Candidate *Treponema pallidum* biomarkers uncovered in urine from individuals with syphilis using mass spectrometry

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Aim: A diagnostic test that could detect *Treponema pallidum* antigens in urine would facilitate the prompt diagnosis of syphilis. **Materials & methods:** Urine from 54 individuals with various clinical stages of syphilis and 6 controls were pooled according to disease stage and interrogated with complementary mass spectrometry techniques to uncover potential syphilis biomarkers. **Results & conclusion:** In total, 26 unique peptides were uncovered corresponding to four unique *T. pallidum* proteins that have low genetic sequence similarity to other prokaryotes and human proteins. This is the first account of direct *T. pallidum* protein detection in human clinical samples using mass spectrometry. The implications of these findings for future diagnostic test development is discussed. Data are available via ProteomeXchange with identifier PXD009707.

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Syphilis, a multistage chronic disease caused by the spirochete *Treponema pallidum* subspecies *pallidum*, remains a major public health problem, particularly in men who have sex with men (MSM) [1] and pregnant women populations [2]. Little has changed in the principles of syphilis diagnostics during the last century; nontreponemal tests (NTTs) [3] that likely measure nonspecific antibodies directed against cardiolipin released from damaged human cells and present in *T. pallidum*'s cell wall [4] remain the only tests that can be used to evaluate post-treatment pathogen eradication and reinfection with *T. pallidum*. These tests suffer from a number of shortcomings in specificity and sensitivity, especially during very early and late stage disease, and are not high-throughput [5]. A systematic review of factors determining serological outcomes following therapy for syphilis found that serological cure as determined by NTT testing was primarily associated with younger age, higher baseline nontreponemal titers and earlier syphilis stage [6]. In our medical center in Antwerp, Belgium, more than half of new infections present as reinfections [7], which are more likely to be asymptomatic in HIV-infected individuals [8]. We, therefore, depend on NTT tests with all their shortcomings for the diagnosis of half the cases of syphilis. New diagnostic tests would, therefore, be of considerable utility. One option would be the detection of *T. pallidum* DNA through polymerase chain reaction (PCR) testing, but this has a low sensitivity. Even in the case of secondary syphilis when there is a high *T. pallidum* load in the blood only 52 % of serum specimens are PCR positive [9–11].

Urine testing offers promise due to the ease of collection, high volume that can be collected and lack of proteome complexity. This was reflected in a recent in-depth analysis of the urinary proteome detailing the detection of 6085

proteins [12] compared with serum and plasma, in which over 10,000 different proteins have been reported [13]. Many urine antigen tests are in widespread use for the diagnosis of human pathogens such as *Cryptococcus* spp. [14] and *Histoplasma capsulatum* [15]. Recent studies [16,17] have demonstrated the clinical usefulness of screening patient urine samples for leptospiral antigens. Rapid immunoassay testing of urine for *Streptococcus pneumoniae* and *Legionella pneumophila* (serogroup 1) is widely implemented in the routine clinical setting with high sensitivity and specificity (74.0 and 97.2%, respectively, for *S. pneumoniae* [18]; 77 and near 100%, respectively, for *L. pneumophila* [19]).

The first phase of diagnostic test development involves biomarker discovery, a step that is increasingly facilitated by proteomics mass spectrometry (MS)-based approaches for the detection of candidate protein biomarkers [20]. During the last 5 years, MS-based diagnostic approaches have been successfully introduced into numerous clinical microbiology laboratories [21]. A missing link, however, is that almost all MS diagnostic techniques are contingent upon culture enrichment to achieve a high enough bacterial load to discriminate pathogen identification. This is likely due to the difficulty of detecting very low concentrations of pathogen antigens respective to human proteins. An exception is a recent study by Young *et al.* that employed a shotgun proteomics approach to investigate urine samples for *Mycobacterium tuberculosis* antigens, whereby multiple proteins were uncovered in patient samples with high confidence using tandem MS analyses [22].

One of the main hurdles in biomarker discovery with MS is the intrinsically stochastic nature of the precursor selection in the context of conventional data-dependent acquisition (DDA) strategies, in other words, the instrument samples precursors based on a set of user-defined parameters. This results in undersampling, primarily of low abundant peptides, and reduced reproducibility between runs [23]. Therefore, data-independent acquisition (DIA) methodologies are becoming increasingly popular. In DIA, the selection of precursors is no longer user defined and all eluting ions are fragmented. Especially approaches that do not use DDA-based spectral libraries for data extraction have great potential to uncover previously undetected peptides. One such strategy is high-definition mass spectrometry (HDMS^E), also called ion mobility-assisted DIA [23]. This mode of acquisition is unique; it continuously alternates between high and low energy scans, spanning the entire mass range. To cope with the increased requirement for peak capacity, ion mobility separation has been implemented on the SynaptG2Si instruments (ESI-Q-IM-TOF). Thus, this method is the most comprehensive acquisition methodology to date. The challenge, however, lies in extracting relevant information from the data. For this, fragments and precursors are linked to one another into pseudo-MS/MS spectra by accurate mass, retention time and drift time alignment and are subsequently searched using a dedicated ion accounting algorithm.

In this study, we combined these complimentary DDA and DIA shotgun MS techniques to search for *T. pallidum* proteins in urine samples from individuals with active syphilis in order to identify with high sensitivity potential biomarkers for antigen assay development.

