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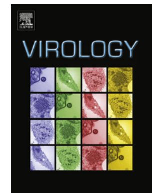

Increased Serpin A5 levels in the cervicovaginal fluid of HIV-1 exposed seronegatives suggest that a subtle balance between serine proteases and their inhibitors may determine susceptibility to HIV-1 infection

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ABSTRACT

HIV-exposed seronegative individuals (HESNs) are persons who remain seronegative despite repeated exposure to HIV, suggesting an in vivo resistance mechanism to HIV. Elucidation of endogenous factors responsible for this phenomenon may aid in the development of new classes of microbicides and therapeutics. We compared cervicovaginal protein abundance profiles between high-risk HESN and two control groups: low-risk HESN and HIV-positives. Four iTRAQ-based quantitative experiments were performed using samples classified based on presence/absence of particular gynaecological conditions. After statistical analysis, two proteins were shown to be differentially abundant between high-risk HESNs and control groups. Serpin A5, a serine proteinase inhibitor and Myeloblastin, a serine protease, were up- and downregulated, respectively. Commercially available ELISA assays were used to confirm differential Serpin A5 levels. These results suggest that HIV resistance in CVF of HESNs is the result of a delicate balance between two complementary mechanisms: downregulation of serine proteinases and upregulation of their inhibitors.

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Introduction

HIV is responsible for one of the most disastrous epidemics throughout human history, and approximately 34 million people are currently infected (UNAIDS, 2013). Unfortunately, the development of a prophylactic vaccine will likely not be available soon. Other strategies include topically applicable (e.g., rectal or vaginal) or oral antiretrovirals (ARV), which are chemical entities that can prevent or reduce HIV transmission. However, although these ARVs are a very promising strategy to reduce HIV spread, some issues remain, like the uptake and adherence of these compounds and the improvement of their efficiency (Cutler and Justman, 2008; Rohan and Sassi, 2009; Baeten and Grant, 2013).

HIV-exposed seronegative individuals

HIV-exposed seronegative individuals (HESNs) are frequently exposed to HIV but are not infected and are thus apparently HIV resistant in vivo. They comprise less than 5% of the general population and can be found among commercial sex workers, haemophiliacs receiving HIV contaminated blood, healthcare workers, children from HIV-infected mothers, intravenous drug users and seronegative partners in a discordant couple (Hirbod and Broliden, 2007; Horton et al., 2010; Broliden, 2010; Lederman et al., 2010; Shearer and Clerici, 2010; Miyazawa et al., 2009; Kulkarni et al., 2003; Shacklett, 2006). Elucidation of endogenous factors that inhibit HIV transmission and prevent the establishment of a productive infection are of high importance as

Abbreviations: CAN, acetonitrile; ARV, antiretroviral; BCA, bicinchoninic acid assay; BV, bacterial vaginosis; CI, confidence interval; CVF, cervicovaginal fluid; FA, formic acid; FC, fold change; HESNs, HIV-1 exposed seronegative individuals; HIV, human immunodeficiency virus; HR, high-risk; IHGT, infection of the higher genital tract; IL, interleukin; iTRAQ, isobaric tags for relative and absolute quantification; LR, low-risk; MALDI, matrix assisted laser desorption/ionisation; MCD, mucopurulent cervical discharge; MCP, monocyte chemotactic protein; NC, not calculated; NI, not infected; RP, reversed phase; SCX, strong cation exchange; TLR, toll-like receptor

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they can be used as a base for the development of new types of ARV and microbicides with higher efficiency. Therefore, many studies have been performed to identify physiological factors correlated with the HESN-status. Mutations of chemokine or Toll-like receptors (TLR), upregulation of chemokines due to genetic polymorphisms, specific human leukocyte antigen haplotypes, natural killer cell activity regulated by the killer Ig-like receptor (KIR)/HLA interaction, presence of autoantibodies and/or alloantibodies, cytotoxic and helper T lymphocyte responses against HIV epitopes, altered cytokine profiles and production of anti-HIV antibodies have all been linked to HIV resistance (Guerini et al., 2011; Ghadially et al., 2012; Arenzana-Seisdedos and Parmentier, 2006; Hirbod and Broliden, 2007; Kulkarni et al., 2003; Marmor et al., 2006; Shacklett, 2006; Lajoie et al., 2012; Choi et al., 2012; Sironi et al., 2012; Tomescu et al., 2011; Turk et al., 2013; Yao et al., 2013; Prodger et al., 2013). In addition, some studies hypothesize that HIV resistance by HESNs occurs at the viral entry gate before HIV interacts with dendritic or other target cells (Belec et al., 2001; Soderlund et al., 2007). Because the cervicovaginal mucosa is the most important entry point for HIV in women, resistance to sexually transmitted HIV infection in female HESNs may be the result of factors present at the lower female genital tract. Among these, the mucosal epithelium and especially proteins or peptides present in the cervicovaginal fluid (CVF) may play an important role (Iqbal et al., 2009; Shen and Smith, 2014).

Cervicalvaginal fluid

The use of CVF as clinical samples has gained interest in recent years because analysis of the CVF proteome can be used for several purposes. Knowledge of the CVF proteome may: (1) yield information about the aetiology of specific gynaecological pathologies, (2) lead to the identification of biomarkers for disease diagnosis and progression or (3) provide insight into physiological phenomena such as HIV resistance. Using antibody-based techniques (e.g., ELISA and Western blotting), a plethora of potential biomarkers for preterm birth, preterm premature rupture of membranes, bacterial vaginosis and cervical cancer have been discovered (Zegels et al., 2010). In addition, several studies on HIV resistance have used CVF from HESNs for the identification of correlates of HIV protection using antibody-based techniques. Anti-HIV IgA and IgG antibodies were detected in CVF obtained from heterosexual HESN women (Archibald et al., 1992; Belec et al., 1994b, 1994a; Beyrer et al., 1999; Devito et al., 2000; Ghys et al., 2000; Mazzoli et al., 1997; Choi et al., 2012). Additionally, the levels of the HIV-suppressive β-chemokine RANTES were found significantly different in CVF from HESNs (Belec et al., 2001; Iqbal et al., 2005; Yao et al., 2013). These results indicate that CVF is an important factor for the establishment of HIV resistance in HESNs. However, the use of antibody-based techniques limits the research to the analysis of only a few selected proteins. Therefore, comprehensive studies on CVF, which take all proteins under consideration, may yield more HIV resistance factors (Zegels et al., 2010).

