Quantitative proteomics of the 2016 WHO *Neisseria gonorrhoeae* reference strains surveys vaccine candidates and antimicrobial resistance determinants

Fadi E. El-Rami[1], Ryszard A. Zielke[1], Teodora Wi[2], Aleksandra E. Sikora[1,3*,**] and Magnus Unemo[4*,**]

[1]Department of Pharmaceutical Sciences, College of Pharmacy, Oregon State University, Corvallis, Oregon, USA; [2]Department of Reproductive Health and Research, World Health Organization, Geneva, Switzerland; [3]Vaccine and Gene Therapy Institute, Oregon Health and Science University, Beaverton, Oregon, USA; [4]World Health Organization Collaborating Centre for Gonorrhoea and other Sexually Transmitted Infections, Department of Laboratory Medicine, Clinical Microbiology, Faculty of Medicine and Health, Örebro University, Örebro, Sweden.

*Corresponding authors:

Aleksandra E. Sikora: Aleksandra.Sikora@oregonstate.edu

Magnus Unemo: magnus.unemo@regionorebrolan.se

**Joint senior authors

Running title: Proteomic mining of gonorrhea antigens and AMR

Author contributions: A.E.S. designed research with input from M.U.; R.A.Z. performed the experiments; F.E.E. performed computational data analysis; F.E.E., A.E.S., T.W., and

M.U. interpreted the data; F.E.E., A.E.S., and M.U. wrote the paper. All authors commented on and approved the final version of the paper.

Keywords: *Neisseria gonorrhoeae*, gonorrhea, WHO reference strain, quantitative proteomics, TMT, vaccine, antimicrobial resistance.

Abstract

The sexually transmitted disease gonorrhea (causative agent: *Neisseria gonorrhoeae*) remains an urgent public health threat globally due to the repercussions on reproductive health, high incidence, widespread antimicrobial resistance (AMR), and absence of a vaccine. To mine gonorrhea antigens and enhance our understanding of gonococcal AMR at the proteome level, we performed the first large-scale proteomic profiling of a diverse panel (*n*=15) of gonococcal strains, including the 2016 World Health Organization (WHO) reference strains. These strains show all existing AMR profiles, previously described in regard to phenotypic and reference genome characteristics, and are intended for quality assurance in laboratory investigations. Herein, these isolates were subjected to subcellular fractionation and labeling with tandem mass tags coupled to mass spectrometry and multi-combinatorial bioinformatics. Our analyses detected 901 and 723 common proteins in cell envelope and cytoplasmic subproteomes, respectively. We identified nine novel gonorrhea vaccine candidates. Expression and conservation of new and previously selected antigens were investigated. In addition, established gonococcal AMR determinants were evaluated for the first time using quantitative proteomics. Six new proteins, WHO_F_00238, WHO_F_00635, WHO_F_00745, WHO_F_01139, WHO_F_01144, and WHO_F_01226, were differentially expressed in all strains, suggesting that they represent global proteomic AMR markers, indicate a predisposition toward developing or compensating gonococcal AMR, and/or act as new antimicrobial targets. Finally, phenotypic clustering based on the isolates' defined antibiograms and common differentially expressed proteins yielded seven matching clusters between established and proteome-derived AMR signatures. Together, our investigations provide a reference proteomics databank for gonococcal vaccine and

AMR research endeavors, which enables microbiological, clinical, or epidemiological projects and enhances the utility of the WHO reference strains.

The abbreviations used are:

ACN	Acetonitrile
AGC	Automatic gain control
AMR	Antimicrobial resistance
C	Cytoplasmic
CDC	Centers for Disease Control and Prevention
CE	Cell envelope
COG	Cluster of orthologous genes
cRAP	Common repository of adventitious proteins
FDR	False discovery rate
GCB	Gonococcal base agar
GCBL	Gonococcal base liquid medium
KEGG	Kyoto encyclopedia of genes and genomes
LPS	Lipopolysaccharide
OMV	Outer membrane vesicle
ORF	Open reading frame
WHO	World Health Organization

INTRODUCTION

Neisseria gonorrhoeae is an obligate human pathogen and the causative agent of the sexually transmitted disease gonorrhea. Gonorrhea is a global public health concern. In 2012 the World Health Organization (WHO) estimated over 78 million new urogenital cases per year in adults (15–49 years of age) worldwide (1, 2). The spread of gonorrhea is facilitated by the high prevalence of asymptomatic infections. Urogenital gonorrhea is asymptomatic in up to 10-15% of infected men and up to 50% of infected women. Pharyngeal and rectal infections, which have increased in prevalence in both sexes and are predominant among men who have sex with men, are primarily asymptomatic (3, 4). Untreated or inappropriately treated gonorrhea can result in serious consequences on reproductive and neonatal health. Women, in particular, are disproportionately affected, as gonococcal infection can ascend from the cervix to the uterus, Fallopian tubes, ovaries, and surrounding tissue, causing pelvic inflammatory disease. Long-term sequelae include ectopic pregnancy, chronic pelvic pain, and infertility. Furthermore, gonorrhea is strongly associated with an increased risk of both the acquisition and transmission of HIV (5).

Antimicrobial therapy is the only mainstay in the effective management and control of gonorrhea. However, *N. gonorrhoeae* exhibits an extraordinary capacity to develop antimicrobial resistance (AMR) through mutations and acquisition of AMR genes. The evolution of AMR in *N. gonorrhoeae* has overcome every therapeutic option since the "miracle drug" penicillin was introduced for gonorrhea treatment. Currently, a dual antimicrobial therapy (mainly ceftriaxone and azithromycin) is recommended for treatment of uncomplicated infections (6). Of grave concern over the past decade is the proliferation of resistance or decreased susceptibility to ceftriaxone worldwide. Azithromycin resistance has also emerged in most settings (7). The first failure of one of the recommended dual

antimicrobial therapies against pharyngeal gonorrhea was reported in 2016 (8), and the first *N. gonorrhoeae* isolates with resistance to ceftriaxone combined with high-level resistance to azithromycin were identified in the United Kingdom (9, 10) and Australia (11, 12) in early 2018. In consideration of dwindling treatment options, scarce therapeutic alternatives, disease prevalence and morbidity, and lack of a vaccine(s), *N. gonorrhoeae* has been categorized by the WHO as a high priority pathogen globally and by the Centers for Disease Control and Prevention (CDC) as an urgent level threat in the USA (13).

Developing an effective gonococcal vaccine is essential because this is the only sustainable solution to quell the spread of gonococcal AMR and gonorrhea in general. The battle against penicillin-nonsusceptible *Streptococcus pneumoniae* exemplifies a successful vaccination strategy. Introduction of a pneumococcal conjugate vaccine in 2010 reduced the number of infections over 45% (14). Unfortunately, despite its public health importance, gonorrhea vaccine development remains in its infancy. Since 1970, only three small-scale vaccine trials using whole cell (15), pilin (16), and porin proteins (17) have been launched. All were unsuccessful in developing immunity against reinfection with gonorrhea. However, recent breakthroughs, including the development of small animal models for evaluating gonorrhea vaccines (18, 19), increased knowledge about *N. gonorrhoeae* immune evasion mechanisms (20-26), and the development of an effective vaccine for the closely related *N. meningitidis* serogroup B, which provided a low level of cross-protection against gonococcal infection (27), have reinvigorated the interest in gonococcal vaccine development (28).

Proteomic technology offers a powerful toolbox to enable vaccine antigen mining (28-32) and AMR proteome analysis (33-35), and to provide insights into host-pathogen interactions (36-39). Proteomic approaches have an advantage over genomics in drug and vaccine discovery endeavors by delivering information pertaining to protein abundance,

post-translational modification(s), structure-function relationships, and protein-protein interactions (40-42). In addition, subcellular fractionation steps preceding proteomic applications reduce sample complexity, increase the likelihood of discovering low-abundance proteins, and aid in defining protein localization, all of which provide further insights into the proteins' functions and interactomes (43, 44). For *N. gonorrhoeae*, proteomic approaches have begun to deliver proteinaceous vaccine candidates (29, 30, 39, 45) and to support elucidation of AMR patterns (46, 47). Current off-gel proteomics, such as isobaric tag labeling (isobaric tagging for absolute quantification, iTRAQ; and tandem mass tags, TMT) coupled with high-pressure liquid chromatography and mass spectrometry techniques (LC-MS/MS), demonstrate superb protein separation and identification and enable detection of proteins in the low femtomole to high attomole range with precision and reliability (29, 48, 49).

To address the need for discovery of additional gonorrhea vaccine and drug candidates and to enhance our understanding of AMR at the proteome level, herein we examined the 2016 WHO *N. gonorrhoeae* reference strains (50) and the FA6140 strain (51) using a global quantitative proteomic approach. The WHO panel consists of 14 *N. gonorrhoeae* reference strains strictly selected and validated internationally to represent the *N. gonorrhoeae* species. All known gonococcal phenotypic and genetic AMR determinants are included for use as quality control strains during phenotypic and genetic laboratory testing. Eight of the strains were initially included in the 2008 WHO reference strains [WHO F, G, K, L, M, N, O, and P; (52)] to which 6 novel strains (U, V, W, X, Y, and Z) were added to constitute the 2016 WHO reference strains (50). All WHO panel strains have been subjected to extensive phenotypic, genomic, and genetic analyses to establish diagnostic markers, molecular epidemiological characteristics, reference genomes, and AMR profiles

(phenotypic and genetic) for all antimicrobials currently and previously used for gonorrhea treatment, in addition to novel antimicrobials considered for future interventions. This panel includes WHO X, the first extensively drug-resistant gonococcal strain identified with high-level resistance to ceftriaxone, as well as additional strains with different levels of resistance to ceftriaxone, azithromycin and any additional therapeutic antimicrobials. Complete genomes with detailed annotations are available for all panel strains, providing a fundamental resource for future molecular studies. Accordingly, the well-characterized 2016 WHO reference strains (50) are ideally suited to provide detailed descriptions of the global *N. gonorrhoeae* proteome, a greater understanding of gonococcal AMR at the proteome level, and a source for the identification of broadly conserved novel vaccine candidates. In addition to the WHO panel strains, we have included in our investigations *N. gonorrhoeae* FA6140, which is a penicillin-resistant, β-lactamase-negative isolate that was originally described after a local epidemic outbreak of 199 gonococcal cases in Durham, North Carolina, USA in 1983 (51). It serves as a model for gonococcal AMR studies and has facilitated the characterization of mutations in genes encoding the “multiple transferable resistance” repressor MtrR (53), ribosomal protein S10 (54), and penicillin-binding protein 2 (55) and their impact on AMR.

Our study is the first to investigate the global proteomic profiles of 15 *N. gonorrhoeae* reference strains using subcellular fractionation to separate cytoplasmic (C) and cell envelope (CE) associated proteomes, which were measured with tandem mass tags coupled to liquid chromatography and tandem mass spectrometry [TMT-LC-MS/MS; (56)], a highly reproducible and sensitive technique. These proteomic studies achieved our three major objectives. First, to enhance progress on gonorrhea vaccine development, novel vaccine candidates were identified, and the expression profiles of currently proposed

antigens were established in diverse clinical isolates. Second, to broaden our understanding of AMR, proteomic signatures associated with AMR were defined by conducting a pairwise analysis of differentially expressed proteins to compare FA6140 and the 2016 WHO panel to WHO F, which possesses the largest genome and is susceptible to all relevant antimicrobials (50). Third, to facilitate the use of the 2016 WHO panel in various types of basic research and quality assurance, the complete reference proteomes of all tested strains were defined.

EXPERIMENTAL PROCEDURES

Bacterial strains and growth conditions. The 2016 WHO *N. gonorrhoeae* reference strains [n=14; (50, 52)] and the *N. gonorrhoeae* FA6140 strain (51) were used in this study. The AMR profiles of all isolates were described previously (50). Gonococcal strains were cultured from frozen stocks (−80°C) onto gonococcal base agar (GCB) medium (Difco) with Kellogg's supplements I and II, diluted 1:100 and 1:1,000, respectively (57). After incubation at 37°C in a 5% CO_2–enriched atmosphere for 18-20 h, nonpiliated and transparent colonies were subcultured onto GCB and incubated as described above. To initiate growth in liquid medium, nonpiliated colonies were collected from GCB and suspended to an OD_{600} of 0.1 in pre-warmed GCB liquid (GCBL) medium supplemented as described above with the addition of 0.042% sodium bicarbonate. Suspensions were incubated at 37°C with shaking at 220 rpm.

***Subcellular fractionation and TMT labeling*.** All 15 *N. gonorrhoeae* strains were simultaneously cultured in GCBL as described above. Cells were collected by centrifugation (20 min, 6,000 × *g*) when the Optical Density (OD_{600}) of each culture reached 0.6 – 0.8, re-suspended in PBS and lysed by passage through a French Press. The cell debris was removed by centrifugation and the crude CE fraction was separated from the C proteins using a sodium carbonate extraction procedure and subsequent ultracentrifugation steps. The fraction enriched with CE proteins was reconstituted in PBS supplemented with 0.1% SDS (29, 30). Experiments were conducted in two biological replicates. Sample quality and the overall sub-proteome profiles were examined by SDS-PAGE coupled with Colloidal Coomassie staining (58, 59). The total protein amount in each fraction was assessed using a Protein Assay Kit (Bio Rad). Each CE and C fraction containing 100 µg of protein in 25 µL volume of triethylammonium bicarbonate buffer was reduced with tris(2-

carboxyethyl)phosphine hydrochloride and the cysteines were alkylated using iodoacetamide. Proteins were digested using trypsin (Promega) at a 1:40 ratio. TMT reagents (ThermoFisher Scientific) were dissolved in acetonitrile (ACN) and used to label proteins in CE and C fractions as follows for the 10-plex experiment (ref 90111, Thermo Fisher Scientific): WHO F strain: TMT^{10}-126, WHO K strain: TMT^{10}-127C, WHO G strain: TMT^{10}-127N, WHO M strain: TMT^{10}-128C, WHO L strain: TMT^{10}-128N, WHO O strain: TMT^{10}-129C, WHO N strain: TMT^{10}-129N, WHO U strain: TMT^{10}-130C, WHO P strain: TMT^{10}-130N, WHO V strain: TMT^{10}-131; for the 6-plex experiment (ref 90402, Thermo Fisher Scientific): WHO F strain: TMT^{6}-126, WHO W strain: TMT^{6}-127, WHO X strain: TMT^{6}-128, WHO Y strain: TMT^{6}-129, WHO Z strain: TMT^{6}-130, FA6140 strain: TMT^{6}-131. Mixtures were incubated for 1 h at room temperature. The reaction was quenched by addition of 8 µL of 5% hydroxylamine. Samples were pooled, dried in a vacuum concentrator and stored at -80°C before separation by high pressure liquid chromatography (HPLC) and MS analysis.

Sample fractionation and MS analysis. Samples were fractionated by strong cation exchange (SCX) with a Paradigm (Michrom Biosciences) HPLC with mobile phases of 5 mM potassium phosphate monobasic in 30% ACN/70% water (v/v) pH 2.7 (buffer A) and 5 mM potassium phosphate monobasic in 30% ACN/70% water (v/v) pH 2.7 with 500 mM potassium chloride (buffer B). The sample was brought up in buffer A (200 µL). The peptides were separated using a 2.1 mm x 100 mm Polysulfoethyl A column (PolyLC) over 60 min at a flow rate of 200 µL/min. The separation profile was as follows: hold 2% B for 5 min, 2% to 8% B in 0.1 min, 8% to 18% B in 14.9 min, 18% to 34% B in 12 min, 34% to 60% B in 18 min, 60% to 98% B in 0.1 min and hold for 10 min. Fractions were collected in 96-well microtiter plates at 1 min/fraction. Sixty fractions were pooled into 12 and dried using a

speed vac. The samples were desalted using Oasis HLB 1cc cartridges. The cartridges were washed with 70% ACN/0.1% trifluoroacetic acid (TFA) and equilibrated with 0.1% TFA. Samples were loaded onto the cartridge in 0.1% TFA, washed with 0.1% TFA, and eluted in 1 mL 70% ACN/0.1% TFA. The samples were dried by vacuum centrifugation.

Desalted SCX fractions were analyzed by liquid chromatography electrospray ionization mass spectrometry (LC/ESI MS/MS) with a Thermo Scientific Easy-nLC II (Thermo Scientific) nano HPLC system coupled to a hybrid Orbitrap Elite ETD (Thermo Scientific) mass spectrometer. In-line de-salting was accomplished using a reversed-phase trap column (100 µm × 20 mm) packed with Magic C_{18}AQ (5-µm 200Å resin; Michrom Bioresources) followed by peptide separations on a reversed-phase column (75 µm × 250 mm) packed with Magic C_{18}AQ (5-µm 100Å resin; Michrom Bioresources) directly mounted on the electrospray ion source. A 90-minute gradient from 7% to 35% ACN in 0.1% formic acid at a flow rate of 400 nL/min was used for chromatographic separations. The heated capillary temperature was set to 300°C and a spray voltage of 2750 V was applied to the electrospray tip. The Orbitrap Elite instrument was operated in the data-dependent mode, switching automatically between MS survey scans in the Orbitrap [automatic gain control (AGC) target value 1,000,000; resolution 120,000; and injection time 250 msec] with MS/MS spectra acquisition in the Orbitrap (AGC target value of 50,000; 15,000 resolution; and injection time 250 msec). The 15 most intense ions from the Fourier-transform full scan were selected for fragmentation in the higher-energy C-trap dissociation (HCD) cell by higher-energy collisional dissociation with a normalized collision energy of 40%. Selected ions were dynamically excluded for 30 sec with a list size of 500 and exclusion mass by mass width +/- 10ppm. HPLC and MS/MS analyses were performed in the Proteomic Facility at the Fred Hutchinson Cancer Center, Seattle.

Proteomic data analysis. Data analysis was performed using Proteome Discoverer 1.4 (Thermo Scientific). The data were searched against WHO_F_CDS with the common Repository of Adventitious Proteins (cRAP, http://www.thegpm.org/crap/) fasta file. Trypsin was set as the enzyme with maximum missed cleavages set to 2. The precursor ion tolerance was set to 10 ppm and the fragment ion tolerance was set to 0.8 Da. Variable modifications included TMT 6Plex (+229.163 Da) on any N-Terminus, oxidation on methionine (+15.995 Da), carbamidomethyl on cysteine (+57.021 Da), and TMT 6Plex on lysine (+229.163 Da). Data were searched using Sequest HT. All search results were run through Percolator for scoring. Quantification was performed using the canned TMT 6plex or TMT 10plex methods through Proteome Discoverer with stringent criteria for protein identification including 1% False Discovery Rate (FDR), at least one unique peptide per protein, each identified peptide restricted to a single protein, and the score for every detected peptide of ≥1. Differential protein expression between CE and C fractions was determined by comparing the normalized total reporter ion intensities of groups using the WHO F protein expression profile as a reference.