Materials & methods

Ethics approval & informed consent

The prospective observational cohort study (“*Treponema pallidum*-specific Proteomic Changes in Patients with Incident Syphilis Infection [SeTPAT]”; ClinicalTrials.gov #NCT02059525) that provided the clinical samples used in this sub-study was approved by the Institutional Review Board of the Institute of Tropical Medicine Antwerp and the Ethics Committee of the University of Antwerp (13/44/426), Belgium. Written permission for participation was provided by all participants prior to study inclusion.

Study participants

Potentially eligible study participants of 18 years and older, in whom a new syphilis diagnosis was made, were screened and recruited between January 2014 and August 2015 at the sexually transmitted infection clinic of the Institute of Tropical Medicine. Syphilis diagnosis and disease staging was performed according to Centers for Disease Control guidelines [24] and clinical stage-appropriate therapy was administered according to European guidelines [25]. HIV-infected controls with negative syphilis serological tests (Rapid plasma reagin [RPR] and Vitros Syphilis TPA chemiluminescence immunoassay negative) were also included during the same time period. Individuals who used beta-lactam, doxycycline or macrolide antibiotics during the 28 days preceding enrollment were excluded from study participation.

After screening 167 potential study participants, 17 individuals were excluded from participating due to recent antibiotic use or unwillingness to participate and 150 individuals were included into the study, 120 with an active episode of syphilis (RPR and TPA-positive and/or *T. pallidum* serum PCR positive) and 30 controls (RPR, TPA

Table 1. Characteristics of individuals included in this study.

Characteristics	Syphilis-positive cases N = 54	Controls N = 6
	N = (%/IQR)	N = (%/IQR)
Gender (male)	54 (100)	6 (100)
MSM	54 (100)	5 (83)
Age (years)	43 (35–50)	40 (37–46)
HIV-infection	50 (93)	6 (100)
Taking ART	48 (89)	4 (67)
Initial syphilis episode	21 (39)	NA
RPR titre at diagnosis	64 (32–128)	0
Serum PCR positive	16 (30)	0
TPA positive	52 (96)	0
IgM EIA positive	43 (80)	0
Syphilis stage at diagnosis		
Primary/secondary stage	36 (67)	NA
Early latent	13 (24)	NA
Late latent	5 (9)	NA

ART: Antiretroviral therapy; EIA: Enzyme immunoassay; IQR: Interquartile range; PCR: Polymerase chain reaction; RPR: Rapid plasma reagin; TPA: *Treponema pallidum* agglutination.

and serum PCR negative). Plasma, serum and urine were collected from all individuals at study inclusion. For this sub-study, 54 participants with syphilis and 6 controls were included based on syphilis staging and, if applicable, PCR positivity for *T. pallidum*. Of the 54 participants with syphilis, 7 had primary, 29 secondary, 13 early latent and 5 late latent syphilis. Approximately two-thirds (36/54; 67%) of participants were symptomatic at the time of diagnosis – henceforth, these individuals will be referred to as primary secondary stage (PSS) since many of the symptomatic individuals (12/36; 33%) presented with clinical symptoms of both primary and secondary clinical stages, for example, rash and a genital ulcer. All individuals with syphilis were treated shortly after diagnosis with intramuscular benzathine penicillin G (BPG). Seven syphilis positive individuals reported dental problems such as dental caries or gingivitis at the time of study inclusion.

The median age of the participants was 42.5 years (interquartile range [IQR]: 35–50). Almost all (56/60; 93%) were HIV-positive, of which 52/56 (93%) were taking antiretroviral therapy at the time of syphilis diagnosis. All except one control subject self-identified as MSM. Of the syphilis-positive individuals, 33/54 (61%) presented as a repeat infection since they had a previous history of syphilis infection. Repeat syphilis was defined as minimal fourfold increase in RPR titre compared with the previous RPR titre measurement regardless of time between testing. Sixteen (30%) of the individuals with syphilis tested PCR-positive for *T. pallidum* DNA in serum. Of the PCR-positive individuals, 75% (12/16) were from primary and secondary stage, the other four were early latent stage. The median RPR titre at the time of syphilis diagnosis was 64 (IQR: 32–128). One subject with secondary syphilis presented with a diffuse maculopapular rash; serum testing revealed a negative RPR test, indeterminate TPA result and the PCR test was positive. Almost all individuals with syphilis were TPA positive, with the exception of one indeterminate and one negative result. Totally, 80% (43/54) of the IgM enzyme immunoassay (EIA) test results were positive for the individuals with syphilis. A summary of the study participant information is provided in Table 1.

Clinical sample processing & serological testing

Immediately before BPG injection, blood was drawn into serum gel and EDTA-coated collection tubes (Sarstedt Monovette, Nümbrecht, Germany) and separated at 2000 × *g* for 10 min at ambient temperature. Sera were subsequently refrigerated at 4 °C until routine clinical syphilis serological testing had been performed, including Macro-Vue RPR Card (Becton Dickinson, MD, USA), TPA immunoassay (Ortho-Clinical Diagnostics, NY, USA) performed on the Vitros 5600 Integrated System (Ortho-Clinical Diagnostics) and SERODIA *T. pallidum* particle agglutination assay (Fujirebio, Inc., Tokyo, Japan) testing following the manufacturers' instructions.