HIV resistance factors

Information from qualitative comprehensive proteomics studies on CVF showed that this biological fluid contains proteins/peptides with intrinsic anti-HIV activity such as defensins, lactoferrin, lysozyme, cathelicidin and SLPI (Cole and Cole, 2008; Hirbod and Broliden, 2007; Kazmi et al., 2006; Zegels et al., 2009). In addition, (Venkataraman et al., 2005) demonstrated that the cationic fraction of CVF has inherent anti-HIV activity and hypothesized that this activity is the result of a complex synergism between different proteins in CVF (Levinson et al., 2012). Later, a study of Levinson et al. (2012) confirmed these findings and pointed to HNP1-3 and LL-37 as possible mediators. In addition, Ghosh et al. (2010) showed that this anti-HIV activity correlated significantly with CVF levels of MIP-3α, HBD-2 and anti-gp160 IgG antibodies. Such factors may contribute to the highly inefficient sexual transmission of HIV, as most unprotected exposures to HIV ($>99.5\%$) do not result in infection (Gray et al., 2001).

Three quantitative proteomics studies for the isolation of HIV resistance biomarkers have been published. Burgener et al. (2008) employed resp. 2D-DIGE and LC-LTQ-FT (Burgener et al., 2011) and compared protein abundance profiles from HESN persons with those from healthy controls. The authors identified resp. 16 and 41 differentially expressed proteins with diverse biological functionalities, including several serine proteinase inhibitors. Iqbal et al. (2009) used surface-enhanced laser desorption/ionization time-of-flight (SELDI-TOF) mass spectrometry for comparison of protein abundance profiles from HESNs with those from control groups and found that the serine proteinase inhibitor elafin/trappin-2 is significantly upregulated in HESNs. In addition, elafin/trappin-2 has recently been identified as a new anti-HIV factor of the innate immune system of the lower female genital tract (Ghosh et al., 2009; Drannik et al., 2012b). However, the use of other proteomics techniques and test populations may help to characterize other CVF proteins correlated with in vivo HIV resistance. To improve the reliability of potential resistance factors that inhibit HIV transmission, it is important to confirm and to validate such factors in different independent HESN cohorts, whether or not with additional (genital) infections. Therefore, we analyzed the CVF from an HESN population of female sex workers from Abidjan, Côte d'Ivoire using iTRAQ-based quantitative proteomics to further unravel in vivo HIV resistance.

Results

Sample population

Three different subgroups were selected from a female commercial sex worker population from Abidjan, Côte d'Ivoire: HR, LR and HIV. We chose the HR group as the test group because these persons remained seronegative despite frequent HIV exposure for a long period of time. Therefore, this group is very likely to be enriched for HESN individuals. Two different control groups were included in the experiments. The individuals from the LR group were not (or extremely exceptionally) exposed to HIV due to the protective measures they have taken and the relatively low number of clients per week. This group is expected to be representative for the general population that includes only a small fraction of HESNs ($<5\%$ (Hirbod and Broliden, 2007; Kulkarni et al., 2003; Shacklett, 2006)). Therefore, these LR individuals act as a low-risk control group. Comparison between HR and LR may lead to the identification of proteins specifically correlated with HIV resistance. However, non-protective HIV-specific immune reactions occur in seropositive persons and HESNs (Biasin et al., 2000). Therefore, it is possible that adaptive immunity-related proteins can be significantly different in the HR and LR group, but not in comparison to HIV-positive persons. This type of result would indicate that the protein is derived from an HIV-specific immune reaction that does not contribute to the observed HIV resistance. Therefore, we incorporated HIV-infected persons as a non-resistant control group.

The large amount of potential biomarkers resulting from one experiment is one of the major downsides of MS-based quantitative methods such as iTRAQ. We conducted four different experiments so that the number of potential biomarkers could be reduced by statistics. As recently suggested in order to prevent confounding and bias, the HESN samples must be well documented, and pathological conditions need to be taken into consideration (Kaul et al., 2011). Indeed, because gynaecological pathologies can induce significant alterations of the CVF proteome (Zegels et

al., 2010), all samples within one experiment should originate from women with no infections or from women with a particular pathology. Enrichment of pathology in a specific subgroup may occur, ultimately resulting in proteome profiles that may be the consequence of the pathology instead of the HESN status. For each experiment, samples were selected and classified according to the presence or absence of a pathological condition as follows: no infections (NI), bacterial vaginosis (BV), infections of the higher genital tract (IHGT) and mucopurulent cervical discharge (MCD).

Qualitative proteome analysis

In the course of the four experiments, a total of 233 different proteins were identified after a stringent scaffold analysis. To correctly calculate the percentage of shared versus total number of identifications, we made use of the previously published calculation (Hattan et al., 2005) that takes into account the redundancy of identifications. The four experiments yielded a total of 572 (161+89+156+166) identifications of which 260 (4×65) were found in all four experiments, resulting in an overlap ratio of 45.5% (260/572). The proteins that were identified in the different experiments are listed in the Supporting information. A FDR was determined for all four experiments and ratios were all below 2% (resp. 1.23; 1.30; 1.35 and 1.32%).

A schematic overview of the number of identifications per experiment and the degree of redundancy is given in Fig. 1. Using DAVID (Dennis et al., 2003; Huang et al., 2009) (version 6.7), we classified the identified proteins based on biological function and cellular localization. We found that the majority of the proteins are involved in the immune system (18%) and protein metabolism and modification (14%). All of the other functional categories contained fewer proteins. In addition, a large fraction of the identified proteins were found in the extracellular region (38%). These results are characteristic for CVF, which is a biological fluid with important immunological functions (Zegels et al., 2010).

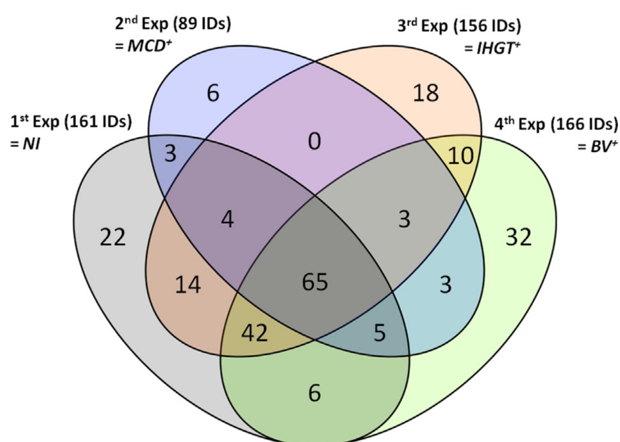

Fig. 1. Venn diagram showing the overlap of protein identifications between the four different experiments.

Quantitative proteome analysis

Proteins associated with gynaecopathological conditions

We initially analyzed the data in order to determine whether gynaecopathological conditions effectively introduce proteome changes that are detectable by our iTRAQ-based proteomics platform. Table 2 summarizes the statistically significant differences of the conditions (compared to NI) and lists the corresponding *p*-values. The abundance level of five proteins was significantly altered in the case of MCD. Transthyretin was the only upregulated protein, whereas leukocyte elastase inhibitor (LEI or Serpin B1), serine proteinase inhibitor Kazal-type 5 (SPINK5), cornulin and suprabasin were significantly downregulated. Proteins S100A8 and S100A9 were expressed at higher levels, and Serpin B3 and dermcidin were downregulated in women with IHGT. Eight proteins were differentially expressed in women with BV. Four proteins were overexpressed (S100A6, S100A12, LEI and Serpin B4), and four were underexpressed (dermcidin, fatty acid binding protein (FABP), suprabasin and cystatin A). These results indicate that certain pathologies affect the proteome composition and may therefore be a confounding variable as mentioned before.

