Proteome profiles of vaginal fluids from women affected by bacterial vaginosis and healthy controls: outcomes of rifaximin treatment

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Objectives: This study was designed to characterize the proteome of vaginal fluid (VF) from women with bacterial vaginosis (BV) in comparison with that from healthy women, and to evaluate the effect exerted by rifaximin vaginal tablets.

Methods: Women with BV (n = 39) and matched healthy controls (n = 41) were included in the study. BV patients were distributed among four groups receiving different doses of rifaximin. Vaginal rinsings were collected at the screening visit from all the participants and at a follow-up visit from BV-affected women. The VF proteome was analysed by tandem mass spectrometry using an Orbitrap mass analyser.

Results: A large number of human proteins were differentially expressed in women with BV in comparison with healthy women (n = 118) and in BV-affected women treated with rifaximin (n = 284). In both comparisons, a high proportion of the dysregulated proteins (≏20%) were involved in the innate immune response. Twenty-one of 24 proteins increased in abundance in women with BV versus healthy women and 31/59 proteins decreased after rifaximin treatment, suggesting a general reduction of the immune response resulting from the therapy. Major changes in protein abundance were found following treatment with 25 mg of rifaximin once daily for 5 days.

Conclusions: BV is associated with a massive change in the VF proteome, mainly regarding the abundance of proteins involved in the innate immune response. Rifaximin at a dosage of 25 mg for 5 days modulated the vaginal proteome, counteracting the alterations associated with the BV condition.

Keywords: antibiotics, vaginal fluid proteome, MS/MS analysis, innate immune response

Introduction

Vaginal fluid (VF) is a complex biological fluid consisting of water, electrolytes, low molecular weight organic compounds (glucose, amino acids and lipids), a vast array of proteins and proteolytic enzymes arising from plasma transudate, vaginal epithelial cells and vaginal microbiota.1–3 VF forms the first line of defence against external pathogens, signals fertility and aids insemination, pregnancy and labour.3 Collection of VF according to standardized procedures4 is minimally invasive and safe, and therefore it is especially interesting and potentially useful as a source of biomarkers for the diagnosis of pathological conditions as well as for the development of prevention strategies.1–3

Bacterial vaginosis (BV) is a common vaginal disorder among women of childbearing age. BV is an imbalance in the ecology of the normal vaginal microbiota that is characterized by the depletion of lactobacilli and the proliferation of anaerobic bacteria such as Gardnerella vaginalis, Mobiluncus species, Prevotella species, Mycoplasma hominis and Atopobium vaginae.5,7 BV affects 10%–15% of women of reproductive age and is associated with genital tract infections8 and pregnancy complications such as preterm birth.9

Conventional treatments for BV are represented by metronidazole and clindamycin, but their effectiveness seems to be limited due to BV recurrence10 and adverse effects associated with their systemic absorption.11 The limits of conventional antibiotics raise the question for alternative therapeutics. Rifaximin is a semisynthetic rifamycin derivative with a broad antimicrobial spectrum and a good safety profile because of its negligible systemic absorption.12 On the basis of these pharmacological features, together with the emerging evidence that rifaximin does not dramatically affect the gut microbiota,13,14 it has recently been proposed as a promising candidate for BV cure and remission maintenance.15
Proteomics is a powerful discipline that has become increasingly important in the study of biological processes by defining cellular functions and networks at the protein effector level. In particular, advances in mass spectrometric technologies have made it possible to comprehensively analyse proteins in very complex mixtures, making feasible the resolution of human biological fluid proteomes. Furthermore, advancements in multidimensional protein separation techniques have allowed the identification of proteins present in trace amounts, thereby increasing the dynamic range of detection in complex biological samples. To date, studies on the identification of potential biomarkers in the VF of women affected by BV have been restricted to the use of antibody-dependent techniques. Only a limited number of proteomic studies using high-throughput mass spectrometry techniques have been performed on VF, and the majority of them have been focused on the search for potential markers for pregnancy-associated conditions such as preterm labour and intra-amniotic infections.

The present study was designed to characterize the proteome profile of VF from BV-affected women in comparison with healthy women, as well as to evaluate the effect exerted by rifaximin administered through vaginal tablets. The VF proteome was analysed by tandem mass spectrometry (MS/MS) using an Orbitrap mass analyser, which is an instrument of choice for many proteomics applications owing to its high mass accuracy, high resolving power and high dynamic range. In addition, the ProteomeSep (MF10) fractionation system was used in order to increase the proteome coverage, particularly by enriching low-mass and low-abundance proteins and peptides.