All fresh sera were also tested with an in-house *T. pallidum* PCR test directed against *polA* [26] performed on the Rotor-Gene 6000 platform (Qiagen, Hilden, Germany). The estimated limit of detection of our assay is two copies per microliter. PCR results were deemed indeterminate if the test result was weakly positive; a second confirmatory

PCR was not performed. EIAs were performed on frozen stored sera (anti-*T. pallidum*-IgM recomWell ELISA, Mikrogen, Neuried, Germany).

Random-void mid-stream urine samples were collected in sterile containers and processed following HUPO guidelines [27], including centrifugation for 10 min at $2000 \times g$ at ambient temperature to remove insoluble contents such as cells and casts. Urine was aliquoted into 15 ml falcon tubes and stored at -80°C until further testing. All urine samples were processed within 3 h of collection. See Supplementary Table 1 for study participant clinical information and urine sample description.

Pooled urine sample preparation, protein extraction & trypsinization for 'proof of concept' MALDI-TOF-TOF analyses to investigate *T. pallidum* biomarkers

Briefly, individual urine samples were thawed at ambient temperature, placed on ice and pooled ($N = 6$ study subjects/pool and ± 1.5 ml urine per subject). Most (55/60) of the urine samples were only subjected to one freeze thaw cycle, with a maximum of two freeze-thaw cycles. Pools were stratified symptomatic (primary and/or secondary) versus asymptomatic (latent stages) since bacterial loads differ considerably during the course of infection. In early infection, especially during secondary stage, *T. pallidum* levels in blood are relatively high (median of 1.4×10^5 cells/ml blood [28]), whereas levels are lower in latent syphilis [29]. However, it is unknown if high *T. pallidum* levels in blood would correlate to high levels in urine. In total, five pools of urine were analyzed (see Supplementary Table 1 for pool details), including $n = 2$ PSS, $n = 1$ early latent and $n = 1$ early/late latent stage and $n = 1$ control pools.

The pooled samples were individually loaded into 50 kDa molecular weight cut-off (MWCO) (Merck Millipore, MA, USA) filters and centrifuged at 4°C at $4000 \times g$ until the sample passed through the filter completely. This step was performed to remove abundant higher molecular weight proteins that could potentially hamper the identification of lower abundant proteins and for the prioritization of smaller size proteins that could be more suitable for EIA detection. The filtrate was then transferred to a 3 kDa MWCO (second step) filter (Merck Millipore) for sample concentration and removal of small nonprotein compounds including urea and centrifuged using the same conditions as the first step. The retentate was transferred into LoBind Eppendorf tubes (Eppendorf, Hamburg, Germany) and six volumes of proteomics grade acetone (Biosolve, Valkenswaard, The Netherlands) was added, followed by overnight incubation at -20°C .

Following protein precipitation, samples were re-suspended in 50 mM Tris-HCl/6 M urea/5 mM dithiothreitol/10% beta-mercaptoethanol (25 μl /100 μg protein) at pH 8.7. For the denaturation and reduction process, all samples were incubated at 56°C for 1 h. Subsequently, samples were diluted with 50 mM Tris-HCl/1 mM CaCl_2 (75 μl /100 μg protein) and alkylated by adding 200 mM iodoacetamide (10 μl /100 μg protein) for 1 h at ambient temperature. Proteomics-grade modified trypsin (Promega, WI, US) was added at an approximate 30:1 protein-to-enzyme ratio. After incubation at 37°C for 18 h the digestion was stopped by freezing the samples.

Peptide separation by reversed phase C18 liquid chromatography at high (1st dimension) & low (2nd dimension) pH

A first dimension peptide separation was performed based on hydrophobicity at high pH using a reversed phase (RP) C18 column (X!Select, CSH, RP-C18, $2.1 \times 150 \text{ mm}^2$, 3.5 μm , Waters Corporation, MA, USA) connected to a Water Alliance e2695 HPLC bio-system and a Waters 996 PDA detector (Waters Corporation). Solvent A contained 200 mM ammonium formate at pH 10 while solvents C and D contained 100% water and 100% acetonitrile (ACN), respectively (ULC/MS grade, Biosolve). During the entire chromatographic separation, 10% of solvent A was continuously added to reach an overall pH of 10. The following gradient was applied at a constant flow rate of 200 $\mu\text{l}/\text{min}$: 5–15% Solvent D over the first 5 min, 15–40% D over 80 min, 40–90% D over 8 min, 5 min 90% D and 90–95% D over 2 min. In total, 30 fractions were collected starting from 10 to 100 min with an interval of 3 min/fraction. The peptide concentration of the different fractions was determined based on the area under the curve at 214 nm.

Peptide fractions were separated in a second dimension by RP-C18 at low pH using an Agilent 1100 series microcapillary HPLC system (Agilent Technologies, Waldbronn, Germany). For each sample, 15 μg of peptides was injected onto a Zorbax 300SB-C18 guard column (0.3 mm $\times$ 5 mm; particle size 3.5 μm ; Agilent Technologies) serially connected to a Zorbax 300SB-C18 analytical RP column (0.3 mm $\times$ 150 mm; particle size 3.5 μm ; Agilent Technologies). Samples were desalted online by loading the peptides on the guard column before the ACN gradient

was started. Solvent A contained 0.1% formic acid (FA) in water while solvent B contained 0.1% FA in 90% ACN/10% water. The following ACN gradient was performed using the microcapillary pump with a constant flow rate of 6 μ l/min: 5–60% B in 56.7 min, ramp to 90% B over 3.3 min, persistent 90% B for 5 min, 85% B for 5 min and back to equilibrating conditions of 3% B. Starting from minute 5 until minute 51.7 of the chromatographic run, 350 spots (800 nl/spot) for each fraction were spotted onto an Opti-TOF MALDI-target (28 columns $\times$ 25 rows; 8 s intervals; 700 spots; 2 runs per target) (Applied Biosystems, Inc., California, USA). Thereafter, each spot was covered with matrix 2 mg/ml α -cyano-4-hydroxycinnamic acid in 70% ACN containing an internal calibrant (93 pmol human [Glu¹]-fibrinopeptide B per ml matrix) by using an external syringe pump with a 4 s interval (800 nl matrix/spot) at a flow rate of 12 μ l/min.

