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ORIGINAL ARTICLE

HspBP1 and anti-HspBP1 levels in the serum of HIV-infected individuals are associated to the disease progression

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Abstract

Aims: The objective of this research was to quantify the levels of circulating HspBP1 and anti-HspBP1 IgG in HIV-infected individuals and to correlate them with CD4 T cell counts and viral load, as well as to determine the kinetics of those proteins during acute phase.
Methods and Results: Sixty serum samples from HIV-positive outpatients, thirty with high viral load and thirty with low viral load were analysed. The HspBP1 and anti-HspBP1 were quantified by ELISA. To investigate the kinetic of HspBP1 and anti-HspBp1 during the acute phase, these proteins and antibodies were quantified in samples of a commercial seroconverting HIV panel. All dosages were compared with the CD4 and CD8 T cell counts and HIV viral load. The results indicated that HIV positive outpatients presented significant increase in HspBP1 and anti-HspBP1 serum levels, compared with uninfected healthy. HspBP1 and anti-HspBP1 were negatively correlated with CD4 counts and CD4:CD8 ratio. In the acute phase, HspBP1 became significantly elevated 15 days after HIV infection.
Conclusions: These results indicate that the quantification of HspBP1 can be associated to others well-established parameters of the HIV progression.
Significance and Impact of the Study: The discovery that HspBp1 and anti-HspBp1 are associated with progression of HIV infection is new and corroborates to validate the quantification of these proteins as an additional strategy in the management of the HIV infection.

Introduction

Heat-shock proteins (HSPs) are intracellular chaperones that facilitate protein folding and trafficking and prevent protein degradation under stress conditions (Thulasiraman *et al.* 1999; Liberek *et al.* 2008). HSPs interact with polypeptides to promote their correct folding or provide an appropriate microenvironment for protein egress (Frydman 2001). Hsp70 is an ATP-dependent molecular chaperone with an ATPase domain in the N-terminus that controls the function of the C-terminus, which is the site for peptide binding (Mayer and Bukau 2005; Liu *et al.* 2010). The process of control is complex and involves other accessory proteins called co-chaperones. Hsp70-binding protein-1 (HspBP1) is a co-chaperone that inhibits the Hsp70 activity. When a peptide is chaperoned by Hsp70, a co-chaperone Hsp40 activates the Hsp70 ATPase domain to convert ATP into ADP. However, HspBP1 inhibits the ATPase function and the ability of Hsp70 to refold denatured proteins (Raynes and Guerriero 1998).

The role of HSPs in viral infection was first identified by infecting *Escherichia coli* by lambda bacteriophages (7). It was observed that HSPs were necessary to release proteins responsible for the initiation of viral replication (Liberek *et al.* 1988). Thereafter, several chaperones and

co-chaperones involved in viral infection and replication have been characterized (Filone *et al.* 2014; Manzoor *et al.* 2014; Abraham *et al.* 2015; Chen *et al.* 2017). In the acute phase of human immunodeficiency virus (HIV) infection, Hsp70 level in $CD4^+$ T cells increases at 3 h after the viral entry, after which it is detectable in the serum (Wainberg *et al.* 1997). Moreover, Hsp70 level markedly increases during HIV infection because of the presence of this protein on the surface of infected cells or within the virion. Hsp70 is one of the most well-characterized chaperones that interacts with HIV during infection (Babaahmady *et al.* 2007; Kumar *et al.* 2011). Enhanced Hsp70 expression on the surface of HIV-infected cells is accompanied by the increased production of anti-Hsp70 IgG, which decreases after the highly active antiretroviral therapy (HAART) (Kocsis *et al.* 2003). Some studies indicate that Hsp70 promotes viral replication (Taguwa *et al.* 2015; Kunihiro *et al.* 2016), whereas other studies indicate that Hsp70 has antiviral properties (Sugiyama *et al.* 2013). These two controversial hypotheses suggest that Hsp70 level alone is not a reliable marker for predicting the evolution and prognosis of HIV infection.

As Hsp70 plays a role during HIV infection, it may be suggested that HspBP1 also affects the immune response to HIV, thus making it a promising tool to assess the progression of HIV infection (Pido-Lopez *et al.* 2007; Kumar *et al.* 2011; Sugiyama *et al.* 2011). Detectable HspBP1 levels are normally present in the human serum; however, HspBP1 levels are elevated in the tissues and sera of patients with rheumatoid arthritis and breast cancer (Raynes *et al.* 2003, 2006; Souza *et al.* 2009; Sedlackova *et al.* 2011). Furthermore, circulating anti-HspBP1 IgG levels are higher in HIV-infected individuals compared with HIV-negative individuals. One study has reported that 53% HIV-negative individuals lack anti-HspBP1 IgG compared with only 24·3% HIV-infected individuals (Papp *et al.* 2005). In the present study, we quantified circulating HspBP1 and anti-HspBP1 IgG levels in HIV-infected individuals and correlated these levels with CD4+ T cell counts and viral load. In addition, we determined the kinetics of HspBP1 in the seroconversion phase of HIV infection. Our data indicate that circulating HspBP1 or anti-HspBP1 IgG levels can be used as a new marker to predict the prognosis and management of HIV infection.

Materials and methods

Samples

Sixty serum samples were obtained from HIV-positive outpatients who visited the viral quantification service centre affiliated to the Clinical Analysis Laboratory, University Hospital of Santa Maria (Santa Maria, Brazil) for viral quantification and T lymphocyte counts. All individuals enrolled in the study are all therapy naive, also at the time of blood collection they declared that they did not present any chronic disease other than HIV and were not undergoing acute phase of other infectious process. All individuals enrolled in the study had never received antiretroviral therapy. The number of $CD4^+$ and $CD8^+$ T cells and viral load in all serum samples obtained from HIV-positive outpatients were quantified at this service centre. Serum samples of HIV-negative donors (tested by enzyme-linked immunosorbent assay (ELISA) and interviewed by a physician) were obtained from the Blood Center Institute of Santa Maria. Individuals with viral load below the detection limit of the assay used for virus quantification were considered as low viral load. Individuals with detectable viral load or above limit detection were classified as high viral load. All the serum samples were kept at −80°C.