Methods

Subjects and sample collection

A total of 80 Belgian pre-menopausal, non-pregnant women, aged between 18 and 50 years were included in the study (Figure 1). At a screening visit (V1), 39 patients were diagnosed for BV as they presented with a Nugent score ≥3 and were positive for at least three of four Amsel's criteria [BV (n = 39)]. These BV-affected women were included in a multicentre, double-blind, randomized, placebo-controlled study (EudraCT: 2009-011826-32) that was performed to compare the efficacy and safety of a multicentre, double-blind, randomized, placebo-controlled study (EudraCT: 2009-011826-32) that was performed to compare the efficacy and safety of 25 mg rifaximin administered through vaginal tablets.

Protein extraction

From each vaginal rinsing, 1 mL was centrifuged at 9500 g for 15 min to separate the pellet from the supernatant, which was used for protein extraction.

Nine volumes of acetone:HCl (10:1) were added to the supernatant of the vaginal rinsing and proteins were precipitated by centrifuging at 12000 g for 10 min. The protein pellet was dissolved in 1 mL of 70% ethanol and the sample was spun at 12000 g for 10 min. Acetone (1 mL) was added and the proteins were further precipitated by centrifugation at 12000 g for 5 min. After removing the supernatant, the pellet was air-dried and stored at −20°C. Each protein extract was resuspended in 50 mM ammonium bicarbonate, 2 M urea and 10 mM dithiothreitol pH 8 and quantified using the 2-D Quant Kit (GE Healthcare, Uppsala, Sweden) according to the manufacturer’s instructions.

Proteomic study design

Proteins extracted from VF of the enrolled women were collected into nine pools (Table S1, available as Supplementary data at JAC Online): H pool, containing proteins from healthy women; BV pool, containing proteins from BV-affected patients collected at V1; A-R pool, containing proteins from BV-affected women belonging to treatment group A who were in remission at V3; A-N pool, containing proteins from BV-affected women belonging to treatment group A who were not in remission at V3; B-R pool, containing proteins from BV-affected women belonging to treatment group B who were in remission at V3; B-N pool, containing proteins from BV-affected women belonging to treatment group B who were not in remission at V3; C-R pool, containing proteins from BV-affected women belonging to treatment group C who were not in remission at V3; C-N pool, containing proteins from BV-affected women belonging to treatment group C who were not in remission at V3; D-R pool, containing proteins from BV-affected women belonging to treatment group D who were not in remission at V3.

In order to identify low-abundance proteins and peptides, the Microflow MF10 system was used to fractionate protein pools H and BV (Table S1, available as Supplementary data at JAC Online). The fractionation was not performed on A-R, A-N, B-R, B-N, C-R, C-N and D-N pools due to the low protein content of samples. Two comparisons were carried out: (i) fractionated pool of proteins from VF of healthy women (H) versus fractionated pool of proteins from women affected by BV (BV); and (ii) whole protein pools from patients affected by BV treated with different doses of rifaximin or placebo before (BV) and after (A-R, A-N, B-R, B-N, C-R, C-N, D-N) treatment (Table S1, available as Supplementary data at JAC Online).

MF10 fractionation of proteins

Prior to fractionation, pools H and BV, containing 1 mg of protein each, were prepared. To constitute these pools, equal quantities of protein from each vaginal sample were mixed, dried down and resuspended in 280 μL of 90 mM Tris/10 mM epsilon aminocaproic acid (EACA) and 1 M urea buffer pH 10.2. MF10 fractionation of proteins was performed using a 5-cartridge assembly. The cathode-end cartridge was fitted with a 5 kDa restriction membrane followed by 125 kDa, 50 kDa, 25 kDa and 5 kDa separation membranes. The anode-end cartridge was fitted with a 1 kDa membrane (regenerated cellulose, Millipore) and a 5 kDa membrane facing the anode-circulating buffer. The resulting assembly generated five chambers. Cartridge assemblies had two lanes of chambers that allowed fractionation of two samples in one run. One hundred mL of 90 mM Tris/10 mM EACA and 1 M urea buffer pH 10.2 was added to the buffer reservoir and circulated around the electrodes. Protein pools (140 μL) were added to the chamber closest to the cathode for separate runs. Fractionations were performed
at 250 V for 30 min. Following fraction collection, the lower fractions (1–5 kDa and 5–25 kDa) were further concentrated and desalted using Stage tips\textsuperscript{C18, 200 mL (Proxeon Biosystems, Odense, Denmark)}, according to the manufacturer’s instructions (pools H\textsubscript{F} and B\textsubscript{VF}).