MALDI-TOF/TOF MS/MS analysis in DDA mode

Spotted fractions were analyzed offline using a MALDI ABI4800 proteomics analyzer (Applied Biosystems). MALDI-TOF MS-analysis (reflectron mode; laser intensity: 3400; 25 $\times$ 20 laser shots per spot; mass-range 800–3000 Da) was performed first, after which precursors were selected with an S/N ratio above or equal to 100. [Glu¹]-fibrinopeptide B (m/z 1570.667) was used as internal standard to calibrate the MS spectra. MALDI-TOF/TOF MS/MS analysis was performed on the selected MS precursors. A maximum of 50 unique precursors per spot were selected for fragmentation, starting from the precursors with the lowest S/N ratio. These precursors were ionized (laser intensity: 4300; 25 $\times$ 20 laser shots per spot) and fragmented by collision induced dissociation (CID, 1 kV collision energy).

Spectra from each sample were extracted by Peak Explorer and screened against a *T. pallidum* database containing the UniProt proteomes IDs UP000014259 (corresponding to the resequenced Nichols strain genome [30]) and human proteome UP000005640 (Accessed March 2015) using the MASCOT search engine (Matrix Science; version 2.1.03, MA, USA) based on the digestion enzyme trypsin. Protein designations are listed as in the currently annotated *T. pallidum* proteome listed in the UniProt database (Proteome ID: UP000014259). Carbamidomethylation of cysteine was listed as fixed modification and oxidation of methionine was set as a variable modification. A maximum of one missed cleavage of trypsin was tolerated. Mass tolerance was set to 50 ppm for the precursors and 0.20 Da for the fragment ions. The MudPIT scoring algorithm of MASCOT was used. Because of its higher stringency Scaffold Q+ (version Scaffold 4.4.5, Proteome Software, Inc., OR, USA) was used instead of MASCOT to validate MS/MS-based peptide and protein identifications. Protein identifications were accepted if they could be established at greater than 95.0% probability according to the protein prophet algorithm.

Pooled urine sample preparation for LC/ESI-IM-Q-TOF/HDMS^E analyses

In total, ten pooled patient urine samples were analyzed, five of which were technical replicates containing the same urine samples as the MALDI-TOF/TOF MS/MS analyses. These were prepared from fresh aliquots of urine, with the exception of three samples (two samples from pool 2 and one control sample), for which there was no additional aliquot available thus previously thawed samples were used in these cases. Five additional pools were prepared, corresponding to primary/secondary stage n = 2, secondary n = 2 and early latent stage n = 1 pools. See Figure 1 for work-flow description and sample summary. The samples were prepared similarly to the procedure used in the aforementioned MALDI-TOF MS/MS analyses, with a few minor procedural modifications. Following protein acetone precipitation, samples were resuspended in 50 mM ammonium bicarbonate buffer and 10 mM dithiothreitol at pH 8.7. For the denaturation and reduction process, all samples were incubated at 60 °C for 1 h. Subsequently, proteins in all fractions were diluted in 50 mM ABC and alkylated by adding 200 mM iodoacetamide in isopropanol for 10 min at ambient temperature in the dark. Proteomics-grade modified trypsin (Promega) was added at a 30:1 protein-to-enzyme ratio and 5% ACN was added to each sample.

LC-Q-IM-TOF HDMS^E analysis of pooled urine samples for *T. pallidum* protein biomarker detection

Prior to the 1D chromatographic peptide separation, dried peptide samples were suspended in 300 μ l 0.1% FA to obtain a concentration of approximately 1 μ g/ μ l. Approximately 400 ng of peptides were separated on a NanoACQUITY System (Waters Corporation) with direct injection on a NanoACQUITY column (UPLC 1.7 μ m BEH130 100 μ m $\times$ 100 mm C18) at a flow rate of 300 nl/min. The LC-gradients were obtained by a combination of mobile phase A (0.1% FA and 3% DMSO) and mobile phase B (80% ACN and 0.1% FA), with a column temperature maintained at 45 °C. All ten pooled samples were run twice, with a 60 and 120 min LC gradient, respectively. All samples were analyzed on a Synapt G2Si instrument (Waters Corporation) by HDMS^E with an

Figure 1. Overview of experimental workflow *Treponema pallidum* urine biomarker experiments.

