Proteomic Profile Associated with Loss of Spontaneous HIV-1 Elite Control

Running head: Proteomic profile of loss HIV-1 control

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Summary of article's main point: Proteomic signature associated with spontaneous loss of virological control which is characterized by inflammation, transendothelial migration and coagulation. This is the first "omics" approach showing potential biomarkers for the prediction of this virological progression and as therapeutic targets in EC.

NOTES

CONFLICT OF INTEREST STATEMENT

All authors report no potential conflicts of interests.

FUNDING STATEMENT

This work was supported by grants from the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Fondos Europeos para el Desarrollo Regional (FEDER) [PI10/02635, PI13/00796, PI16/00503 to FV and, PI12/02283, PI16/00684, CPII014/00025 to E.R-M; FI14/00431 to L.T-D; CP14/00198 to N.R.]; Programa de Suport als Grups de Recerca AGAUR [2017SGR948 and 2014SGR250]; the Gilead Fellowship Program [GLD14/293]; the Red de Investigación en Sida (RIS) [RD12/0017/0005, RD16/0025/0006, RD12/0017/0031, RD16/0025/0013]. [SAF2015-66193-R] from MINECO (Spain). The Spanish Ministry of Education [FPU13/02451 to B.D.M]. FV is supported by a grant from the Programa de Intensificación de Investigadores, Instituto de Salud Carlos III [INT11/240; INT12/282; INT15/226]. AR is supported by a grant from the Acció Instrumental d'incorporació de científics i tecnòlegs [PERIS SLT002/16/00101], Departament de Salut, Generalitat de Catalunya.

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ABSTRACT

Background: Elite Controllers (EC) spontaneously control plasma HIV-1-RNA without antiretroviral therapy. However, 25% lose virological control over time. The aim of this work was to study the proteomic profile that preceded this loss of virological control to identify potential biomarkers.

Methods: Plasma samples from EC who spontaneously lost virological control (Transient Controllers, TC), at two and one year before the loss of control, were compared with a control group of EC who persistently maintained virological control during the same follow-up period (Persistent Controllers, PC). Comparative plasma shotgun proteomics was performed with TMT isobaric tag labeling and nanoflow liquid chromatography coupled to Orbitrap mass spectrometry.

Results: Eighteen proteins exhibited differences comparing PC and pre-loss TC time points. These proteins were involved in proinflammatory mechanisms and some of them play a role in HIV-1 replication and pathogenesis and interact with structural viral proteins. Coagulation factor XI, Alpha-1-antichymotrypsin, Ficolin-2, 14-3-3 protein and Galectin-3-binding protein were considered potential biomarkers.

Conclusion: The proteomic signature associated with the spontaneous loss of virological control was characterized by higher levels of inflammation, transendothelial migration and coagulation. Galectin-3-binding protein could be considered as potential biomarker for the prediction of virological progression and as therapeutic target in EC.

Keyword: Biomarkers; Elite Controllers; HIV-1; Loss of control; Proteomic profile.

INTRODUCTION

The so-called Elite Controllers (EC), who represent a minority group of subjects in the scenario of human immunodeficiency virus type 1 (HIV-1) infection (ca. 1%), are able to maintain an undetectable viral load (VL) in the absence of combined antiretroviral treatment [1]. Due to this characteristic, EC have been proposed as a model of functional cure, eradication strategies and also HIV vaccine development [2]. Knowledge of the mechanisms involved in the controller phenomenon is highly relevant for the identification of the virological and host determinants involved in the spontaneous control.

Host genetic factors, mainly HLA class I molecules, such as HLA-B*57 [3], as well as immunological mechanisms, such as the HIV-1-specific T-cell response characterized by increased production of cytokines, chemokines and cytolytic enzymes (named poly-functionality), and HIV-1-suppression capacity [4–6] have been associated with this clinical situation.

