an additional link mediated by CysLT with TNF-α, IL-10 and IL-4. Interestingly, a progressive loss of links between pairs of chemokines can be observed from AC to HAM/TSP clinical groups.

Establishing an algorithm of serum biomarkers for follow up/diagnosis of disease morbidity

The logistic regression analysis selected IFN-γ, TNF-α, IL-10, IL-6, IL-4 and CysLT biomarkers for modeling ($p < 0.25$). Table 1 summarizes the results of first stereotype regression analysis. Among these selected biomarkers, the best settings were with IL-6 and CysLT biomarkers. According to the $\log\left[\pi_j/\pi_{AC}\right] = \alpha_j + \emptyset_j\beta_i x_i$, j=1, 2 and i=IL-6 and CysLT model (Anderson, 1984) with identifiability constrains $\left(\emptyset_{HAM/TSP} = 1, \emptyset_{AC} = 0\right)$, we estimate $\emptyset_{pHAM} = 0.70$ and β_i values, and set models $\log\left[\pi_{HAM/TSP}/\pi_{AC}\right] = 2.34 + (-0.83)x_{IL6} + (-1.06)x_{CYST}$ for HAM/TSP and $\log\left[\pi_{pHAM}/\pi_{AC}\right] = 2.77 + (-0.83)x_{IL6} + (-1.06)\ x_{CYST}$ for pHAM. The model log-likelihood was −81.92 on 168 degrees of freedom. Deviance residuals of model indicated general goodness of fit of the model to the data obtained. Table 2 summarizes the results of final stereotype regression analysis.

Table 1. Estimated coefficients, standard errors, *z*-scores, and two-tailed *p*-values for the fitted stereotype regression model.

Coefficients	Estimate	SE	*z*-Score	*p* Value
$\emptyset_{AC}$	0[a]	–	–	–
$\emptyset_{pHAM}$	0.80	0.18	4.38	<0.001
$\emptyset_{HAM/TSP}$	1[a]	–	–	–
α_{pHAM}	2.77	0.90	3.07	0.0002
$\alpha_{HAM/TSP}$	2.57	0.87	2.95	0.0003
β_{IFN}	−0.46	0.29	−1.56	0.1184
β_{TNF}	−0.15	0.30	−0.51	0.6074
β_{IL10}	0.63	0.41	1.54	0.1232
β_{IL6}	−0.70	0.38	−1.83	0.0667
β_{IL4}	−0.27	0.29	−0.92	0.3572
β_{CYST}	−1.04	0.32	−3.19	0.0001
Log-likelihood: −79.82 on 164 degrees of freedom				

SE, standard error.
[a]Identifiability constraints.

Table 2. Estimated coefficients, standard errors, z-scores, and two-tailed *p*-values for the final fitted stereotype regression model.

Coefficients	Estimate	SE	*z*-Score	*p* Value
$\emptyset_{AC}$	0[a]	–	–	–
$\emptyset_{pHAM}$	0.70	0.17	3.94	<0.0001
$\emptyset_{HAM/TSP}$	1[a]	–	–	–
α_{pHAM}	2.77	0.82	3.37	<0.0001
$\alpha_{HAM/TSP}$	2.34	0.82	2.82	0.0004
β_{IL6}	−0.83	0.30	−2.75	0.0007
β_{CYST}	−1.06	0.32	−3.33	<0.0001
Log-likelihood: -81.92 on 168 degrees of freedom				

SE, standard error.
[a]Identifiability constraints proposed by Anderson (1984).

Table 3. Results of the logistic regression analysis involving HTLV disease and IL6 and CYST predictors.

	OR pHAM	OR HAM/TSP
IL-6	0.55	0.43
CysLT	0.47	0.34

Table 4. Summary of quadratic discriminant analysis between HTLV disease groups for predictors IFN, TNF, IL10, IL6, IL4 and CYST.

Put into Group	True group AS	pHAM	HAM/TSP
Classification			
AS	22	10	8
pHAM	3	16	4
HAM/TSP	2	6	16
Total *N*	27	32	28
N correct	22	16	16
Proportion	0.815	0.500	0.571
N=87; *N* correct = 54; proportion correct = 0.621			
Classification with cross-validation			
AS	19	12	9
pHAM	4	11	6
HAM/TSP	4	9	13
Total N	27	32	28
N correct	19	11	13
Proportion	0.704	0.344	0.464
N=87; *N* correct = 43; proportion correct = 0.494			

From the final model above, we calculated the odds ratio (OR) of the IL-6 and CysLT predictors by the $e^{\emptyset\beta}$ formula (Lunt, 2004). Table 3 represents the OR values for the selected predictors, with confidence intervals and values *p*. The results indicate substantial decreases in pHAM and HAM/TSP odds of the development related to both IL-6 as CysLT, suggesting a protective role of these biomarkers.