Proteins associated with HIV resistance

After establishing that the proteome composition is influenced by certain pathologies, we compared the protein abundance profiles of the different subgroups. First, HR was compared with LR and HIV in the first experiment (absence of other gynaecopathological conditions) to identify proteins that showed clear up- or downregulation in HR. Twenty-one possible marker candidates were identified, as shown in Table 3, including several proteinase inhibitors (SPINK5, Serpin A5 and calpastatin), proteinases (cathepsin B and Myeloblastin), structural proteins from the cornified envelope (periplakin, involucrin, envoplakin and suprabasin) and several other proteins with diverse functions. The largest abundance difference was found for semenogelin-2, a protein that is abundantly present in semen coagulum. This protein was clearly overrepresented in HR compared to HIV (fold change: 16.00) and LR (fold change: 14.93).

We next wanted to investigate whether proteins from this list also showed differential abundance in the three other experiments performed on samples from women with pathological conditions. As the presence of infections results in changes to the CVF proteome, inclusion of these samples introduces an additional degree of stringency. Hence, proteins that show a different abundance profile between HR and LR/HIV in all four experiments are more confident HIV resistance markers because they overcome the variabilities of the proteome introduced by the pathologies.

The quantitative data from the four experiments were statistically analyzed to isolate markers showing significantly different protein abundances in the HR group (Fig. 2; Table 3). Serpin A5

Table 1
Overview of the experimental setup. Four different iTRAQ experiments were performed. In each experiment, three different subgroups differing in HIV exposure and HESN status, plus an internal standard were compared. The samples for each experiment were matched according to the presence (+) or absence of bacterial vaginosis (BV), infections of the higher genital tract (IHGT) and mucopurulent cervical discharge (MCD). NI means *no (additional) infection*. A total of 48 samples were used.

	Type of individual in terms of HIV infection and HESN status			**Standard**	**iTRAQ experiment**
	LR	**HR**	**HIV**		
NI	Samples 1–4	Samples 17–20	Samples 33–36	Samples 1–48	Samples pooled during iTRAQ **experiment 1**
MCD+	Samples 5–8	Samples 21–24	Samples 37–40	Samples 1–48	Samples pooled during iTRAQ **experiment 2**
IHGT+	Samples 9–12	Samples 25–28	Samples 41–44	Samples 1–48	Samples pooled during iTRAQ **experiment 3**
BV+	Samples 13–16	Samples 29–32	Samples 45–48	Samples 1–48	Samples pooled during iTRAQ **experiment 4**

Table 2
Overview of CVF proteins that are significantly up- or downregulated in different pathologies as compared to healthy controls. Fold change (fc) versus healthy controls and the statistical significance (*p*-value) of the difference are shown.

MCD/NI			IHGT/NI			BV/NI		
Protein	fc	*p*-Value	Protein	fc	*p*-Value	Protein	fc	*p*-Value
LEI	0.52	0.048	S100A8	1.79	0.036	LEI	1.56	0.024
Transthyretin	2.97	0.034	S100A9	1.98	0.035	S100A6	2.32	0.017
SPINK5	0.35	0.041	Serpin B3	0.50	0.020	S100A12	2.14	0.032
Cornulin	0.47	0.023	Dermcidin	0.60	0.044	Dermcidin	0.57	0.035
Suprabasin	0.34	0.040				Serpin B4	2.33	0.023
						FABP	0.65	0.028
						Suprabasin	0.22	0.020
						Cystatin A	0.44	0.036

Table 3
Statistical analysis of the protein abundance differences over the four different experiments. The first column shows the proteins that had a significant change of abundance in HR as compared to HIV and LR in the not infected (NI) group. Fold changes of these differences are shown in the column “NI”. For each protein, the increased (> 1) or decreased (< 1) fold change (fc) between two different subgroups (HR/LR or HR/HIV) is given. Data about these proteins from all groups is presented in the column “NI, IHGT, BV and MCD combined”. For each protein, the average (av) normalized ion ratio of the reporter group over the four experiments is presented for the different types (HR, LR, HIV) with a 95% confidence interval (CI). Furthermore, the fold change (fc; HR/HIV or HR/LR) and statistical significance (*p*) of the abundance difference are presented. NC: not calculated due to too few data points.

Protein name	NI		NI, IHGT, BV and MCD combined									
	HR/LR	HR/HIV	HR		LR		HIV		HR/LR		HR/HIV	
	fc	fc	av	CI	av	CI	av	CI	fc	*p*	fc	*p*
SPINK 5	1.52	2.00	1.04 ± 0.64		1.07 ± 0.69		0.70 ± 0.23		0.97	0.95	1.48	0.43
Serpin A5	**1.32**	**1.62**	**1.88 ± 0.23**		**1.15 ± 0.07**		**1.12 ± 0.13**		**1.64**	**< 0.001**	**1.69**	**< 0.001**
Calpastatin	1.74	2.30	1.67 ± 1.89		1.37 ± 0.28		1.11 ± 0.08		1.22	0.73	1.51	0.53
Periplakin	1.41	2.46	1.06 ± 0.41		0.81 ± 0.24		0.76 ± 0.17		1.30	0.27	1.39	0.19
Involucrin	2.14	3.03	1.31 ± 0.83		0.99 ± 0.57		0.69 ± 0.28		1.32	0.48	1.90	0.19
Envoplakin	1.32	2.46	1.68 ± 1.20		1.25 ± 0.96		0.82 ± 0.22		1.35	0.55	2.05	0.27
Suprabasin	1.52	2.64	1.00 ± 0.80		0.75 ± 0.66		0.67 ± 0.42		1.35	0.59	1.51	0.49
Fibrinogen α chain	0.71	0.38	0.97 ± 0.30		1.12 ± 0.35		1.00 ± 0.66		0.86	0.66	0.97	0.93
Cathepsin B	1.87	2.00	1.18 ± 0.14		0.71	NC	0.99 ± 0.33		1.67	NC	1.19	NC
Myeloblastin	**0.33**	**0.33**	**0.81 ± 0.31**		**1.49 ± 0.17**		**1.36 ± 0.13**		**0.55**	**0.01**	**0.60**	**0.02**
Plastin-2	0.23	0.25	0.74 ± 0.31		1.29 ± 0.75		0.89 ± 0.23		0.57	0.15	0.83	0.67
Vitamin D-binding protein	0.71	0.50	1.29 ± 0.80		0.92 ± 0.23		1.14 ± 0.22		1.40	0.33	1.13	0.69
Haptoglobin	0.71	0.31	1.16 ± 0.51		0.91 ± 0.04		1.34 ± 0.85		1.28	0.56	0.87	0.68
Prolactin-inducible protein	3.03	4.29	2.03 ± 1.20		0.81 ± 0.11		0.66 ± 0.09		2.49	0.1	3.06	0.07
Histone H2A type 1-A	0.47	0.54	0.73 ± 0.24		1.29 ± 0.50		1.18 ± 0.48		0.57	0.10	0.62	0.18
Dermcidin	2.14	1.32	1.45 ± 0.55		1.08 ± 0.52		1.19 ± 0.37		1.34	0.32	1.22	0.48
Semenogelin-2	14.93	16.00	6.50	NC	0.44	NC	0.41	NC	16.0	NC	14.9	NC
Peptidyl-prolylcis-trans isomerase A	1.32	1.74	0.91 ± 0.62		0.92 ± 0.3		1.04 ± 0.24		0.99	0.99	0.88	0.70
Galectin-3	0.35	0.41	0.99 ± 0.48		1.23 ± 0.47		1.33 ± 0.29		0.80	0.45	0.74	0.29
Ras GTPase-activating-like protein IQGAP1	0.44	0.18	0.67 ± 0.45		0.71 ± 0.3		1.36 ± 0.16		0.95	0.88	0.49	0.03
Protein FAM25	4.92	2.83	1.53 ± 0.93		0.53 ± 0.18		0.78 ± 0.23		2.90	0.06	1.96	0.14