New evidence suggests that these subjects have heterogeneous clinical outcomes including a variable proportion who loses HIV control overtime [7,8]. Although recent findings have identified Transient Controllers (TC) as subjects with low Gag-specific T-cell polyfunctionality, high viral diversity) [9], high proinflammatory cytokine levels [7] and T-cell homeostasis disturbances [10], to date, no longitudinal study elucidating the proteomic profile associated with the loss of spontaneous HIV-1 elite control has been performed. In the HIV-infection scenario, proteomics has emerged for identifying proteins involved in virus pathogenesis in several models of HIV-1 disease [11]. A better understanding of HIV-1 and human protein interactions might be used as a

starting point for further functional analysis that may also be translated into novel therapeutic strategies [12].

Thus, the aim of this work was to study the plasma proteomic profile that preceded the loss of spontaneous virological control in EC to identify the involved mechanistic pathways that can be further explored to find the potential interventional targets and also to identify the potential biomarker predictors of virological progression in these subjects.

METHODS

Patients and study design

EC were defined as subjects with VL determinations below the detection limit (<50 HIV-1-RNA copies/mL) in the absence of antiretroviral treatment for at least 12 months [13]. Subjects were included based on frozen plasma sample availability according to the study design (Supplementary Figure 1). Samples were received, processed and stored at the Spanish HIV HGM BioBank belonging to the AIDS Research Network (RIS) [14] and data were registered in the RIS cohort of the HIV Controllers Study Group (ECRIS) (Annex I, Supplementary Data). The cohort's characteristics were previously described in detail [8,9,13]. A total of 16 subjects were retrospectively selected and analyzed: eight were EC who had experienced loss of spontaneous virological HIV-1 control (at least two consecutive measurements of VL above the detection limit over 12 months), named Transient Controllers (TC), and another group of eight EC who persistently maintained virological control during the same follow-up period, named Persistent Controllers (PC) (see study design in Supplementary Figure 1). In TC, up to four determinations were assessed: at one and two years before the loss of virological control, called the "pre-loss-of-control period", (–T1 and –T2, respectively), and up to two more in the "post-loss-of-control period" including the closest time point (T0) and one year (+T1) after the loss of virological control. In PC, up to two determinations were performed at one-year intervals.

We also assessed three other subsets of individuals: uninfected controls, viremic HIV-infected patients with progressive disease and virological-supressed HIV-infected patients on ART (see Western blot section). Samples were stored at the BioBanc IISPV following standard procedures and with the appropriate approval of the Ethical and Scientific Committees.

All subjects provided their informed consent, and the protocols were approved by the institutional ethical committees. Detailed information about laboratory procedures can be found in Supplementary Data.

Proteomic analysis

For proteomic analysis, the seven most abundant plasma proteins (albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin and fibrinogen) were depleted using a Human-7 MARS cartridge (Agilent) following the manufacturer's protocol and the flow-through fractions were concentrated and buffer exchanged to approximately 100 µl of 50 mM ammonium bicarbonate (ABC) using 5K MWCO spin columns (Agilent). This immunoafinity depletion enhances the detection of lower abundance proteins and improves the subsequent analysis of serum samples expanding the dynamic range of the analysis.

Then, 65 µg of protein (quantified by Bradford's method) were incubated at 96ºC for 3 minutes, reduced with 4 mM 1.4-Dithiothreitol (DTT) for 25 minutes at 56°C and alkylated with 8 mM iodoacetamide (IAA) for 30 min at 25ºC in the dark. Afterwards, samples were digested overnight (pH 8.0, 37ºC) with sequencing-grade trypsin (Promega) at an enzyme:protein ratio of 1:50. Peptides were desalted on a C18 SPE column (BondElut, Agilent) and labelled with TMT 10-plex reagents (Thermo Fisher) following the manufacturer's instructions. To normalize all samples in the study, a pool containing all of the samples was labeled with TMT-126 tag and included in each TMT batch.