Quantification of serum HspBP1 levels

Serum HspBP1 levels were quantified by capture ELISA following a protocol previously described (Raynes *et al.* 2006) with some modifications. The immunogenic HspBP1 fragment (amino acids 84–359) and anti-Hsp BP1 IgG used in this study was kindly provided by Dr. Vince Guerriero (University of Arizona, USA). Briefly, a 96-well plate (COSTAR; Sigma-Aldrich, St. Louis, MO) was coated with 100 μl of 1 μl ml^{-1} sheep anti-HspBP1 IgG in phosphate buffer saline (PBS) at 4°C. After incubation, the plate was washed three times with PBS/ 0·05% Tween-20 and was blocked with 300 μl PBS/ 0·05% Tween-20 containing 5% nonfat dry milk (blocking solution). Next, the plate was incubated for 1 h at room temperature (RT) with shaking and washed three times with PBS/0·05% Tween-20. A standard curve in serial dilution of HspBP1 (0·625–20 ng ml^{-1}) was prepared in duplicate. The serum samples were diluted at a ratio of 1 : 5 and were added to each well. The plate was then incubated for 2 h at RT with shaking and was washed five times with PBS/0·05% Tween-20. Next, 0·01 μg ml^{-1} rabbit anti-HspBP1 IgG (100 μl per well) (Delta Biolabs, Campbell, CA) was diluted in blocking solution and added to the wells, the plate was incubated for 1 h at RT. After incubation, the plate was washed five times with PBS/0·05% Tween-20 and was incubated with 100 μl horseradish peroxidase (HRP)-conjugated goat anti-rabbit antibody (dilution, 1 : 10·000) in a blocking buffer. Next, the plate was incubated for 1 h at RT with shaking and washed five times with PBS/0·05%

Tween-20. The reaction was observed using 3,3′,5,5′-tetramethylbenzidine (TMB; Bio-Rad, Hercules, CA) and was stopped by adding 100 μl 1 mol l^{-1} H_2SO_4. Absorbance was read at 450 nm using a microplate reader (Bio-Rad). The robustness of the assay was considered that previously published by other group (Raynes *et al.* 2006).

Quantification of anti-HspBP1 IgG levels

Serum anti-HspBP1 IgG levels were determined using a protocol previously described (Papp *et al.* 2005). For this, each well of a 96-well plate was coated with 100 μl solution containing 1 μg ml^{-1} HspBP1 and was incubated overnight at 4°C. Following, the wells were washed three times with a wash solution and were blocked with a blocking solution for 1 h at RT with shaking. The serum samples were diluted with PBS at a ratio of 1 : 5, and 100 μl serum samples were added to each well. The plate was then incubated at 4°C for 4 h and was washed five times with the wash solution. Next, 100 μl of HRP-conjugated anti-human IgG (dilution, 1 : 500; Sigma-Aldrich) in a blocking buffer was added to each well, and the plate was incubated for 1 h at RT. The reaction was observed using TMB and was stopped using 100 μl H_2SO_4. Absorbance was read at 450 nm using the microplate reader (Bio-Rad).

Sensitivity and specificity of the HspBP1 and anti-HspBP1 ELISA test

The sensitivity and specificity of the ELISA assays were calculated following the parameters of Lalkhen and McCluskey (2008). The cut-off for HspBP1 and anti-HspBP1 were determined as the mean of the dosages of the 30 HIV-negative subjects. The data for true positive, false negative, true negative and false positive were from the HIV positive high VL and low VL outpatients. For the calculation of sensitivity and specificity the following formulas were used:

$$\text{Sensitivity} = \frac{\text{True pos}}{\text{True pos} + \text{False neg}}$$

$$\text{Specificity} = \frac{\text{True neg}}{\text{True neg} + \text{False pos}}$$

True pos: High VL and HspBP1 or anti-HspBP1 $\geq$ cut-off

False neg: High VL and HspBP1 or anti-HspBP1 $<$ cut-off

True neg: Low VL and HspBP1 or anti-HspBP1 $<$ cut-off

False pos: Low VL and HspBP1 or anti-HspBP1 $\geq$ cut-of

HIV seroconversion panel and HspBP1 kinetics

To determine the alterations in serum HspBP1 levels in the acute phase of HIV infection, we quantified the HspBP1 levels in samples of a commercial seroconversion panel of anti-HIV (PRB931). This panel consisted of sera from a single uninfected patient who became infected and seroconverted. It covers days 0, 2, 7, 9, 15, 28, 33, 35 and 42 after the infection, and each point of the seroconversion curve represents a specific titre of anti-HIV antibodies. In the present study, anti-HIV antibody (IgG, IgM and IgA) titres at all days of the seroconverting curve were measured using a third-generation ELISA (Bioclin, Belo Horizonte, Brasil). A cut-off value was determined to add the absorbance of a negative control to 0·160, and an index was expressed as the ratio between the absorbance of the sample and the cut-off. Results were expressed as negative when the index was <0·9, as positive when the index was >1·1, and as indeterminate when the index was between 0·9 and 1·1.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 6 software (GraphPad Software, San Diego, CA). Results are representative of three independent experiments, which means that all serum samples were tested three times and each value used for each sample represents the mean of these experiments. Data are expressed as mean ± standard error mean or median and interquartile range. Parametric variables were analysed using one-way ANOVA (analysis of variance) followed by Bonferroni test, and nonparametric variables were analysed using Mann–Whitney *U* test. Pearson correlation test was performed by assuming that the presented data followed a Gaussian distribution. Differences were considered statistically significant at $*P < 0.05$, $**P < 0.01$ and $***P < 0.001$.

Ethical statement

This study was evaluated and approved by the Human Research and Ethics Committee of the Universidade Franciscana under the number 028.2010.2. The work described here was performed in accordance with The Code of the World Medical Association (Declaration of Helsinki), and the informed consent was obtained from all participants.