was overexpressed in HR as compared to HIV (fold change: 1.69; $p < 0.001$) and LR (fold change: 1.64; $p < 0.001$). Myeloblastin (leukocyte proteinase 3) was underexpressed in HR compared to HIV (fold change: 0.60; $p = 0.02$) and LR (fold change: 0.55; $p = 0.01$) (Fig. 2; Table 3). The statistical test (i.e., ANOVA) could only be applied on proteins that were identified in at least three out of four experiments. Because semenogelin-2 was only identified in the first experiment, no statistical significance could be calculated for this protein. We believe that the semenogelin-2 values in the first experiment can be considered as outliers, possibly due to recent sexual contact of at least one of the HR individuals (see Discussion).

Confirmation of proteomics experiments by ELISA

To confirm the above experiments, samples from women from the 3 subgroups with or without MCD were selected (LR: 5 MCD− and 4 MCD+, HIV: 6 MCD− and 4 MCD+, HR: 3 MCD− and 4 MCD+). These samples were not included in the differential proteomics experiment described above, but originate from the same study population. The protein concentration of the samples was measured three times because the BCA assay often gives variable results and only slight differences (1.66 for Serpin A5 and 0.57 for Myeloblastin) were expected. Only samples with high protein concentrations (> 100 μg/ml) were selected because BCA is suboptimal in measuring low protein concentrations and a high concentration (1 mg/ml) was needed for detection of Serpin A5. Results (Fig. 3A) show a statistical difference between Serpin A5 levels from HR (median value of 6.59 ng/ml) vs LR (median value of 4.05 ng/ml) samples (1.63-fold; $p = 0.031$) and HR (median value of 6.59 ng/ml) vs. HIV positive (median value of 3.83 ng/ml) samples (1.72-fold; $p = 0.014$). Moreover, levels of Serpin A5 levels did not vary significantly in samples from women with MCD (median value of 4.29 ng/ml) vs. samples from women without MCD (median value of 5.12 ng/ml) (Fig. 3B) confirming the above mentioned observation that MCD did not influence Serpin A5 levels as it did not show up in the list of differentially expressed proteins from MCD- and MCD+CVF samples (Table 2).

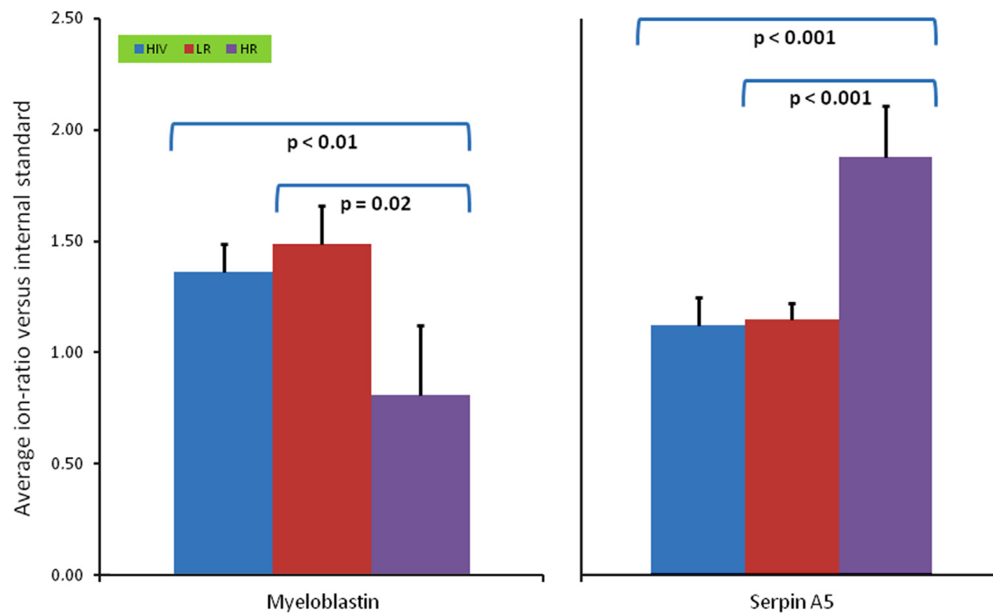

Fig. 2. Abundance difference between HR, LR and HIV for the proteins Myeloblastin and Serpin A5. Significance levels (*p*-values) of the differences are shown.