Bioinformatic Analysis. To detect potential homologous proteins, amino acid sequences of each identified *N. gonorrhoeae* vaccine candidate were downloaded and compared against the GenBank proteome database (https://www.ncbi.nlm.nih.gov/genbank/) using our in-house designed program based on the Reciprocal Best Blast Hit approach (60) using BLASTP with the following parameters: percentage identity ≥50%, and E-value ≤1.0 e-5.

Differential protein expression in four proteomics data sets (CE and C fractions in two biological replicates) was designated by fold changes ≥1.5 or ≤0.667 in reference to strain WHO F. Due to the variable nature of protein expression in *N. gonorrhoeae*, we took a conservative approach to designate protein expression and a protein was categorized as

273 "up-regulated" or "down-regulated" solely when the fold change abundance was higher than
274 1.5 or lower than 0.667, respectively, to that of WHO F consistently in two biological
275 experiments. A protein was designated as "ubiquitous" when its abundance was between
276 0.667-1.5-fold compared to WHO F in both experiments, or "variable" when its protein levels
277 were not consistent between experiments.

278 A comprehensive assessment of predicted subcellular protein localization was
279 accomplished by using the CELLO (61), PsortB 3.0.2 (62), SOSUI-GramN (63), SignalP 4.1
280 (64), LipoP 1.0 (65), and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) prediction
281 algorithms and a majority voting strategy. Furthermore, for proteins whose subcellular
282 localization was not predicted using the aforementioned algorithms, we relied on the
283 difference between their unique peptide counts in the CE and C fractions as follows:

284 *Unique Peptide Count Difference (UPCD)* = $\sum_{i=1}^{2} x\ CE\ \sum_{\mathrm{i=1}}^{2} \mathrm{x\ CE}\text{-}\ \sum_{i=1}^{2} x\ C$

285 where "i" is the sequential number assigned for samples and "x" is the total number of
286 peptides detected in each fraction. Cytoplasmic proteins had more of their unique peptides
287 detected in the C fraction (UPCD < 0), while membrane proteins had unique peptides
288 enriched in the CE fraction (UPCD > 0). Proteins with UPCD=0 were excluded from analysis
289 using this UPCD formula. Proteins were categorized as follows: outer membrane,
290 periplasmic, inner membrane, C proteins, and proteins with unknown localization.

291 The phenotypic and proteotypic clusters of all strains were constructed using as
292 variables both their AMR (50) and proteomic profiles obtained in this study. These clusters
293 were designed based on the Hamming distance between tested strains, which counts how
294 many elements differ between two vectors, and is equivalent to Manhattan distance on
295 binary data. Average linkage was used to determine distances between clusters.

296 Graphs were generated with GraphPad Prism version 7 for Mac (GraphPad
297 Software). The proteotypes of strains that belong to the same phenotypic cluster were
298 compared, highlighting proteins that are significantly up- or down-regulated with respect to
299 those proteins of WHO F.
300 ***Data Availability***. The raw mass spectrometry data have been deposited to the
301 ProteomeXchange Consortium via the PRIDE (66) partner repository with the data set
302 identifier PXD008412.
303

RESULTS and DISCUSSION

***Study rationale*.** In our study design (Fig. 1), all 15 strains were cultured concurrently to mid-logarithmic growth, harvested, and subjected to subcellular fractionation to separate CE (outer membrane, periplasmic, inner membrane) and C proteins. We utilized TMT reagent technology for protein identification and quantitation as it provides a highly sensitive method for peptide labeling (56) and allows up to 10 biological samples to be analyzed in a single experiment (67). TMT-labeling, two-dimensional liquid chromatography fractionation, and subsequent MS/MS analyses were conducted on every 6-plex and 10-plex experiment pertaining to the CE and C fractions derived from each strain (Fig. 1). We selected WHO F as the reference strain for protein identification and quantitation because it has the largest genome (2,292,467 bp) and proteome (2,450 ORFs) among the 2016 WHO reference strains (50) and FA6140 (68), and it is susceptible to most antimicrobials currently or historically used for gonorrhea treatment.

Sub-cellular fractionation experiments coupled with proteomics repeatedly show cytoplasmic proteins associated with the membranes, which are commonly regarded as "contaminating" or "moonlighting" proteins (29, 30, 69, 70). Therefore, to focus solely on the enriched proteins in individual subproteomes, we first eliminated C and CE proteins that were detected in the CE and C protein fractions, respectively, from further analyses. Complete lists of all identified proteins are in Supplemental Tables S1-S2. Subsequently, we performed two-armed proteomic data analyses: 1) for vaccine antigen mining, we focused on common proteins identified in all strains in the CE fraction with the overarching goal to discover omnipresent *N. gonorrhoeae* proteins; 2) to profile AMR signatures, we performed a pairwise comparison of individual strains to WHO F to enhance the discovery of strain-specific feature(s).

328 ***Overview of cell envelope and cytoplasmic proteomes*.** The 10-plex biological replicate
329 experiments identified a total of 1150 proteins in the CE fraction of all ten strains, of which
330 1010 were common in both sets (Fig. 2 A). In the two 6-plex experiments, 1194 proteins
331 were identified; 975 were shared in all six isolates (Fig. 2 A). Taken together, the 10-plex
332 and the 6-plex experiments resulted in identification of 1084 proteins in the CE fractions, of
333 which 901 were common among all examined *N. gonorrhoeae* strains (Fig. 2 A). The
334 proteome coverage per strain ranged from 41.22% (981 proteins) for WHO Y to 45.32%
335 (1042 proteins) for WHO G (Supplemental Table S3).

336 Proteomics of the C fraction in the 10-plex set conducted in biological replicates
337 yielded 904 proteins that were shared among all 10 strains, of which 747 were common in
338 both experiments (Fig. 2 B). The two 6-plex experiments identified 1023 shared proteins,
339 with 852 common among the two replicates (Fig. 2 B). Cumulatively, C fraction profiling
340 resulted in identification of 876 proteins with 723 common in all 15 *N. gonorrhoeae* strains
341 (Fig. 2 B). Proteome coverage ranged from 31.37% (746 proteins) in WHO U to 38.43%
342 (852 proteins) in FA6140 (Supplemental Table S3).

343 Subsequently, we allocated common proteins that were identified in all 15 *N.*
344 *gonorrhoeae* strains to outer membrane, inner membrane, periplasm, cytoplasm, or
345 unknown localization categories based on PSORTb 3.0.2 (62), SOSUIGramN (63), and
346 CELLO (61) predictions and the majority-voting strategy. We used these software packages
347 to take advantage of their different algorithms and statistical approaches for the prediction
348 of protein subcellular localization. As expected from our subcellular fractionation approach
349 (49, 69, 71), the CE fraction was enriched in membrane proteins in comparison to the C
350 sample, with outer membrane (26 vs. 8), periplasmic (51 vs. 38), and inner membrane
351 proteins (145 vs. 6) that were also identified with considerably higher peptide counts (Fig. 3

352 A-C, and Supplemental Tables S1-S2, and S4-S5). The C preparations yielded 592
353 cytoplasmic proteins that were identified with greater peptide counts in comparison to the
354 cytoplasmic proteins associated with the CE fraction (Fig. 3 D, Supplemental Tables S5-
355 S6). Furthermore, to increase the discovery of potential vaccine candidates, we searched
356 the 149 proteins of unknown localization identified in the CE fraction (Fig. 3 D) for the
357 presence of signal peptides and transmembrane motifs using SignalP 4.1 (64), LipoP 1.0
358 (65), and TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/). The results of all software
359 programs and the majority votes strategy revealed six additional CE proteins, four of which
360 were found in the majority of examined strains (Supplemental Table S6). In addition,
361 literature searches for experimental evidence of protein surface exposure allowed
362 assignment of BamE [NGO1780; (72)], SliC [NGO1063; (73)], MetQ [NGO2139; (30, 74)],
363 Ng-MIP [NGO1225; (30, 75)], and BamG [NGO1985; (76)] to the cell surface.
364 ***Expression patterns of common identified proteins in comparison to WHO F*****.** Proteins
365 were categorized as ubiquitous, up- or down-regulated, or variable based on their
366 abundance in relation to the corresponding protein in WHO F in biological duplicate
367 experiments. We investigated expression patterns of detected proteins in both sub-
368 proteome fractions that were shared among all strains (Figs. 4-5, Supplemental Tables S4-
369 5). Annotated cell envelope proteins were predominantly ubiquitous in the CE fraction. The
370 proportion of ubiquitous CE outer membrane proteins (*n*=26) ranged from 73% (*n*=19 in
371 WHO N) to 46.15% (*n*=12 in WHO L; Fig. 4 A). Ubiquitous periplasmic proteins ranged from
372 76.47% (*n*=39 in WHO N) to 35.2% (*n*=18 in WHO U) of the total number of proteins
373 annotated to localize to the periplasm (*n*=51; Fig. 4 B). Finally, between 83.4% (*n*=121 in
374 WHO M) and 44.1% (*n*=64 in WHO U) of inner membrane proteins (*n*=145) were
375 ubiquitously expressed (Fig. 4 C). Up-regulated outer membrane (0 – 19.23%), periplasmic

(0 – 8%) and inner membrane (0 – 4.8%) proteins made up the smallest proportion of proteins in the CE fraction (Supplemental Table S4), whereas down-regulated outer membrane (3.85 – 26.9%), periplasmic (0 – 23.53%) and inner membrane (1.38 – 24.8%) proteins were moderately prevalent (Supplemental Figure S4). Further analysis of the CE fraction detected 121 common proteins with unknown localization (Fig. 3 E, Supplemental Table S4). Ubiquitous expression was the dominant pattern for these proteins in WHO G, K, M, N, V, W, and X and ranged from 42.97% (*n*=52, WHO V) to 66.94% (*n*=81, WHO W). Variable expression of proteins with unknown localization dominated in WHO L, O, P, Y, Z, and FA6140. WHO U had the highest number of down-regulated proteins (39.67%, *n*=48) with respect to those in WHO F (Fig. 4 D).

In contrast to the CE expression pattern, we observed a striking increase in the number of variably expressed cytoplasmic proteins in the C fraction of analyzed strains in comparison to WHO F (Fig. 5 A, Supplemental Table S5). The percentage of variable proteins ranged from 32.9% to 82.6% for WHO G and WHO Y, respectively (Supplemental Table S5). Ubiquitous proteins were the next most common category and oscillated from 15.5% in WHO Y to 56.4% in WHO G. The third group contained up-regulated proteins (0 – 21.28%), and down-regulated proteins ranged from 0.5 – 3.88% (Supplemental Table S5). For proteins with no assigned localization, variable expression was the most prevalent pattern in WHO K, L, O, P, U, W, X, Y, Z, and FA6140 (Fig. 5 B), ranging from 79.75% (*n*= 63, WHO Y) to 46.83% (*n*= 37, WHO L). Ubiquitous proteins were the dominating group in WHO G, M, N, and V (Fig. 5 B). Finally, up- and down-regulated proteins constituted up to 11.39% and 7.59%, respectively, of the total proteins with unknown localization in the C fraction.

Together, the first quantitative proteomic profiling of the 15 *N. gonorrho*eae strains demonstrated distinct differences in their proteomes and showed that a pattern of ubiquitous protein expression was prevalent in the CE fraction, whereas variably expressed proteins were the dominant group in the C subproteome.

Antigen mining decision tree. To identify novel gonorrhea antigens and to gain information about expression of previously identified vaccine candidates (49, 71, 77, 78), we designed an antigen mining decision tree (Fig. 6). We included in this process 25 outer membrane proteins (all outer membrane proteins identified except RmpM) and 121 proteins of unknown localization identified in CE proteomic profiling (Fig. 6 and Supplemental Table S1). The latter group of proteins was subjected to signal sequence and transmembrane motif analyses to increase the coverage of potential vaccine candidates. Together, these investigations yielded nine novel antigens including NGO0282, NGO0425, NGO0439, NGO0778, NGO1251, NGO1688, NGO1889, NGO1911a, and NGO2105 in addition to previously discovered proteomics-derived antigens [(29, 30); Table 1] and vaccine candidates identified by other means (Table 2).

Further bioinformatics and literature searches were performed to gain insights into the new proteomics-derived vaccine candidates. The putative lipoprotein NGO0282 is a homolog of the outer membrane localized LptE, which is a component of the trans-envelope LptA-G machinery involved in the transport of lipopolysaccharide/lipooligosaccharide (LPS/LOS) molecules to the *E. coli* and *N. meningitidis* outer membrane, respectively. LptE's chaperone-like role in LptD biogenesis is conserved in both bacteria but LptE works in concert with LptD to translocate LPS to the cell surface only in *E. coli* (79, 80). LptD is essential for *E. coli* and *N. gonorrhoeae* viability but is dispensable for *N. meningitidis* (81-83). Therefore, the function of *N. gonorrhoeae* LptE in both LOS transport and LptD

biogenesis needs to be elucidated. NGO0425 contains a tetratricopeptide repeat–like domain and a transmembrane helix. Together with its *E. coli* homolog, YfgM, NGO0425 belongs to the UPF0070 family. In *E. coli*, YfgM was proposed to act within the β-barrel trafficking chaperone network and its depletion in Δ*skp* and Δ*surA* knockout backgrounds contributed to further alterations in outer membrane integrity (84). The vaccine candidate protein NGO0439 is homologous to the *E. coli* outer membrane protein LolB, which is involved in lipoprotein trafficking to the outer membrane (85, 86). We consider *N. gonorrhoeae* LolB to be a vaccine candidate antigen because its surface localization should be experimentally verified. Differences in the localization of homologous proteins exist. For instance, fHbp is a surface-displayed lipoprotein in *N. meningitidis* but not in *N. gonorrhoeae* (87), while BamE is on the surface of gonococci but faces the periplasmic side of the outer membrane in *E. coli* (72). Protein NGO1688, annotated as OmpU, is a putative iron uptake outer membrane protein that is positively regulated by the oxygen-sensing transcription factor, FNR (88). NGO1911a is a predicted pilus assembly protein that is associated with the adhesin PilY (89). Finally, NGO0778, NGO1251 (a putative lipoprotein), and NGO1889 are hypothetical proteins. NGO1889 belongs to the LprI family (PFO7007) that comprises bacterial proteins of ~120 amino acids in length that contain four conserved cysteine residues. LprI from *Mycobacterium tuberculosis* acts as a lysozyme inhibitor (90), providing the exciting possibility that *N. gonorrhoeae* LprI contributes to residual lysozyme resistance observed in gonococci deprived of surface-exposed lysozyme inhibitors SliC and ACP (73, 91). Lastly, NGO2105 contains peptidase S6 (residues 43-310) and autotransporter (residues 1215-1468) domains potentially involved in proteolytic activity and auto-translocation, respectively, suggesting that this is a newly identified autotransporter protein in *N. gonorrhoeae*. In support of this notion, the NGO2105 locus, also known as adhesion

447 and penetration protein or “NEIS1959 (iga2)” in the PubMLST database, encodes IgA2
448 protease (AidA) and has homologs in other *Neisseria* (Table 1) as well as *Haemophilus*
449 *influenzae* (92).

450 Together, our investigations yielded nine novel gonorrhea vaccine candidates,
451 including proteins with implications in pathogenesis such as IgA2 (AidA) and LprI, and
452 provided valuable information regarding the expression patterns of previously selected
453 vaccine candidates.

454 ***Expression and homologs of gonorrhea vaccine candidates***. We first evaluated
455 expression profiles of extensively studied gonorrhea vaccine candidates including MtrE (93-
456 95), PorB (96, 97), PilQ (98), TbpA (99, 100), Opa (101, 102), and AniA (19, 103-105). MtrE
457 and PorB were up- and down-regulated, respectively, in 12 isolates (Table 2). Compared to
458 WHO F, PorB was present at similar levels only in WHO G and N. PilQ (98) was ubiquitously
459 expressed in 10 strains, whereas expression of Opa proteins was widely variable, as
460 expected (106, 107). The TbpA level was similar in 8 strains; however, we did not detect
461 TbpB (108). Nor did we detect ACP (109, 110) or OpcA (111, 112) under the standard
462 growth conditions used in our studies, which suggested that they might be specifically
463 regulated. AniA was present at different levels in 7 strains, ubiquitous in five, and up-
464 regulated in two isolates. Immunoblotting experiments with anti-AniA antisera corroborated
465 these findings (105). The cellular pool of NspA (113) varied in ten isolates, while lactoferrin
466 binding protein LbpA (114) was variable in five strains and was ubiquitous in WHO L and G
467 (Table 2).

468 Strikingly, most of the proteome-derived vaccine candidates showed ubiquitous
469 expression among numerous strains (Table 1). In particular, SliC, PldA, BamE, BamA, and
470 BamG were ubiquitous in all 15 isolates. Similar results for these proteins were obtained by

iTRAQ-MS/MS applied to the proteomic profiling of cell envelopes and outer membrane vesicles (OMVs) isolated from four different strains of *N. gonorrhoeae* (29). Further, LolB, Ng-MIP, NGO1559, and NGO2054 were unvaryingly expressed in at least 12 isolates. Among the novel vaccine candidates identified in our study, LptE, LolB, IgA2, and NGO1251 were found ubiquitous in at least 13 strains. In addition, LprI and NGO0778 were similarly expressed in 12 and 9 isolates, respectively. In support of our proteomics data, immunoblotting analyses demonstrated similar cellular levels of BamA, MetQ, TamA, LptD, NGO2054 (30), BamE-D (72), SliC (73), and BamG (76) in whole cell lysates of the 2016 WHO strains as well as geographically and temporally diverse clinical isolates of *N. gonorrhoeae* from Baltimore (*n*=5) and Seattle (*n*=13). Our previous studies showed that PorB, PilQ, BamA-D, SliC, MafA, PldA, MetQ, IgA1 protease, and LptD are cargo proteins present at similar levels in naturally released gonococcal OMVs (29, 72, 73), which further highlights their potential as vaccine antigens considering the success of *N. meningitidis* OMV-based vaccines (26, 115).