Results

Sample profile: viral load and $CD4^+$ and $CD8^+$ T cell counts

This study included the serum samples obtained from HIV-positive outpatients (with low viral load (lower than

the minimum limit of detection—50 copies per mL) and high viral load—higher than 50 copies per ml) and HIV-negative individuals. In the HIV-positive group, the mean age of the outpatients with low viral load was 41·73 years and that of the outpatients with high viral load was 42·83 years (Table 1). Regarding gender, the number of men with high viral load was higher than the number of women. The mean of $CD4^+$ T cell count in the HIV-positive outpatients with high viral load was 85 cells per μl and that in the HIV-positive outpatients with low viral load was 681 cells per μl. The mean of $CD8^+$ T cell count decreased in the HIV-positive outpatients with high viral load (142·50 cells per μl) compared with that in those with low viral load (718·50 cells per μl). However, this decrease in the CD8+ T cell counts was not sufficient to rescue the CD4 : CD8 ratio, which was 0·11 in the HIV-infected outpatients with high viral load and 0·98 in those with low viral load. Viral load, $CD4^+$ T cell counts, and CD4:CD8 ratio are the well-established parameters for assessing the clinical prognosis of HIV infection and were used as associated variables to study HspBP1 and anti-HspBP1 IgG levels in the HIV-infected outpatients in the present study. More detailed information from groups of study is presented in supporting information (Tables S1 and S2).

Comparison of serum levels of HspBP1 and anti-HspBP1 between HIV positive and negative subjects

HspBP1 serum levels in 30 adult HIV-negative individuals, 30 adult HIV-infected outpatients with low viral load and 30 adult HIV-infected outpatients with high viral load were determined by ELISA. The minimum and maximum HspBP1 levels observed in HIV-negative individuals were 0·53 and 7·95 ng ml^{-1}, respectively. The minimum and maximum HspBP1 levels found in HIV-infected outpatients with low viral load were 0·52 and 12·51 ng ml^{-1}, respectively, while these values found for subjects with high viral load was 1·25 and 11·10 ng mL^{-1} respectively. The mean of circulating HspBP1 levels in the HIV-infected outpatients with high viral load (5·44 ± 0·60 ng ml^{-1}) was significantly higher (by 2·1-fold) than those found in the HIV-negative individuals (2·54 ± 0·66 ng ml^{-1}) (Fig. 1a). Moreover, the mean of circulating HspBP1 levels were significantly different ($P = 0{\cdot}032$) between groups of HIV-infected outpatients with high viral and low viral load, being 1·7-fold higher in the group with high viral load in relation subjects with low viral load (3·19 ± 0·41 ng ml^{-1}) (Fig. 1a). The sensitivity and specificity for the HspBP1 ELISA test were 73·3 and 70%, respectively.

As we observed that HspBP1 levels increased in the serum of the HIV-infected outpatients and autoantibodies against HspBP1 have been previously detected in patients with other diseases, we determined the anti-HspBP1 IgG levels in groups with high and low viral load. Anti-HspBP1 IgG levels were quantified by ELISA using the HspBP1 (84–359) fragment as antigen. Similarly to observed for HspBP1, anti-HspBP1 IgG levels increased significantly in the HIV-infected outpatients with high viral load compared with those with low viral load or HIV-negative individuals (Fig. 1b). The optical density (OD) ranged from 0·100 to 0·272 in HIV-negative group, 0·100 to 0·351 in HIV-infected outpatients with low viral load, and 0·110 to 0·488 in group of HIV-infected outpatients with high viral load. The mean OD values were 0·160 ± 0·019, 0·178 ± 0·016, and 0·257 ± 0·007 for HIV-negative individuals, HIV-infected outpatients with low viral load and with high viral load, respectively. The sensitivity determined for the anti-HspBP1 ELISA was 60% and the specificity was 66%. Together, these results indicate that HspBP1 and anti-HspBP1 IgG levels are significantly increased in the serum of HIV-infected individuals and are possibly associated with the viral load.

Table 1 Subject's characteristics

	Low viral load (n = 30)	High viral load (n = 30)	P
Age (years)	41·73 ± 11·70	42·86 ± 11·32	0·57
Gender (male/female)	11/19	20/10	$0{\cdot}039^{NS}$
CD4 T cells (cells per μl)	681·00 (254·75)	85·50 (137·00)	<0·001***
CD4 T cells (%)	34·50 (8·00)	7·00 (9·00)	<0·001***
CD8 T cells (cells per μl)	718·50 (381·50)	142·50 (700·61)	0·008**
CD8 T cells (%)	37·00 (7·50)	63·50 (15·75)	<0·001***
CD4 : CD8 ratio	0·98 (0·38)	0·11 (0·15)	<0·001***

Parametric data are presented as mean ± standard deviation. Variables between groups were compared through unpaired Student *t*-test (P < 0·05). *P < 0.05; **P < 0.01; ***P < 0.001; NS, not significant.

Nonparametric data are presented as median (interquartile range) and differences between groups were compared through Mann–Whitney *U* test (P < 0·05).

Age was analysed by qui-squared.

HspBP1 and anti-HspBP1 IgG levels are inversely correlated with the $CD4^+$ T cell count

The $CD4^+$ T cell count is a valuable parameter for assessing the prognosis of HIV infection and response to therapeutic intervention. Therefore, we compared the $CD4^+$ T cell count with HspBP1 and anti-HspBP1 IgG levels in the serum of the HIV-infected and HIV-negative individuals. The participants were subdivided according to their