**MS/MS analysis**

Liquid chromatography (LC)–MS/MS analysis was carried out for the H\textsubscript{F} and B\textsubscript{VF} pools and for the unfractionated BV, A\textsubscript{R}, A\textsubscript{N}, B\textsubscript{R}, B\textsubscript{N}, C\textsubscript{R}, C\textsubscript{N} and D\textsubscript{N} pools containing 50 μg of protein each (Table S1, available as Supplementary data at JAC Online). Each fraction or pool was resuspended in 50 μL of 50 mM ammonium bicarbonate, 2 M urea and 10 mM dithiothreitol pH 8. Trypsin (1 μg) was added and the reaction was incubated at 37°C overnight. The digestion was halted by addition of 5 μL of formic acid and the samples dried. Digested samples were resuspended in 10 μL of buffer A (0.1% formic acid), and 0.2 μL of each sample in triplicate was analysed with blanks in between (buffer A).

Digested peptides were separated by nano-LC using an Ultimate 3000 HPLC and autosampler system (Dionex, Amsterdam, the Netherlands). Samples (0.2 μL) were concentrated and desalted onto a micro C\textsubscript{18} precolumn (500 μm x 2 mm, Michrom Bioresources, Auburn, CA, USA) with H\textsubscript{2}O:CH\textsubscript{3}CN (98:2, 0.05% trifluoroacetic acid, v/v) at 10 μL/min. After a 4 min wash, the precolumn was switched (Valco 10 port valve, Dionex) into line with a fritless C\textsubscript{18} nano column (75 μm i.d. x 10 cm containing 5 μm, 200 Å media, Michrom Bioresources) manufactured according to Gatlin et al.\textsuperscript{26}. Peptides were eluted using a linear gradient of H\textsubscript{2}O:CH\textsubscript{3}CN (98:2, 0.1% formic acid, v/v) to H\textsubscript{2}O:CH\textsubscript{3}CN (64:36, 0.1% formic acid, v/v) at 250 nL/min over 30 min. High voltage (2000 V) was applied to low volume tee (Upchurch Scientific, Oak Harbor, WA, USA) and the column tip positioned 0.5 cm from the heated capillary (T=280°C) of an LTQ-Orbitrap Velos (Thermo Electron, Bremen, Germany) mass spectrometer. Positive ions were generated by electrospray and the Orbitrap operated in data-dependent acquisition mode. A survey scan of 350–1750 m/z was acquired (resolution = 30000 at 400 m/z). Up to the 10 most abundant ions (>5000 counts) with charge states ≥2 were sequentially isolated and fragmented within the linear ion trap using collisionally induced dissociation. Mass-to-charge ratios selected for MS/MS were dynamically excluded for 45 s.

MS peak intensities were analysed using Progenesis LC–MS data analysis software v4 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Ion intensity maps from each run were aligned to a reference sample and ion feature

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**Figure 1.** Study design and participants throughout the study.
matching was achieved by aligning consistent ion m/z and retention times. The peptide intensities were normalized against total intensity (sample-specific log-scale abundance ratio scaling factor) and compared between groups by one-way analysis of variance (P ≤ 0.05 for statistical significance) and post hoc multiple comparison procedures. Type I errors were controlled for by false discovery rate with q value significance set at 0.01. Results are reported as mean ± SD (normalized ion intensity score). Peptides exhibiting a statistically significant 1.5-fold or greater difference in abundance between groups were identified using the database search program Mascot (Matrix Science, London, UK, www.matrixscience.com). MS/MS spectra of differentiating peptides were searched against the Swiss-Prot database (version 15) using Mascot. Parent and fragment ions were searched with tolerances of ±4 ppm and ±0.5 Da, respectively. Peptide charge states were set at +2 and +3. ‘No enzyme’ was specified. Proteins and peptides were considered confidently identified when matches had a high ion score >20 and were statistically significant and at least semitryptic. Following identification, a filter was applied to select proteins of human origin and those produced by microorganisms associated with the vaginal environment.