Finally, we examined the presence of homologs of the gonorrhea vaccine candidates among non-gonococcal *Neisseria* species, other commensal bacteria (116, 117), and co-infecting microbes (118-121) that inhabit the same ecological niche as *N. gonorrhoeae.* Antigens conserved between these pathogens and preferably not in commensals have the potential to eradicate several sexually transmitted infections, if formulated into a protective vaccine(s). Our comparative analyses showed that all of the proteomics-based antigens have homologous proteins in the majority of investigated *N. meningitidis* strains, and none are present in *M. hominis* (Table 1). In addition, Ng-MIP-like proteins exist in *C. trachomatis*, *G. vaginalis*, and *P. ruminicola;* BamA and NGO1559 homologs were found in *C. trachomatis* and *P. ruminicola*. MetQ, a methionine transporter (74), was the only

proteomics-derived vaccine candidate with homologs across all examined bacteria with the exception of *C. trachomatis* and *P. ruminicola*. Further, we detected protein homologs of PilQ in two *C. trachomatis* strains and MtrE and ZnuD in *P. ruminicola*; these three proteins were absent in commensal species.

Together, our investigations provide pioneering information into newly identified and existing gonorrhea vaccine candidates. We have established each candidate's expression pattern in diverse *N. gonorrhoeae* isolates and identified homologs among other pathogenic and/or commensal bacteria that share the same ecological niche. Stable expression in the WHO gonococcal panel coupled to presence in *N. meningitidis* and co-infecting agents – but rarely in urogenital commensals – further highlights the importance of including these antigens in gonorrhea vaccine(s).

Proteomics profiling of N. gonorrhoeae antimicrobial resistance. Various genome-based AMR determinants have been deciphered in the gonococcus over the past decades (51, 122-127). However, many AMR determinants remain to be identified and characterized, e.g. the chromosomally-encoded penicillin and cephalosporin resistance determinant "factor X" (128-130) and the AMR mechanisms that contribute to a large proportion of azithromycin resistance (131). The uncertainty behind these AMR determinants illustrates the need for alternative approaches to enhance our understanding of gonococcal AMR complexity. At the proteomic level, only two studies have attempted to address this challenge, both of which used 2D-SDS PAGE exclusively (47, 132). Therefore, we focused on identifying proteomic AMR signatures that exist in the absence of antimicrobial pressure during standard *in vitro* growth conditions by performing a pairwise comparison of all identified proteins in each individual strain to the WHO F reference strain (Supplemental Tables S1-S2). As expected, we identified different numbers of proteins in the CE and C fractions in each comparison set

519 due to differences in the number of open reading frames (ORFs) between the gonococcal
520 strains (Supplemental Table S3). Similarly to our previous analysis, we excluded typical
521 cytoplasmic proteins from the CE subproteome and cell envelope proteins from the C
522 fraction. We solely focused on proteins with significantly different expression in the
523 examined strains compared to the fully antimicrobial-susceptible strain WHO F with the
524 rationale that these proteins may provide clues about the proteomic basis of AMR. For
525 instance, we identified MtrE as up-regulated in many strains with increased resistance to
526 numerous antimicrobials even in the absence of antimicrobial exposure, which represents
527 an up-regulation of the multidrug MtrCDE efflux pump and possibly additional efflux pumps
528 for which MtrE acts as the outer membrane channel (29, 94, 133-135). Overall, we identified
529 162 (including 21 known AMR determinants) and 95 proteins with known and unpredicted
530 subcellular locations, respectively (Figure 6). Peptide counting performed for the latter group
531 of proteins yielded 55 and 36 proteins that are likely localized to the CE and C, respectively,
532 and four proteins with ambiguous localization. Next, we separated proteins that have been
533 previously verified as *N. gonorrhoeae* AMR determinants (Table 3) from new potential
534 proteomic AMR signatures (Tables 4-5).

535 ***Proteomic signature of previously verified gonococcal antimicrobial resistance***
536 ***determinants.*** Our proteomic analysis detected subcomponents of all the five efflux pumps
537 described in *N. gonorrhoeae,* i.e., MtrCDE, MtrF, FarAB, MacAB, and NorM (Table 3). The
538 outer membrane-barrel protein MtrE serves as the channel for the tripartite MtrCDE pump
539 and likely fulfills this same function in the MacAB and FarAB efflux pumps (133, 135). The
540 MtrCDE complex is the most studied efflux pump system in *N. gonorrhoeae*. The multiple
541 transferable resistance (*mtr*) locus contains the *mtrCDE* operon (136) that is negatively
542 regulated by the repressor MtrR (137). Mutations that abrogate *mtrR* activity result in an

543 over-expression of the MtrCDE efflux pump and decreased susceptibility to numerous
544 antimicrobials, e.g. macrolides, penicillins, cephalosporins, and tetracycline (53, 135). AMR
545 mutations in the *mtrR* promoter were previously identified in WHO G, K, M, O, P, V, W, X,
546 Y, and Z and within the *mtrR* gene (G45D or a frame shift mutation resulting in a truncated
547 peptide) in WHO K, L, M, P, and W (50), suggesting an over-expression of the MtrCDE efflux
548 pump. Our proteomic profiling also verified that the levels of MtrE were significantly
549 increased in all isolates with the exception of two strains lacking any type of *mtrR* AMR
550 determinant (WHO U and N; Table 3). Accordingly, MtrE proved to be an effective indicator
551 of expression of the MtrCDE efflux pump. Our findings were further supported by the down-
552 regulation of MtrR in all examined strains except WHO L (Table 3). WHO L is also the only
553 examined strain that contains an mtr_{120} mutation, which generates a novel promoter for
554 *mtrCDE* transcription and further enhances the expression of the MtrCDE efflux pump (50,
555 138). The second efflux system that showed differential expression was the MacAB efflux
556 pump, which can decrease macrolide susceptibility (139). MacA expression varied across
557 the isolates. Expression of the inner membrane component, MacB, was enhanced in the
558 azithromycin resistant strains WHO P and V, but also in the azithromycin susceptible strains
559 WHO N and K, as well as WHO L, which is intermediately susceptible to azithromycin.
560 Interestingly, MacB was the most highly expressed in WHO V, the only strain with high-level
561 azithromycin resistance [MIC>256 µg/mL; due to the 23S rRNA A2059G mutation in all four
562 alleles (50)], which indicates that over-expression of the MacAB efflux pump may contribute
563 to the high MICs of azithromycin and other macrolides in WHO V. The FarA component of
564 the FarAB efflux pump system, which exports long-chain fatty acids and other hydrophobic
565 agents (140), was not over-expressed in any of the examined strains and was instead
566 ubiquitously expressed in seven WHO strains (M, N, K, L, X, Z, and V) and down-regulated

in WHO U, O, FA6140. Our proteomic profiling also revealed that the NorM and MtrF efflux pumps, which can decrease the susceptibility to fluoroquinolones and sulfonamides, respectively (141, 142), were not over-expressed in any of the examined strains.

Among other established AMR determinants that were differentially expressed was the major porin of *N. gonorrhoeae*, PorB (143, 144), which was down-regulated in all strains with the exception of WHO G and N (Table 4). This down-regulation suggests reduced import of antimicrobials such as penicillins, cephalosporins and tetracyclines, which can contribute to a decreased antimicrobial susceptibility. Furthermore, the WHO F, G, and N express PorB1a, which is associated with a lack of the AMR determinant *penB* and consequently high-level chromosomally-mediated resistance to penicillins and cephalosporins (127), while all other strains express PorB1b. All WHO strains with PorB1b (*n*=11), except WHO U, contained the AMR determinant *penB*. Consequently, our proteomic data suggest that *penB* may be associated with also a decreased expression of PorB1b in addition to the previously documented decreased penetration through PorB1b, resulting in a decreased susceptibility to several antimicrobials. The expression of penicillin-binding protein 1 (PBP1) was significantly down-regulated in nine out of the twelve WHO strains that possess the *ponA1* resistance determinant, which encodes a L421P amino acid substitution in PBP1 that contributes to high-level chromosomally-mediated penicillin resistance (50). Accordingly, our proteomic data indicate that the PBP1 L421P amino acid alteration, in addition to decreased expression of PBP1, might contribute to high-level chromosomally mediated penicillin resistance. In contrast, PBP2 (the main lethal target for penicillins and cephalosporins) was ubiquitously expressed in 13 of the 14 WHO strains. Similarly, GyrA expression was ubiquitous in 13 of the 14 WHO strains. No association between GyrA expression and the main fluoroquinolone resistance mutations [amino acids S91 and D95

591 (50)] was identified. Both GyrB and the second fluoroquinolone target, ParC, were over-
592 expressed in the four ciprofloxacin-resistant WHO strains G, N, V and X, which may suggest
593 that these strains upregulate GyrB and ParC to compensate for the mutated main
594 fluoroquinolone target GyrA (Table 3).

595 ***New potential proteomic-derived antimicrobial resistance signatures***. In the CE
596 fraction, two hypothetical proteins predicted to localize to the inner membrane,
597 WHO_F_00238c and WHO_F_01226, were down-regulated in all examined strains
598 compared to the antimicrobial-susceptible WHO F strain (Table 4). WHO_F_00238c, which
599 corresponds to NGO0222 in the FA1090 genome, is a small protein with a predicted
600 molecular weight of 8.32 kDa that contains two predicted transmembrane domains but no
601 signal peptide. WHO_F_01226 lacks a homologous protein in FA1090. This is also a small
602 protein (5.39 kDa) with no peptides predicted to be recognized by signal peptidase I or II. In
603 the C fraction, no protein was differentially expressed in all strains, but two cytoplasmic
604 proteins, NGO0597 and NGO0701, were up-regulated in all strains except WHO L.
605 NGO0597 is a nucleoside diphosphate kinase (Ndk; 15.4 KDa) involved in DNA and RNA
606 synthesis (145), regulation of gene transcription (146), and peptide chain elongation during
607 translation (147), all processes that are targets for different antimicrobials. NdK is secreted
608 from *Pseudomonas aeruginosa* (146), *M. tuberculosis* (148), *and Leishmania* (149) to
609 modulate interaction with host cells, block phagosome maturation in macrophages (148,
610 150), and promote host cell apoptosis and necrosis (151). It remains to be investigated
611 whether the gonococcal Ndk is secreted during infection and whether it may serve as an
612 anti-virulence or antimicrobial target. Finally, we detected two proteins with undefined
613 subcellular localization displaying global differential expression. WHO_F_01139 and
614 WHO_F_01144, which have no homologs in the FA1090 genome, were down-regulated in

all strains. Our use of UPCD predicted WHO_F_01139 to localize to the cell envelope. WHO_F_01139 is a putative lipoprotein (16.9 KDa) with a predicted signal peptide II domain. Based on UPCD, in addition to the lack of a predicted signal peptide and the absence of transmembrane domains, we predict the hypothetical protein WHO_F_01144 (7.4 kDa) is cytoplasmic. The impact of these six proteins on AMR is yet to be elucidated; however, our data suggest that they may represent general proteomic markers for gonococcal AMR, a predisposition toward developing or compensating for gonococcal AMR, and/or new antimicrobial targets.

Phenotypic clustering based on antibiograms and common differentially expressed proteins. To link AMR phenotypes with proteomic signatures, we performed phenotypic clustering of gonococcal strains based on their defined antibiograms (50, 53-55, 152) and common differentially expressed proteins (Tables 4-5). We additionally investigated each protein's Cluster of Orthologous Genes (COG) annotations and inferred the functional relevance to the observed phenotypes. These analyses generated seven phenotypic clusters that matched between established and proteome-derived AMR signatures (I-VII; Tables 4-6).

Cluster I strains, WHO P and U, exhibit resistance to azithromycin and the majority of up-regulated proteins identified were involved in ribosomal biogenesis: 30S ribosomal proteins S15 and S19; 50S ribosomal proteins L1, L2, and L22; the small GTPase EngA; pseudouridine synthase; RNA helicase; and ribonuclease E. In contrast, proteins involved in cell envelope biogenesis – PilE, LolA, and PglB – were down-regulated in both strains, which may be associated with the strains' decreased susceptibility to penicillin G.

Cluster II strains, WHO M and N, exhibit resistance to penicillin G, tetracycline, and ciprofloxacin. Up-regulated proteins included DNA repair factors (DnaE, a putative type I-

site specific deoxyribonuclease NGO0407, and exonuclease UvrB). Proteins involved in amino acid metabolism (NGO0269, NGO0679); and translation (NGO0803, NGO1870) were also up-regulated, which suggested strains in Cluster II possess compensatory mechanisms for ciprofloxacin and tetracycline resistance, respectively. Hypothetical proteins represented the majority of down-regulated proteins and included WHO_F_00875c, NGO1299, NGO1945, NGO1967, NGO1969, NGO1970, and NGO2089.

Cluster III, IV, and V are comprised of WHO X and L, WHO W and K, and WHO Y and Z, respectively, and exhibit resistance to at least four different antimicrobials, with ciprofloxacin and tetracycline in common (Table 4-5). Three proteins in common between strains in Cluster III and IV were identified. Of these, homoserine dehydrogenase and holo-ACP synthase – involved in amino acid and lipid metabolism, respectively – were down-regulated, while thioredoxin, which is involved in defense against oxidative stress and protein turnover, was up-regulated (153, 154). The NADP quinone reductase (MdaB, modulator of drug activity B) was up-regulated in Cluster IV and V strains. In *E. coli,* MdaB protects against polyketide compound toxicity (155), while overproduction of this protein defends *P. aeruginosa* from oxidative stress (156).

Strains in Cluster VI (WHO V and G) are resistant to penicillin G (WHO G intermediate susceptible), tetracycline and ciprofloxacin. Differentially regulated proteins in this cluster were strikingly similar to Cluster II, with 13 proteins in common (Table 4-6). Seven proteins involved in DNA repair, amino acid metabolism and translation were up-regulated, further strengthening a possible compensatory mechanism for the resistance to ciprofloxacin and tetracycline. Six proteins functioning in coenzyme metabolism (NGO2056) and with unknown functions (NGO1299, NGO1945, NGO1969, NGO1970, NGO2089) were down-regulated (Table 6).

663 Finally, the cluster VII strains (WHO O and FA6140), displaying resistance to penicillin
664 G and tetracycline, had nine common differentially expressed proteins (Table 6). Among
665 these proteins, two metabolic coenzymes (NGO0360 and NGO2056) and a putative
666 cytochrome *b*561 involved in energy production were down-regulated. This cluster
667 possessed a similar expression profile to strains in Cluster I that are intermediately
668 susceptibility to penicillin G and tetracycline. Finally NGO2017, a putative integral inner
669 membrane protein; NGO0452, a potassium proton/antiporter; PilW; and PilE were also
670 down-regulated in the cluster VII strains (Table 6).

671 Our proteomic findings elucidate many differentially regulated proteins as potential
672 general proteomic markers for gonococcal AMR, a predisposition toward developing or
673 compensating for gonococcal AMR, and/or new antimicrobial targets, e.g. NGO0222,
674 WHO_F_01226, NGO0597, NGO0701, WHO_F_01139, WHO_F_011144. Deeper analysis
675 of gonococcal proteotypes that relied on AMR-based phenotypic clustering identified
676 additional proteomic markers potentially associated with (or compensating for) AMR in
677 clusters I, II, VI, and VII. Further studies should examine the proteomic profiles of wild type
678 and AMR gonococcal strains during exposure to varying levels of different antimicrobials. In
679 line with this, the expression of eight outer membrane proteins was enhanced in ampicillin
680 resistant *E. coli* strains upon exposure to the minimal inhibitory concentration of ampicillin
681 (33). Additionally, the functional role(s) of the differentially regulated hypothetical proteins
682 potentially involved in gonococcal AMR need to be elucidated, which would help decode the
683 intricate AMR network and promote the design of ways to curb the spread of AMR among
684 *N. gonorrhoeae* strains.

685

686 **CONCLUSIONS**

687 The present study provides the first global quantitative proteomic characterization of the
688 2016 WHO *N. gonorrhoeae* reference strains (50) and FA6140 (51) to identify new vaccine
689 candidates, gain information about expression of previously identified antigens, and
690 enhance our understanding of AMR in *N. gonorrhoeae*. To our knowledge, this is also the
691 largest quantitative proteomics study performed on bacterial sub-proteomes to date.
692 Importantly, nine novel vaccine candidates have been identified, significantly broadening
693 the gonorrhea antigen repertoire. Further, expression of 21 previously verified AMR
694 determinants at the proteome level was investigated and six new proteomic signatures that
695 may be associated with AMR or may indicate a strain's likelihood of developing or
696 compensating for the physiological consequences of gonococcal AMR. The proteomic
697 signatures we identified may also represent new antimicrobial targets. Expression patterns
698 of antimicrobial targets and AMR determinants provide proteomic signatures that can
699 complement, verify, and enhance our phenotypic- and genetic-derived understanding of
700 gonococcal AMR complexity. Cumulatively, our studies provide a wealth of information
701 regarding gonococcal proteomic profiles and will contribute to ongoing efforts in
702 vaccine/drug development as well as elucidation of AMR mechanisms in *N. gonorrhoeae.*

703 **ACKNOWLEDGEMENTS**

704 This work was supported by the National Institute of Allergy and Infectious Diseases R01-
705 AI117235 to A.E.S., and the World Health Organization, Geneva, Switzerland (2015) and
706 the Foundation for Medical Research at Örebro University Hospital, Sweden (2016) to M.U.
707 We thank Benjamin I. Baarda for critical reading of this manuscript.