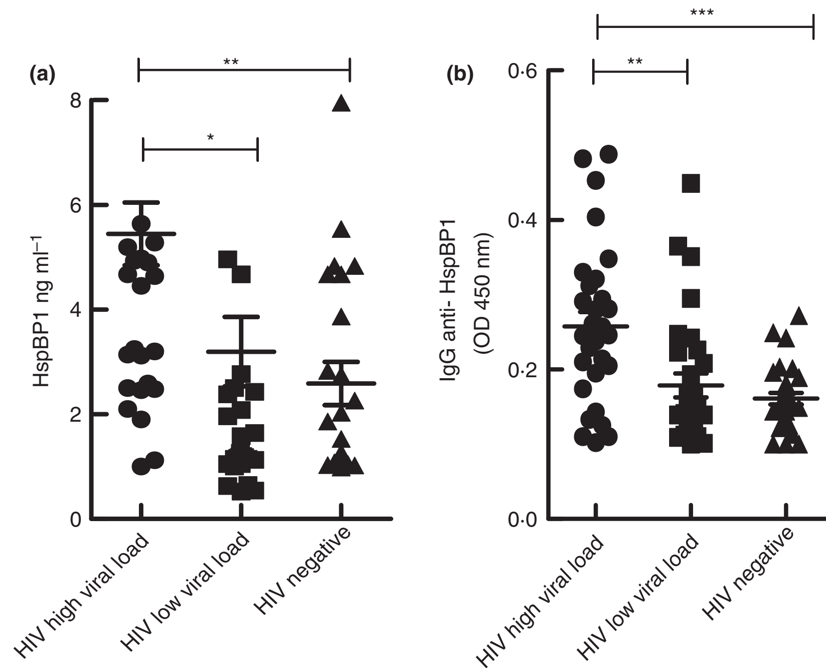

Figure 1 (a) Serum levels of HspBP1. Levels of HspBP1 were quantified by capture ELISA in serum from HIV-infected outpatients (low and high viral load) and uninfected (HIV negative). (b) Serum levels of anti-HspBP1. Levels of IgG anti-HspBP1 were quantified by indirect ELISA in serum from HIV-infected outpatients (low and high viral load) and uninfected (HIV negative). Data are expressed as means ± standard error mean (SEM) and are representative of three independent experiments (the same serum was evaluated three times). $*P < 0{\cdot}05$, $**P < 0{\cdot}01$, $***P < 0{\cdot}001$ (one way ANOVA followed by Bonferroni test).

HIV infection status (uninfected or infected) and CD4$^+$ T cell counts (<350 or >350 cells per μl). These criteria were also in accordance to the Clinical Protocol and Therapeutic Guidelines for the Management of HIV Infection in Adults (Ministério da Saúde do Brasil, 2018). Circulating HspBP1 levels increased in the outpatients with the lowest CD4$^+$ T cell count compared with those with CD4$^+$ T cell counts >350 cells per μl or with HIV-negative individuals (Fig. 2a). The mean circulating HspBP1 levels were 5·44, 3·19 and 2·59 ng ml^{-1} in the outpatients with CD4$^+$ T cell count <350 cells per μl, >350 cells per μl, and HIV-negative individuals, respectively. These data are consistent with the data presented in Fig. 1a that showed that serum levels of HspBP1 are higher in individuals presenting indications of disease progression., i.e., high viral load and low CD4$^+$ T cell count. We also compared the CD4$^+$ T cell count with the levels of anti-HspBP1 IgG. Consistently, we observed that the anti-HspBP1 IgG levels also increased in the outpatients with low CD4$^+$ T cell count (Fig. 2b).

HspBP1 and anti-HspBP1 are positively correlated with each other and negatively correlated with T cell parameters

As we observed a significant increase in HspBP1 and anti-HspBP1 IgG levels in the serum of the HIV-infected outpatients and an association of HspBP1 and anti-HspBP1 IgG levels with CD4$^+$ T cell counts and CD4 : CD8 ratio, we analysed the correlation among these variables using all HIV-infected groups (high and low viral load). A significant positive correlation was observed between circulating HspBP1 and anti-HspBP1 IgG levels ($r = 0{\cdot}2699$, $P = 0{\cdot}037^*$; Fig. 3a). As expected, a significant inverse correlation was observed between circulating HspBP1 levels and CD4$^+$ T cell count ($r = -0{\cdot}2595$, $P = 0{\cdot}0453^*$; Fig. 3b). Moreover, a negative correlation was observed between HspBP1 levels and CD4: CD8 ratio ($r = -0{\cdot}2664$, $P = 0{\cdot}0397$; Fig. 3c). Anti-HspBP1 IgG levels increased with a decrease in the CD4$^+$ T cell count during HIV infection progression, indicating a negative correlation among these variables ($r = -0{\cdot}38827$, $**P = 0{\cdot}0025$). Together, these analyses indicate that increased circulating HspBP1 and anti-HspBP1 IgG levels are associated with parameters related to unfavourable prognosis of patients with HIV infection.

HspBP1 levels after anti-HIV seroconversion

All the serum samples obtained from the HIV-infected outpatients included in this study were collected in the chronic phase of HIV infection. Therefore, we used a commercial HIV seroconversion panel to determine the kinetics of HspBP1 and anti-HspBP1 IgG during HIV infection. First, we determined the anti-HIV antibody (IgM, IgG and IgA) reactivity with the seroconversion panel. The reactivity of each sample was determined as a ratio between the absorbance and the cut-off and was presented as an index. The tested serum maintained a nonreactive status until point four in the seroconvertion

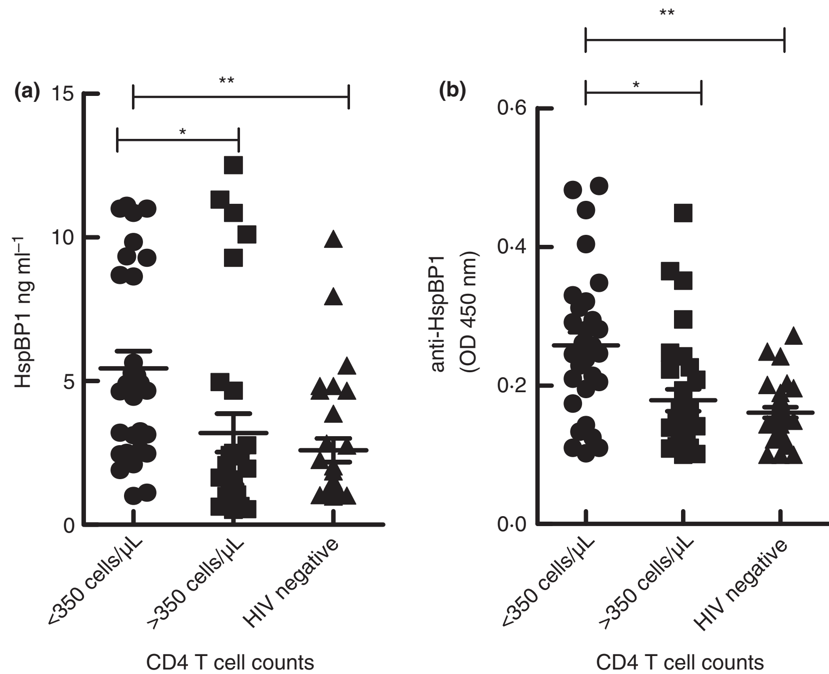

Figure 2 Association between HspBP1, anti-HspBP1 and CD4 T cell counts. (a) Levels of HspBP1 were quantified by capture ELISA in serum from HIV-infected outpatients and associated with CD4 T counts according <350 cells per μl, >350 cells per μl or HIV negative. (b) Levels of anti-HspBP1 IgG were quantified by indirect ELISA in serum from HIV-infected outpatients and associated with CD4 counts according <350 cells per μl, >350 cells per μl or HIV negative. Data are expressed as means ± SEM and are representative of three independent experiments (The same serum was evaluated three times). $*P < 0{\cdot}05$, $**P < 0{\cdot}01$ (one way ANOVA followed by Bonferroni test).