Multiplexed samples were on-line fractionated in a strong cation exchange nano-column (350 µm x 3.5 cm; 3.5 µm, Agilent) coupled to a C18 trap nano-column (100 µm x 2 cm; 5 µm, Thermo Fisher, USA) and a C-18 analytical nano-column (75 µm x 15 cm; 3

μm, Nikkyo Technos, Japan) on an EASY-II nanoLC chromatograph (Thermo Fisher) by a gradient salt pulsed sequential elution using ammonium acetate (0, 12.5, 25, 37.5, 50, 75, 100, 250 and 500 mM NH4AcO) followed by a continuous water-acetonitrile (0.1% formic acid) elution gradient at 300 nl/min.

Mass spectrometry analyses were performed on an LTQ-Orbitrap Velos Pro (Thermo Fisher) by acquiring an enhanced FT-resolution spectrum (R=30,000 FHMW) followed by two data-dependent MS/MS scan events from the most intense ten-parent ions with a charge state rejection of one and a dynamic exclusion of 0.5 min. Thus, an HCD fragmentation (40% NCE) with FT-MS/MS acquisition (R=15,000 FHMW) was conducted for peptide quantification, followed by a CID fragmentation (35% NCE) from the same parent ions with IT-MS/MS acquisition for peptide identification.

Protein identification/quantification was performed on Proteome Discoverer software v.1.4.0.288 (Thermo Fisher) by Multidimensional Protein Identification Technology (MudPIT). For protein identification, the MS and MS/MS spectra were analyzed using Mascot search engine (v.2.5) with SwissProt_2016_07.fasta database (551,705 entries), restricted for human taxonomy (20,198 sequences) and assuming trypsin digestion. Two missed cleavages and an error of 0.02 Da for FT-MS/MS, 0.8 Da for IT-MS/MS and 10.0 ppm for a FT-MS spectra were allowed. The TMT-10plex was set as quantification modification, oxidation of methionine and acetylation of N-termini were set as dynamic modifications, whereas carbamidomethylation of cysteine was set as static modification. The false discovery rate (FDR) and protein probabilities were calculated by Percolator.

For protein quantification, the ratios between each TMT label against the 126-TMT label were used, and the quantification results were normalized based on the protein median to reduce experimental bias and, Log2 transformed and mean centered for

variance stabilization, data range compression, and making the data more normally distributed before statistical analysis. Detailed information about laboratory procedures can be found in Supplementary Data. In addition, Supplementary Figure 2 shows concentration distribution graph and PCA before and after normalization steps.

Western blot

Protein samples were submitted to SDS-PAGE, transferred to nitrocellulose membranes. The membrane was then blocked, incubated with a primary antibody followed by an appropriate secondary antibody conjugated with horseradish peroxidase (HRP) and developed by chemiluminescence using Versadoc (Bio-Rad Laboratories). Finally, the proteins were quantified by ImageJ software.

The primary antibodies used were: LGALS3BP (ab67353), Ficolin 2 (ab56225), Anti-alpha 1 Antichymotrypsin (EPR14118(B)), Factor XI (MM0193-7C38) all from Abcam and 14-3-3 ζ/δ (#7413, Cell Signaling Technology). Secondary antibodies were polyclonal goat anti-rabbit (Pierce) and polyclonal goat anti-mouse (Pierce). Ponceau S staining was used as loading control.

We also compared the levels of the proteins of interest in PC and TC individuals to the levels seen in uninfected controls, viremic individuals with progressive disease and virological-supressed HIV-infected patients on ART (n=8 of each group) to see if the protein profiles in TC were more similar to those of viremic individuals.

Protein function and pathway analysis

Protein function was elucidated by a SWISS-PROT database (http://www.expasy.org) search. Interaction between the differentially expressed proteins and HIV proteins was investigated with the HIV interaction database

(http://www.ncbi.nlm.nih.gov/RefSeq/HIVInteractions) [15]. Signaling pathway analysis was defined with STRING v.10 (http://string.embl.de/) using proteins identified in this work and data from the SWISS-PROT function annotation as input [16].