Gene ontology (GO) and network analysis
Identified proteins were submitted for GO analysis (AmiGO version 1.8, database release 3 November 2012; http://amigo.geneontology.org) to define biological processes, molecular functions and subcellular localizations. Protein accession numbers and their corresponding fold changes were imported into MetaCore™, a web-based computational platform (v6.8 build 30387; Thomson Reuters, St Joseph, MI, USA) for pathway enrichment and network analysis. The network building among dysregulated proteins and the MetaCore database proteins was performed using the shortest path algorithm and its variants. Networks were ranked according to their statistical significance (P < 0.001) and interpreted in terms of GO. Major hubs were identified based on the connections and edges within the networks.

Results
Clinical outcome
The clinical outcome of rifaximin treatment is shown in Figure 1. The highest percentage of therapeutic remission was found for treatment group B (5/10, 50%), while lower percentages were found among the other treatment groups (A: 2/10, 20%; C: 4/9, 44%). No women treated with placebo were found in remission among the other treatment groups (A: 2/10, 20%; C: 4/9, 44%).

Multivariate analysis of MS data
A multivariate analysis (principal component analysis) was performed on the extracted ions of differentially expressed peptides (P < 0.05) obtained from MS analysis (Figure 2). According to the x-axis, which explained 93.04% of the overall variance in the dataset, the proteomic profiles from healthy women and BV-affected women were dramatically different from each other (Figure 2a). A less clear separation was shown by the peptides of BV-affected women before and after treatment with rifaximin or placebo (Figure 2b). Along the x-axis, which accounted for 39.14% of the total variance, the pools of peptides of VF samples collected after rifaximin treatment were closely grouped but segregated from the pools of peptides of BV-affected women and women treated with placebo. According to the y-axis, which showed a variation of 18.36%, the peptide pools of VF collected after cure were distinct from each other, on the basis of the dose of antibiotic and the response to treatment. Interestingly, pools A-N and B-N were in line with the BV pool and near to the C-R and C-N pools, suggesting a similarity among the proteomic profiles of BV-affected women, women who were not in remission after rifaximin treatment and women who received the antibiotic for only 2 days. Moreover, according to the y-axis, the B-R pool was the most distant from the BV pool, demonstrating significant changes in protein pattern. The A-R and D-N pools occupied an intermediate position between the B-R and BV pools.

Comparison of protein profiles between healthy and BV-affected women
Following a Mascot database search using acquired MS/MS data, a total of 131 human and microbial proteins were successfully identified in the Hf and BVf pools (Table S2, available as Supplementary data at JAC Online). The vast majority of the identified human proteins (84/118, 71%) were increased in the BVf pool, with a median 5.5-fold ratio (range 1.5-fold to 521.1-fold). A significant reduction, ranging from −1.5 to −5645.4-fold (median, −7.0), occurred for 34/118 (29%) human proteins.

Each human protein was assigned to a biological process, a cellular localization and a molecular function based on information from the GO database (Figure 3). Most of the differentially expressed proteins (24/118, 20%) were involved in the innate immune response and complement activation (Figure 3a). Interestingly, this GO category grouped 14 immunoglobulin chain regions that were almost all over-represented in BV (median 7.1-fold ratio). Epidermis development and keratinization accounted for 15/118 (13%) identified proteins whereas 14/118 (12%) were classified as involved in small-molecule metabolic process. Only 6/118 (5%) proteins were involved in the inflammatory response. More than half of the identified proteins were localized in the extracellular space (41/118, 35%) or associated with the plasma membrane (18/118, 15%) (Figure 3b). Nearly a quarter of the identified proteins were cytoplasmic (26/118, 22%). According to molecular function (Figure 3c), as many as 48% (57/118) of the differentially expressed proteins were classified as having binding activity. Among these, protein binding (22/118, 19%) was the most represented GO category, followed by calcium ion (15/118, 13%) and antigen binding (14/118, 12%). Nineteen (22/118) and 16% (19/118) of identified proteins were related to enzymatic and structural molecule activity, respectively.