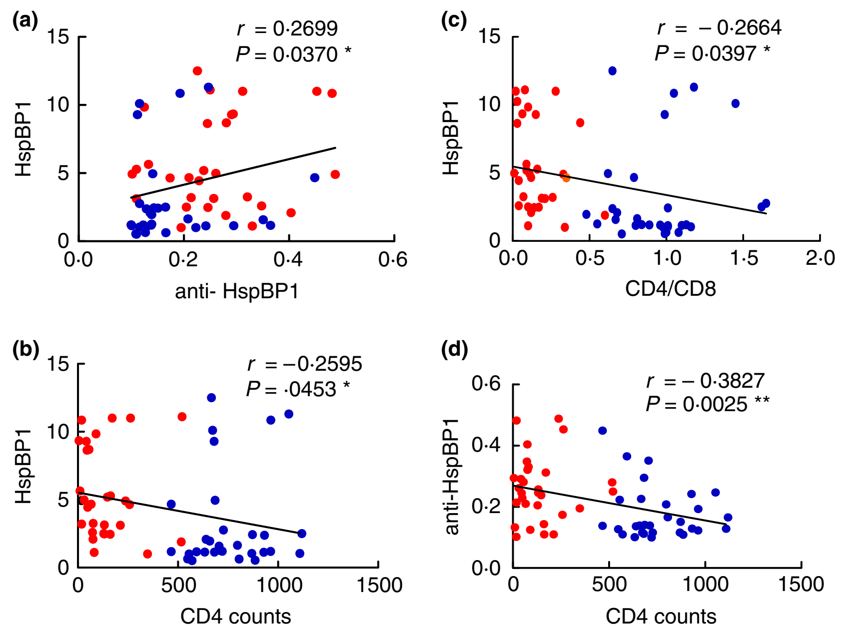

Figure 3 Correlation among the variables HspBP1, anti-HspBP1, CD4 T cell counts and the CD4/CD8 ratio. The data of 60 HIV positive outpatients and HIV negative individuals were correlated using the Pearson correlation test. (a) Correlation between HspBP1 and anti-HspBP1. (b) Correlation between HspBP1 and CD4 counts. (c) Correlation between HspBP1 and CD4:CD8 ratio. (d) Correlation between anti-HspBP1 and CD4 T cell counts. Differences were considered statistically significant when $P < 0{\cdot}05$. Red rods: high viral load; blue rods: low viral load. [Colour figure can be viewed at wileyonlinelibrary.com]

curve (day 9 after the infection) (Fig. 4a). At point six (day 28 after the infection), the status of the serum changed to anti-HIV reactive Moreover, anti-HIV antibody levels significantly increased from days 28 to 42 after the infection.

When HspBP1 levels were quantified at each point of the seroconversion curve we obtained an interesting data (Fig. 4b). From points one to four (days 0–9 after infection), the mean of HspBP1 levels was $1{\cdot}066 \pm 0{\cdot}051$ ng ml^{-1}, which was almost similar to that found in the HIV-negative group ($2{\cdot}54 \pm 0{\cdot}66$ ng ml^{-1}). In contrast, at point nine (day 42 after the infection), the mean of HspBP1 levels was $4{\cdot}04 \pm 0{\cdot}28$ ng ml^{-1}, which was almost similar to that found in the HIV-infected outpatients with high viral load ($5{\cdot}44 \pm 0{\cdot}60$ ng ml^{-1}). We did not observe any significant alteration in anti-HspBP1