Statistical analysis

To find the significant protein changes between the different conditions Mass Profiler Professional software v.14.5 (Agilent Technologies) was used. For statistical purposes, only those proteins that were quantified in more than 70% of the samples and in at least one of the groups were considered and protein quantification was normalized based on protein median and Log2 transformed. Firstly, a paired Student's t-test for PC samples was performed to check if a time effect existed in this group. Due to the lack of change in proteins in the PC group, we simplified the following analysis to a single variable, which is expressed as the mean value of two consecutive longitudinal determinations. Next, a Student's t-test between PC and TC in the pre-loss-of-control-period was performed to find early biomarkers of control loss. In this sense, we focused on pre-loss-of-control time points from TC and compared them with PC. For all of the comparisons, a mean value of –T1 and –T2 was calculated. Finally, a paired t-test between pre- and post-loss of the control period in the TC group was conducted to find protein changes over time. In all of the comparisons, a multivariate analysis, such as Hierarchical Clustering Analysis (HCA) and Principal Component Analysis (PCA), was performed. To select and evaluate the performance of the potential biomarker, Random Forest analysis, PCA and characteristic operating curves (ROC) were conducted using the 'R' program (http://cran.r-project.org) and the SPSS 21.0 package (IBM, Madrid, Spain). *P*-values <0.05 were considered statistically significant.

RESULTS

Characteristics of the studied subjects

Clinical and demographic characteristics of the EC at baseline are shown in Table 1. No differences were observed in age, sex, transmission route, or HCV coinfection; in CD4+ or CD8+ T-cell counts; or in the CD4:CD8 ratio between the TC and PC. The viral load (VL) evolution after that time of follow-up is only partially known because some of these patients were rapidly treated with antiretrovirals. The median [interquartile range] VL from TC was 539 [295-1,120] HIV-RNA copies/mL at T0 and 2,740 [985-22,250] HIV-RNA copies/mL at +T1. No co-morbidities such as cardiovascular diseases, malignancies, hepatic diseases, metabolic disorders, bacterial pneumonia, renal diseases or osteonecrosis were observed in any patient during the follow-up. Moreover, injecting drug users abandoned this habit and they were not under methadone treatment. Finally TC group presented a shorter time since diagnosis than PC (3 [2-8] vs. 13 [10-17] years; p=0.005). No differences were found in HLA-B frequencies.

Proteomic profile preceding the loss of natural HIV-1 control in Transient Controllers

Using a shotgun proteomic approach, we were able to identify and quantify a total of 293 proteins in plasma samples. To evaluate protein changes as an effect of time in the PC group, we applied PCA analysis and a Student's t-test in paired samples in that group. No proteins significantly differed along time in PC (Supplementary Figure 3).

As initial information regarding all of the quantified proteins, a partial least squares-discriminant analysis (PLS-DA) applied before statistical analysis is shown in Supplementary Figure 4. The PLS-DA model demonstrated that there was a great difference between PC and TC, using the complete result quantified set of proteins that

defined the model with an accuracy of 100%, which suggested that some of these quantified proteins may become a potential biomarker for the selected conditions.

Eighteen proteins were found to exhibit statistically significant differences in plasma levels between TC before the loss of HIV-1 control and PC, within the complete set of important proteins defining the model (Table 2). As illustrated in the heatmap representation (Figure 1A), seven of them were downregulated and 11 were upregulated in TC compared with PC. These proteins also showed good clusterization (Figure 1B) and a good differentiation between the studied groups (Figure 1C). In addition, a reliable separation between TC's follow-up time points with those of PC group could be observed in Figure 1C, despite there was some clustering between four samples of the PC T2 with TC -T1. All together, these data indicate the feasibility of these proteins to be potential biomarkers for the loss of HIV-1 control.