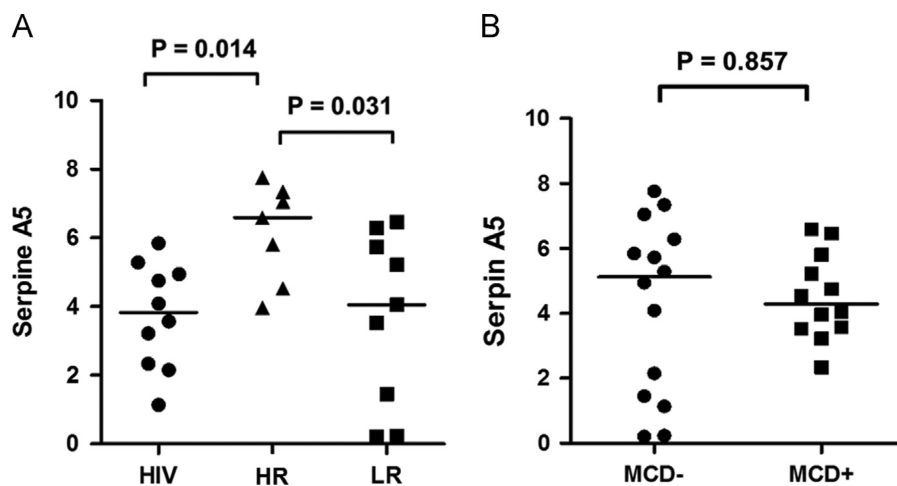

Fig. 3. (A) Abundance levels of Serpin A5 (ng/ml) for the three populations (all including with and without MCD) HIV, HR and LR. (B) Difference in Serpin A5 abundance levels between samples from women that had MCD (HIV, HR and LR) vs. samples from women without MCD (HIV, HR and LR). Calculation of statistical difference was done by the non-parametric Mann Witney *U*-test, due to the restricted number of samples.

Unfortunately, Myeloblastin concentration could not be accurately determined with the two ELISA's from different manufacturers we have tested. Problems may be due to matrix effects since the difference in ELISA signal during titration did not follow the extend of the dilution.

Discussion

Study design

Analysis of the cervicovaginal proteome from high-risk HESNs could result in isolation of potent anti-HIV proteins and peptides that can block viral entry in humans in vivo. A few studies have already been performed on CVF to identify such proteins. However, most have used antibody-based methods, such as ELISA or Western blotting, which restricts the study to the analysis of proteins for which antibodies are available (Zegels et al., 2010). Moreover, antibody-based techniques are sometimes incapable of discriminating between different subtle protein forms (Buhimschi et al., 2008, 2005) or have difficulties in analyzing certain proteins due to interference from other proteins (Van de et al., 1994). Therefore, we decided to use proteome-wide techniques that allowed for analysis and comparison of protein abundance profiles from HESNs with control groups in an unbiased manner. Besides, it is important to analyse and confirm potential resistance factors in several independent cohorts, if possible in combination with additional genital infections. Until now, all comprehensive proteomic studies were performed by using CVF samples from the same "Pumwani" cohort (Iqbal et al., 2009; Burgener et al., 2008, 2011) and confirmation of potential correlates of protection by other research groups and in other cohorts are urgently needed. Therefore, we analysed the cervicovaginal proteome of a HESNs population originating from female sex workers located at Côte d'Ivoire, many of which suffered from additional genital infections.

Four iTRAQ experiments were performed in order to identify proteins and peptides that showed statistically significant altered abundance patterns in the CVF from HESNs as compared to control groups. Three subgroups were compared in every experiment: LR (HIV seronegative individuals, commercial sex work for less than 12 months, under 25 clients/week and dedicated use of condoms),

HIV (seropositive commercial sex workers, no further criteria) and HR (seronegative persons, commercial sex work for more than 48 months, no or sporadic condom use). The samples ($n=4$) were pooled as to level out variability between individual samples. To limit the chance for false positives, the pooled samples were composed in such a way that stratification according to pathological conditions (NI, BV, IHGT and MCD) was possible. As such, each experiment was performed four-fold, each time on samples originating from women with a different pathological condition (total of 48 samples). Hence, correction for these confounding factors adds another level of stringency to the selection such that more reliable markers may come out of the procedure.

Effect of additional genital infections

As mentioned above, confounders were included with the aim to identify trustworthy candidate markers correlated with the HESN-status without being influenced by the additional pathological conditions. We decided to stratify our samples according to the site of infection and we selected pathologies that are often caused by a plurality of pathogens resulting in a strong activation of the immune system at different regions in the female genital tract (BV: vagina, MCD: cervix and IHGT: uterus and higher). Although the influence of these additional infections on the CVF proteome is definitely worth further investigation, our main purpose was to outselect proteins for which the expression levels were influenced by genital infections and thus could not be qualified as markers of HIV susceptibility. Fourteen proteins were identified, from which five proteins (LEI, transthyretin, SPINK5, cornulin and suprabasin) were differentially expressed in the case of MCD. However, the link between those proteins and the pathological condition remains vague. The inflammation-related proteins S100A8 and S100A9 (Ehrchen et al., 2009) were upregulated in IHGT, while two other S100 proteins, S100A6 and S100A12 (Pietzsch and Hoppmann, 2009; Lesniak et al., 2009; Eckert et al., 2004), were upregulated in BV. Because these proteins promote HIV infection (Ryckman et al., 2002; Hashemi et al., 2001), they may be partially responsible for increased HIV susceptibility caused by IHGT and BV (Cohen et al., 1999; Atashili et al., 2008). Indeed, disruption of the vaginal flora (like in the case of BV, IHGT and MCD), influences innate immunity, leading to a higher susceptibility for HIV-infection and -transmission (Kaul et al., 2008; Mirmonsef et al., 2012). The serine proteinase inhibitor Serpin B3 was downregulated in IHGT, but Serpin B4 was upregulated in BV. Dermcidin was downregulated both in IHGT and BV. In addition, four other proteins were differentially expressed in BV: LEI was overexpressed, while FABP, suprabasin and cystatin were downregulated. However, their exact correlation with this pathology is uncertain. Nevertheless, these results indicate that the CVF proteome is altered due to the presence of certain pathologies.

Unravelling HIV resistance mechanism

We next analyzed the protein abundance profiles between the different subgroups. Protein abundance profiles from HR were compared with those from HIV and LR in the first experiment (samples obtained from women with no infection (NI)) (Kaul et al., 2011). For the quantification of our proteins we used an iTRAQ labeling method. For this we selected one label as reference, while the other labels are relatively expressed as fold change compared to the reference. Since pooled samples were used for the proteomic analysis, no quantitative cut off value could be calculated. Although in one experiment, semenogelin-2 showed a high differential expression, this could not be reproduced in other experiments. We therefore arbitrarily choose for a cut off value of >1.3 or <0.77 because it selected the second most differential abundant proteins. We realized that these fold changes of Serpin A5 and Myeloblastin are rather small, however they are consistent over all the experiments. Therefore, given the fact that in some biological processes small changes in abundance can result in a large effect, especially in the case of enzyme cascades (e.g. blood coagulation), we reasoned that small changes in protease-antiprotease levels could be biologically relevant. Moreover, we previously experienced that differential protein expression levels analysed by iTRAQ are sometimes more distinct when measured by ELISA.

Twenty-one proteins showed an increased or decreased abundance in HR individuals compared to the control groups and were listed as potential biomarkers of the HESN status. Of these, those that may influence HIV infection are discussed here.