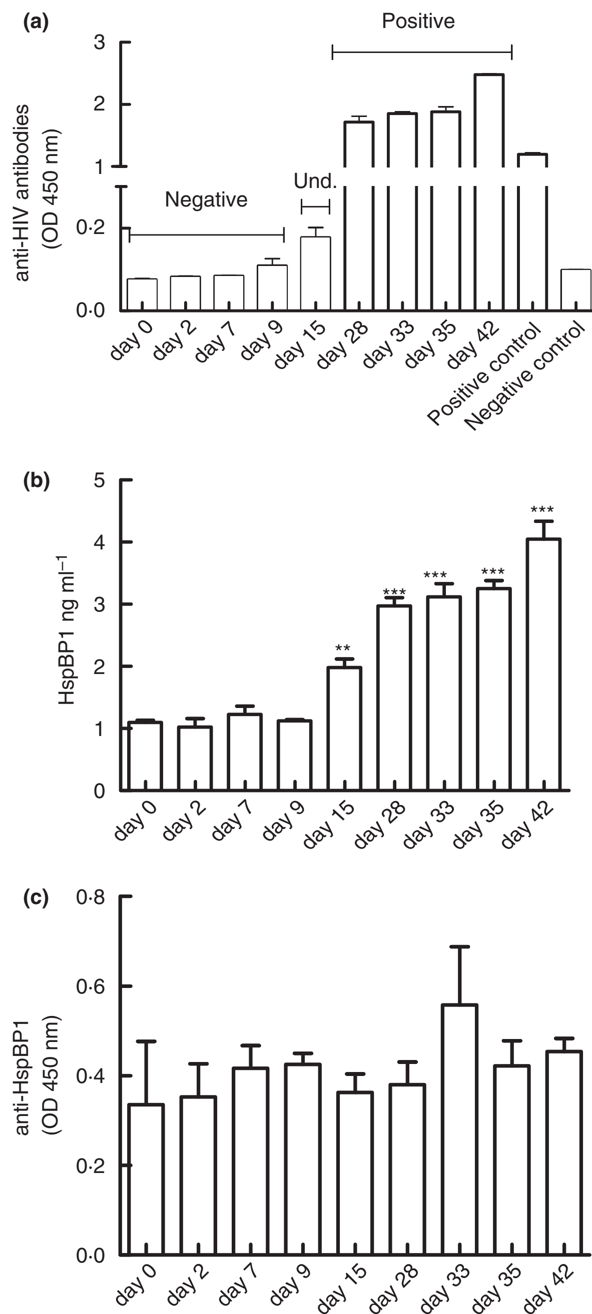

Figure 4 Kinetics of HspBP1 and anti-HspBP1 in HIV seroconversion panel. (a) Levels of anti-HIV antibodies (IgM, IgG and IgA) along the days of the HIV seroconversion panel. Index: day zero: 0·293; day 2: 0·317, day 7: 0·325; day 15: 0·686; day 28: 6·64; day 33: 7·17; day 35: 7·29; day 42: 9·6; positive control: 4·63; negative control: 0·39. Results were interpreted as negative <0·9; positive >1·1; undetermined 0·9 to 1·1. (b) Levels of HspBP1 in each day of the seroconversion panel. The significance was calculated using day zero as a parameter. (c) Levels of anti-HspBP1 in each day of the seroconversion panel. Data are expressed as means ± SEM and are representative of three independent experiments (The same serum was evaluated three times). $^{**}P < 0{\cdot}01$, $^{***}P < 0{\cdot}001$.

IgG levels at any point of the seroconversion curve (Fig. 4c). Together, these data indicate that serum HspBP1 levels are significantly increased and that HspBP1 and anti-HspBP1 IgG levels are correlated with the viral load in the acute phase of HIV infection.

Discussion

In this study, we found that serum HspBP1 and anti-HspBP1 IgG levels were significantly increased in HIV-infected outpatients and that this increase was associated with viral load, HspBP1 and anti-HspBP1 IgG levels, being higher in the outpatients with a high viral load compared with those with low viral load. The presence of HspBP1 in the blood of healthy individuals and its increase in several pathologies have been reported (Raynes *et al.* 2003, 2006; Yokoyama *et al.* 2008; Grebenyuk *et al.* 2010). However, the present study is the first to quantify the HspBP1 and anti-HspBP1 serum levels in the same samples of HIV-infected patients and to compare these levels with the parameters of HIV infection (i.e., $CD4^+$ T cell count, viral load, and acute and chronic phases of infection). Since, HspBP1 is a nucleotide-exchange factor of Hsp70, its expression, function and clinical relevance are closely associated with the previously reported immunological mechanisms of Hsp70.

During the HIV infection cycle, host cells are under stress due to the degradation or unfolding of several proteins, that significantly increases Hsp70 expression (Brenner and Wainberg 2001). In HIV-infected patients, Hsp70 is expressed in lymphocytes. Moreover, these patients have significantly higher serum anti-Hsp70 IgG levels than HIV-negative individuals (Agnew *et al.* 2003). Anti-Hsp70 IgG levels decrease significantly after HAART (Füst *et al.* 2005) because of the concomitant virus clearance and the augment in circulating $CD4^+$ T cell counts (Kocsis *et al.* 2003). In contrast, systemic Hsp70 levels increase by 3·5-fold in patients showing HIV virological failure (viral resistance or therapy discontinuation) compared with those viroimmunologically stable patients. Increased Hsp70 expression is important for the folding and refolding of cell and viral proteins; moreover, increased intracellular Hsp70 levels are associated with the optimization of delivery peptides by antigen-presenting cells (Zheng and Li 2004).

The increased HspBP1 levels in the serum of HIV-positive outpatients indicate that the presence of an extracellular protein (secreted or released after cell lysis), could be due to the intense activity of Hsp70. HIV replication induces stress as well as the synthesis of new proteins, including viral proteins, that lead to HspBP1 expression, which controls the intense activity of Hsp70 in this environment. Thus, the increased serum HspBP1 levels are an

indicator of an intense viral replication in cells in the bloodstream. The viral replication induces cellular stress, activation of immunological system, and sometimes production of reactive oxygen species. This condition promotes the induction of HSPs, including Hsp70 and consequently the co-chaperone HspBP1. Antibodies against HSPs are normally present in the serum of healthy individuals (Cohen 2013; Shi *et al.* 2017). The increased anti-HspBP1 IgG levels observed in the present study may be associated with B cell activation because of the increased serum level of HspBP1.

In the present study, the HIV-infected outpatients were subdivided into two groups, i.e., patients with CD4+ T cell count <350 and those with CD4+ T cell count >350 cells per μl, according to the criteria recommended by the Clinical Protocol and Therapeutic Guidelines for the Management of HIV Infection in Adults (Ministry of Health of Brazil, 2018). High levels of serum HspBP1 may be due to the HIV replication accompanied by a decrease in the $CD4^{+}$ T cell count. Surprisingly, a significant increase in serum HspBP1 levels was observed only in patients with a very low $CD4^{+}$ T cell count (immunosuppressed patients), indicating that increased circulating HspBP1 levels are associated with the parameters of HIV severity. This observation is corroborated by the fact that serum HspBP1 levels show a significant negative correlation with the CD4 : CD8 ratio. Alterations in CD4 : CD8 ratio is an indicator of some type of immune dysfunction and is a marker of disease progression, treatment response, morbidity and mortality in HIV-infected individuals. Moreover, alteration in that ratio is associated with HIV DNA integration in host cells (Serrano-Villar and Deeks 2015; McBride and Striker 2017). Therefore, during HIV infection, high serum HspBP1 levels can be associated with a low CD4 : CD8 ratio and consequently a highly impaired immune system.