STRING was used to analyze specific pathways and protein networks involving the differentially expressed proteins for biological interpretation. This tool determined two related inflammatory signaling pathways: leukocyte transendothelial migration and complement and coagulation cascade (Figure 1D). As shown in Figure 1D, the host proteins Galectin-3-binding protein (LG3BP) and Ficolin-2 (FCN2) were found to interact with *gp120* (HIV function protein) and were upregulated (FC=2 and FC=1.59, respectively), whereas Intercellular adhesion molecule 1 (ICAM1), which interacts with *tat* protein, was downregulated in TC before the loss of natural HIV-1 control (FC=1.34).

To select and evaluate the performance of the potential biomarkers, we conducted a Random Forest analysis (Figure 2A), which elucidated that Coagulation factor XI (FA11), Alpha-1-antichymotrypsin (AACT), Ficolin-2, Galectin-3-binding protein and

14-3-3 protein zeta/delta (1433Z) were the main differentiators in a ranked list of the most significant proteins in order of their classification capability. It is important to highlight that these proteins were also the most significant variables in the univariate test.

After PCA analysis with these five proteins a good differentiation between both groups was observed, which corroborates their predictive strength of virological progression in EC (Figure 2B). Using logistic regression and ROC curves, although all of them have a statistically significant area under the curve, only Galectin-3-binding protein could discriminate PC and TC patients with 100% sensitivity and specificity, which suggests that this protein could be the most reliable biomarker for the prediction of the spontaneous loss of control in EC (Figure 2C).

Finally, as shown in Supplementary Figure 5, there were not any differences in these proteins before and after the loss of control in TC group, so we could dismiss that the virus is changing the protein profile after the change of status.

Validation by Western blot

Western blot analyses were performed to verify differentially expressed proteins between PC and TC in the pre-loss-of-control-period and in searching for potential biomarkers. For this ascertainment, we selected the five main protein differentiators mentioned before (Figure 3A). The immunoblotting results confirmed the observations of the shotgun proteomic approach: Galectin-3-binding protein, Coagulation factor XI and 14-3-3 protein zeta/delta showed the highest differences between groups. Galectin-3-binding protein and 14-3-3 protein zeta/delta were upregulated in TC, whereas Coagulation factor XI was downregulated in this group of EC (Figure 3B). We also

316 confirmed that, as suggested by the previous analyses, Galectin-3-binding showed the
317 greatest difference between groups.

318 Finally, when we compared the levels of these five proteins to the levels in uninfected
319 controls, viremic individuals with progressive disease and individuals on ART we could
320 see that TC and viremic HIV-infected patients showed a similar trend in the most of
321 cases. However, protein profile in PC were comparable to uninfected patients and
322 somehow to virological-supressed HIV-infected patients on ART (Figure 4).

323 *Protein changes in Transient Controllers as an effect of the loss of control*

324 We also determined protein changes in TC as an effect of detectable viremia, in other
325 words, we wanted to see if there was any change between pre and post the loss of
326 control. In this comparison, nine proteins showed statistically significant differences in
327 plasma levels between TC pre-loss and post-loss time points (Supplementary Table 1).
328 The heatmap representation showed that 5 proteins were upregulated after the loss of
329 control, whereas 4 proteins were downregulated after the loss of virological HIV-1
330 control (Figure 5A). Of note, SH3 domain-binding glutamic acid-rich like protein
331 expression was almost 3-fold higher after the loss of control (Figure 5B).

332 Interestingly, the pathway analysis showed that three of them were involved in
333 complement activation, which suggests an impaired regulation of that signaling pathway
334 (scheme in Supplementary Figure 6).

DISCUSSION

In this study, we analyzed the proteomic profile of plasma associated with the virological progression of EC. A specific proteomic signature that included mainly proteins involved in proinflammatory pathways identified subjects who were going to lose natural HIV-1 control. Thus, these proteins could be considered potential biomarkers to rapidly screen for future loss of natural control as well as members of mechanistic pathways to be further explored to discover potential drug targets in EC for achieving persistent control.