DDA: Data-dependent acquisition; ESI-Q-TOF: Electrospray ionization quadrupole time-of-flight; HDSM^E: High-definition mass spectrometry; HE: High collision energy; IMS: Ion mobility separation; LC: Liquid chromatography; LE: Low collision energy; MALDI: Matrix-assisted laser desorption/ionization time-of-flight; MWCO: Molecular weight cut-off.

in-house optimized collision energy look up table (ultrdefinition MS^E) [23]. Data were processed using Symphony (Waters). After peak picking (Apex3D), ion sticks were cleaned using Select3D and pseudo MSMS spectra were generated using Peptide3D. The data were searched using IAdB (ion accounting) against a database concatenating *T. pallidum* database containing the UniProt proteomes IDs UP000014259 (corresponding to the resequenced Nichols strain genome [30]), a UniProt human proteome (Accessed June 2016) and contaminants from the cRAP database (<http://www.thegpm.org/crap/>), with methylthio (C) as fixed modification and deamidation (NQ) and oxidation (M) as variable modifications. The enzyme specificity was set to trypsin with a maximum of one missed cleavage.

The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE [31] partner repository with the dataset identifier PXD009707.

Statistical & bioinformatic analyses

All analyses were performed in Stata version 12.1 (StataCorp. 2011. Stata Statistical Software: Release 12, StataCorp LP, TX, USA). Values are summarized as medians and IQR. *Treponema pallidum* proteins detected in the MS analyses were checked for homology to proteins found in other organisms in order to confirm specificity, BLASTp [32] was run with a 90% identity cut-off to check for whole protein and peptide sequence similarity against the NR database of NCBI (accessed 31 January 2018). This was done against all of NR excluding *endemicum*, *carateum* and *pertenue*, in addition to *T. paraluiscuniculi* as these have very high (>98%) sequence similarity with *T. pallidum* subspecies *pallidum* [33,34]. This allowed for substantial discriminatory power between other commensal and disease-causing prokaryotes, human proteins and the peptides/proteins detected in this study.

Table 2. Pooled urine samples prepared for the MALDI-TOF/TOF and Q-IM-TOF MS/MS analyses.

Pool number	MALDI-TOF MS/MS DDA analysis	Q-TOF MS/MS DIA analysis	Clinical stages participants in pools	N = individuals per clinical stage/control in each pool				
				Primary	Secondary	Early latent	Late latent	Control
1 [†]	Yes	Yes	Primary/secondary	3	3	0	0	0
2 [†]	Yes	Yes	Secondary	0	6	0	0	0
3 [†]	Yes	Yes	Control	0	0	0	0	6
4 [†]	Yes	Yes	Early latent	0	0	6	0	0
5 [†]	Yes	Yes	Early/late latent	0	0	1	5	0
6	No	Yes	Primary/secondary	1	5	0	0	0
7	No	Yes	Primary/secondary	3	3	0	0	0
8	No	Yes	Secondary	0	6	0	0	0
9	No	Yes	Secondary	0	6	0	0	0
10	No	Yes	Early latent	0	0	6	0	0

[†] Technical replicate samples that were analyzed by both MS techniques.

DDA: Data-dependent acquisition; DIA: Data-independent acquisition; MALDI TOF: Matrix-assisted laser desorption/ionization time-of-flight MS/MS analysis; Q-TOF: Electrospray-Waters Synapt G2Si traveling-wave ion mobility MS/MS analysis.

Results

Four unique *T. pallidum* proteins detected by MALDI-TOF/TOF MS/MS & LC-ESI-Q-IM-TOF MS/MS HDMS^E analyses

To investigate the presence of *T. pallidum* proteins in urine during infection we conducted a multiplatform shotgun proteomics analysis on pooled urine samples from individuals with syphilis and controls. First, 2D-LC-MALDI TOF/TOF MS/MS (henceforth referred to as MALDI-TOF) analyses were performed on five pooled urine samples as initial ‘proof of concept’ experiments. This resulted in the detection of two unique proteins, Borrelia-like antigen p83/100 (Tp0486) and GTPase Obg (Tp0742) in two pooled samples corresponding to PSS individuals. Protein Tp0486 was detected in one sample with one peptide identification and protein Tp0742 was detected in two samples, each sample with one unique peptide. These peptides were identified by the stringent ProteinProphet algorithm that is far more stringent than MASCOT, hence these peptides were considered to be highly confident. Details of the MALDI-TOF experimental results is provided in Table 3 & Supplementary Table 2. The latent stage urine pools (n = 2) and control samples were negative for *T. pallidum* proteins. A median of 314 (IQR: 299–317) unique human proteins was found in each sample, details are provided in Supplementary Table 2.

Following the success of initial experiments, ESI-Q-IM-TOF HDMS^E experiments (henceforth referred to as HDMS^E) were performed on ten pooled urine samples, five of which were technical replicates of the initial MALDI-TOF experimental set (see Table 2 for sample inclusion overview). In total, these experiments contained urine samples from 54 unique individuals with different active syphilis clinical stages and 6 control samples. A short 60 min LC gradient run preceding the MS analysis resulted in the detection of protein Tp0486 in a PSS pooled sample with a total of seven unique peptide identifications, two of which were high confidence hits. This sample was a technical replicate of the same sample that was MALDI-TOF positive for one peptide (TLADALPR) from Tp0486. Next, analyses were performed using a longer 120 min LC gradient preceding MS analyses. This resulted in the detection of 5 peptides (1 of which was high confidence) from a protein Sugar ABC superfamily ATP binding cassette transporter (Tp0804) in a latent stage pooled sample and 11 peptides (4 of which were high confidence) corresponding to an uncharacterized protein (Tp0369) in a PSS pooled sample. No *T. pallidum* peptides were detected in the other samples analyzed, including the control and 6 *T. pallidum* positive pooled samples. Details of these analysis results are provided in Supplementary Table 3. A median of 128 (IQR: 68–178) human proteins were detected in the HDMS^E analysed samples (Supplementary Table 3).