A recent study carried out by Chaudhary *et al.* (2016) showed that HspBP1 *in vitro* directly inhibited viral gene expression. During HIV infection, host cell Hsp70 is induced and HspBP1 levels are increased to inhibit chaperone performance; moreover, HspBP1 directly binds to the viral promoter during HIV infection (Chaudhary *et al.* 2016). Although in that study they have observed that HIV downregulated HspBP1, this observation was made *in vitro* and in a specific lymphocyte lineage. In our study, we observed that HspBP1 levels were elevated in HIV-infected outpatients with high viral load. In this situation many other cells are producing HspBP1 as an element direct linked to stress response that occurs during viral infection.

The advantages of the application of HspBP1 and anti-HspBP1 dosages as complementary prospective biomarkers are several. The measurement of HspBP1 and anti-HspBP1 in serum is not expensive and is easy to be performed in any Immunology or Clinical laboratory, serum dosages of HspBP1 and anti-HspBP1 can be used in the monthly monitoring of patients under antiretroviral therapy and since HspBP1 is involved in immunological processes, the serum dosage of HspBP1 and anti-HspBP1 can be used as a complement to assess the immune compromise or indicate other opportunistic infection disease or tumours. The sensitivity and specificity of HspBP1 ELISA were 73·3 and 70%, respectively, and of anti-HspBP1 were 60 and 66%, respectively. Based on sensitivity and specificity values, the dosage of HspBP1 and anti-HspBP1 does not replace the conventional tests for diagnosis and follow-up of HIV infection, but it can be used to optimize the evaluation of the disease progression.

In summary, we characterized HspBP1 as a promising new complementary biomarker for determining the prognosis of HIV-infected patients. HspBP1 or anti-HspBP1 IgG levels could be easily quantified in the peripheral blood by ELISA and could be used as an auxiliary tool to follow the progression of HIV infection. The results also contribute to the understanding of the immunobiological effects of HspBp1. However, further studies are needed to investigate whether HspBP1 serum levels can be used to predict the evolution of HIV infection, to monitor the immune response of HIV-positive patients under antiretroviral therapy, or as a marker of other viral infections and immunosuppressive conditions.

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Conflict of Interest

The authors have no conflict of interest.

References

Abraham, R., Mudaliar, P., Jaleel, A., Srikanth, J. and Sreekumar, E. (2015) High throughput proteomic analysis

and a comparative review identify the nuclear chaperone, nucleophosmin among the common set of proteins modulated in chikungunya virus infection. *J Proteomics* **120**, 126–141.

Agnew, L.L., Kelly, M., Howard, J., Jeganathan, S., Batterham, M., Ffrench, R.A., Gold, J. and Watson, K. (2003) Altered lymphocyte heat shock protein 70 expression in patients with HIV disease. *AIDS* **17**, 1985–1988.

Babaahmady, K., Oehlmann, W., Singh, M. and Lehner, T. (2007) Inhibition of human immunodeficiency virus type 1 infection of human CD4 + T cells by microbial HSP70 and the peptide epitope 407-426. *J Virol* **81**, 3354–3360.

Brenner, B.G. and Wainberg, Z. (2001) Heat shock proteins: novel therapeutic tools for HIV-infection? *Expert Opin Biol Ther* **1**, 67–77.

Chaudhary, P., Khan, S.Z., Rawat, P., Augustine, T., Raynes, D.A., Guerriero, V. and Mitra, D. (2016) HSP70 binding protein 1 (HspBP1) suppresses HIV-1 replication by inhibiting NF-kappaB mediated activation of viral gene expression. *Nucleic Acids Res* **44**, 1613–1629.

Chen, Z., Eggerman, T.L., Bocharov, A.V., Baranova, I.N., Vishnyakova, T.G., Kurlander, R. and Patterson, A.P. (2017) Heat shock proteins stimulate APOBEC-3–mediated cytidine deamination in the hepatitis B virus. *J Biol Chem* **292**, 13459–13479.

Cohen, I.R. (2013) Autoantibody repertoires, natural biomarkers, and system controllers. *Trends Immunol* **34**, 620–625.

Filone, C.M., Caballero, I.S., Dower, K., Mendillo, M.L., Cowley, G.S., Santagata, S., Rozelle, D.K., Yen, J. *et al.* (2014) The master regulator of the cellular stress response (HSF1) is critical for orthopoxvirus infection. *PLoS Pathog* **10**, e1003904.

Frydman, J. (2001) Folding of newly translated proteins in vivo: the role of molecular chaperones. *Annu Rev Biochem* **70**, 603–647.

Füst, G., Beck, Z., Bánhegyi, D., Kocsis, J., Bíró, A. and Prohászka, Z. (2005) Antibodies against heat shock proteins and cholesterol in HIV infection. *Mol Immunol* **42**, 79–85.

Grebenyuk, E.S., Stupnikova, T.V., Sakharov, D.A., Shleptsova, V.A., Sashchenko, L.P. and Tonevitsky, E.A. (2010) Long-term exercises increase the concentration of HspBP1, a Co-Chaperone of 70-KDa heat shock protein. *Bull Exp Biol Med* **149**, 640–644.

Kocsis, J., Prohászka, Z., Biró, A., Füst, G. and Bánhegyi, D. (2003) Elevated levels of antibodies against 70 kDa heat shock proteins in the sera of patients with HIV infection. *J Med Virol* **71**, 480–482.

Kumar, M., Rawat, P., Khan, S.Z., Dhamija, N., Chaudhary, P., Ravi, D.S. and Mitra, D. (2011) Reciprocal regulation of human immunodeficiency virus-1 gene expression and replication by heat shock proteins 40 and 70. *J Mol Biol* **410**, 944–958.

Kunihiro, M., Fujii, H., Miyagi, T., Takahashi, Y., Tanaka, R., Fukushima, T., Ansari, A.A. and Tanaka, Y. (2016) Heat shock enhances the expression of the human T cell leukemia virus type-I (HTLV-I) trans-activator (Tax) antigen in human HTLV-I infected primary and cultured T cells. *Viruses* **8**, 1–16.

Lalkhen, A.G. and McCluskey, A. (2008) Clinical tests: sensitivity and specificity. *Contin Educ Anaesthesia, Crit Care Pain* **8**, 221–223.

Liberek, K., Georgopoulos, C. and Zylicz, M. (1988) Role of the *Escherichia coli* DnaK and DnaJ heat shock proteins in the initiation of bacteriophage lambda DNA replication. *Proc Natl Acad Sci U S A* **85**, 6632–6636.