This is the first extensive analysis of the proteomic profile in plasma in EC with different viral load evolution. From the 18 proteins differentially expressed before the loss of natural control in TC compared to PC, three were related with the cytoskeleton: Keratin type I cytoskeletal 9 (K1C9), Vinculin (VINC) and Actin (ACTB). ACTB is involved in HIV-1 pathogenesis, including entry [17], viral assembly and budding [18,19] and cell-to-cell HIV-1 transmission [20]. In myeloid cells [21], HIV-1 *Nef* protein induced a marked polarization of VINC, which directly binds ACTB and is over-expressed in apoptotic cells [22]. VINC knockdown has also been related to increased rates of infection [23], and it has been considered a potential biomarker for HIV-1-infection [24]. The higher abundance of VINC and ACTB found in TC might reflect low residual viral replication levels and cell-to-cell HIV-1 transmission. In addition, VINC and ACTB, as well as, intercellular adhesion molecule 1 (ICAM1), have also been shown to be involved in leukocyte transendothelial migration, which suggests an inflammatory state in TC that may explain the higher immune activation previously reported in EC [7].

Further evidence observed in our study supporting this hypothesis was the downregulation of complement and coagulation cascade components (Carboxipeptidase B2 (CBPB2), Coagulation factor XI (FA11) and Complement component C8 gamma chain (CO8G)) before the loss of spontaneous HIV-1 elite control. This deregulation remained after the loss of virological control, as can be observed with the abundance of three complement activation proteins: Complement C1r subcomponent (C1R), Complement factor H (CFAH) and Clusterin (CLUS) compared with the under-control-period in TC. The role of complement activation proteins in viral pathogenesis has widely been studied in the HIV-1 and HIV/HCV-coinfection scenario [25,26].

Random Forest analysis, including the combination of the selected proteins, highlighted the importance of Ficolin-2 (FCN2) and 14-3-3 protein zeta/delta (1433Z). Recent findings have demonstrated a protective role of FCN2 against HIV-1 infection, blocking the entry of the virus into the target cells by direct interaction with gp120 [27]. The overexpression of FCN2 found in TC before the loss of control could be a reflection of partially restricted viral replication despite undetectable VL in TC. Regarding 1433Z, it plays an important role in cell-cycle checkpoint regulation and has previously been suggested as a potential biomarker for HIV-1-related neurodegeneration [28].

Of special interest is Galectin-3-binding protein (LG3BP), which was the most remarkable biomarker for group discrimination after ROC curve analysis with a concentration that was 2-fold higher in the TC group than PC. LG3BP is a glycoprotein known to be implicated in macrophage activation, cell signaling and migration. Regarding the HIV-1 scenario, LG3BP has been associated with increased HIV-1 replication and transmission through a direct interaction with viral *gp120* and host CD4+ T-cells [29–31]. This result not only identifies LG3BP as a reliable biomarker for the rapid screening of potential EC with virological loss of control but also suggests the

immunomodulation of this glycoprotein as a therapeutic target in EC and extensive to HIV remission in the general population.

In this work, a differential proteomic profile in plasma allows us to discriminate two EC phenotypes. This finding enhances the recent idea that suggest that HIV-1 controllers might be a heterogeneous group of subjects with different characteristics that remain partially unknown [9,32]. In addition, our data provide assistance for the design of new therapeutics aiming to achieve sustained virological remission.

The main limitation of this work is the small sample size. However, it has to be highlighted that this type of patients are rare and it is difficult to have this follow up with stored sample and that this cohort has been used in previous published own works [9,32]. Despite the relatively low number of participants, our highly sensitive method shows a wide spectrum of proteins (293 proteins were identified and quantified), and the most relevant proteins were confirmed by Western blot. However, further studies using larger cohorts are need for the establishment of the proposed proteins as biomarkers for the loss of virological control in EC. Moreover, the lower time of diagnosis observed in TC may be considered an inherent characteristic of this group because of the faster loss of EC status.

In conclusion, our study determined multiple pathways and deregulated proteins that lead to an inflammatory state that precedes the loss of spontaneous HIV-1 control and which is also maintained after the loss of control. These proteins could be considered potential predictive biomarkers to rapidly screen future loss of natural control and they provide new clues for a more accurate definition of EC, which will help in the identification of important determinants for the persistent natural control of viral replication and disease progression.