The average length of the *T. pallidum* peptides detected during all of the analyses was 21 amino acids (IQR: 14–25). Three peptides were shorter than nine amino acids. No posttranslational modifications (PTMs) were detected on the *T. pallidum* proteins and peptides detected. An overview of detected *T. pallidum* proteins and highest confidence peptides is provided in Table 3.

Table 3. *Treponema pallidum* proteins and corresponding highest confidence[†] peptides detected in pooled urine samples through MALDI-TOF/TOF MS/MS and ESI Q-IM-TOF- MS/MS high-definition mass spectrometry analyses.

Pool	Syphilis stage pooled subjects	Number of technical replicates	Protein name UniProt ID. TP number	<i>T. pallidum</i> peptides identified by MALDI-TOF analyses	<i>T. pallidum</i> peptides identified by Q-TOF analyses 60 min LC gradient	<i>T. pallidum</i> peptides identified by Q-TOF analyses 120 min LC gradient
1	PSS	2	GPase Obg R9UVA5 Tp0486	VLVGTKLDLPNAR	ND	ND
2	PSS	2	Borrelia-like antigen p83/100 R9UT45 Tp0742	TLADALPR	ANATVEFENFAGTHTDVSAAAIRR LLYSEPHALVAIADTTGNGTVR	ND
			GPase Obg R9UVA5 Tp0486	LSDAGAGALRSPVWR	ND	ND
3	Control [†]	2	ND	ND	ND	ND
4	Early latent	2	ND	ND	ND	ND
5	Early/late latent	2	ABC sugar protein O83782 Tp0804	ND	ND	IVIMKDCVQQIGSPLHIYQHPANTFVAQFIGSPPMNCFVTIVK
6	PSS	1	ND	NA	ND	ND
7	PSS	1	Uncharacterized protein R9UUC1 Tp0369	NA	ISPAQSPSAAPIEAHVPASAHKEGQEK QFCAQGNARDALASLGDFFAQFPSSHER MDEAWFLRGOAYEINGAQ VPESQEREQKPESKPVQVEPVSASPVKPR	
8	PSS	1	ND	NA	ND	ND
9	PSS	1	ND	NA	ND	ND
10	Early latent	1	ND	NA	ND	ND

[†] Controls were syphilis-negative (RPR/TPA test negative).

The median Random pepScore and Std Random pepScore are calculated for each candidate peptide within 10 ppm for each ion. Only peptides achieving 'green threshold' are reported in this table, implying peptides that have an ion accounting score that is at least four standard deviations away from the scoring distribution of all considered precursors [35].

MALDI TOF: Matrix-assisted laser desorption/ionization time-of-flight MS/MS analysis; NA: Not applicable; ND: None detected; PSS: Primary/secondary stage syphilis; RPR: Rapid plasma reagin; TPA: *Treponema pallidum* agglutination.

Table 4. Characteristics of four detected proteins.

Tp number	Protein name	Molecular weight (kDa)	Predicted sub-cellular localization	Predicted protein function	COG category	Other comments	Found in previous MS analysis Osbak et al. [34] NSAF value [†]	Transcription expression cDNA/DNA ratio signal [‡]
Tp0742	GTPase Obg	41	Cytoplasm	GTPase	None	NA	2.32	0.997
Tp0804	ABC protein	43	NK	Transport	G	NA	3.39	0.258
Tp0369	Unknown protein	56	NK	NK	S	Signal peptide	Not found	4.254
Tp0486	Borrelia-like antigen p83/100	59	Lipoprotein; unknown submembrane localization	NK	None	Lipoprotein	3.72	3.327

[†] Previous MS analysis of *Treponema pallidum* expression during rabbit *T. pallidum* infection [40], average NSAF value.

[‡] Transcription expression data extracted from reference [39].

Clusters of orthologous groups S, unknown function and G, carbohydrate transport and metabolism.

NK: Not known; NSAF: Normalized spectral abundance factor.

T. pallidum protein description

The main characteristics of the four identified proteins are provided in Table 4. An extensive BLAST analysis was performed at both the protein and peptide levels for all four proteins and their corresponding MS detected peptides. Three peptides were excluded from the BLAST analysis since they were too short to provide a reliable result; two from protein Tp0486 (TLADALPR/EAQEGTR) and one from Tp0804 (VQMR). The remaining 23 peptides did not show any significant sequence matching with other prokaryotic and human proteins, excluding *T. paraluiscuniculi*. The proteins identified by peptide identification in this study are nearly identical among the all of the *T. pallidum* subspecies. For example, Tp0742 is identical in all subspecies and strains examined, Tp0804 and Tp0486 each differ only by one amino acid and Tp0369 differs by one to three amino acids in this group. The proteins also had low sequence similarity with other prokaryotes and human proteins. The closest matching bacterium for all four proteins was *T. phagedenis*, the cause of an emerging digital dermatitis in cattle and rarely found as a commensal on human skin [36,37], with a median sequence similarity of 57%. All peptides detected had low sequence similarity with this commensal bacterium. A previous protein array ELISA study investigating the reactivity of recombinant *T. pallidum* proteins with human sera from syphilitic individuals did not find significant reactivity with regards to the four proteins detailed in this study [38].