SPINK5, which was found to be overexpressed in HR individuals, inhibits several serine proteinases such as kallikreins, cathepsin G and trypsin. In addition, it acts in the regulation of epithelial desquamation and contributes to the integrity and protective barrier function of the skin (Meyer-Hoffert, 2009; Deraison et al., 2007). The role of serine proteinases and their inhibitors in protection against HIV infection will be explained in more detail below with the discussion of Myeloblastin and Serpin A5. Galectin-3, a β-galactoside-binding lectin, was found to be downregulated in HR individuals. Galectin has been reported to be upregulated upon HIV infection and stabilizes the interaction between virions and HIV target cells (Ouellet et al., 2005; Fogel et al., 1999). Therefore, downregulation may hinder cellular infection. Prolactin-inducible protein was also found to be overexpressed in HR. Because this protein binds CD4, it may antagonize the interaction between this receptor and gp120 and thus prevent viral adhesion and entry of target cells (Debily et al., 2009). The largest fold change was noted for semenogelin-2. Although semenogelin has been shown to have intrinsic anti-HIV activity and to bind HIV virions, this protein is abundantly present in semen (Martellini et al., 2009). In contrast to women from the LR and HIV group, women from the HR group used no condoms or much less frequently. Therefore, the increase in the semenogelin-2 abundance may be indicative of the unprotected sexual intercourse of HR women. Notably, this protein was not detected in the three other experiments, suggesting that the values for semenogelin-2 in the first experiment are outliers. Indeed, the amount of seminal proteins in the cervicovagina is inversely correlated with the time passed between the last unprotected sexual intercourse and sample collection (Carballada and Esponda, 1997). The abundance of these proteins is likely to be highly variable and/or too low to detect reproducibly, and they will thus unlikely be associated with HESN status. Nevertheless, this observation points to the variability of the data obtained from experiments with this kind of samples and underscores the necessity of performing several experiments to filter out outliers.

Validation of potential HIV resistance mechanism

For this reason, three additional experiments were performed. These experiments showed that many of the potential markers did not exhibit a statistically significant change in abundance. These results do not imply that these proteins are all false positives. Our experimental setup results in large interindividual variation because samples were collected from women with different gynaecopathological conditions. The HESN-related proteins in our study could therefore be identified with higher confidence (high specificity), but more false negatives are present due to limited statistical power (low sensitivity). In this comparison, two proteins still showed a significantly different abundance in the HR-HESN group as compared to the control groups, namely the serine proteinase inhibitor Serpin A5 (increased abundance in HR) and the serine proteinase Myeloblastin (decreased abundance in HR). Identification and quantification of these two proteins was reliable as they were based on more than one peptide. Although the difference in abundance levels of Myeloblastin could not be confirmed by ELISA - possibly due to matrix effects - a similar slight difference in Serpin A5 levels was observed in a Serpin A5 ELISA (RES/

LR: 1.56-fold (iTRAQ) vs. 1.63-fold (ELISA) and RES/HIV: 1.59-fold (iTRAQ) vs. 1.72-fold (ELISA)).

Serpin A5: A serine proteinase inhibitor

Serpin A5 is expressed in a wide variety of tissues and biological fluids and is an inhibitor of several serine proteinases such as activated protein C and proteinases that are involved in fertilization (Suzuki, 2008). It is also present in seminal plasma; therefore, we initially speculated that CVF concentrations of Serpin A5 are correlated with the frequency of unprotected sexual intercourse instead of HIV resistance. However, the abundance of seminal proteins in the CVF is highly variable as demonstrated by the filtering out of semenogelin-2. Moreover, since each of the four HIV groups contained samples from female sex workers reporting never or rare use of condoms, it is expected that levels of proteins originating from semen would also be increased in the HIV group. However, this was not the case for Serpin A5. Therefore, because Serpin A5 was identified to be differentially abundant in all four iTRAQ experiments and these results were confirmed on additional samples by using ELISA, it most likely originates from the female genital tract and not from seminal contamination.

In addition to its function in fibrinolysis and reproduction, Serpin A5 has been reported to play a role in tissue regeneration, vascular permeability and tumor invasion (Suzuki, 2008). Malmstrom et al. demonstrated that Serpin A5 has broad antimicrobial activity towards Gram-negative and -positive bacteria and is resistant to degradation by host and bacterial proteinases. It has an affinity for negatively charged lipids, allowing it to adhere to bacterial cell walls, leading to bacterial destruction (Malmstrom et al., 2009). As shown for other serine proteinase inhibitors, Serpin A5 can bind enveloped virions such as HIV and inhibit viral transmission (Burgener et al., 2008; Sallenave, 2002; Moriuchi et al., 2000). Furthermore, Serpin A5 can be internalized by neutrophils, thereby promoting phagocytosis (Baumgartner et al., 2007).

Our findings are interesting in light of recent research suggesting that serine proteinase inhibitors overexpressed in the CVF may be involved in HIV resistance. In two independent studies, (Burgener et al., 2008, 2011) identified 16 and 41 differentially expressed proteins including several serine proteinase inhibitors, such as those from the Serpin family (A1, A3, B1, 3, 4, 13, C1, G1) and alpha 2-macroglobulin like-1 protein. Recently, a study from the same authors demonstrated that abundance levels of serpins such as Serpin A1 and A3 may vary according to the menstrual cycle but are independent to epidemiological sexual confounders (Rahman et al., 2013). Although Serpin A5 was not mentioned in this paper, it is highly unlikely that the difference in protein abundance of Serpin A5 described in our study is influenced by the menstrual cycle because for each of the four iTRAQ experiments we used pooled samples originating from 4 women. A study of our group also showed that Serpin A1 levels were influenced by the menstrual cycle, while Myeloblastin levels were not. Unfortunately, Serpin A5 could not be identified in this study, possibly because another LC-MS/MS platform was used (data not shown). Nevertheless, validation of differences in Serpin A5 levels by means of ELISA on individual samples showed very similar results, indicating that our findings are most probably independent of the menstrual cycle.

Iqbal et al. (2009), Drannik et al. (2012b) found a significant correlation between elafin, a serine proteinase inhibitor, and the HESN status. Increased CVF concentrations of another serine proteinase inhibitor, the secretory leukocyte proteinase inhibitor (SLPI), have been shown to be correlated with reduced rates of perinatal HIV-1 transmission (Pillay et al., 2001). Serine proteinase inhibitors are a large protein family and have a plethora of biological functions including regulation of inflammation and immune response, aid in wound repair, extracellular matrix remodelling and maintaining the integrity of the epithelial barrier (Mangan et al., 2008). The role of serine proteinase inhibitors in HIV resistance has not been determined. Many serine proteinase inhibitors (e.g., Serpin B1, Serpin B4 and elafin) have anti-inflammatory activities and prevent disruption of the epithelial barrier by host and bacterial-derived proteinases, thereby ensuring the structural integrity of the mucosal layer (Benarafa et al., 2007; Sallenave, 2002; Young et al., 2010). Thus, they may prevent HIV entry via lesions in the mucosal layer. SLPI has been postulated to bind the membrane proteins scramblase (Tseng and Tseng, 2000) and/or annexin II (Ma et al., 2004), thereby preventing fusion and interaction of the HIV virus with the host cell membrane, respectively. Serine proteinase inhibitors often inhibit cathepsin G, which is an inflammatory proteinase that enhances HIV replication in vitro (Moriuchi et al., 2000). Furthermore, some serine proteinase inhibitors such as elafin, Serpin A1 and C1 directly interact with HIV to inhibit replication (Elmaleh et al., 2005; Ghosh et al., 2009; Young et al., 2010). Finally, Serpin A1 has been shown to have in vivo anti-HIV activity and is downregulated in HIV infection, suggesting an HIV-suppressing role for this proteinase inhibitor (Bryan et al., 2010). The results from these studies indicate that serine proteinase inhibitors may be important for the establishment of in vivo HIV resistance.