ACKNOWLEDGMENT

The constructive comments and criticisms of the editors and the reviewers helped us to improve the manuscript and are greatly appreciated. All authors report no potential conflicts of interests. This work was supported by grants from the Fondo de Investigación Sanitaria, Instituto de Salud Carlos III, Fondos Europeos para el Desarrollo Regional (FEDER) [PI10/02635, PI13/00796, PI16/00503 to FV and, PI12/02283, PI16/00684, CPII014/00025 to E.R-M; FI14/00431 to L.T-D; CP14/00198 to N.R.]; Programa de Suport als Grups de Recerca AGAUR [2017SGR948 and 2014SGR250]; the Gilead Fellowship Program [GLD14/293]; the Red de Investigación en Sida (RIS) [RD12/0017/0005, RD16/0025/0006, RD12/0017/0031, RD16/0025/0013]. [SAF2015-66193-R] from MINECO (Spain). The Spanish Ministry of Education [FPU13/02451 to B.D.M]. FV is supported by a grant from the Programa de Intensificación de Investigadores, Instituto de Salud Carlos III [INT11/240; INT12/282; INT15/226]. AR is supported by a grant from the Acció Instrumental d'incorporació de científics i tecnòlegs [PERIS SLT002/16/00101], Departament de Salut, Generalitat de Catalunya.

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FIGURE LEGENDS

Figure 1. Proteomic analysis comparing TC before the loss of natural HIV-1 control and PC, and a heatmap representation of the fold change of each quantified statistically significant proteins (A). Fold change was calculated as A/B-1, where A was the variable mean in the PC group and B was the variable mean in the TC group. The scale from green (low abundance) to red (high abundance) represents the normalized abundance in arbitrary units.. Hierarchical combined tree showing the clusterization of proteins (B), and PCA showing that these proteins allow differentiation between the studied groups and also between different time points (C), PC T1 (red, n=8), PC T2 (green, n=5), TC –T1 (blue, n=8), TC –T2 (purple, n=6), . STRING network analysis of differentially expressed proteins in TC before the loss of natural HIV-1 control and host-virus protein interactions. Line colors among proteins represent the interactions according to the legend (top left). The line symbol indicates the directionality of the effect in the case that it is known. The abbreviations for proteins are listed in Table 2 (D).

Figure 2. Proteins as potential biomarkers of the loss of virological control. Random Forest analysis plot of the 18 proteins ordered by importance of classification. Only the top five were considered potential biomarkers (A). Score plot of the PCA using this top five entities showed good differentiation between TC (blue, n=8) and PC (red, n=8) (B). Logistic regression and receiver operator characteristic (ROC) curves elucidated Galectin-3-binding protein (LG3BP) as the most reliable potential biomarker for the prediction of the spontaneous loss of virological control in EC (C).

Figure 3. Protein validation by Western blot analyses. Representative immunoblot of selected proteins in PC or TC using Ponceau S staining as loading control (A). Changes in protein levels between individuals were calculated by immunoblot densitometry; n=8

patients of each group (B). LG3BP, Galectin-3-binding protein; FA11, Coagulation factor XI; 1433Z, 14-3-3 protein zeta/delta; FCN2, Ficolin-2; AACT, Alpha-1-antichymotrypsin; PC, Persistent Controllers; TC, Transient Controllers.

Figure 4. Representative immunoblot of selected proteins using Ponceau S staining as loading control (A). Changes in protein levels between individuals were calculated by immunoblot densitometry; n=8 patients of each group (B). LG3BP, Galectin-3-binding protein; FA11, Coagulation factor XI; 1433Z, 14-3-3 protein zeta/delta; FCN2, Ficolin-2; AACT, Alpha-1-antichymotrypsin; PC, Persistent Controllers; TC, Transient Controllers; VIR, viremic HIV-infected patients with progressive disease; ART, virological-supressed HIV-infected patients on ART; UN, uninfected individuals.