Discussion

A multiplatform shotgun proteomics approach was used to mine for *T. pallidum* proteins in pooled human urine samples collected from individuals with active syphilis; this resulted in the detection of four *T. pallidum* proteins in four different pooled samples. With the exception of protein Tp0804 that was uncovered in the latent stage sample, the other three pools contained urine samples from individuals with PSS stage syphilis. Protein Tp0742 was positive in two separate samples from PSS stage subjects and Tp0486 was uncovered by both complementary types of MS analyses used. The remaining five urine pools and control sample were negative for *T. pallidum* proteins. To our knowledge, this is the first account of *T. pallidum* protein detection in human biofluid samples using MS-based proteomics methods. These findings could have important implications for diagnostic test development.

This study focused on the discovery of novel *T. pallidum* biomarker proteins and peptides in urine. In order to assess the sensitivity and specificity of these biomarkers another experimental design is required. This includes sampling from larger patient cohorts and omitting the pooling of the samples, as well as using adapted, quantitative data acquisition and analytical approaches. This study also only included men, and future studies should include women with active syphilis.

It is not too surprising that the proteins we detected were not detected consistently in each sample analyzed and with both MS approaches. The stochastic nature of discovery DDA workflows limits its reproducibility between replicate injections when it comes to identifying the same peptides [23,41]. Moreover, the dynamic range of a DDA measurement is limited to the higher abundant human proteins. To minimize these issues, we pooled patient samples and used a 50 kDa filter to help exclude the higher molecular weight proteins that proportionally generate more tryptic peptides per protein.

Data processing has a major impact on the target peptides that are found in each run. One very relevant example from this study is the ion accounting algorithm used to identify proteins from HDMS^E (for details refer to the header of Supplementary Table 3 and to [41]). Briefly, the algorithm tries to account for all the ions using an iterative algorithm that increasingly allows more unexpected alterations on the ions that have not been accounted for, such as by looking for PTMs, in source decay fragments, neutral losses and cation adducts. This effort to increase protein coverage based on the knowledge that the protein is present in the sample (i.e. an unmodified peptide was already found in a previous iteration) inclines toward an error tolerant search in MASCOT, wherein unexpected PTMs are considered in a second pass on only a subset database of detected proteins. This in turn results in the issue of PTMs that are very dynamic, variable and can be both of biological and technical origin. This can hamper the detection of any biomarker in both LC–MS and antibody-based assays. With very little information available on *T. pallidum* PTMs [42], a dedicated targeted LC–MS work-flow to elucidate their prevalence on these biomarker candidates will need to be performed in the future. Another phenomenon that is difficult to control for is protein and peptide degradation during sample preparation. Unfortunately, it is impossible to account for all of these proteolytic events during MS data acquisition and analysis. Last, we noticed considerable amount of spontaneous peptide degradation in the samples upon re-analysis and targeted data mining (data not shown). This most probably also accounts for the relatively low number of protein IDs in the HDMS^E approach, as these samples were subjected to several freeze-and-thaw cycles before the final data acquisition. While some studies have alluded to this phenomenon [43–45], peptides are generally still considered very stable biomolecules and further work is needed to quantify the impact of this on a larger scale [46], especially from a biomarker perspective.

The possibility that the detected biomarker peptides actually arise from an undescribed spirochete commensal in the human microbiome can be considered very unlikely based on the repeated detection of a large number of peptides from the same proteins using different MS-detection techniques in individuals with syphilis but not in controls. The low homology of the detected peptides with those from humans and commensal bacteria further increases the probability that the detected peptides are from *T. pallidum*. Further supportive evidence comes from the fact that three of the four proteins were previously found in a shotgun proteomics analysis of rabbit cultured *T. pallidum* [40] at above average expression levels (above average normalized spectral abundance factor value of 3.39 [IQR 2.3–3.7]). This included the protein Tp0486, which was found in both MALDI-TOF/TOF and ESI-Q-IM-TOF HDMS^E analyses that are very different in ionization, data acquisition and data analysis. This, and other considerations, make this the most promising biomarker candidate detected in this study.

Several considerations should be kept in mind when developing downstream diagnostic applications. During sample preparation, the most important aspects to be optimized are the urine volume collected and the use of MWCO filters. Importantly, our study design aimed at peptide detection and the subsequent inference of proteins based on this. From a bottom-up approach one cannot confirm the presence of the complete protein, therefore peptides should be considered to be the main target for future assay development. This in turn limits the specificity of the sequence, making it important to assess cross-species reactivity of future antibody-assays. Fortunately, BLAST analyses of the proteins revealed low sequence similarity with other prokaryotes and human proteins, excluding other *T. pallidum* species, whereby there is extensive or complete sequence similarity with all proteins detected in this study. The spirochete commensal bacterium *T. phagedenis* had moderate sequence similarity to the four proteins varying from 37.5% (Tp0369) to 65.8% (Tp0804). Moreover, *T. denticola*, a treponeme frequently implicated in periodontitis [47], also exhibits moderate sequence similarity for the proteins detected in this study (range 31.3% [Tp0369]–57.5% [Tp0742]). Approximately a tenth of syphilis positive patients reported dental problems, including an individual in the positive latent stage pool 5; however, the peptide sequence detected by HDMS^E analyses was not homologous to *T. denticola*. Future studies should consider the possible role of cross-reactivity with *T. denticola* and *T. pallidum* peptide/protein detection. It is unclear if this commensal bacterium is extensively present in the human body and a study showed no cross-reaction of treponemal antibody testing with this organism [48]. As for the endemic treponemes, since they are pathogenic and treated in a similar way to subspecies *pallidum* it could be considered an advantage if a syphilis antigen test detects these organisms in addition to *T. pallidum* subspecies *pallidum*.