MetaCore enrichment analysis revealed that the majority of enriched pathways were related to cytoskeleton remodelling, complement activation (classical, alternative and lectin-induced pathways) and blood coagulation (data not shown). Based on the functional subnetworks built using the ‘analyse network’ algorithm, the proteins differentially expressed in the HF and BVf pools were primarily involved in developmental processes (P = 1.22 × 10⁻¹¹), immune system processes (P = 3.93 × 10⁻²²) and response to chemical stimulus (P = 1.71 × 10⁻²⁰) (Table S3, available as Supplementary data at JAC Online). Figure 4 shows a high-significance subnetwork linked to immune response and complement activation. Most of the identified proteins mapped in this network were immunoglobulins and the majority of them were significantly overexpressed in BV (range 3.7-fold to 12.6-fold; median, 7.6). Notably, in this network as well as in
other top-scoring networks, a highly linked hub was represented by the transcription factor SP1. According to the transcriptional regulation networks, SP1 was ranked #1, with 42 targets among the 118 identified human proteins ($P = 7.10 \times 10^{-115}$). Among the 13 microbial proteins that were differentially expressed between the HF and BVF pools, 9 (69%) were derived from *Lactobacillus* strains (belonging to *Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus gasseri* and *Lactobacillus helveticus*) and were mainly involved in glucose metabolism and protein synthesis (Table S2, available as Supplementary data at JAC Online). Three proteins from *Staphylococcus aureus, Staphylococcus epidermidis* and *Candida glabrata* were significantly increased in the BVF pool, even though none of these bacteria is known to be associated with BV.

**Impact of rifaximin on the proteome of BV-affected women**

A total of 314 human and microbial proteins were successfully identified in VF samples of BV-affected women before and after rifaximin/placebo treatment (Table S4, available as Supplementary data at JAC Online). In general, most of the proteins differentially expressed in response to rifaximin/placebo treatment were reduced (192/314, 61%) rather than increased in abundance (122/314, 39%). Of the 284 identified human proteins, 48 (17%) were dysregulated in all pools from rifaximin-treated women compared with the BV pool, regardless of both antibiotic dosage and clinical outcome. Interestingly, the greatest variation for these dysregulated proteins occurred in the B-R pool, followed by the A-R pool, while few or no changes were observed after placebo administration. Opposite trends of expression for BV versus H and rifaximin versus BV were observed for 46 of the 89 proteins that differentially changed their abundance in both datasets. In particular, 26 of these proteins were up-regulated (6) or down-regulated (20) in at least four of the six pools from rifaximin-treated women, contrary to what was found in the BV versus H comparison. For 17/26 (65%) proteins, the greatest fold changes were associated with the B-R pool. Interestingly, group B showed the largest total number of differentially expressed human proteins, with 214 and 155 dysregulated proteins in the B-R and B-N pools, respectively. Moreover, the fold changes of 83 proteins in the B-R pool were the highest among all pools, suggesting a major

Figure 2. Multivariate analysis of MS data. (a) Principal component analysis of the peptides in the fractionated pools of healthy women (HF) and women affected by BV at V1 (BV). (b) Principal component analysis of the peptides in the unfractionated pools of women affected by BV before treatment (BV) and after treatment with 100 mg of rifaximin once daily for 5 days (remission, A-R; no remission, A-N), 25 mg of rifaximin once daily for 5 days (remission, B-R; no remission, B-N), 100 mg of rifaximin once daily for 2 days (remission, C-R; no remission, C-N) and placebo for 5 days (D-N). Samples were run in triplicate.
Figure 3. Pie charts showing the GO categorization of the MS/MS-identified proteins differentially expressed between healthy and BV-affected women. Classification was performed according to keyword categories [(a) biological process, (b) cellular component, (c) molecular function]. When proteins were associated with more than one functional category, one GO term was chosen arbitrarily. NA, not available.
impact of this treatment regimen on the BV-related proteome. Conversely, placebo administration was associated with the lowest number of differentially expressed proteins (207) and the expression variation was often in the opposite direction with respect to the trend observed in the other pools.

Each human protein was assigned to a biological process, a cellular localization and a molecular function based on information from the GO database (Figure 5). Similar to the BV versus H comparison, most proteins were involved in the innate immune response and complement activation (59/284, 21%) and small-molecule metabolic process (41/284, 14%), whereas only 9/284 (3%) were involved in the inflammatory response (Figure 5a). Interestingly, the most represented GO category grouped 17 proteins that were identified as dysregulated also in the BVF versus the HF pool. Ten of them exhibited a trend toward underexpression, contrary to what was found in the BV versus the H dataset. As expected, a large number of proteins were localized in the extracellular space (108/284, 38%) and plasma membrane (34/284, 12%) (Figure 5b). As many as 19% (55/284) of the differentially expressed proteins were cytoplasmic. The main represented molecular functions were structural molecule activity (50/284, 18%), antigen binding (39/284, 14%) and protein binding (36/284, 13%) (Figure 5c).