708

REFERENCES

1. Newman L, Rowley J, Vander Hoorn S, Wijesooriya NS, Unemo M, Low N, et al. Global Estimates of the Prevalence and Incidence of Four Curable Sexually Transmitted Infections in 2012 Based on Systematic Review and Global Reporting. PloS one. 2015;10(12):e0143304.
2. (CDC) CfDCP. Gonorrhea statistics 2017 [Available from: https://www.cdc.gov/std/stats16/gonorrhea.htm.
3. Kent CK, Chaw JK, Wong W, Liska S, Gibson S, Hubbard G, et al. Prevalence of rectal, urethral, and pharyngeal chlamydia and gonorrhea detected in 2 clinical settings among men who have sex with men: San Francisco, California, 2003. Clin Infect Dis. 2005;41(1):67-74.
4. Newman LM, Dowell D, Bernstein K, Donnelly J, Martins S, Stenger M, et al. A tale of two gonorrhea epidemics: results from the STD surveillance network. Public Health Rep. 2012;127(3):282-92.
5. Cohen MS, Hoffman IF, Royce RA, Kazembe P, Dyer JR, Daly CC, et al. Reduction of concentration of HIV-1 in semen after treatment of urethritis: implications for prevention of sexual transmission of HIV-1. AIDSCAP Malawi Research Group. Lancet. 1997;349(9069):1868-73.
6. Unemo M, Bradshaw CS, Hocking JS, de Vries HJC, Francis SC, Mabey D, et al. Sexually transmitted infections: challenges ahead. Lancet Infect Dis. 2017;17(8):e235-e79.
7. Wi T, Lahra MM, Ndowa F, Bala M, Dillon JR, Ramon-Pardo P, et al. Antimicrobial resistance in Neisseria gonorrhoeae: Global surveillance and a call for international collaborative action. PLoS medicine. 2017;14(7):e1002344.
8. Fifer H, Natarajan U, Jones L, Alexander S, Hughes G, Golparian D, et al. Failure of Dual Antimicrobial Therapy in Treatment of Gonorrhea. N Engl J Med. 2016;374(25):2504-6.
9. England PH. UK case of Neisseria gonorrhoeae with high-level resistance to azithromycin and resistance to ceftriaxone acquired abroad. 2018 29 March 2018. Contract No.: 11.
10. Eyre DW, Sanderson ND, Lord E, Regisford-Reimmer N, Chau K, Barker L, et al. Gonorrhoea treatment failure caused by a Neisseria gonorrhoeae strain with combined ceftriaxone and high-level azithromycin resistance, England, February 2018. Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin. 2018;23(27).
11. Murphy B. Multi-drug resistant gonorrhoea Australia: Australian Government Department of Health; 2018 [updated 17 April 2018.
12. Whiley DM, Jennison A, Pearson J, Lahra MM. Genetic characterisation of Neisseria gonorrhoeae resistant to both ceftriaxone and azithromycin. Lancet Infect Dis. 2018;18(7):717-8.
13. Alirol E, Wi TE, Bala M, Bazzo ML, Chen XS, Deal C, et al. Multidrug-resistant gonorrhea: A research and development roadmap to discover new medicines. PLoS medicine. 2017;14(7):e1002366.
14. Hampton LM, Farley MM, Schaffner W, Thomas A, Reingold A, Harrison LH, et al. Prevention of antibiotic-nonsusceptible Streptococcus pneumoniae with conjugate vaccines. J Infect Dis. 2012;205(3):401-11.
15. Greenberg L, Diena BB, Ashton FA, Wallace R, Kenny CP, Znamirowski R, et al. Gonococcal vaccine studies in Inuvik. Can J Public Health. 1974;65(1):29-33.

16. Boslego JW, Tramont EC, Chung RC, McChesney DG, Ciak J, Sadoff JC, et al. Efficacy trial of a parenteral gonococcal pilus vaccine in men. Vaccine. 1991;9(3):154-62.
17. Tramont EC. Gonococcal vaccines. Clinical microbiology reviews. 1989;2 Suppl:S74-7.
18. Taylor-Robinson D, Furr PM, Hetherington CM. Neisseria gonorrhoeae colonises the genital tract of oestradiol-treated germ-free female mice. Microb Pathog. 1990;9(5):369-73.
19. Rice PA, Shafer WM, Ram S, Jerse AE. Neisseria gonorrhoeae: Drug Resistance, Mouse Models, and Vaccine Development. Annual review of microbiology. 2017;71:665-86.
20. Normark S, Albiger B, Jonsson AB. Gonococci cause immunosuppression by engaging a coinhibitory receptor on T lymphocytes. Nat Immunol. 2002;3(3):210-1.
21. Lewis LA, Choudhury B, Balthazar JT, Martin LE, Ram S, Rice PA, et al. Phosphoethanolamine substitution of lipid A and resistance of Neisseria gonorrhoeae to cationic antimicrobial peptides and complement-mediated killing by normal human serum. Infection and immunity. 2009;77(3):1112-20.
22. Packiam M, Wu H, Veit SJ, Mavrogiorgos N, Jerse AE, Ingalls RR. Protective role of Toll-like receptor 4 in experimental gonococcal infection of female mice. Mucosal immunology. 2012;5(1):19-29.
23. Packiam M, Yedery RD, Begum AA, Carlson RW, Ganguly J, Sempowski GD, et al. Phosphoethanolamine decoration of Neisseria gonorrhoeae lipid A plays a dual immunostimulatory and protective role during experimental genital tract infection. Infection and immunity. 2014;82(6):2170-9.
24. Sintsova A, Wong H, MacDonald KS, Kaul R, Virji M, Gray-Owen SD. Selection for a CEACAM receptor-specific binding phenotype during Neisseria gonorrhoeae infection of the human genital tract. Infection and immunity. 2015;83(4):1372-83.
25. Liu Y, Liu W, Russell MW. Suppression of host adaptive immune responses by Neisseria gonorrhoeae: role of interleukin 10 and type 1 regulatory T cells. Mucosal immunology. 2014;7(1):165-76.
26. Liu Y, Hammer LA, Liu W, Hobbs MM, Zielke RA, Sikora AE, et al. Experimental vaccine induces Th1-driven immune responses and resistance to Neisseria gonorrhoeae infection in a murine model. Mucosal immunology. 2017;10(6):1594-608.
27. Petousis-Harris H, Paynter J, Morgan J, Saxton P, McArdle B, Goodyear-Smith F, et al. Effectiveness of a group B outer membrane vesicle meningococcal vaccine against gonorrhoea in New Zealand: a retrospective case-control study. Lancet. 2017.
28. Unemo M, Sikora AE. Infection: Proof of principle for effectiveness of a gonorrhoea vaccine. Nat Rev Urol. 2017;14(11):643-4.
29. Zielke RA, Wierzbicki IH, Weber JV, Gafken PR, Sikora AE. Quantitative proteomics of the Neisseria gonorrhoeae cell envelope and membrane vesicles for the discovery of potential therapeutic targets. Mol Cell Proteomics. 2014;13(5):1299-317.
30. Zielke RA, Wierzbicki IH, Baarda BI, Gafken PR, Soge OO, Holmes KK, et al. Proteomics-driven Antigen Discovery for Development of Vaccines Against Gonorrhea. Mol Cell Proteomics. 2016;15(7):2338-55.
31. Baarda BI, Sikora AE. Proteomics of Neisseria gonorrhoeae: the treasure hunt for countermeasures against an old disease. Frontiers in microbiology. 2015;6:1190.
32. Otto A, Becher D, Schmidt F. Quantitative proteomics in the field of microbiology. Proteomics. 2014;14(4-5):547-65.
33. Xu C, Lin X, Ren H, Zhang Y, Wang S, Peng X. Analysis of outer membrane proteome of Escherichia coli related to resistance to ampicillin and tetracycline. Proteomics. 2006;6(2):462-73.

804 34. Al-Haroni M, Skaug N, Bakken V, Cash P. Proteomic analysis of ampicillin-resistant
805 oral Fusobacterium nucleatum. Oral Microbiol Immunol. 2008;23(1):36-42.
806 35. Giddey AD, de Kock E, Nakedi KC, Garnett S, Nel AJ, Soares NC, et al. A temporal
807 proteome dynamics study reveals the molecular basis of induced phenotypic resistance in
808 Mycobacterium smegmatis at sub-lethal rifampicin concentrations. Scientific reports.
809 2017;7:43858.
810 36. Len AC, Harty DW, Jacques NA. Stress-responsive proteins are upregulated in
811 Streptococcus mutans during acid tolerance. Microbiology. 2004;150(Pt 5):1339-51.
812 37. Liebeke M, Dorries K, Zuhlke D, Bernhardt J, Fuchs S, Pane-Farre J, et al. A
813 metabolomics and proteomics study of the adaptation of Staphylococcus aureus to glucose
814 starvation. Molecular bioSystems. 2011;7(4):1241-53.
815 38. Alreshidi MM, Dunstan RH, Macdonald MM, Smith ND, Gottfries J, Roberts TK.
816 Metabolomic and proteomic responses of Staphylococcus aureus to prolonged cold stress.
817 Journal of proteomics. 2015;121:44-55.
818 39. Baarda BI, Emerson S, Proteau PJ, Sikora AE. Deciphering the function of new
819 gonococcal vaccine antigens using phenotypic microarrays. J Bacteriol. 2017.
820 40. Christodoulides M. Neisseria proteomics for antigen discovery and vaccine
821 development. 2014.
822 41. Otto A, Bernhardt J, Hecker M, Becher D. Global relative and absolute quantitation
823 in microbial proteomics. Curr Opin Microbiol. 2012;15(3):364-72.
824 42. Adamczyk-Poplawska M, Markowicz S, Jagusztyn-Krynicka EK. Proteomics for
825 development of vaccine. Journal of proteomics. 2011;74(12):2596-616.
826 43. Zielke RA, Sikora AE. Isolation of Cell Envelopes and Naturally Released Membrane
827 Vesicles of Neisseria gonorrhoeae. Curr Protoc Microbiol. 2014;34:4A 3 1-4A 3 17.
828 44. Olaya-Abril A, Jimenez-Munguia I, Gomez-Gascon L, Rodriguez-Ortega MJ.
829 Surfomics: shaving live organisms for a fast proteomic identification of surface proteins.
830 Journal of proteomics. 2014;97:164-76.
831 45. Zielke RA, Wierzbicki IH, Jerse AE, Gafken PR, Soge OO, Holmes KK, et al., editors.
832 Proteomics-Driven Reverse Vaccinology for Gonorrhea. American Society for Microbiology;
833 2015 6/2/2015; New Orleans, Louisiana2015.
834 46. Yoo JS, Seong WK, Kim TS, Park YK, Oh HB, Yoo CK. Comparative proteome
835 analysis of the outer membrane proteins of in vitro-induced multi-drug resistant Neisseria
836 gonorrhoeae. Microbiol Immunol. 2007;51(12):1171-7.
837 47. Nabu S, Lawung R, Isarankura-Na-Ayudhya P, Isarankura-Na-Ayudhya C, Roytrakul
838 S, Prachayasittikul V. Reference map and comparative proteomic analysis of Neisseria
839 gonorrhoeae displaying high resistance against spectinomycin. Journal of medical
840 microbiology. 2014;63(Pt 3):371-85.
841 48. Smith SM. Strategies for the Purification of Membrane Proteins. Methods Mol Biol.
842 2017;1485:389-400.
843 49. Zielke RA, Gafken PR, Sikora AE. Quantitative proteomic analysis of the cell
844 envelopes and native membrane vesicles derived from gram-negative bacteria. Curr Protoc
845 Microbiol. 2014;34:1F 3 1-F 3 16.
846 50. Unemo M, Golparian D, Sanchez-Buso L, Grad Y, Jacobsson S, Ohnishi M, et al.
847 The novel 2016 WHO Neisseria gonorrhoeae reference strains for global quality assurance
848 of laboratory investigations: phenotypic, genetic and reference genome characterization. J
849 Antimicrob Chemother. 2016;71(11):3096-108.

850 51. Faruki H, Kohmescher RN, McKinney WP, Sparling PF. A community-based
851 outbreak of infection with penicillin-resistant Neisseria gonorrhoeae not producing
852 penicillinase (chromosomally mediated resistance). N Engl J Med. 1985;313(10):607-11.
853 52. Unemo M, Fasth O, Fredlund H, Limnios A, Tapsall J. Phenotypic and genetic
854 characterization of the 2008 WHO Neisseria gonorrhoeae reference strain panel intended
855 for global quality assurance and quality control of gonococcal antimicrobial resistance
856 surveillance for public health purposes. The Journal of antimicrobial chemotherapy.
857 2009;63(6):1142-51.
858 53. Veal WL, Nicholas RA, Shafer WM. Overexpression of the MtrC-MtrD-MtrE efflux
859 pump due to an mtrR mutation is required for chromosomally mediated penicillin resistance
860 in Neisseria gonorrhoeae. Journal of bacteriology. 2002;184(20):5619-24.
861 54. Hu M, Nandi S, Davies C, Nicholas RA. High-level chromosomally mediated
862 tetracycline resistance in Neisseria gonorrhoeae results from a point mutation in the rpsJ
863 gene encoding ribosomal protein S10 in combination with the mtrR and penB resistance
864 determinants. Antimicrobial agents and chemotherapy. 2005;49(10):4327-34.
865 55. Powell AJ, Tomberg J, Deacon AM, Nicholas RA, Davies C. Crystal structures of
866 penicillin-binding protein 2 from penicillin-susceptible and -resistant strains of Neisseria
867 gonorrhoeae reveal an unexpectedly subtle mechanism for antibiotic resistance. The
868 Journal of biological chemistry. 2009;284(2):1202-12.
869 56. Thompson A, Schafer J, Kuhn K, Kienle S, Schwarz J, Schmidt G, et al. Tandem
870 mass tags: a novel quantification strategy for comparative analysis of complex protein
871 mixtures by MS/MS. Analytical chemistry. 2003;75(8):1895-904.
872 57. Spence JM, Wright L, Clark VL. Laboratory maintenance of Neisseria gonorrhoeae.
873 Curr Protoc Microbiol. 2008;Chapter 4:Unit 4A 1.
874 58. Candiano G, Bruschi M, Musante L, Santucci L, Ghiggeri GM, Carnemolla B, et al.
875 Blue silver: a very sensitive colloidal Coomassie G-250 staining for proteome analysis.
876 Electrophoresis. 2004;25(9):1327-33.
877 59. Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M. In-gel digestion for mass
878 spectrometric characterization of proteins and proteomes. Nature protocols.
879 2006;1(6):2856-60.
880 60. Moreno-Hagelsieb G, Latimer K. Choosing BLAST options for better detection of
881 orthologs as reciprocal best hits. Bioinformatics. 2008;24(3):319-24.
882 61. Yu CS, Chen YC, Lu CH, Hwang JK. Prediction of protein subcellular localization.
883 Proteins. 2006;64(3):643-51.
884 62. Yu NY, Wagner JR, Laird MR, Melli G, Rey S, Lo R, et al. PSORTb 3.0: improved
885 protein subcellular localization prediction with refined localization subcategories and
886 predictive capabilities for all prokaryotes. Bioinformatics. 2010;26(13):1608-15.
887 63. Imai K, Asakawa N, Tsuji T, Akazawa F, Ino A, Sonoyama M, et al. SOSUI-GramN:
888 high performance prediction for sub-cellular localization of proteins in gram-negative
889 bacteria. Bioinformation. 2008;2(9):417-21.
890 64. Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal
891 peptides from transmembrane regions. Nature methods. 2011;8(10):785-6.
892 65. Juncker AS, Willenbrock H, Von Heijne G, Brunak S, Nielsen H, Krogh A. Prediction
893 of lipoprotein signal peptides in Gram-negative bacteria. Protein science : a publication of
894 the Protein Society. 2003;12(8):1652-62.
895 66. Vizcaino JA, Csordas A, del-Toro N, Dianes JA, Griss J, Lavidas I, et al. 2016 update
896 of the PRIDE database and its related tools. Nucleic acids research. 2016;44(D1):D447-56.

67. Senko MW, Remes PM, Canterbury JD, Mathur R, Song Q, Eliuk SM, et al. Novel parallelized quadrupole/linear ion trap/Orbitrap tribrid mass spectrometer improving proteome coverage and peptide identification rates. Analytical chemistry. 2013;85(24):11710-4.
68. Abrams AJ, Trees DL, Nicholas RA. Complete Genome Sequences of Three Neisseria gonorrhoeae Laboratory Reference Strains, Determined Using PacBio Single-Molecule Real-Time Technology. Genome Announc. 2015;3(5).
69. Papanastasiou M, Orfanoudaki G, Koukaki M, Kountourakis N, Sardis MF, Aivaliotis M, et al. The Escherichia coli peripheral inner membrane proteome. Mol Cell Proteomics. 2013;12(3):599-610.
70. Jan AT. Outer Membrane Vesicles (OMVs) of Gram-negative Bacteria: A Perspective Update. Frontiers in microbiology. 2017;8:1053.
71. Zielke RA, Wierzbicki IH, Baarda BI, Gafken PR, Soge OO, Holmes KK, et al. Proteomics-driven antigen discovery for development of vaccines against gonorrhea. Mol Cell Proteomics. 2016.
72. Sikora AE, Wierzbicki IH, Zielke RA, Ryner RF, Korotkov KV, Buchanan SK, et al. Structural and functional insights into the role of BamD and BamE within the beta-barrel assembly machinery in Neisseria gonorrhoeae. The Journal of biological chemistry. 2018;293(4):1106-19.
73. Zielke RA, Le Van A, Baarda BI, Herrera MF, Acosta CJ, Jerse AE, et al. SliC is a surface-displayed lipoprotein that is required for the anti-lysozyme strategy during Neisseria gonorrhoeae infection. PLoS pathogens. 2018;14(7):e1007081.
74. Semchenko EA, Day CJ, Seib KL. MetQ of Neisseria gonorrhoeae Is a Surface-Expressed Antigen That Elicits Bactericidal and Functional Blocking Antibodies. Infection and immunity. 2017;85(2).
75. Leuzzi R, Serino L, Scarselli M, Savino S, Fontana MR, Monaci E, et al. Ng-MIP, a surface-exposed lipoprotein of Neisseria gonorrhoeae, has a peptidyl-prolyl cis/trans isomerase (PPIase) activity and is involved in persistence in macrophages. Molecular microbiology. 2005;58(3):669-81.
76. Wierzbicki IH, A. ZR, Jerse AE, Begum AA, Unemo M, Sikora AE, editors. NGO1985 is a surface-exposed outer membrane lipoprotein involved in Neisseria gonorrhoeae cell envelope homeostasis and a novel gonorrhea vaccine candidate. International Pathogenic Neisseria Conference; 2016; Manchester, UK.
77. Liu Y, Hammer LA, Liu W, Hobbs MM, Zielke RA, Sikora AE, et al. Experimental vaccine induces Th1-driven immune responses and resistance to Neisseria gonorrhoeae infection in a murine model. Mucosal immunology. 2017.
78. Lomholt H, Lind I, Kilian M. Neisseria gonorrhoeae IgA1 proteases share epitopes recognized by neutralizing antibodies. Vaccine. 1995;13(13):1213-9.
79. Malojcic G, Andres D, Grabowicz M, George AH, Ruiz N, Silhavy TJ, et al. LptE binds to and alters the physical state of LPS to catalyze its assembly at the cell surface. Proc Natl Acad Sci U S A. 2014;111(26):9467-72.
80. Bos MP, Tommassen J. The LptD chaperone LptE is not directly involved in lipopolysaccharide transport in Neisseria meningitidis. The Journal of biological chemistry. 2011;286(33):28688-96.
81. Bos MP, Tefsen B, Geurtsen J, Tommassen J. Identification of an outer membrane protein required for the transport of lipopolysaccharide to the bacterial cell surface. Proc Natl Acad Sci U S A. 2004;101(25):9417-22.