Liberek, K., Lewandowska, A. and Ziętkiewicz, S. (2008) Chaperones in control of protein disaggregation. *EMBO J* **27**, 328–335.

Liu, Y., Gierasch, L.M. and Bahar, I. (2010) Role of Hsp70 ATPase domain intrinsic dynamics and sequence evolution in enabling its functional interactions with NEFs. *PLoS Comput Biol* **6**, e1000931.

Manzoor, R., Kuroda, K., Yoshida, R., Tsuda, Y., Fujikura, D., Miyamoto, H., Kajihara, M., Kida, H. *et al.* (2014) *Heat shock protein 70 modulates influenza A virus polymerase activity*. *J Biol Chem* **289**, 7599–7614.

Mayer, M.P. and Bukau, B. (2005) Hsp70 chaperones: cellular functions and molecular mechanism. *Cell Mol Life Sci* **62**, 670–684.

McBride, J.A. and Striker, R. (2017) Imbalance in the game of T cells: what can the CD4/CD8 T-cell ratio tell us about HIV and health? *PLoS Pathog* **13**, e1006624.

Ministério da Saúde do Brasil. (2018) *Protocolo Clínico e Diretrizes Terapêuticas para Manejo da Infecção pelo HIV em Adultos/ Secretaria de Vigilância em Saúde*, p. 412. Brasília: Departamento de Vigilância, Prevenção e Controle das Infecções Sexualmente Transmissíveis, do HIV e das Hepatites Viraus-Brasília.

Papp, D., Prohászka, Z., Kocsis, J., Füst, G., Bánhegyi, D., Raynes, D.A. and Guerriero, V. (2005) Development of a sensitive assay for the measurement of antibodies against heat shock protein binding protein 1 (HspBP1): increased levels of anti-HspBP1 IgG are prevalent in HIV infected subjects. *J Med Virol* **76**, 464–469.

Pido-Lopez, J., Whittall, T., Wang, Y., Bergmeier, L.A., Babaahmady, K., Singh, M. and Lehner, T. (2007) Stimulation of cell surface CCR5 and CD40 molecules by their ligands or by HSP70 up-regulates APOBEC3G expression in CD4 + T cells and dendritic cells. *J Immunol.* **178**, 1671–1679.

Raynes, D.A. and Guerriero, V. (1998) Inhibition of Hsp70 ATPase activity and protein renaturation by a novel Hsp70-binding protein. *J Biol Chem* **273**, 32883–32888.

Raynes, D.A., Graner, M.W., Bagatell, R., McLellan, C. and Guerriero, V. (2003) Increased expression of the Hsp70 cochaperone HspBP1 in tumors. *Tumor Biol.* **24**, 281–285.

Raynes, D.A., Thomson, C.A., Stroster, J., Newton, T., Cuneo, P. and Guerriero, V. (2006) Human serum

contains detectable levels of the Hsp70 cochaperone HspBP1 and antibodies bound to HspBP1. *J Immunoass Immunochem* **27**, 251–264.

Sedlackova, L., Sosna, A., Vavrincova, P., Frýdl, J., Guerriero, V., Raynes, D.A. and Hromadnikova, I. (2011) Heat shock protein gene expression profile may differentiate between rheumatoid arthritis, osteoarthritis, and healthy controls. *Scand J Rheumatol* **40**, 354–357.

Serrano-Villar, S. and Deeks, S.G. (2015) CD4/CD8 ratio: an emerging biomarker for HIV. *Lancet HIV* **2**, e76–e77.

Shi, L., Chevolot, Y., Souteyrand, E. and Laurenceau, E. (2017) Autoantibodies against heat shock proteins as biomarkers for the diagnosis and prognosis of cancer. *Cancer Biomarkers* **18**, 105–116.

Souza, A.P., Albuquerque, C., Torronteguy, C., Frasson, A., Maito, F., Pereira, L., Duval da Silva, V., Zerwes, F. *et al.* (2009) HspBP1 levels are elevated in breast tumor tissue and inversely related to tumor aggressiveness. *Cell Stress Chaperones* **14**, 301–310.

Sugiyama, R., Naganuma, H., Nishitsuji, H. and Takaku, H. (2011) Human immunodeficiency virus-1 Nef suppresses Hsp70-mediated Tat activation. *FEBS Lett* **585**, 3367–3371.

Sugiyama, R., Abe, M., Nishitsuji, H., Murakami, Y., Takeuchi, H. and Takaku, H. (2013) Induction of heat-shock protein 70 by prostaglandin A1 inhibits HIV-1 Vif-mediated degradation of APOBEC3G. *Antiviral Res* **99**, 307–311.

Taguwa, S., Maringer, K., Li, X., Bernal-Rubio, D., Rauch, J.N., Gestwicki, J.E., Andino, R., Fernandez-Sesma, A. *et al.* (2015) Defining Hsp70 subnetworks in dengue virus replication reveals key vulnerability in flavivirus infection. *Cell* **163**, 1108–1123.

Thulasiraman, V., Yang, C.F. and Frydman, J. (1999) In vivo newly translated polypeptides are sequestered in a protected folding environment. *EMBO J* **18**, 85–95.

Wainberg, Z., Oliveira, M., Lerner, S., Tao, Y. and Brenner, B.G. (1997) Modulation of stress protein (hsp27 and hsp70) expression in CD4 + lymphocytic cells following acute infection with human immunodeficiency virus type-1. *Virology* **233**, 364–373.

Yokoyama, Y., Kuramitsu, Y., Takashima, M., Fujimoto, M., Iizuka, N., Terai, S., Okita, K., Sakaida, I. *et al.* (2008) Increased expression of heat shock protein-binding protein 1 and heat shock protein 70 in human hepatocellular carcinoma tissues. *Mol Med Rep* **1**, 197–201.

Zheng, H. and Li, Z. (2004) Cutting edge: cross-presentation of cell-associated antigens to MHC class I molecule is regulated by a major transcription factor for heat shock proteins. *J Immunol* **173**, 5929–5933.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Profile of HIV + samples with high viral load.

Table S2. Profile of HIV + samples with low viral load.