According to the MetaCore enrichment analysis, the most enriched pathways were related to cytoskeleton remodelling, blood coagulation and complement activation (lectin-induced

Figure 4. Protein network of differentially expressed proteins in Vf of BV-affected women (BVF) in comparison with healthy women (Hf). MetaCore pathway analysis software was used to generate a network of connections between all identified proteins with altered expression levels. Network proteins and connections are visualized by symbols that specify the functional nature of the protein or interaction. Blue or red circles flank experimentally identified proteins and indicate, respectively, a significant down- or up-regulation of the protein in the BVF pool compared with the Hf pool. The mixed coloured circle for IgM indicates mixed protein expression between files. This figure appears in colour in the online version of JAC and in black and white in the print version of JAC.
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Figure 5. Pie charts showing the GO categorization of the MS/MS-identified proteins differentially expressed between BV-affected women before and after rifaximin/placebo treatment. Classification was performed according to keyword categories [(a) biological process, (b) cellular component, (c) molecular function]. When proteins were associated with more than one functional category, one GO term was chosen arbitrarily. NA, not available.
and classical pathways) (data not shown), similar to the previous analysis of the HF and BVF pools. Based on the functional subnetworks built using the ‘analyse network’ algorithm, the proteins differentially expressed in response to rifaximin/placebo treatment were primarily involved in cell differentiation ($P = 1.44 \times 10^{-45}$), complement activation ($P = 4.10 \times 10^{-39}$) and response to chemical stimulus ($P = 4.34 \times 10^{-34}$) (Table S5, available as Supplementary data at JAC Online). Figure 6 shows the MetaCore map of five dysregulated proteins (complement C3, complement C4-A, immunoglobulin γ1, immunoglobulin μ and plasma protease C1 inhibitor) in the classical complement pathway. All the proteins were generally down-regulated after antibiotic administration and the highest decreases were observed in the B-R pool, confirming the major impact exerted by group B treatment on the vaginal environment.

More than half (16/30, 53%) of the microbial proteins that were differentially expressed in BV-affected women before and after rifaximin/placebo treatment were from Lactobacillus species (L. acidophilus, Lactobacillus brevis, L. casei, Lactobacillus delbrueckii subsp. bulgaricus, L. gasseri, L. helveticus, Lactobacillus johnsonii), and were mainly involved in glucose metabolism, replication and protein synthesis. Interestingly, only trigger factor from L. brevis was found to be down-regulated in all pools after rifaximin treatment, with a median 2.5-fold ratio. Contrasting expression patterns among pools were observed for nearly half (7/16, 44%) of the proteins from lactobacilli, suggesting a lack of correlation with the antibiotic treatment. Fourteen (47%) dysregulated microbial proteins were from other microorganisms that are associated with the vaginal environment, Oenococcus oeni, Pichia guilliermondii, Bifidobacterium longum subsp. infantis, Saccharomyces cerevisiae.
S. epidermidis, Ureaplasma parvum, Mycoplasma genitalium, Escherichia coli and S. aureus.

Discussion

In the last decade, many studies have focused on the complex pathophysiological processes underlying BV, with the aim of finding efficient strategies for prevention and cure of this vaginal condition.11,15,29 Nonetheless, the root cause of BV remains poorly understood, even if the polymicrobial nature of the vaginal microbiota has been shown to be implicated in the disease aetiology.30–33 The present study reports for the first time, to our knowledge, the proteome profiling of VF from BV-affected women in comparison with healthy women, and discusses the potential of rifaximin in restoring a healthy condition.

Profound changes were detected in the VF proteome of BV-affected women compared with healthy women, as well as following rifaximin/placebo treatment. In particular, according to principal component analysis, the major separation from the BV condition was observed following treatment with 25 mg of rifaximin once daily for 5 days, suggesting that this dosage exerted the major impact on the vaginal proteome. This finding is in agreement with both the clinical11 and microbiological response,15 which has demonstrated that this dosage is the most effective in inducing a decline of BV-associated bacteria without affecting the normal population of lactobacilli.