Myeloblastin: A serine proteinase

In contrast to other proteomics studies on CVF of HESNs, which pointed to the involvement of serine protease inhibitors in the mechanism of HIV resistance, we also demonstrated that decreased abundance of the serine proteinase Myeloblastin may contribute to HIV resistance. Although validation by ELISA was so far unsuccessful, possibly due to matrix effects, literature points to a role of Myeloblastin in HIV resistance. Indeed, the activity of Myeloblastin is counteracted by elafin (Ghosh et al., 2009; Iqbal et al., 2009; Zani et al., 2009), and SLPI binds to this protein, although with low affinity (Moreau et al., 2008). Therefore, the reduction of Myeloblastin could be considered as the 'mirror part' of the anti-HIV mechanisms in the CVF of HESNs, suggesting that simultaneous upregulation of serine proteinase inhibitors and downregulation of serine proteinases is involved in the anti-HIV mechanism.

Myeloblastin (also known as leukocyte proteinase 3) is a serine proteinase that is expressed in the azurophilic granules of neutrophils and in the granules of monocytes and is secreted at sites of inflammation. Myeloblastin has several distinct physiological roles such as eliminating phagocytised micro-organisms and promoting the influx of inflammatory cells to the site of infection. It can degrade extracellular matrix proteins and basement membrane proteins (e.g., elastin, collagen and laminin), thus disrupting the mucosal barrier. This improves cellular movement over the epithelial layer but also enhances HIV transmission through gaps between the epithelial cells (Hladik and Hope, 2009). In addition, the protein enhances the production of IL-8 and MCP-1 by endothelial cells, which contributes to continued inflammation, and recruits neutrophils, monocytes and T cells (van der Geld et al., 2001). Because many of these cells are in fact target cells for HIV, Myeloblastin could also indirectly promote HIV infection. Finally, secretion of Myeloblastin by neutrophils is increased in HIV-infected persons, which further supports the hypothesis that Myeloblastin exhibits HIV-promoting activity (Trial et al., 2004).

HESN studies

While we identified several proteins that were differentially expressed in the first study of Burgener et al. (2008), we generally did not find correlations of these protein levels with the HESN status. Only haptoglobin was found to be downregulated in HESN individuals in both studies (Burgener et al., 2008). A better overlap was observed with the second study of Burgener et al. (2011) as SPINK 5, suprabasin, haptoglobin, fibrinogen and dermcidin were also overexpressed in

HR-HESNs in this study. However, our study setup shows that care must be taken in pointing some of these proteins (SPINK5, suprabasin and dermcidin) as correlates of HIV resistance since variation in abundance may also occur as a result of other gynaecopathological conditions (Table 2). In contrast, cathepsin B was found to be down-regulated in Burgener et al. (2011) while it was overexpressed in our study. Furthermore, although we identified elafin, we did not find the same correlation between this protein and HIV resistance, as documented in the study of Iqbal et al. (2009). The discrepancies between our results and these two studies may be due to different techniques and HESN cohorts (Wu et al., 2006; Burgener et al., 2010), once more stressing the importance of investigating several cohorts during the search for correlates of HIV resistance. Indeed, the HIV-resistance mechanisms may differ due to particular genetic polymorphisms. For example, several polymorphisms of the gene coding for Serpin A5 have been described for different ethnic populations (Radtke et al., 1996; Bungum et al., 2010; Larget-Piet et al., 1993). Therefore, the HESN status of the women we examined may be associated with a particular polymorphism of the Serpin A5 gene, which is absent in other HESNs. Further analysis of other different HESN cohorts and use of diverse experimental procedures may therefore yield new potential markers of HIV resistance.

Conclusions

We hypothesize that a reduction of the total serine proteinase activity in CVF may result in increased resistance to HIV. We suggest that this decreased activity is not only achieved by an upregulation of serine proteinase inhibitors (e.g., Serpin A5, elafin, SLPI, Serpin B, C and G and alpha 2-macroglobulin like-1 protein) but may also be obtained by a decrease in the abundance of serine proteinases (e.g., Myeloblastin). During the last decades, numerous correlates of HIV protection were described but rarely confirmed in independent studies. Therefore, it is important to note that findings of this study were now observed by different research teams and in different cohorts. The anti-HIV activity in the CVF of HESN individuals may be the result of several mechanisms that work cooperatively (Benarafa et al., 2007; Sallenave, 2002; Young et al., 2010) and at multiple levels (Drannik et al., 2012a) such as preservation of the epithelial layer integrity, binding of serine proteinase inhibitors to cellular membrane proteins to prevent virus infection, direct and indirect deactivation of the virus by binding to it or by attenuation of the innate or adaptive immune system (Drannik et al., 2012a; Lajoie et al., 2012; Chege et al., 2012; Tomescu et al., 2011). Finally, the role of the complex vaginal microflora and the reciprocity with inflammatory mucosal anti-HIV response, needs further investigation (Schellenberg and Plummer, 2012).

Materials and methods

Sample collection

Vaginal washings were collected in phosphate buffered saline (PBS) from a female sex worker population attending the confidential clinic of the Projet-RETRO-CI in Abidjan, Côte d'Ivoire from 1998 to 2001 (Jennes et al., 2003; Ghys et al., 2002). All the collected samples were directly frozen, transported in liquid nitrogen and stored at minus 80 °C until analysis to avoid protein degradation. The study was approved by the ethical committee of the Ministry of Health, Côte d'Ivoire, the ethical committee of the Institute of Tropical Medicine, Antwerp, Belgium, and by the Institutional Review Board of the Centers for Disease Control and Prevention, Atlanta, GA. A written informed consent was obtained from all subjects prior to their participation in the study. Women enrolled in the study were subjected to a gynaecological examination and were interviewed by trained social assistants to gain information about demographical, socio-economical, physiological and pathological parameters. These epidemiological data were used to create relevant study groups in a very strict way. Although no available measurable physiological parameters are indicative for HIV resistance, the HESN status of individuals in the population was estimated using three reported factors: (1) the duration of the commercial sex work, (2) the use of condoms and (3) the number of clients (Camara et al., 2010; Jennes et al., 2004).