The results of the discriminant analysis of predictors selected by logistic regression are shown in Table 4. Sixteen elements of pHAM group and 14 elements of HAM/TSP group were classified incorrectly, resulting in the following percentage of correct answers: 50% for pHAM group and 57% for HAM/TSP group. These hit ratios indicate limited quality of the discriminant function of IFN-γ, TNF-α, IL-10, IL-6, IL-4 and CysLT predictors between pHAM and HAM/TSP groups.

The method CART proposed for clinical practice classification of the IFN-γ, TNF-α, IL-10, IL-6, IL-4 and CysLT biomarkers created a rule among these predictors to identify the three clinical groups of HTLV disease: AC, pHAM and HAM/TSP (Figure 5). The first initialization variable (''root node'') was CysLT. Using the method of CART, it was possible to propose a classification for each of the biomarkers, meaning that a rule was created to identify the three clinical groups of HTLV disease: AC, pHAM and HAM/TSP. If we count the number of patients with categorized values of CysLT, which are less than 1.5 and at the same time have IL-6 less than 0.5, we will find 11 AC patients. However, if we count the number of patients with categorized values of CysLT, which are greater than 1.5 and at the same time have IFN-γ greater than 0.5, we will find 18 HAM/TSP patients. Between the extremes AC and HAM/TSP shown in Figure 5, combinations of values of TNF-α and IL-4 biomarkers greater than 2.5 and 0.5, respectively, with IL-6 values greater than 0.5 favored the pHAM group.

Discussion

During the chronic infection by HTLV-1, the virus induces a strong activation and proliferation of many subsets of cells, especially T cells. HTLV-1 preferentially persists in the host by clonal expansion of $CD4^+$ T-cells of the infected host and by viral synapses (Sibon et al., 2006; Umeki et al., 2009). There are evidences that the immunological profile of HAM/TSP patients is composed by a robust pro-inflammatory response (Zane et al., 2009), contrasting with ATL patients. Increased levels of Type-1/2 cytokines such as IFN-γ, TNF-α, IL-1β, IL-2, IL-6, IL-9 and IL-13 are found in HAM/TSP (Bangham & Osame, 2005; Goon et al., 2002, 2003; Jacobson et al., 1990; Montanheiro et al., 2009), whereas asymptomatic HTLV-1 carriers display a balanced response characterized by high frequency of IL-10 secreted by $CD4^+$ and $CD8^+$ T-cells (Brito-Melo et al., 2001).

Doubtless, there is an essential role of pro-inflammatory and regulatory mediators involved in the progression of disease as well as a protection against the development of inflammatory symptoms. Even though the immunological status of HAM/TSP and the AC have been scrutinized, some patients that have borderline diagnosis are difficult to

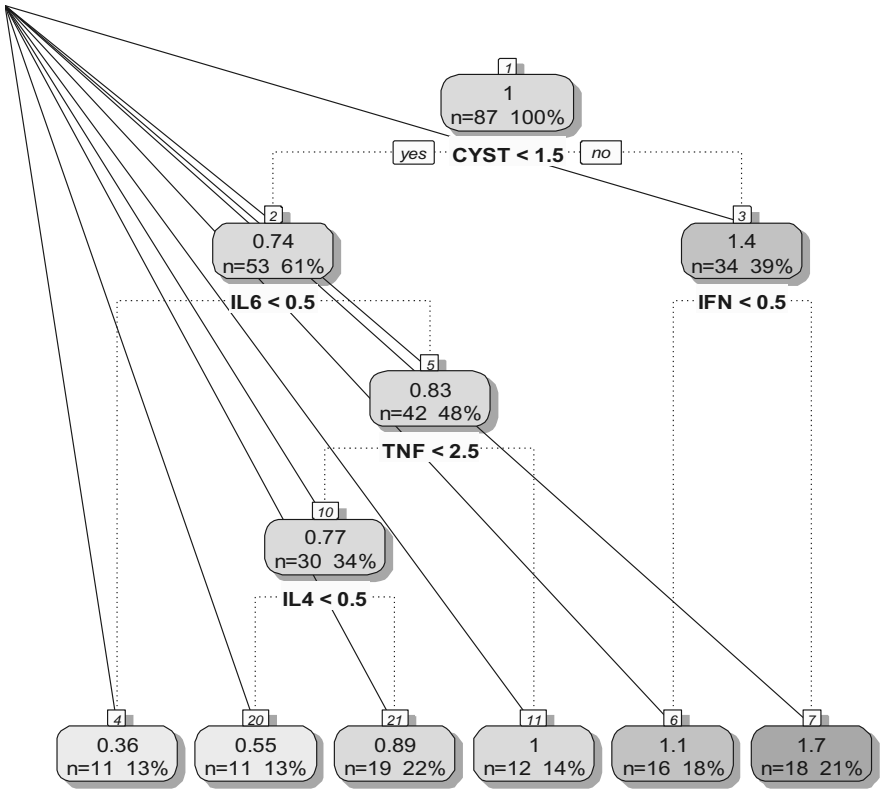

Figure 5. Classification and regression trees (CARTs) of the selected biomarkers: IFN-γ, TNF-α, IL-10, IL-6, IL-4 and also the leukotriene CysLT in the identification of AC, pHAM and HAM/TSP groups. *N*=sample size; the number on top correspond to odds ratio and the percentage corresponds to the group frequency in that condition.