944 82. Zielke RA, Simmons RS, Park BR, Nonogaki M, Emerson S, Sikora AE. The type II
945 secretion pathway in Vibrio cholerae is characterized by growth-phase dependent
946 expression of exoprotein genes and is positively regulated by sigmaE. Infection and
947 immunity. 2014.
948 83. Braun M, Silhavy TJ. Imp/OstA is required for cell envelope biogenesis in Escherichia
949 coli. Molecular microbiology. 2002;45(5):1289-302.
950 84. Gotzke H, Palombo I, Muheim C, Perrody E, Genevaux P, Kudva R, et al. YfgM is an
951 ancillary subunit of the SecYEG translocon in Escherichia coli. J Biol Chem.
952 2014;289(27):19089-97.
953 85. Matsuyama S, Yokota N, Tokuda H. A novel outer membrane lipoprotein, LolB
954 (HemM), involved in the LolA (p20)-dependent localization of lipoproteins to the outer
955 membrane of Escherichia coli. EMBO J. 1997;16(23):6947-55.
956 86. Grabowicz M, Silhavy TJ. Redefining the essential trafficking pathway for outer
957 membrane lipoproteins. Proc Natl Acad Sci U S A. 2017;114(18):4769-74.
958 87. Jongerius I, Lavender H, Tan L, Ruivo N, Exley RM, Caesar JJ, et al. Distinct binding
959 and immunogenic properties of the gonococcal homologue of meningococcal factor h
960 binding protein. PLoS Pathog. 2013;9(8):e1003528.
961 88. Whitehead RN, Overton TW, Snyder LA, McGowan SJ, Smith H, Cole JA, et al. The
962 small FNR regulon of Neisseria gonorrhoeae: comparison with the larger Escherichia coli
963 FNR regulon and interaction with the NarQ-NarP regulon. BMC genomics. 2007;8:35.
964 89. Alm RA, Hallinan JP, Watson AA, Mattick JS. Fimbrial biogenesis genes of
965 Pseudomonas aeruginosa: pilW and pilX increase the similarity of type 4 fimbriae to the
966 GSP protein-secretion systems and pilY1 encodes a gonococcal PilC homologue. Mol
967 Microbiol. 1996;22(1):161-73.
968 90. Sethi D, Mahajan S, Singh C, Lama A, Hade MD, Gupta P, et al. Lipoprotein LprI of
969 Mycobacterium tuberculosis Acts as a Lysozyme Inhibitor. The Journal of biological
970 chemistry. 2016;291(6):2938-53.
971 91. Ragland SA, Humbert MV, Christodoulides M, Criss AK. Neisseria gonorrhoeae
972 employs two protein inhibitors to evade killing by human lysozyme. PLoS pathogens.
973 2018;14(7):e1007080.
974 92. van Ulsen P, Tommassen J. Protein secretion and secreted proteins in pathogenic
975 Neisseriaceae. FEMS Microbiol Rev. 2006;30(2):292-319.
976 93. Jerse AE, Sharma ND, Simms AN, Crow ET, Snyder LA, Shafer WM. A gonococcal
977 efflux pump system enhances bacterial survival in a female mouse model of genital tract
978 infection. Infection and immunity. 2003;71(10):5576-82.
979 94. Delahay RM, Robertson BD, Balthazar JT, Shafer WM, Ison CA. Involvement of the
980 gonococcal MtrE protein in the resistance of Neisseria gonorrhoeae to toxic hydrophobic
981 agents. Microbiology. 1997;143 (Pt 7):2127-33.
982 95. Warner DM, Folster JP, Shafer WM, Jerse AE. Regulation of the MtrC-MtrD-MtrE
983 efflux-pump system modulates the in vivo fitness of Neisseria gonorrhoeae. J Infect Dis.
984 2007;196(12):1804-12.
985 96. Edwards JL, Brown EJ, Uk-Nham S, Cannon JG, Blake MS, Apicella MA. A co-
986 operative interaction between Neisseria gonorrhoeae and complement receptor 3 mediates
987 infection of primary cervical epithelial cells. Cellular microbiology. 2002;4(9):571-84.
988 97. Ling AE, Bash MC, Lynn F, Lister NA, Zhu P, Garland SM, et al. Evaluation of PorB
989 variable region typing of Neisseria gonorrhoeae using PCR-ELISA in samples collected from
990 men who have sex with men. J Clin Lab Anal. 2007;21(4):237-43.

991 98. Haghi F, Peerayeh SN, Siadat SD, Zeighami H. Recombinant outer membrane
992 secretin PilQ(406-770) as a vaccine candidate for serogroup B Neisseria meningitidis.
993 Vaccine. 2012;30(9):1710-4.
994 99. Price GA, Masri HP, Hollander AM, Russell MW, Cornelissen CN. Gonococcal
995 transferrin binding protein chimeras induce bactericidal and growth inhibitory antibodies in
996 mice. Vaccine. 2007;25(41):7247-60.
997 100. Noto JM, Cornelissen CN. Identification of TbpA residues required for transferrin-iron
998 utilization by Neisseria gonorrhoeae. Infection and immunity. 2008;76(5):1960-9.
999 101. Callaghan MJ, Lewis S, Sadarangani M, Bailey SE, Chan H, Ferguson DJ, et al.
1000 Potential of recombinant opa proteins as vaccine candidates against hyperinvasive
1001 meningococci. Infection and immunity. 2011;79(7):2810-8.
1002 102. Cole JG, Jerse AE. Functional characterization of antibodies against Neisseria
1003 gonorrhoeae opacity protein loops. PloS one. 2009;4(12):e8108.
1004 103. Boulanger MJ, Murphy ME. Crystal structure of the soluble domain of the major
1005 anaerobically induced outer membrane protein (AniA) from pathogenic Neisseria: a new
1006 class of copper-containing nitrite reductases. J Mol Biol. 2002;315(5):1111-27.
1007 104. Shewell LK, Ku SC, Schulz BL, Jen FE, Mubaiwa TD, Ketterer MR, et al.
1008 Recombinant truncated AniA of pathogenic Neisseria elicits a non-native immune response
1009 and functional blocking antibodies. Biochem Biophys Res Commun. 2013;431(2):215-20.
1010 105. Sikora AE, Mills RH, Weber JV, Hamza A, Passow BW, Romaine A, et al. Peptide
1011 Inhibitors Targeting the Neisseria gonorrhoeae Pivotal Anaerobic Respiration Factor AniA.
1012 Antimicrobial agents and chemotherapy. 2017;61(8).
1013 106. Jerse AE, Bash MC, Russell MW. Vaccines against gonorrhea: current status and
1014 future challenges. Vaccine. 2014;32(14):1579-87.
1015 107. Bhat KS, Gibbs CP, Barrera O, Morrison SG, Jahnig F, Stern A, et al. The opacity
1016 proteins of Neisseria gonorrhoeae strain MS11 are encoded by a family of 11 complete
1017 genes. Molecular microbiology. 1991;5(8):1889-901.
1018 108. Noinaj N, Buchanan SK, Cornelissen CN. The transferrin-iron import system from
1019 pathogenic Neisseria species. Molecular microbiology. 2012;86(2):246-57.
1020 109. Humbert MV, Awanye AM, Lian LY, Derrick JP, Christodoulides M. Structure of the
1021 Neisseria Adhesin Complex Protein (ACP) and its role as a novel lysozyme inhibitor. PLoS
1022 Pathog. 2017;13(6):e1006448.
1023 110. Hung MC, Heckels JE, Christodoulides M. The adhesin complex protein (ACP) of
1024 Neisseria meningitidis is a new adhesin with vaccine potential. mBio. 2013;4(2).
1025 111. Keiser PB, Biggs-Cicatelli S, Moran EE, Schmiel DH, Pinto VB, Burden RE, et al. A
1026 phase 1 study of a meningococcal native outer membrane vesicle vaccine made from a
1027 group B strain with deleted lpxL1 and synX, over-expressed factor H binding protein, two
1028 PorAs and stabilized OpcA expression. Vaccine. 2011;29(7):1413-20.
1029 112. Moore J, Bailey SE, Benmechernene Z, Tzitzilonis C, Griffiths NJ, Virji M, et al.
1030 Recognition of saccharides by the OpcA, OpaD, and OpaB outer membrane proteins from
1031 Neisseria meningitidis. J Biol Chem. 2005;280(36):31489-97.
1032 113. Li G, Jiao H, Jiang G, Wang J, Zhu L, Xie R, et al. Neisseria gonorrhoeae NspA
1033 induces specific bactericidal and opsonic antibodies in mice. Clinical and vaccine
1034 immunology : CVI. 2011;18(11):1817-22.
1035 114. Pettersson A, Kortekaas J, Weynants VE, Voet P, Poolman JT, Bos MP, et al.
1036 Vaccine potential of the Neisseria meningitidis lactoferrin-binding proteins LbpA and LbpB.
1037 Vaccine. 2006;24(17):3545-57.

115. Holst J, Oster P, Arnold R, Tatley MV, Naess LM, Aaberge IS, et al. Vaccines against meningococcal serogroup B disease containing outer membrane vesicles (OMV): lessons from past programs and implications for the future. Hum Vaccin Immunother. 2013;9(6):1241-53.
116. Fredricks DN, Fiedler TL, Marrazzo JM. Molecular identification of bacteria associated with bacterial vaginosis. N Engl J Med. 2005;353(18):1899-911.
117. Nelson DE, Dong Q, Van der Pol B, Toh E, Fan B, Katz BP, et al. Bacterial communities of the coronal sulcus and distal urethra of adolescent males. PloS one. 2012;7(5):e36298.
118. Lim RB, Wong ML, Cook AR, Brun C, Chan RK, Sen P, et al. Determinants of Chlamydia, Gonorrhea, and Coinfection in Heterosexual Adolescents Attending the National Public Sexually Transmitted Infection Clinic in Singapore. Sexually transmitted diseases. 2015;42(8):450-6.
119. Oriel JD, Reeve P, Thomas BJ, Nicol CS. Infection with Chlamydia group A in men with urethritis due to Neisseria gonorrhoeae. J Infect Dis. 1975;131(4):376-82.
120. Moller BR, Mardh PA, Ahrons S, Nussler E. Infection with Chlamydia trachomatis, Mycoplasma hominis and Neisseria gonorrhoeae in patients with acute pelvic inflammatory disease. Sexually transmitted diseases. 1981;8(3):198-202.
121. Vonck RA, Darville T, O'Connell CM, Jerse AE. Chlamydial infection increases gonococcal colonization in a novel murine coinfection model. Infection and immunity. 2011;79(4):1566-77.
122. Sparling PF. Antibiotic resistance in Neisseria gonorrhoeae. Med Clin North Am. 1972;56(5):1133-44.
123. Maness MJ, Sparling PF. Multiple antibiotic resistance due to a single mutation in Neisseria gonorrhoeae. J Infect Dis. 1973;128(3):321-30.
124. Sarubbi FA, Jr., Blackman E, Sparling PF. Genetic mapping of linked antibiotic resistance loci in Neisseria gonorrhoeae. Journal of bacteriology. 1974;120(3):1284-92.
125. Sparling PF, Sarubbi FA, Jr., Blackman E. Inheritance of low-level resistance to penicillin, tetracycline, and chloramphenicol in Neisseria gonorrhoeae. Journal of bacteriology. 1975;124(2):740-9.
126. Biswas G, Comer S, Sparling PF. Chromosomal location of antibiotic resistance genes in Neisseria gonorrhoeae. Journal of bacteriology. 1976;125(3):1207-10.
127. Unemo M, Shafer WM. Antimicrobial resistance in Neisseria gonorrhoeae in the 21st century: past, evolution, and future. Clinical microbiology reviews. 2014;27(3):587-613.
128. Ohnishi M, Golparian D, Shimuta K, Saika T, Hoshina S, Iwasaku K, et al. Is Neisseria gonorrhoeae initiating a future era of untreatable gonorrhea?: detailed characterization of the first strain with high-level resistance to ceftriaxone. Antimicrobial agents and chemotherapy. 2011;55(7):3538-45.
129. Lindberg R, Fredlund H, Nicholas R, Unemo M. Neisseria gonorrhoeae isolates with reduced susceptibility to cefixime and ceftriaxone: association with genetic polymorphisms in penA, mtrR, porB1b, and ponA. Antimicrobial agents and chemotherapy. 2007;51(6):2117-22.
130. Zhao S, Duncan M, Tomberg J, Davies C, Unemo M, Nicholas RA. Genetics of chromosomally mediated intermediate resistance to ceftriaxone and cefixime in Neisseria gonorrhoeae. Antimicrobial agents and chemotherapy. 2009;53(9):3744-51.
131. Grad YH, Harris SR, Kirkcaldy RD, Green AG, Marks DS, Bentley SD, et al. Genomic Epidemiology of Gonococcal Resistance to Extended-Spectrum Cephalosporins,

Macrolides, and Fluoroquinolones in the United States, 2000-2013. J Infect Dis. 2016;214(10):1579-87.
132. Nabu S, Lawung R, Isarankura-Na-Ayudhya P, Roytrakul S, Dolprasit S, Sengyee S, et al. Comparative proteomics analysis of Neisseria gonorrhoeae strains in response to extended-spectrum cephalosporins. EXCLI J. 2017;16:1207-29.
133. Golparian D, Shafer WM, Ohnishi M, Unemo M. Importance of multidrug efflux pumps in the antimicrobial resistance property of clinical multidrug-resistant isolates of Neisseria gonorrhoeae. Antimicrobial agents and chemotherapy. 2014;58(6):3556-9.
134. Veal WL, Yellen A, Balthazar JT, Pan W, Spratt BG, Shafer WM. Loss-of-function mutations in the mtr efflux system of Neisseria gonorrhoeae. Microbiology. 1998;144 (Pt 3):621-7.
135. William M. Shafer EWY, Corinne Rouquette-Loughlin,Daniel Golparian,Ann E. Jerse,Magnus Unemo. Efflux Pumps in Neisseria gonorrhoeae: Contributions to Antimicrobial Resistance and Virulence. In: Xian-Zhi Li CAE, Helen I. Zgurskaya, editor. Efflux-Mediated Antimicrobial Resistance in Bacteria: Mechanisms, Regulation and Clinical Implications: Springer; 2016. p. 439-69.
136. Hagman KE, Pan W, Spratt BG, Balthazar JT, Judd RC, Shafer WM. Resistance of Neisseria gonorrhoeae to antimicrobial hydrophobic agents is modulated by the mtrRCDE efflux system. Microbiology. 1995;141 (Pt 3):611-22.
137. Zarantonelli L, Borthagaray G, Lee EH, Shafer WM. Decreased azithromycin susceptibility of Neisseria gonorrhoeae due to mtrR mutations. Antimicrobial agents and chemotherapy. 1999;43(10):2468-72.
138. Ohneck EA, Zalucki YM, Johnson PJ, Dhulipala V, Golparian D, Unemo M, et al. A novel mechanism of high-level, broad-spectrum antibiotic resistance caused by a single base pair change in Neisseria gonorrhoeae. mBio. 2011;2(5).
139. Rouquette-Loughlin CE, Balthazar JT, Shafer WM. Characterization of the MacA-MacB efflux system in Neisseria gonorrhoeae. The Journal of antimicrobial chemotherapy. 2005;56(5):856-60.
140. Lee EH, Shafer WM. The farAB-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. Molecular microbiology. 1999;33(4):839-45.
141. Rouquette-Loughlin C, Dunham SA, Kuhn M, Balthazar JT, Shafer WM. The NorM efflux pump of Neisseria gonorrhoeae and Neisseria meningitidis recognizes antimicrobial cationic compounds. Journal of bacteriology. 2003;185(3):1101-6.
142. Su CC, Bolla JR, Kumar N, Radhakrishnan A, Long F, Delmar JA, et al. Structure and function of Neisseria gonorrhoeae MtrF illuminates a class of antimetabolite efflux pumps. Cell Rep. 2015;11(1):61-70.
143. Deo P, Chow SH, Hay ID, Kleifeld O, Costin A, Elgass KD, et al. Outer membrane vesicles from Neisseria gonorrhoeae target PorB to mitochondria and induce apoptosis. PLoS Pathog. 2018;14(3):e1006945.
144. Zhu W, Tomberg J, Knilans KJ, Anderson JE, McKinnon KP, Sempowski GD, et al. Properly folded and functional PorB from Neisseria gonorrhoeae inhibits dendritic cell stimulation of CD4(+) T cell proliferation. The Journal of biological chemistry. 2018.
145. Levit MN, Abramczyk BM, Stock JB, Postel EH. Interactions between Escherichia coli nucleoside-diphosphate kinase and DNA. J Biol Chem. 2002;277(7):5163-7.
146. Yu H, Xiong J, Zhang R, Hu X, Qiu J, Zhang D, et al. Ndk, a novel host-responsive regulator, negatively regulates bacterial virulence through quorum sensing in Pseudomonas aeruginosa. Scientific reports. 2016;6:28684.

147. Mukhopadhyay S, Shankar S, Walden W, Chakrabarty AM. Complex formation of the elongation factor Tu from Pseudomonas aeruginosa with nucleoside diphosphate kinase modulates ribosomal GTP synthesis and peptide chain elongation. J Biol Chem. 1997;272(28):17815-20.
148. Sun J, Wang X, Lau A, Liao TY, Bucci C, Hmama Z. Mycobacterial nucleoside diphosphate kinase blocks phagosome maturation in murine RAW 264.7 macrophages. PloS one. 2010;5(1):e8769.
149. Kolli BK, Kostal J, Zaborina O, Chakrabarty AM, Chang KP. Leishmania-released nucleoside diphosphate kinase prevents ATP-mediated cytolysis of macrophages. Mol Biochem Parasitol. 2008;158(2):163-75.
150. Sun J, Singh V, Lau A, Stokes RW, Obregon-Henao A, Orme IM, et al. Mycobacterium tuberculosis nucleoside diphosphate kinase inactivates small GTPases leading to evasion of innate immunity. PLoS pathogens. 2013;9(7):e1003499.
151. Chopra P, Singh A, Koul A, Ramachandran S, Drlica K, Tyagi AK, et al. Cytotoxic activity of nucleoside diphosphate kinase secreted from Mycobacterium tuberculosis. Eur J Biochem. 2003;270(4):625-34.
152. Tomberg J, Unemo M, Davies C, Nicholas RA. Molecular and structural analysis of mosaic variants of penicillin-binding protein 2 conferring decreased susceptibility to expanded-spectrum cephalosporins in Neisseria gonorrhoeae: role of epistatic mutations. Biochemistry. 2010;49(37):8062-70.
153. Archibald FS, Duong MN. Superoxide dismutase and oxygen toxicity defenses in the genus Neisseria. Infection and immunity. 1986;51(2):631-41.
154. Belenky P, Ye JD, Porter CB, Cohen NR, Lobritz MA, Ferrante T, et al. Bactericidal Antibiotics Induce Toxic Metabolic Perturbations that Lead to Cellular Damage. Cell Rep. 2015;13(5):968-80.
155. Adams MA, Jia Z. Modulator of drug activity B from Escherichia coli: crystal structure of a prokaryotic homologue of DT-diaphorase. J Mol Biol. 2006;359(2):455-65.
156. Green LK, La Flamme AC, Ackerley DF. Pseudomonas aeruginosa MdaB and WrbA are water-soluble two-electron quinone oxidoreductases with the potential to defend against oxidative stress. J Microbiol. 2014;52(9):771-7.