There is still a paucity of published studies with regards to the application of MS to the detection of pathogen peptides directly in human biofluid samples, therefore we can mostly speculate about the detectability of pathogen proteins in urine based on studies that employed lateral flow assays and ELISA methods. The urinary shedding of leptospires is well established since they preferentially reside and multiply in the proximal convoluted tubules of the kidney. A recent study by Chaurasia *et al.* [16] described the successful detection of *L. interrogans* proteins

in the urine of patients with leptospirosis using ELISA analyses to differentiate dengue infection. Antigen tests are widely used for the diagnosis of lung infections such as legionellosis and pneumococcal pneumonia [15]. The fact that these infections usually have limited hematogenous spread, hints that a highly invasive organism such as *T. pallidum* would also likely be detectable in urine samples. The kidneys act as a filtration and concentrative system for pathogenic proteins, therefore the likelihood of protein in urine may even be higher than in plasma samples. Moreover, healthy kidneys typically do not allow for the passage of large proteins, thus smaller peptide fractions of pathogen proteins would be permitted to pass through the glomerulus. Hence, focusing on peptide detection and smaller protein epitopes rather than large sections of protein would likely be more effective for testing. Few studies have investigated PCR detection *T. pallidum* in urine. One study, for example, suggested the sensitivity was estimated to be 16% [49]. There are sporadic reports of kidney disease such as glomerulonephritis [50] and glomerulosclerosis [51] related to syphilis, suggestive of kidney involvement during various disease stages. With regards to *T. pallidum* protein secretion, to our knowledge there is little evidence of protein secretion by *T. pallidum*.

Conclusion

This study details the first steps of biomarker discovery using multiplatform shotgun proteomic techniques applied to human urine samples. Syphilis is a multistage disease with varying systemic bacterial loads during the course of infection with especially low bacteraemia during latent stages [29]. Therefore, diagnosing the latent clinical stages will be the most challenging for antigen testing strategies. If biomarkers are found that are stably expressed during all clinical stages, then enrichment strategies may be able to increase the sensitivity sufficient for reliable testing. Future studies could employ antibody affinity enrichment strategies to increase sensitivity, such as used in the Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA) method that has demonstrated promising clinical utility in recent studies [52,53].

Future perspective

Infectious disease diagnostics is one the most unpredictable areas of medicine, with novel keystone technologies periodically emerging that revolutionize how clinicians diagnose disease. During the last decade, MS-based protein detection methods have revolutionized clinical microbiology. We believe it is only a matter of time before these or other more sensitive methods can be reliably applied to detect *T. pallidum*-specific peptides from patient urine/blood in a high-throughput, cost effective and accurate manner. Antibody detection and rapid test lateral flow assays will also likely remain a diagnostic mainstay for syphilis, especially for low resource settings where MS would be difficult to implement.

Summary points

- A molecular diagnostic test that could detect *Treponema pallidum* antigens in urine would facilitate the prompt diagnosis of syphilis and would be useful for posttreatment serological follow-up.
- Urine samples from 54 individuals with various clinical stages of syphilis and 6 syphilis negative controls were used to create 10 different pooled samples according to disease stage.
- Protein extracts from the pooled samples were interrogated using a high-resolution multiplatform proteomics approach.
- Twenty-six unique peptides corresponding to 4 unique *T. pallidum* proteins (Tp0486/Tp0804/Tp0369/Tp0742) were uncovered in 4 different urine pools. Treponemal proteins detected have low genetic sequence similarity to other prokaryotes and human proteins.
- This is the first account of *T. pallidum* protein detection in human biofluid samples using mass spectrometry.
- These findings could have important implications for future diagnostic test development.

Supplementary data

To view the supplementary data that accompany this paper please visit the journal website at: www.futuremedicine.com/doi/full/10.2217/fmb-2018-0182

Author contributions

KK Osbak contributed in study design, experimental work and manuscript writing; GA Van Raemdonck contributed in study design, experimental work and final draft manuscript review; M Dom contributed in experimental work and final draft manuscript

review; CE Cameron contributed in results interpretation/analysis and final draft manuscript review; CJ Meehan contributed in bioinformatics analyses and final draft manuscript review; D Deforce contributed in supervision, results interpretation and final draft manuscript review; X Van Ostade contributed in supervision, study design and final draft manuscript review; CR Kenyon contributed in supervision, study design, study funding acquisition and manuscript writing; M Dhaenens contributed in experimental work, results interpretation, study design and manuscript writing.

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No writing assistance was utilized in the production of this manuscript.

Ethical disclosure

The prospective observational cohort study (*Treponema pallidum*-specific Proteomic Changes in Patients with Incident Syphilis Infection (SeTPAT); ClinicalTrials.gov #NCT02059525) that provided the clinical samples used in this sub-study was approved by the Institutional Review Board of the Institute of Tropical Medicine (ITM) Antwerp and the Ethics Committee of the University of Antwerp (13/44/426), Belgium. Written permission for participation was provided by all participants prior to study inclusion.

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