The MS/MS analysis of protein fractions allowed the identification of 118 human proteins differentially expressed between VF from BV-affected and healthy women. A high percentage of proteins (100/118, 85%) had already been identified in human VF and cervical mucus, indicating good consistency with the data reported in the literature.3 The vast majority of the differentially expressed proteins were up-regulated in BV-affected women, suggesting that BV is characterized by global stimulation of protein expression by the vaginal mucosa. Based on GO classification, a high proportion of these proteins are involved in the innate immune response and metabolic processes and are localized in the extracellular space and cytoplasm, in line with the classification of VF proteins reported by Zegels et al.3 Interestingly, almost all immunoglobulins and other immune molecules were up-regulated in the presence of BV, suggesting a major role for the immune system in the pathophysiological process underlying this vaginal condition, as previously hypothesized.34,35 By contrast, only 5% of identified proteins were involved in the inflammatory response, in accordance with the assumption that BV is a non-inflammatory vaginal infection.15,36 as compared with aerobic vaginitis,37 even if few studies correlate BV with altered levels of certain pro-inflammatory cytokines.38 A high number of intracellular and cytoskeleton proteins and keratins were identified, probably resulting from the disruption of the epithelial cell layer.3 Interestingly, according to MetaCore analysis of the networks involving the dysregulated proteins, the transcription factor SP1 was shown to be a highly linked hub in top-scoring networks, built with the shortest path algorithm. SP1 can activate or repress transcription in response to physiological and pathological stimuli, regulating the expression of a large number of genes involved in a variety of processes, such as cell differentiation, cell growth, apoptosis, the immune response, response to DNA damage and chromatin remodelling.39 However, whether and to what extent SP1 may participate in the onset and progression of BV need to be further explored.

To date, studies aimed at identifying potential protein markers for BV have been conducted using traditional ELISA techniques. In these studies, different markers, such as antimicrobial peptides18,19 and cytokines19 have been evaluated. By contrast, our proteomic study, conducted with a high-throughput approach without seeking specific protein targets, allowed us to characterize the entire protein profile of VF under BV conditions and provided novel information that could be integrated with data coming from traditional analyses.

Following rifaximin/placebo treatment, 284 human proteins in VF were differentially expressed compared with the BV condition. More than 200 proteins (223/284, 79%) had already been identified in previous reports.3 Most of the dysregulated proteins were down-regulated in patients treated with rifaximin, suggesting a role for the antibiotic in counteracting the protein profile alterations observed in BV-affected women. Similarly to the BV versus H comparison, the main categories resulting from GO classification referred to the innate immune response and complement activation, and small-molecule metabolic process, whereas only a small percentage (3%) was involved in the inflammatory response. Notably, immunoglobulins and other immune molecules exhibited a trend towards under-representation, contrary to findings in the BV versus H dataset comparison, indicating a general shutdown of the immune response after antibiotic treatment.

Our proteomic study also highlighted a different modulation of the vaginal proteome according to the antibiotic dosage. The largest number of differentially expressed proteins and the greatest fold changes in protein expression were indeed identified following treatment with 25 mg of rifaximin once daily for 5 days, further confirming the major impact of this treatment regimen on the BV-related proteome. This dosage has also recently been shown to result in the best clinical response in a larger group of women.11 Conversely, placebo administration was associated with the lowest number of differentially expressed proteins and the expression variation was often in the opposite direction to the trend observed in rifaximin-treated women.

With regard to the microbial proteins whose expression was found to be dysregulated in the BV versus the H group or following rifaximin treatment, >50% were of Lactobacillus origin, as expected considering the abundance of lactobacilli in the vaginal microbiota. However, no correlation was observed between the expression levels of these proteins and the development of the pathology or the effects of antibiotic treatment. Despite this, the identification of microbial proteins in VF described in our work represents an important step forward in the knowledge of the vaginal ecology because until now only one microbial protein has been detected in VF.3

In conclusion, our study demonstrates for the first time that BV is associated with profound changes in the VF proteome, mainly with respect to the innate immune response, and suggests the ability of rifaximin, especially at a dosage of 25 mg once daily for 5 days, to modulate the vaginal proteome by counteracting the alterations associated with the BV condition. The proteomic data reported in this work support the clinical and microbiological results previously reported11,15 and open the way to further exploration of the real advantages of rifaximin in comparison with metronidazole and clindamycin in the treatment of BV.
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Author contributions
F. Cruciani, V. W. and S. T. performed the experiments and statistical analysis of the data. G. D. enrolled the subjects and collected the vaginal samples. F. Calanni and P. B. supervised the study. B. V. conceived and designed the experiments.

Supplementary data
Tables S1 to S5 are available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

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