TABLES

TABLE 1. Expression and homologs of proteomics-derived *Neisseria gonorrhoeae* vaccine candidates[#].

Accession	Protein homolog in *Neisseria gonorrhoeae* FA1090	Localization	Protein description	Protein name	Protein expression: WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z	FA6140	Protein conservation: *Gardnerella vaginalis* ATCC14019	*Chlamydia trachomatis* E/11023	*Chlamydia trachomatis* J/6276tet1	*Prevotella ruminicola*	*Mycoplasma hominis* ATCC27545	LBD-4	*Neisseria meningitidis* MC58	*Neisseria meningitidis* serogroup A Z2491	*Neisseria meningitidis* serogroup C 053442	*Neisseria gonorrhoeae* F62	*Neisseria gonorrhoeae* FA1090	*Neisseria gonorrhoeae* NCCP11945	*Neisseria gonorrhoeae* MS11	*Neisseria lactamica* 020-06	*Neisseria weaveri* NCTC13585	*Lactobacillus crispatus* ST1	*Lactobacillus gasseri* ATCC 33323 JCM 1131	*Lactobacillus jensenii* strain SNUV360	*Lactobacillus iners* DSM 13335
WHO_F_00053c	NGO0055	OM	Pilus assembly protein	PilC																						+	+	+	+	+	+	+	+				
WHO_F_00104c	NGO0097	CE	Pilus assembly protein	PilN																					+	+	+	+	+	+	+	+	+				
WHO_F_00296c**	NGO0277	CE	Outer membrane biogenesis protein	BamD																					+	+	+	+	+	+	+	+	+				
WHO_F_00301***	NGO0282	CE	LPS-assembly lipoprotein	LptE																					+	+	+	+	+	+	+	+	+				
WHO_F_00453c**	NGO0425	CE	Hypothetical protein																						+	+	+	+	+	+		+	+				
WHO_F_00467*	NGO0439	OM	Outer membrane lipoprotein LolB	LolB																					+	+	+	+	+	+	+	+	+				
WHO_F_00839c	NGO0778	CE	Membrane protein																						+	+	+	+	+	+	+	+	+				
WHO_F_00962	NGO0834	OM	Membrane protein	CsgG																					+	+	+	+	+	+	+	+	+				
WHO_F_01229c***	NGO1063	OM	Surface-exposed lysozyme inhibitor	SliC																						+	+	+	+	+	+	+	+				
WHO_F_01388	NGO1205	OM	Zinc uptake component D protein	ZnuD																		+			+	+	+	+	+	+	+	+	+				
WHO_F_01407c*	NGO1225	OM	Ng-MIP	Ng-MIP															+	+	+	+			+	+	+	+	+	+	+	+	+				
WHO_F_01438c*	NGO1251	CE	Lipoprotein																						+	+		+	+	+	+	+	+				
WHO_F_01539	NGO1344	CE	AsmA-like protein																						+	+	+	+	+	+	+	+	+				
WHO_F_01599c	NGO1393	CE	Adhesin	MafA																					+	+	+	+	+	+	+	+					
WHO_F_01745c***	NGO1492	OM	Phospholipase A1	PldA																					+	+	+	+	+	+	+	+	+				
WHO_F_01817*	NGO1559	CE	OmpA/MotB domain-containing protein																	+	+	+						+	+	+	+		+				
WHO_F_01959c	NGO1688	OM	Outer membrane protein	OmpU																					+	+	+	+	+	+	+	+	+				
WHO_F_01995c**	NGO1715	OM	LPS-assembly protein	LptD																					+	+	+	+	+	+	+	+	+				
WHO_F_02071***	NGO1780	OM	Outer membrane biogenesis protein	BamE																					+	+	+	+	+	+	+	+	+				
WHO_F_02094***	NGO1801	OM	Outer membrane biogenesis protein	BamA																+	+	+			+	+	+	+	+	+	+	+	+				
WHO_F_02195c*	NGO1889	CE	Lipoprotein																						+	+	+	+	+	+	+	+	+				
WHO_F_02224c	NGO1911a	OM	Pilus assembly protein																							+	+	+	+	+	+	+				
WHO_F_02269c**	NGO1956	OM	Translocation and assembly module A	TamA																					+	+	+	+	+	+	+	+	+				
WHO_F_02304c***	NGO1985	OM	Lipoprotein	BamG																					+	+	+	+	+	+	+	+	+				
WHO_F_02385c*	NGO2054	OM	Hypothetical protein																						+		+	+	+	+		+	+				
WHO_F_02440c	NGO2092	CE	Iron ABC transporter protein																						+	+	+	+	+	+	+	+				
WHO_F_02455c*	NGO2105	CE	Adhesion and penetration protein	AidA																					+	+	+	+	+	+	+	+				
WHO_F_02462	NGO2111	OM	Hypothetical protein																						+	+	+	+	+	+	+	+	+				
WHO_F_02473	NGO2121	CE	Lipoprotein component	MlaA																					+	+	+	+	+	+	+	+	+				
WHO_F_02490**	NGO2139	OM	Methionine ABC transporter protein	MetQ																+					+	+	+	+	+	+	+	+	+	+	+	+	+

[#]Color legends for protein expression: Ubiquitous (green), up-regulated (red), down-regulated (blue), variable (grey). Ubiquitous expression among all 15 strains is marked with (***), 14 strains with (**), 12-13 strains with (*). Abbreviations: OM: Outer membrane, CE: cell envelope, +: protein homolog detected.

TABLE 2. Expression and homologs of *Neisseria gonorrhoeae* vaccine candidates identified by other means[#].

Protein expression:

Accession	Protein homolog in *Neisseria gonorrhoeae* FA1090	Localization	Protein description	Protein name	WHO G	WHO K	WHO L	WHO M	WHO N	WHO O	WHO P	WHO U	WHO V	WHO W	WHO X	WHO Y	WHO Z	FA6140
WHO_F_00101c	NGO0094	OM	Type IV pilus biogenesis and competence protein	PilQ	green	green	green	green	blue	grey	grey	green	green	green	grey	green	green	green
WHO_F_00249c	NGO0233	OM	NspA protein	NspA	grey	grey	grey	grey	green	grey	grey	grey	grey	green	grey	green	green	grey
WHO_F_00279		OM	Lactoferrin binding protein A	LbpA	green	blue	green	red	grey	grey	grey	grey	grey					
WHO_F_00294c	NGO0275	OM	IgA-specific protease	IgA1	grey	green	green	green	green	green	green	green	green	green	green	grey	green	grey
WHO_F_00995	NGO0868	OM	OpcA protein	OpcA														
WHO_F_01461	NGO1276	OM	Copper-containing nitrite reductase	AniA	green	red	green	grey	grey	green	green	grey	red	green	grey	grey	grey	grey
WHO_F_01561c	NGO1363	OM	Multidrug transporter	MtrE	red	red	red	red	green	grey	red	green	grey	red	grey	red	grey	red
WHO_F_01749c	NGO1495	OM	Transferrin-binding protein A	TbpA	green	red	green	green	green	grey	green	green	grey	grey	grey	green	grey	green
WHO_F_01750c	NGO1496	OM	Transferrin-binding protein B	TbpB														
WHO_F_02106	NGO1812	OM	Porin	PorB	green	blue	blue	blue	green	blue	blue	blue	blue	blue	blue	blue	blue	blue
WHO_F_02300c	NGO1981	OM	Adhesin complex protein	ACP														
WHO_F_01083	*Multiple	OM	PIIC_3 opacity protein	PiiC_3	grey	blue	red	grey	grey	grey	grey	blue	grey	blue	blue	blue	grey	blue
WHO_F_01464	*Multiple	OM	PIIC_6 opacity protein	PiiC_6	grey	green	red	red	grey	red	green	red	green					
WHO_F_01766	*Multiple	OM	PIIC_9 opacity protein	PiiC_9	grey	grey								green	grey	green	grey	grey
WHO_F_02168c	*Multiple	OM	PIIC_11 opacity protein	PiiC_11	green	red	grey	grey	green	grey	grey	green	grey	red	grey	red	green	grey

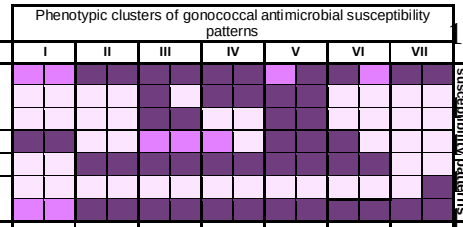
Protein conservation:

Accession	*Gardnerella vaginalis* ATCC14019	*Chlamydia trachomatis* E/11023	*Chlamydia trachomatis* J/6276tet1	*Prevotella ruminicola*	*Mycoplasma hominis* ATCC27545 LBD-4	*Neisseria meningitidis* MC58	*Neisseria meningitidis* serogroup A Z2491	*Neisseria meningitidis* serogroup C 053442	*Neisseria gonorrhoeae* F62	*Neisseria gonorrhoeae* FA1090	*Neisseria gonorrhoeae* NCCP11945	*Neisseria gonorrhoeae* MS11	*Neisseria lactamica* 020-06	*Neisseria weaveri* NCTC13585	*Lactobacillus crispatus* ST1	*Lactobacillus gasseri* ATCC 33323 JCM 1131	*Lactobacillus jensenii* strain SNUV360	*Lactobacillus iners* DSM 13335
WHO_F_00101c		+	+			+	+	+	+	+	+		+	+				
WHO_F_00249c						+	+	+	+	+	+	+	+	+				
WHO_F_00279						+	+	+	+		+	+	+					
WHO_F_00294c						+	+	+	+	+	+	+						
WHO_F_00995						+	+	+	+	+	+		+					
WHO_F_01461						+	+		+	+	+	+	+	+				
WHO_F_01561c				+		+	+	+	+	+	+	+	+	+				
WHO_F_01749c						+	+	+	+	+	+		+	+				
WHO_F_01750c						+	+	+	+	+	+	+	+	+				
WHO_F_02106						+	+	+	+	+	+	+	+	+				
WHO_F_02300c						+	+	+	+	+	+	+	+	+				
WHO_F_01083						+	+	+	+	+	+	+						
WHO_F_01464						+	+	+	+	+	+	+						
WHO_F_01766						+	+	+	+	+	+	+						
WHO_F_02168c						+	+	+	+	+	+	+	+					

[#]Color legends for protein expression in cell envelope fraction: Ubiquitous (green), up-regulated (red), down-regulated (blue), and variable (grey). *Multiple protein homologs in *Neisseria gonorrhoeae* FA1090 are detected: NGO0066a, NGO0070, NGO0950a, NGO1040a, NGO1073a, NGO1277a, NGO1463a, NGO1513, NGO1553a, NGO1861a, NGO2060a. Abbreviations: OM: Outer membrane, +: protein homolog detected.

TABLE 3. Proteomic signature of previously verified gonococcal antimicrobial resistance determinants[#].

Antimicrobial agent	Main antimicrobial target
Penicillin G	PBP2 (PBP1)
Cefixime	
Ceftriaxone	
Azithromycin	50S ribosome
Ciprofloxacin	DNA gyrase, topoisomerase IV
Spectinomycin	30S ribosome
Tetracycline	



Accession	Protein homolog in *N. gonorrhoeae* FA1090	Protein name	Localization	Antimicrobial specificity	Protein description
WHO_F_00422c	NGO0395	NorM	IM	Fluoroquinolones	Multidrug efflux protein
WHO_F_01562c	NGO1364	MtrD (MexB)	IM	Macrolides, tetracycline, penicillin, fluoroquinolones, cephalosporins	MtrD (MexB)
WHO_F_01563c	NGO1365	MtrC (MexA)	P		MtrC (MexA)
WHO_F_01561c	NGO1363	MtrE	OM		Multiple transferable resistance pump, component E
WHO_F_01564	NGO1366	MtrR	C		Repressor of multiple transferable resistance pump
WHO_F_01566	NGO1368	MtrF	IM	Sulfonamides	AbgT transporter
WHO_F_01653c	NGO1439	MacB	IM	Macrolide	MacB Macrolide export ATP-binding/permease protein
WHO_F_01654c	NGO1440	MacA	P		MacA ABC transporter periplasmic protein
WHO_F_01953c	NGO1683	FarA	P	Fatty acids	Fatty acid resistance MFS efflux transporter adaptor subunit
WHO_F_02142c	NGO1841	RpsJ	C	Tetracyclines	RpsJ 30S ribosomal protein S10
WHO_F_00106	NGO0099	PBP1 (PonA1)	P	Penicillin (cephalosporins)	Penicillin-binding protein 1
WHO_F_01799c	NGO1542	PBP2 (PenA)	IM		Penicillin-binding protein 2
WHO_F_02106	NGO1812	PorB	OM		Porin 1B (PorB)
WHO_F_01865	NGO1603	MtgA	P		MtgA Penicillin-binding protein 4
WHO_F_00668c	NGO0629	GyrA	C	Fluoroquinolone	DNA gyrase subcomponent GyrA
WHO_F_02057	NGO1772	GyrB	C		DNA gyrase subcomponent GyrB
WHO_F_01444	NGO1259	ParC	C		Topoisomerase IV subcomponent C
WHO_F_01528c	NGO1333	ParE	C		Topoisomerase IV subcomponent E
WHO_F_01537c	NGO1342	FolP	C	Sulfonamide	7,8-dihydropteroate synthase

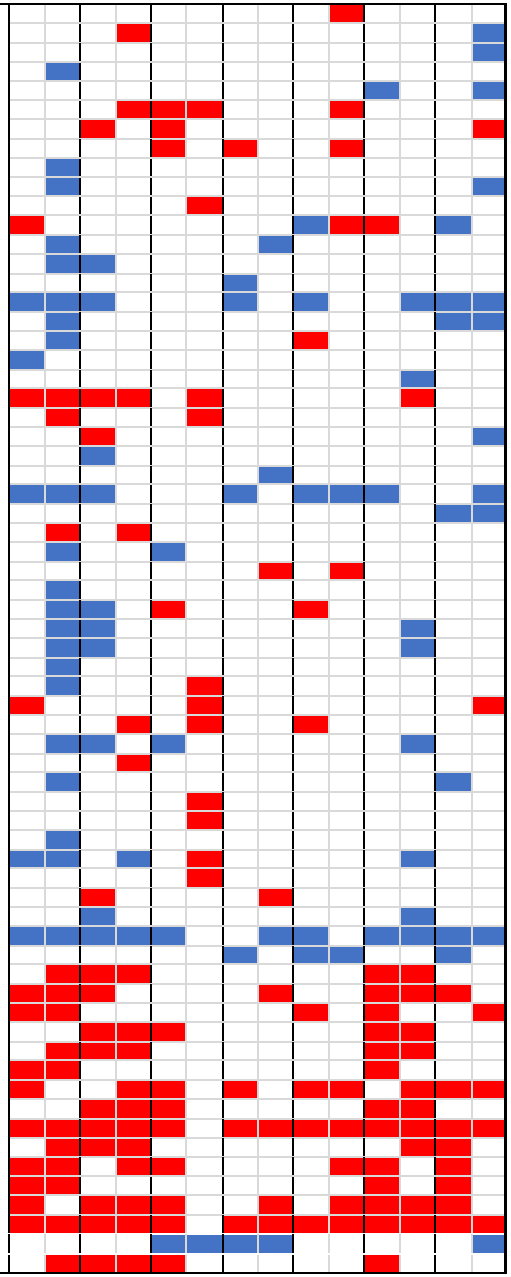
#Color legends for protein expression: Ubiquitous (green), up-regulated (red), down-regulated (blue), variable (grey), and undetected (white). Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purple). Abbreviations: OM: Outer membrane, IM: inner membrane, C: cytoplasmic, P: periplasmic.

TABLE 4. New potential proteomic-derived antimicrobial resistance signatures with defined subcellular localizations[#].