Figure 5. Proteomic analysis comparing TC before and after the loss of natural HIV-1 control. Heatmap representation of the fold change of the differential and significant protein expression as an effect of the loss of control (A). The scale from green (low abundance) to red (high abundance) represents the normalized abundance in arbitrary units. Fold change was calculated as A/B-1, where A was the mean value of –T1 and –T2 and B was the mean of T1 and T2. The most remarkable change was the increased levels of SH3 domain-binding glutamic acid-rich like protein (SH3L3) after the virological progression in TC (B).

Table 1. Patients' characteristics.

	Transient Controllers, TC (n=8)	**Persistent Controllers, PC (n=8)**	***p***
Age (years)	41 [34-60]	44 [40-46]	0.635
Male sex, n (%)	5 (62.5)	4 (50)	0.614
Transmission route, IDU, n (%)	3 (37.5)	4 (50)	0.198
Time since diagnosis (years)	3 [2-8]	13 [10-17]	**0.005**
HCV RNA detected, n (%)	3 (37.5)	3 (37.5)	0.999
CD4+ T-cells (cell/µL)	676 [623-963]	724 [609-985]	0.817
CD8+ T-cells (cell/µL)	787 [553-1162]	636 [432-1026]	0.482
CD4:CD8 Ratio	0.86 [0.53-1.55]	1.08 [0.93-1.47]	0.406
HLA B57, n (%)	3 (37.5)	1 (12.5)	0.248
HLA B27, n (%)	1 (12.5)	1 (12.5)	0.999
HLA B35, n (%)	0 (0)	0 (0)	0.999

Values from TC are taken from –T2. Values are given as percentage for categorical variables or median and interquartile range for continuous variables. The Mann–Whitney U and Chi-squared tests were used. All *p* values <0.05 were considered significant and are highlighted in bold. IDU, Intravenous drug users.

Table 2. List of the differentially plasma protein levels comparing PC and TC at pre-loss of control time-point.

Swiss-Prot Accession Number	Swiss-Prot Entry	Protein description	Gene name	*p*
Q08380	LG3BP_HUMAN	Galectin-3-binding protein	LGALS3BP	2.83E-04
P03951	FA11_HUMAN	Coagulation factor XI	F11	2.59E-03
Q15485	FCN2_HUMAN	Ficolin-2	FCN2	4.53E-03
P01011	AACT_HUMAN	Alpha-1-antichymotrypsin	SERPINA3	1.06E-02
P63104	1433Z_HUMAN	14-3-3 protein zeta/delta	YWHAZ	1.21E-02
Q96IY4	CBPB2_HUMAN	Carboxypeptidase B2	CPB2	1.21E-02
Q86UX7	URP2_HUMAN	Fermitin family homolog 3	FERMT3	1.93E-02
O43866	CD5L_HUMAN	CD5 antigen-like	CD5L	2.47E-02
P07360	CO8G_HUMAN	Complement component C8 gamma chain	C8G	3.05E-02
P02753	RET4_HUMAN	Retinol-binding protein 4	RBP4	3.32E-02
P35527	K1C9_HUMAN	Keratin, type I cytoskeletal 9	KRT9	3.33E-02
P18206	VINC_HUMAN	Vinculin	VCL	3.62E-02

Q6P4Q7	CNNM4_HUMAN	Metal transporter CNNM4	CNNM4	3.69E-02
P60709	ACTB_HUMAN	Actin, cytoplasmic	ACTB	3.92E-02
P05362	ICAM1_HUMAN	Intercellular adhesion molecule 1	ICAM1	3.99E-02
P04114	APOB_HUMAN	Apolipoprotein B-100	APOB	4.05E-02
P17936	IBP3_HUMAN	Insulin-like growth factor-binding protein 3	IGFBP3	4.23E-02
P02775	CXCL7_HUMAN	Platelet basic protein	PPBP	4.73E-02

Differences between groups were calculated using a Student's t test.