characterize by the laboratorial methods currently available. The HTLV-1 proviral load has been largely utilized as a prognostic laboratorial biomarker, however, HTLV-1-infected individuals without myelopathy can present high HTLV-1 proviral load, which prevents the use of this molecular test from being the gold standard to define HAM/TSP (Taylor, 1998). In addition, despite the clear evidence of a vigorous inflammatory response in HAM/TSP, there is no clear standardization of the immunological markers that are suitable for the follow-up of either HTLV-1-ACs or putative HAM/TSP individuals.

In the present study, the cytokine, chemokine and leukotriene secretion profile of healthy blood donors, ACs, putative HAM/TSP and HAM/TSP were evaluated and refined diagnostic measurements were performed in order to establish an immunological signature that would lead to the design of a panel of markers for monitoring disease progression. Networks composed of the correlations among cytokines, chemokines and leukotrienes were built for each group in order to verify the differences in the interactions. In addition, the CARTs proposed for clinical practice allowed the construction of an algorithm to discriminate AC, pHAM and HAM/TSP individuals with the elected biomarkers: IFN-γ, TNF-α, IL-10, IL-6, IL-4 and CysLT. To the best of our knowledge, this is the first proposal of an algorithm based on serum biomarkers applied to the clinical management of HTLV-1-infected people.

The results obtained in the present study confirm previous results from other cohorts (Muniz et al., 2006), showing that there is a strong pro-inflammatory response in HAM/TSP. The secretion profile of inflammatory cells produced by HAM/TSP group was established in order to create an immunological signature of these subjects.

The cumulative frequency of the serum concentration of IL-6, TNF-α, IFN-γ and CXCL10 was higher in the HAM/TSP when compared to the AC and pHAM groups. A higher cumulative frequency of IL-4, LTB_4 and CysLT in the HAM/TSP when compared to AC group was observed. The immunological signature delineated for the HAM/TSP group was mostly composed by increased secretion of the cytokines IL-4, IL-6, IFN-γ, TNF-α, IL-10, and also LTB_4 and CysLT, which supports the present findings obtained by the cumulative frequency of the serum concentration of these molecules.

These cytokines have important pro-inflammatory activity and performs a central role in the coordination of the inflammatory response. Moreover, classical studies have already demonstrated that higher concentration of IL-6 and TNF-α can be observed in HTLV-1-infected individuals (Jeffery et al., 1999; Lal & Rudolph, 1991).

We have found that IL-4, a cytokine involved in controlling inflammation, was higher in the HAM/TSP group. This finding is not in accordance with previous studies, which showed that HAM/TSP evolves to a prominent and uncontrolled pro-inflammatory process (Brito-Melo et al., 2001; Jeffery et al., 1999; Nishimoto et al., 1990). The serum concentration of IL-10, another regulatory cytokine, was high in HAM/TSP group as well as in the other HTLV-1 groups. It is known that the HTLV-1-ACs have an immune-modulated response that controls inflammation (Starling et al., 2013). Maybe, the HAM/TSP-signature of pro-inflammatory profile associated to IL-4 and IL-10 is yet an attempt of the

immunological system of keeping the control of the inflammation.

When taking into consideration the interaction between molecules, the presence of CysLT reinforces the role of the inflammatory axis since this molecule is involved in events such as increased vascular permeability and cell trafficking. Interestingly, the pHAM group demonstrates an intermediate profile between AC and HAM/TSP. Even without strong correlations and leukotriene interactions, the increased production of RANTES and IL-2 in this group may suggest a possible switching to a pro-inflammatory profile.

The analysis of the HTLV-1 groups with and without myelopathy clarified the essential role of pro-inflammatory and regulators mediators involved in the control of viral load and progression of the disease. Besides, the presence of CysLT composing the inflammatory net may contribute to the events such as increased vascular permeability and cell trafficking. Considering that leukotrienes and TNF-α are key biomolecules in the development of the inflammatory process that leads to HAM/TSP as identified in this study, it is essential to consider the possibility of testing the selective leukotriene receptor antagonists and anti-TNF-α antiboby therapy for the treatment of patients in future larger studies.

The present results demonstrated that studying the cytokine microenvironment is important to the analysis of the balance of proinflammatory/regulatory biomarkers. The correlation among these mediators brought more information about the role of this network of molecules analyzed as possible biomarkers of neurological disease progression during HTLV-1 infection.

Conclusion

The molecules IFN-γ, TNF-α, IL-10, IL-6, IL-4 and also the leukotriene cysteinyl leukotrienes are demonstrated as important biomarkers of HAM/TSP and should be taken together as a combined algorithm to follow and identify the HTLV-1-AC that may evolve to neurologic damage.

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Declaration of interest

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