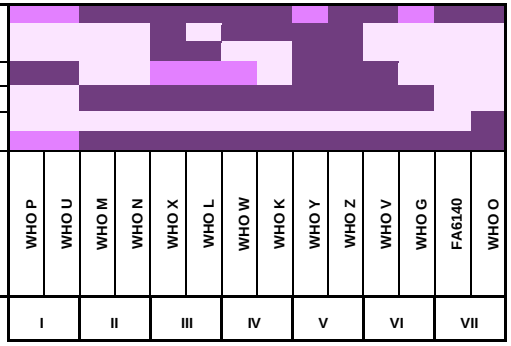
Accession	Protein homolog in GC FA1090	Localization	Protein description	COG	Function	WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O
WHO_F_02461c		E	Putative hemoglobin receptor component HpuA																
WHO_F_00057	NGO0057	IM	Thioredoxin	COG0526OC	Protein turnover														
WHO_F_00073	NGO0071	IM	Lipoprotein signal peptidase II	COG0597MU	Cell wall/membrane/envelope biogenesis														
WHO_F_00089c	NGO0083	IM	Pilin glycosylation protein	COG1086MG	Cell wall/membrane/envelope biogenesis														
WHO_F_00091c	NGO0085	IM	UDP-glucose lipid carrier transferase	COG2148M	Cell wall/membrane/envelope biogenesis														
WHO_F_00139c	NGO0127	IM	Ribonuclease BN-like family	COG1295S	Function unknown														
WHO_F_00155	NGO0143	IM	Sodium/proton antiporter	COG1757C	Energy production and conversion														
WHO_F_00191c	NGO0178	IM	Lipid A core - O-antigen ligase	COG3307M	Cell wall/membrane/envelope biogenesis														
WHO_F_00211	NGO0196	IM	Putative spermidine/putrescine transport system permease,	COG1177E	Amino acid transport and metabolism														
WHO_F_00213c	NGO0198	IM	Ammonia transporter	COG0004P	Inorganic ion transport and metabolism														
WHO_F_00238c	NGO0222	IM	Hypothetical protein																
WHO_F_00246c	NGO0230	IM	Potassium transporter	COG0168P	Inorganic ion transport and metabolism														
WHO_F_00303	NGO0284	IM	Membrane protein																
WHO_F_00311	NGO0291	IM	Potassium/proton antiporter	COG3263P	Inorganic ion transport and metabolism														
WHO_F_00328	NGO0307	IM	Phage T7 F exclusion suppressor FxsA	COG3030R	General function prediction only														
WHO_F_00385	NGO0360	IM	Putative uroporphyrinogen-III C-methyltransferase	COG2959H	Coenzyme transport and metabolism														
WHO_F_00386	NGO0361	IM	Uncharacterized enzyme of heme biosynthesis	COG3071H	Coenzyme transport and metabolism														
WHO_F_00425c	NGO0399	IM	Putative Zn-dependent protease	COG0501O	Protein turnover														
WHO_F_00585	NGO0551	IM	Predicted membrane protein	COG3671S	Function unknown														
WHO_F_00627c	NGO0589	IM	Uracil transporter	COG2233F	Nucleotide transport and metabolism														
WHO_F_00666c	NGO0627	IM	Site-specific recombinase	COG4389L	Replication, recombination and repair														
WHO_F_00696	NGO0656	IM	Oxalate/formate antiporter family transporter	COG2807P	Inorganic ion transport and metabolism														
WHO_F_00816c	NGO0753	IM	Nitrate/nitrite sensor protein	COG3850T	Signal transduction mechanisms														
WHO_F_00996	NGO0869	IM	Hypothetical protein	COG0586S	Function unknown														
WHO_F_01057c	NGO0923	IM	Putative succinate dehydrogenase cytochrome	COG2009C	Energy production and conversion														
WHO_F_01100c	NGO0968	IM	Glutamine transport system permease protein glnP	COG0765E	Amino acid transport and metabolism														
WHO_F_01106c	NGO0974	IM	Lysophospholipid transporter lplT																
WHO_F_01110c	NGO0978	IM	Thiol:disulfide interchange protein DsbD	COG4232OC	Protein turnover														
WHO_F_01126		IM	Hypothetical protein																
WHO_F_01200c	NGO1032	IM	Inner membrane transport protein yajR	COG2814G	Carbohydrate transport and metabolism														
WHO_F_01225c	NGO1059	IM	Membrane protein	COG0861P	Inorganic ion transport and metabolism														
WHO_F_01371	NGO1188	IM	Magnesium transporter	COG2239P	Inorganic ion transport and metabolism														
WHO_F_01381	NGO1198	IM	Phosphoethanolamine transferase eptB	COG2194R	General function prediction only														
WHO_F_01398c	NGO1216	IM	Diacylglycerol kinase	COG0818M	Cell wall/membrane/envelope biogenesis														
WHO_F_01428c	NGO1246	IM	Signal peptide peptidase SppA	COG0616OU	Protein turnover														
WHO_F_01445	NGO1260	IM	Transcriptional regulatory protein ZraR	COG3829KT	Transcription														
WHO_F_01460c	NGO1275	IM	Nitric oxide reductase subunit B	COG3256P	Inorganic ion transport and metabolism														
WHO_F_01535c	NGO1340	IM	DedA-family integral membrane protein	COG0586S	Function unknown														
WHO_F_01568c	NGO1370	IM	Uncharacterized iron-regulated membrane protein	COG3182S	Function unknown														
WHO_F_01572c	NGO1374	IM	Cbb3-type cytochrome oxidase, subunit 1	COG3278O	Protein turnover														
WHO_F_01579	NGO1380	IM	Zn-dependent proteases	COG1994R	General function prediction only														
WHO_F_01621	NGO1410	IM	Inner membrane protein ybaN	COG2832S	Function unknown														
WHO_F_01623	NGO1411	IM	Citrate transporter	COG1055P	Inorganic ion transport and metabolism														
WHO_F_01626	NGO1414	IM	Na( )-translocating NADH-quinone reductase subunit B	COG1805C	Energy production and conversion														
WHO_F_01629	NGO1417	IM	Na( )-translocating NADH-quinone reductase subunit E	COG2209C	Energy production and conversion														
WHO_F_01669c	NGO1455	IM	Manganese transport protein MntH	COG1914P	Inorganic ion transport and metabolism														
WHO_F_01739c	NGO1485	IM	Inner membrane protein ybhI	COG0471P	Inorganic ion transport and metabolism														
WHO_F_01797c	NGO1540	IM	Putative phosphoethanolamine transferase ybiP	COG2194R	General function prediction only														
WHO_F_01811	NGO1552	IM	Proline:sodium symporter PutP	COG0591ER	Amino acid transport and metabolism														
WHO_F_01835c	NGO1574	IM	Phosphatidylglycerophosphatase A	COG1267I	Lipid transport and metabolism														
WHO_F_01979c	NGO1699	IM	Inner membrane protein ypjD	COG4137R	General function prediction only														
WHO_F_01990c	NGO1710	IM	O-acetyltransferase OatA	COG1835I	Lipid transport and metabolism														
WHO_F_01998	NGO1718	IM	Virulence factor MviN	COG0728R	General function prediction only														
WHO_F_02013	NGO1732	IM	Putative multidrug export ATP-binding/permease protein	COG1132V	Defense mechanisms														

1252

WHO_F_02018c	NGO1737	IM	NADH dehydrogenase subunit N	COG1007C	Energy production and conversion
WHO_F_02019c	NGO1738	IM	NADH dehydrogenase subunit M	COG1008C	Energy production and conversion
WHO_F_02021c	NGO1740	IM	NADH:ubiquinone dehydrogenase, L subunit	COG1009CP	Energy production and conversion
WHO_F_02025c	NGO1744	IM	NADH-quinone oxidoreductase subunit H	COG1005C	Energy production and conversion
WHO_F_02033c	NGO1751	IM	NADH dehydrogenase I subunit A	COG0838C	Energy production and conversion
WHO_F_02034c	NGO1752	IM	Putative integral membrane protein	COG0421E	Amino acid transport and metabolism
WHO_F_02035c	NGO1753	IM	Putative integral membrane protein	COG4262R	General function prediction only
WHO_F_02053	NGO1768	IM	Integral membrane protein	COG1971S	Function unknown
WHO_F_02078	NGO1787	IM	Na /alanine symporter	COG1115E	Amino acid transport and metabolism
WHO_F_02091	NGO1798	IM	Phosphatidate cytidylyltransferase	COG0575I	Lipid transport and metabolism
WHO_F_02174c	NGO1867	IM	Two-component system sensor kinase	COG5000T	Signal transduction mechanisms
WHO_F_02207c	NGO1900	IM	Membrane protein	COG1297S	Function unknown
WHO_F_02222	NGO1910	IM	Inner membrane protein yccS	COG1289S	Function unknown
WHO_F_02262c	NGO1948	IM	Membrane protein	COG2259S	Function unknown
WHO_F_02267c	NGO1954	IM	Amino acid/peptide transporter (Peptide:H symporter)	COG3104E	Amino acid transport and metabolism
WHO_F_02308c		IM	Putative cytochrome B561	COG3038C	Energy production and conversion
WHO_F_02419	NGO2071	IM	Putative integral membrane protein	COG1368M	Cell wall/membrane/envelope biogenesis
WHO_F_02432	NGO2084	IM	Integral membrane protein	COG0670R	General function prediction only
WHO_F_02468	NGO2116	IM	ABC-type spermidine/putrescine transport systems	COG1127Q	Secondary metabolites biosynthesis
WHO_F_02481	NGO2128	IM	Inner membrane protein	COG1807M	Cell wall/membrane/envelope biogenesis
WHO_F_01084	NGO0952	OM	TonB-dependent heme/hemoglobin receptor family protein	COG1629P	Inorganic ion transport and metabolism
WHO_F_02460c	NGO2109	OM	Outer membrane cobalamin receptor protein	COG1629P	Inorganic ion transport and metabolism
WHO_F_00222	NGO0206	P	Spermidine/putrescine ABC transporter	COG0687E	Amino acid transport and metabolism
WHO_F_00233c	NGO0217	P	ABC-type thiamine transport system	COG1840P	Inorganic ion transport and metabolism
WHO_F_00242	NGO0225	P	Protein of unknown function		
WHO_F_00268c	NGO0250	P	Cryptic protein cnp1		
WHO_F_00397	NGO0372	P	Lysine-arginine-ornithine-binding periplasmic protein	COG0834ET	Amino acid transport and metabolism
WHO_F_00650c	NGO0613	P	Hypothetical protein		
WHO_F_00702c	NGO0662	P	Aspartyl/glutamyl-tRNA amidotransferase subunit A	COG0154J	Translation, ribosomal structure and biogenesis
WHO_F_00717c	NGO0678	P	Lipoprotein		
WHO_F_00728c	NGO0690	P	Lipoprotein		
WHO_F_00809	NGO0747	P	Lipoprotein	COG1729S	Function unknown
WHO_F_00828	NGO0766	P	Peptidyl-prolyl cis-trans isomerase D		
WHO_F_01000	NGO0873	P	DNA modification methylase	COG0270L	Replication, recombination and repair
WHO_F_01209c	NGO1044	P	Hypothetical protein		
WHO_F_01214	NGO1049	P	Nickel uptake substrate protein	COG5266P	Inorganic ion transport and metabolism
WHO_F_01249		P	Putative serotype-1-specific antigen		
WHO_F_01256	NGO1080	P	C-type cytochrome	COG3909C	Energy production and conversion
WHO_F_01289		P	Predicted transcriptional regulator	COG1396K	Transcription
WHO_F_01440c	NGO1253	P	Putrescine-binding periplasmic protein	COG0687E	Amino acid transport and metabolism
WHO_F_01652	NGO1438	P	Probable thiol:disulfide interchange protein DsbC	COG1651O	Protein turnover
WHO_F_01747	NGO1494	P	Spermidine/putrescine ABC transporter substrate-binding	COG0687E	Amino acid transport and metabolism
WHO_F_01754	NGO1502	P	N-acetylmuramoyl-l-alanine amidase I	COG0860M	Cell wall/membrane/envelope biogenesis
WHO_F_01757c	NGO1505	P	Hypothetical protein		
WHO_F_01925c	NGO1655	P	Putative peptidyl-prolyl isomerase		
WHO_F_01926c	NGO1656	P	Cell-binding factor	COG0760O	Protein turnover
WHO_F_01981	NGO1701	P	Hypothetical protein		
WHO_F_02051	NGO1767	P	Catalase	COG0753P	Inorganic ion transport and metabolism
WHO_F_02387	NGO2056	P	Thiamine transporter substrate binding subunit	COG4143H	Coenzyme transport and metabolism
WHO_F_00060	NGO0059	C	4-hydroxyphenylacetate 3-monooxygenase reductase	COG1853R	Coenzyme transport and metabolism
WHO_F_00082c	NGO0078	C	DNA polymerase III subunit alpha	COG0587L	Replication, recombination and repair
WHO_F_00198c	NGO0185	C	Phosphoribosyl-ATP pyrophosphatase	COG0140E	Amino acid transport and metabolism
WHO_F_00206	NGO0191	C	30S ribosomal protein S15	COG0184J	Translation, ribosomal structure and biogenesis
WHO_F_00288c	NGO0269	C	Amino acid ABC transporter ATP-binding protein	COG1126E	Amino acid transport and metabolism
WHO_F_00434	NGO0407	C	type I restriction-modification system endonuclease	COG0610V	Defense mechanisms
WHO_F_00452c	NGO0424	C	GTP-binding protein EngA	COG1160R	General function prediction only
WHO_F_00481c		C	SodB_2 superoxide dismutase	COG0605P	Inorganic ion transport and metabolism
WHO_F_00610	NGO0573	C	Excinuclease uvr subunit B	COG0556L	Replication, recombination and repair
WHO_F_00635c	NGO0597	C	Ndk nucleoside diphosphate kinase	COG0105F	Nucleotide transport and metabolism
WHO_F_00663	NGO0624	C	Acyl-CoA dehydrogenase	COG1960I	Lipid transport and metabolism
WHO_F_00690c	NGO0650	C	ATP-dependent RNA helicase	COG0513LK	Replication, recombination and repair
WHO_F_00697c	NGO0657	C	Pseudouridine synthase	COG1187J	Translation, ribosomal structure and biogenesis
WHO_F_00718c	NGO0679	C	Isopropylmalate isomerase large subunit	COG0065E	Amino acid transport and metabolism
WHO_F_00745	NGO0701	C	Hypothetical protein		
WHO_F_00840c	NGO0779	C	Homoserine dehydrogenase	COG0460E	Amino acid transport and metabolism
WHO_F_00930c	NGO0803	C	GTPases - translation elongation factors	COG1217T	Signal transduction mechanisms

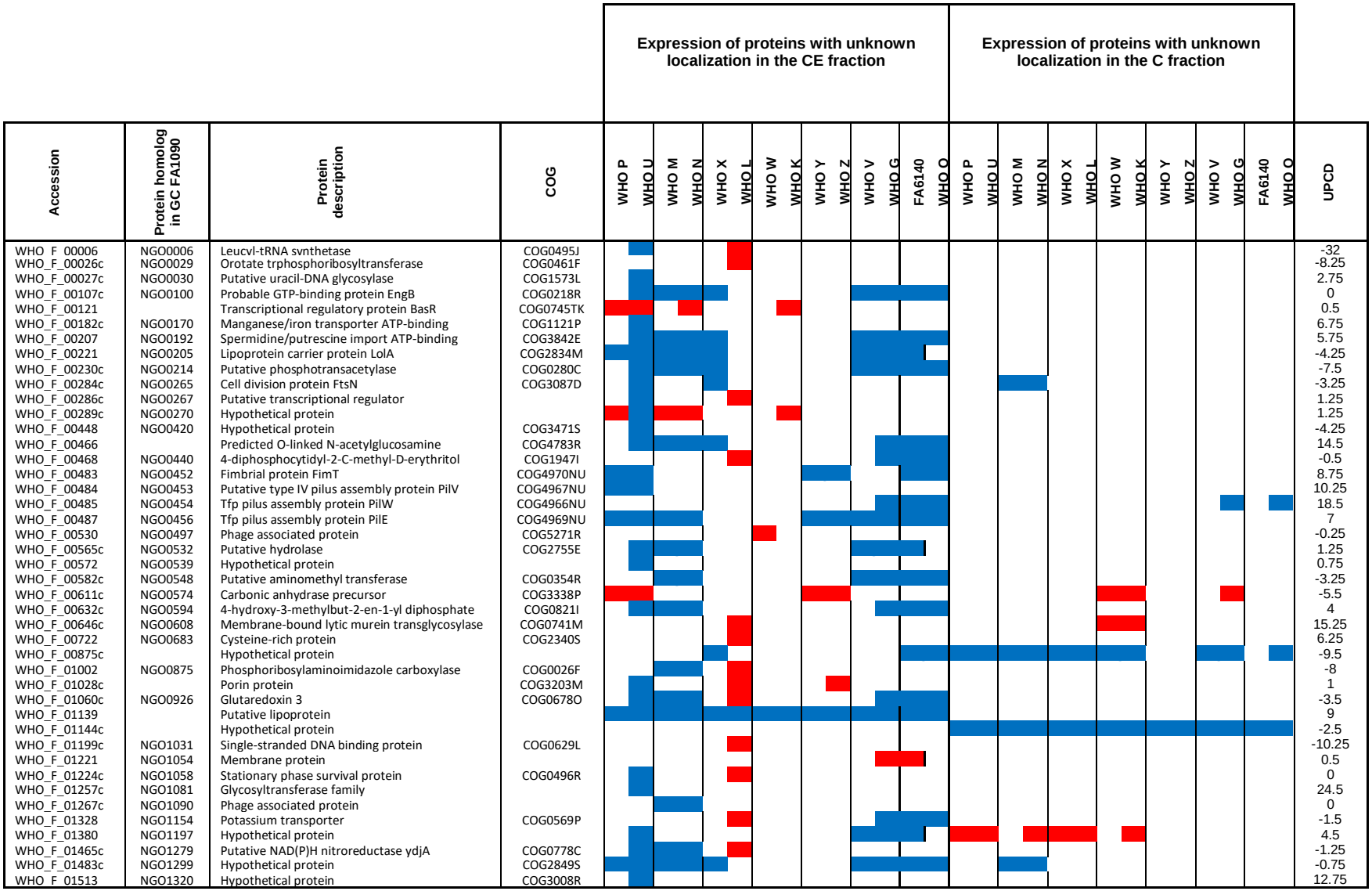


WHO_F_01222	NGO1055	C	Acyl-CoA hydrolase	COG1607I	Lipid transport and metabolism
WHO_F_01314c	NGO1141	C	Hypothetical protein	COG3680S	Function unknown
WHO_F_01421	NGO1239	C	Hypothetical protein	COG3680S	Function unknown
WHO_F_01425	NGO1243	C	Hgd 3-hydroxyacid dehydrogenase	COG2084I	Lipid transport and metabolism
WHO_F_01585c	NGO07315	C	Uncharacterized protein		
WHO_F_01684c	NGO1473	C	NADPH quinone reductase	COG2249R	Coenzyme transport and metabolism
WHO_F_01759c	NGO1507	C	Holo-ACP synthase	COG0736I	Lipid transport and metabolism
WHO_F_01996	NGO1716	C	Phosphotransferase	COG3178R	General function prediction only
WHO_F_02076	NGO1785	C	Ribonuclease E	COG1530J	Translation, ribosomal structure and biogenesis
WHO_F_02135c	NGO1833	C	50S ribosomal protein L22	COG0091J	Translation, ribosomal structure and biogenesis
WHO_F_02136c	NGO1834	C	30S ribosomal protein S19	COG0185J	Translation, ribosomal structure and biogenesis
WHO_F_02137c	NGO1835	C	50S ribosomal protein L2	COG0090J	Translation, ribosomal structure and biogenesis
WHO_F_02156c	NGO1854	C	50S ribosomal protein L1	COG0081J	Translation, ribosomal structure and biogenesis
WHO_F_02177c	NGO1870	C	Methionyl-tRNA formyltransferase	COG0223J	Translation, ribosomal structure and biogenesis
WHO_F_02198c		C	Restriction endonuclease		DNA repair
WHO_F_02422	NGO2074	C	Ubiquinone biosynthesis O-methyltransferase	COG2227H	Coenzyme transport and metabolism
WHO_F_02437c	NGO2089	C	Hypothetical protein	COG2191C	Energy production and conversion



#Color legends for protein expression: up-regulated (red), down-regulated (blue), and undetected (white). Abbreviations: OM: Outer membrane, IM: inner membrane, C: cytoplasmic, COG: cluster of orthologous genes, PBP: penicillin binding protein. Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purpl

1256 **TABLE 5. New potential proteomic-derived antimicrobial resistance signatures with undefined localization[#]**