Experimental setup

Based on these criteria, three different study populations could be distinguished: (1) A low-risk group (LR) consisting of HIV-seronegative individuals who were doing commercial sex work for less than 12 months, had less than 25 clients per week and always used a condom. Although a non sex worker population could be used for this group, the experimental setup described here is more relevant because this group of LR female sex workers may have more variables in common with the other groups, which consequently reduces the potential variabilities in the CVF proteome. (2) A long-term exposed high-risk group (HR) consisting of women who were active for over 48 months and did not or sporadically use condoms. During these years (late 1990s) no ARVs were available in Côte d'Ivoire. Therefore, this group was expected to be enriched for HIV resistance factors because the individuals were exposed to HIV for a long time in the absence of HIV infection. (3) An HIV-positive group of the same cohort of female sex workers (HIV) was included as an additional control group (Burgener et al., 2008; Jennes et al., 2004; Marmor et al., 2006; Kulkarni et al., 2003; Iqbal et al., 2009).

Forty-eight samples were obtained from women who were not menstruating, were not pregnant and did not have bartholonitis at the time of the sample collection. In addition, selected women did not engage in vaginal practices such as the use of herbs or vaginal douching using African homeopathic drugs or Dettol. Women reporting douching with water or soap were not excluded because this is a common practice among female sex workers. For each subgroup (LR, HR or HIV), 16 samples were selected and divided over four different experiments (i.e., a pooled sample from four samples per subgroup per experiment) according to urogenital clinical manifestations located at different sites in the female genital tract: the absence of infections (not infected (NI)), the presence or absence of mucopurulent cervical discharge (MCD, located in cervix), infections of the higher genital tract (IHGT, located in uterus and higher) and bacterial vaginosis (BV, located in vagina). The first experiment (NI) included samples obtained from not infected (other than HIV infection) individuals. In the second experiment (MCD+), all samples were from women with MCD but lacking BV and IHGT. The third experiment (IHGT+) consisted of samples from females devoid of MCD, with IHGT and without BV. Experiment four (BV+) included samples from women with or without MCD but lacking IHGT and with BV. Stratification of these additional infections was based on a gynaecological examination of the individuals by trained health workers. Table 1 gives an overview of the experimental setup. The protein concentration of every sample was determined using a BCA assay. For each experiment, 150 μg was taken from the four samples per subgroup and pooled in order to create one sample per subgroup per experiment, resulting in a total of twelve pooled samples. In order to be able to compare the different experiments with each other, an internal standard was included in each experiment. This standard was composed of a mixture of aliquots from all 48 samples and was subsequently divided into four fractions.

iTRAQ labelling and proteomic analysis

After pooling, samples were digested and labelled according to the iTRAQ protocol (HIV: label 115; LR: label 117; HR: label 119; internal standard: label 121). In a first dimension peptides were separated by using a strong cation exchange (SCX) column. During SCX separation 20 fractions were offline collected. Each of these fractions was loaded and separated in a second dimension on a reverse phase (RP-C18) micro capillary column. Eluting peptides were spotted on a MALDI target by using an automated fraction collector. After applying matrix, mass spectrometric analysis was performed by using MALDI-ToF/ToF. Tandem mass spectra were screened against a human SwissProt database by using MASCOT. See Appendix B for more detailed information about iTRAQ labelling and proteomic analysis.

Data-analysis

Scaffold (version Scaffold 3.00, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 95.0% probability as specified by the stringent Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least 1 identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Applying these stringent algorithms result in highly confident identifications. False discovery rates (FDR) were performed for every experiment based on the peptide level by screening the spectra against a target-decoy database (Elias and Gygi, 2007).

Scaffold Q+ (version Scaffold 3.00, Proteome Software Inc., Portland, OR) was used for the relative quantification based upon detected iTRAQ reporter ions during the tandem mass spectrometric analysis of the four different experiments. For each experiment, the obtained values were normalized by dividing them with the value of the internal standard (label 121). As a result, abundance profiles could be compared, and inter- and intra-experimental and statistical testing could be applied.

In the first experiment, we used samples from women who had no BV, MCD or IHGT and determined that a set of proteins showed pronounced up- or down regulation in the HR individuals compared to the HIV and LR groups. We performed three other experiments using samples grouped according to the presence and absence of specific pathological conditions (BV, MCD and IHGT). After performing a Kolmogorov-Smirnov test to check the normality, a parametric one-way ANOVA combined with post-hoc LSD testing was done in order to isolate those proteins showing a statistically significant abundance difference between HR and LR/HIV. The same statistical methods were used to determine whether the gynaecopathological conditions significantly influence the composition of the CVF proteome by pairwise comparison between NI and BV+, MCD+ and IHGT+. A significance threshold of 0.05 was applied. Due to the limited number of samples and the explorative character of the iTRAQ study, no correction for multiple testing was performed. This way, elimination of potential useful results was largely avoided, but confirmation of positive results by an orthogonal assay (ELISA, see below) is necessary. Statistical analysis was performed using SPSS version 15 (SPSS Inc, Chicago, IL, USA).

ELISA assay

Variations in sample protein content determination were minimalized by calculating the average of three independent BCA assays. Since we noticed that BCA measurements were not confident below concentrations of 100 μg/ml, only samples with protein contents above this value were taken into consideration. 26 individual samples (LR, HR and HIV) originating from the same cohort but differing from the samples used for the proteomics experiments, were additionally tested by means of ELISA. Serpin A5 levels were measured according to manufacturer's guidelines of the ELISA (BlueGene Biotech, Shanghai, China). In short, 50 μl of undiluted samples were pipetted onto a 96-well microtiter plate precoated with an antibody specific for Serpin A5. A Standardized preparation of horseradish peroxidase (HRP)-conjugated polyclonal antibody specific for Serpin A5 (100 μl) was added to the wells and incubated for 1 h at 37 °C. The wells were washed and 100 μl substrate solution was added. Intensity of colour development was measured spectrophotometrically at a wavelength of 450 nm (2103 EnVision Multilabel Plate Reader, PerkinElmer, Zaventem, Belgium). The Serpin A5 concentration in each sample was interpolated from the standard curve. The sensitivity of the assay is 1.0 ng/ml for Serpin A5. The intra-assay precision (coefficient of variation expressed in %) was determined to be $<9\%$. Due to the restricted number of samples (LR: 9; HR: 7; HIV: 10), non-parametric statistical testing (Mann Witney *U*-test) was performed to detect significant difference in abundances between subgroups.

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Appendix A and B. Supporting information

Lists of all the proteins identified in the four experiments and detailed information about the peptide identifications are given in Appendix A.

Extensive methodological details about labeling and applied LC-MS/MS techniques (Appendix B).

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.04.015.

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