Accession	Protein homolog in GC FA1090	Protein description	COG	Expression of proteins with unknown localization in the CE fraction: WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O	Expression of proteins with unknown localization in the C fraction: WHO P	WHO U	WHO M	WHO N	WHO X	WHO L	WHO W	WHO K	WHO Y	WHO Z	WHO V	WHO G	FA6140	WHO O	UPCD
WHO_F_00006	NGO0006	Leucyl-tRNA synthetase	COG0495J																												-32
WHO_F_00026c	NGO0029	Orotate trphosphoribosyltransferase	COG0461F																												-8.25
WHO_F_00027c	NGO0030	Putative uracil-DNA glycosylase	COG1573L																												2.75
WHO_F_00107c	NGO0100	Probable GTP-binding protein EngB	COG0218R																												0
WHO_F_00121		Transcriptional regulatory protein BasR	COG0745TK																												0.5
WHO_F_00182c	NGO0170	Manganese/iron transporter ATP-binding	COG1121P																												6.75
WHO_F_00207	NGO0192	Spermidine/putrescine import ATP-binding	COG3842E																												5.75
WHO_F_00221	NGO0205	Lipoprotein carrier protein LolA	COG2834M																												-4.25
WHO_F_00230c	NGO0214	Putative phosphotransacetylase	COG0280C																												-7.5
WHO_F_00284c	NGO0265	Cell division protein FtsN	COG3087D																												-3.25
WHO_F_00286c	NGO0267	Putative transcriptional regulator																													1.25
WHO_F_00289c	NGO0270	Hypothetical protein																													1.25
WHO_F_00448	NGO0420	Hypothetical protein	COG3471S																												-4.25
WHO_F_00466		Predicted O-linked N-acetylglucosamine	COG4783R																												14.5
WHO_F_00468	NGO0440	4-diphosphocytidyl-2-C-methyl-D-erythritol	COG1947I																												-0.5
WHO_F_00483	NGO0452	Fimbrial protein FimT	COG4970NU																												8.75
WHO_F_00484	NGO0453	Putative type IV pilus assembly protein PilV	COG4967NU																												10.25
WHO_F_00485	NGO0454	Tfp pilus assembly protein PilW	COG4966NU																												18.5
WHO_F_00487	NGO0456	Tfp pilus assembly protein PilE	COG4969NU																												7
WHO_F_00530	NGO0497	Phage associated protein	COG5271R																												-0.25
WHO_F_00565c	NGO0532	Putative hydrolase	COG2755E																												1.25
WHO_F_00572	NGO0539	Hypothetical protein																													0.75
WHO_F_00582c	NGO0548	Putative aminomethyl transferase	COG0354R																												-3.25
WHO_F_00611c	NGO0574	Carbonic anhydrase precursor	COG3338P																												-5.5
WHO_F_00632c	NGO0594	4-hydroxy-3-methylbut-2-en-1-yl diphosphate	COG0821I																												4
WHO_F_00646c	NGO0608	Membrane-bound lytic murein transglycosylase	COG0741M																												15.25
WHO_F_00722	NGO0683	Cysteine-rich protein	COG2340S																												6.25
WHO_F_00875c		Hypothetical protein																													-9.5
WHO_F_01002	NGO0875	Phosphoribosylaminoimidazole carboxylase	COG0026F																												-8
WHO_F_01028c		Porin protein	COG3203M																												1
WHO_F_01060c	NGO0926	Glutaredoxin 3	COG0678O																												-3.5
WHO_F_01139		Putative lipoprotein																													9
WHO_F_01144c		Hypothetical protein																													-2.5
WHO_F_01199c	NGO1031	Single-stranded DNA binding protein	COG0629L																												-10.25
WHO_F_01221	NGO1054	Membrane protein																													0.5
WHO_F_01224c	NGO1058	Stationary phase survival protein	COG0496R																												0
WHO_F_01257c	NGO1081	Glycosyltransferase family																													24.5
WHO_F_01267c	NGO1090	Phage associated protein																													0
WHO_F_01328	NGO1154	Potassium transporter	COG0569P																												-1.5
WHO_F_01380	NGO1197	Hypothetical protein																													4.5
WHO_F_01465c	NGO1279	Putative NAD(P)H nitroreductase ydjA	COG0778C																												-1.25
WHO_F_01483c	NGO1299	Hypothetical protein	COG2849S																												-0.75
WHO_F_01513	NGO1320	Hypothetical protein	COG3008R																												12.75

WHO_F_01578c	NGO1379	Gram-negative bacterial tonB protein	COG0810M	3
WHO_F_01627	NGO1415	Na( )-translocating NADH-quinone reductase	COG2869C	3
WHO_F_01633	NGO1421	Hypothetical protein	COG2991S	0.5
WHO_F_01655	NGO1441	Tfp pilus assembly protein PilE	COG4968NU	3.25
WHO_F_01685c		Modulator of drug activity		-0.5
WHO_F_01735c	NGO1482	Hypothetical protein	COG2830S	2.75
WHO_F_01860		Hypothetical protein		8.5
WHO_F_01894c	NGO1628	Hypothetical protein		-2.75
WHO_F_01987	NGO1707	Deoxyribodopyrimidine photolyase	COG0415L	2
WHO_F_01989	NGO1709	Hypothetical protein	COG2979S	8
WHO_F_02044	NGO1763	3-oxoacyl-[acyl-carrier-protein] synthase 2,3-	COG0304IQ	-6.75
WHO_F_02047	NGO1765	Pilin glycosyl transferase A	COG0438M	1
WHO_F_02081c	NGO1790	Septum formation inhibitor-activating ATPase	COG1192D	1.25
WHO_F_02092	NGO1799	1-deoxy-D-xylulose 5-phosphate	COG0743I	1
WHO_F_02095	NGO1802	Putative membrane protein	COG2825M	-4
WHO_F_02105	NGO1811	tRNA pseudouridine synthase A	COG0101J	2
WHO_F_02139c	NGO1837	rplD 50S ribosomal protein L4	COG0088J	0.75
WHO_F_02140c	NGO1838	rplC 50S ribosomal protein L3	COG0087J	-2
WHO_F_02146c	NGO1845	rpsL 30S ribosomal protein S12	COG0048J	1.75
WHO_F_02154c	NGO1852	rplL 50S ribosomal protein L7/L12	COG0222J	-2.75
WHO_F_02157c	NGO1855	rplK 50S ribosomal protein L11	COG0080J	-1
WHO_F_02166c	NGO1860	Putative DNA methylase	COG0742L	1.25
WHO_F_02218c	NGO1906	Putative lipoprotein		0.5
WHO_F_02235c	NGO1922	Cell division ATP-binding protein FtsE	COG2884D	4
WHO_F_02238	NGO1924a	Hypothetical protein	COG1434S	0.75
WHO_F_02253	NGO1940	Putative translation factor	COG0009J	1.75
WHO_F_02255c	NGO1942	Hypothetical protein	COG2849S	7.5
WHO_F_02259c	NGO1945	Hypothetical protein	COG3219S	-2.5
WHO_F_02263	NGO1949	Hypothetical protein	COG1752R	9.25
WHO_F_02271	NGO1958	Hypothetical protein	COG3219S	-0.25
WHO_F_02281	NGO1966	Hypothetical protein	COG1752R	-2.25
WHO_F_02283c	NGO1967	Hypothetical protein		-0.75
WHO_F_02286c	NGO1969	Hypothetical protein		-1
WHO_F_02287c	NGO1970	Hypothetical protein		0
WHO_F_02303c	NGO1984	Hypothetical protein	COG5373S	6
WHO_F_02306c	NGO1987	Predicted endonuclease	COG0792L	-0.25
WHO_F_02318c	NGO1999	5,10-methylene-detetrahydrofolate	COG0190H	-3.25
WHO_F_02346	NGO2023	Hypothetical protein	COG3027S	-0.25
WHO_F_02390c	NGO2059	Thioredoxin/methionine sulfoxide reductase	COG0225O	10
WHO_F_02394c		Tfp pilus assembly protein	COG4969NU	1
WHO_F_02446c	NGO2097	Hypothetical protein	COG2849S	0.5
WHO_F_02453	NGO2104	Hypothetical protein	COG3177S	1.75
WHO_F_02488	NGO2137	Methionine import ATP-binding protein MetN	COG1135P	3
WHO_F_02506		Putative glycosyl transferase	COG0463M	13.75
WHO_F_02508		Lipooligosaccharyl-alpha-1 transferase	COG1442M	-0.75
WHO_F_02509		Lipopolysaccharide 1,3-galactosyltransferase	COG1442M	0.25
WHO_F_02510		Poly-beta-1,6-N-acetyl-D-glucosamine synthase	COG0463M	0.25
WHO_F_02511	NGO2158	Glycosyl transferase family protein	COG1216R	1.5
WHO_F_02515c	NGO2162	Hypothetical protein	COG3012S	-0.75
WHO_F_02516c	NGO2163	3-ketoacyl-ACP reductase,3-oxoacyl-[acyl-	COG1028IQ	1.25

Antimicrobial agent	Main antimicrobial target
Penicillin G	PBP2 (PBP1)
Cefixime	PBP2
Ceftriaxone	PBP2
Azithromycin	50S ribosome
Ciprofloxacin	DNA gyrase
Spectinomycin	30S ribosome
Tetracycline	30S ribosome

WHO P WHO U WHO M WHO N WHO X WHO L WHO W WHO K WHO Y WHO Z WHO V WHO G FA6140 WHO O

I II III IV V VI VII

[#] Color legends for protein expression: Up-regulated (red), down-regulated (blue). Color legends for phenotype against antimicrobial agents are as follows: resistant (dark purple), intermediate susceptible/resistant (purple), susceptible (light purple). Abbreviations: UPCD: unique peptide count difference; PBP: penicillin binding protein.

TABLE 6. Common differentially expressed proteins in phenotypically clustered *Neisseria gonorrhoeae* strains.

	Class I		Class II		Class III		Class IV		Class V		Class VI		Class VII	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Amino acid metabolism	NGO0185		NGO0269 NGO0679		NGO1752	NGO0779		NGO0779			NGO0185 NGO0269 NGO0679	NGO0192		
Cell envelope biogenesis		NGO0085 NGO0456 LolA												NGO2071
Coenzyme metabolism	NGO2074	NGO2056 NGO0360		NGO2056			MdaB		MdaB	NGO0059	NGO2074	NGO2056		NGO2056 NGO0360
DNA repair		NGO1707	NGO0078 NGO0407 UvrB						WHO_F_02198c		NGO0078 NGO0407 UvrB			
Energy production		WHO_F_02308c								NGO0143				WHO_F_02308
Hypothetical protein	NGO1239 NGO1958	WHO_F_00875c, NGO1299 NGO1969 NGO1970 NGO2089	NGO1942 NGO2097	WHO_F_00875c, NGO1299 NGO1945 NGO1967 NGO1969 NGO1970 NGO2089		WHO_F_00875c		NGO1141	NGO1239	NGO1628 NGO1966 NGO7315		NGO1299 NGO1945 NGO1969 NGO1970 NGO2089		
Inorganic ion metabolism	NGO0952	NGO1059 NGO2137	NGO0952 NGO1188						NGO1411 WHO_F_00481c			NGO2137	WHO_F_00481	NGO0291

1281

	Class I		Class II		Class III		Class IV		Class V		Class VI		Class VII	
	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated	Up-regulated	Down-regulated
Intracellular and trafficking secretion				PilE	WHO_F_02394c	PilE						NGO0456		NGO0452 NGO0454 PilE
Lipid metabolism	NGO1243 NGO1906	NGO1763	NGO0624 NGO1055 NGO1243	NGO1507		NGO1507		NGO1507 NGO1710			NGO1243			
Protein turnover		NGO0399 NGO1655		NGO0926	NGO0057		NGO0057							
Translation and Ribosomal biogenesis	S15, S19, EngA, RNA helicase, Ribonucl-ease E, L1, L2, L22 , NGO0657		NGO0803 NGO1870								NGO1870			

FIGURE LEGENDS

Figure 1. Experimental paradigm of quantitative proteomic profiling of the *N. gonorrhoeae* 2016 WHO reference strains and the FA6140 strain. All gonococci were cultured concurrently in liquid medium until reaching mid-logarithmic growth. Bacterial cells were harvested, lysed, and subjected to subcellular fractionation to separate the crude cell envelope (CE) and cytoplasmic (C) proteomes. CE proteins were enriched using a sodium carbonate wash and ultracentrifugation. The obtained CE and C protein samples (100 µg) were denatured, reduced, alkylated, trypsinized, and the peptides from each strain were labeled using 10-plex and 6-plex Tandem mass tag (TMT) reagents, as indicated. Finally, samples were pooled, fractionated by strong cation exchange, and analyzed by liquid chromatography electrospray ionization mass spectrometry. Experiments were performed in biological duplicates.

Figure 2. Venn diagrams illustrating the distribution of proteins identified in cell envelope and cytoplasmic fractions in two independent proteomic experiments. (A) Cell envelope proteomes derived from the 2016 WHO reference strains and FA6140 were analyzed in 10-plex and 6-plex experiments performed in biological duplicates. A total of 1079 and 1081 proteins was identified in Experiments 1 and 2, respectively, and 1010 common proteins were found in both 10-plex experiments. The 6-plex TMT labeling revealed 975 common proteins as well as 197 and 22 unique proteins in Experiments 1 and 2, respectively. Further analyses were applied to 901 proteins mutually identified in both 10-plex and 6-plex experiments. **(B)** The proteomic profiling of cytoplasmic fractions yielded 904 proteins shared among all 10 strains, of which 747 were common in both experiments. The 6-plex TMT identified 904 and 971 proteins in Experiments 1 and 2, respectively; of

which 852 were common between replicates. In further analyses solely the 723 proteins shared between both experiments were included. Exp 1 – experiment 1; Exp 2 – experiment 2.

Figure 3. Subcellular localization of proteins identified in cell envelope and cytoplasmic subproteomes. Proteins identified in the cell envelope (blue circle) and cytoplasmic (red circle) fractions were subjected to comprehensive assessments of subcellular localization using different prediction algorithms and were allocated into the outer membrane **(A)**, periplasm **(B)**, inner membrane **(C)**, cytoplasm **(D)**, or unknown localization **(E)**.

Figure 4. Expression patterns of common proteins identified in the cell envelope fraction. Outer membrane **(A)**, periplasmic **(B)**, inner membrane **(C)**, or proteins with unknown localization **(D)** are shown. Expression of each protein in each gonococcal strain was compared to the protein level in the reference WHO F isolate. Protein expression is categorized as ubiquitous (green bars); up-regulated (red bar); down-regulated (blue bar); and variable (grey bar).

Figure 5. **Expression patterns of common proteins identified in the cytoplasmic proteome.** Cytoplasmic **(A)** and proteins with unknown localization **(B)** are shown. Protein levels in individual gonococcal strains were compared to the protein level in the reference

WHO F isolate. Protein expression is categorized as ubiquitous (green bars); up-regulated (red bar); down-regulated (blue bar); and variable (grey bar).

Figure 6. Decision tree designed for proteomic mining of *Neisseria gonorrhoeae* vaccine candidates and antibiotic resistance markers. Detailed description is provided in the text.

Figure 1

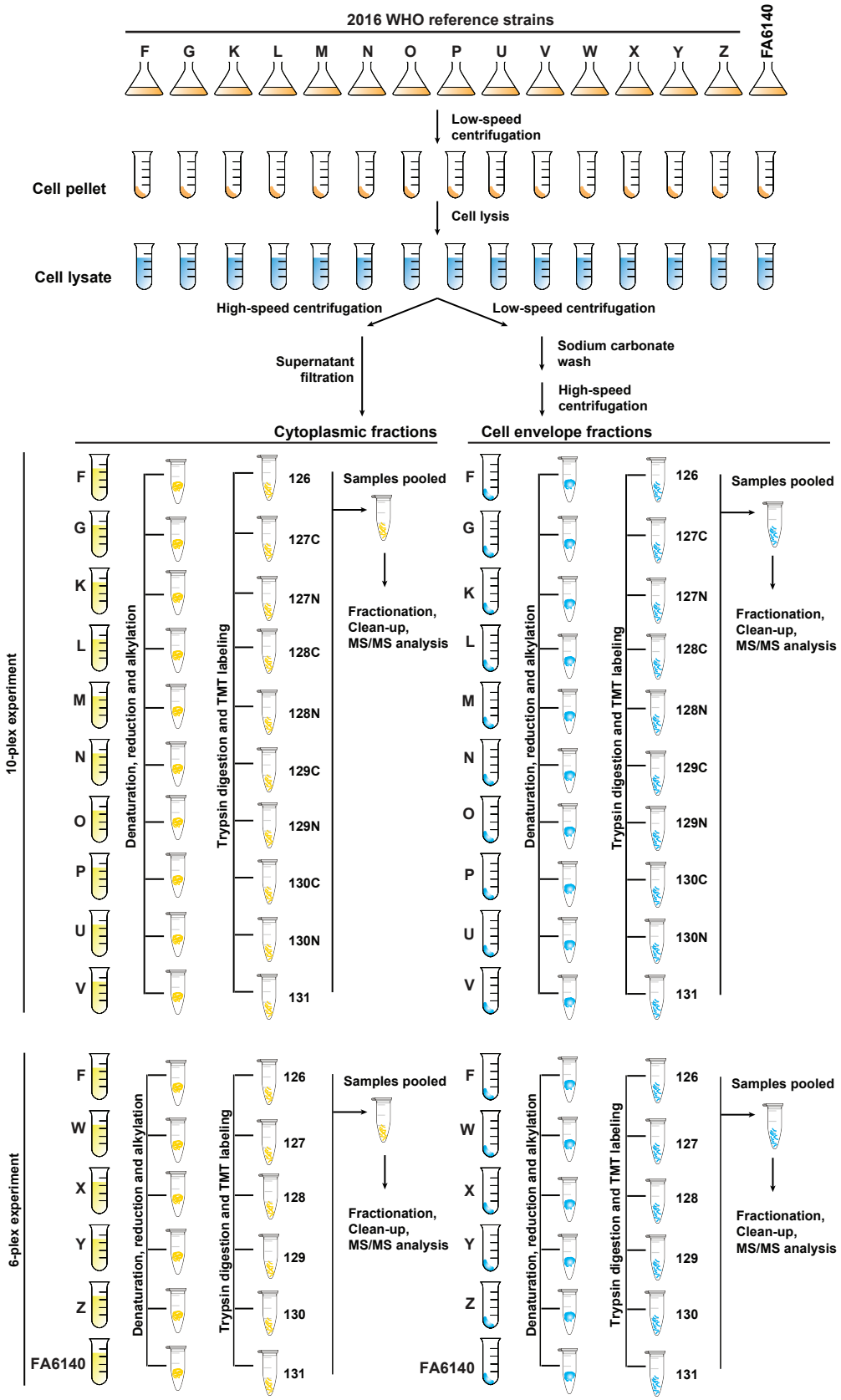


Figure 2

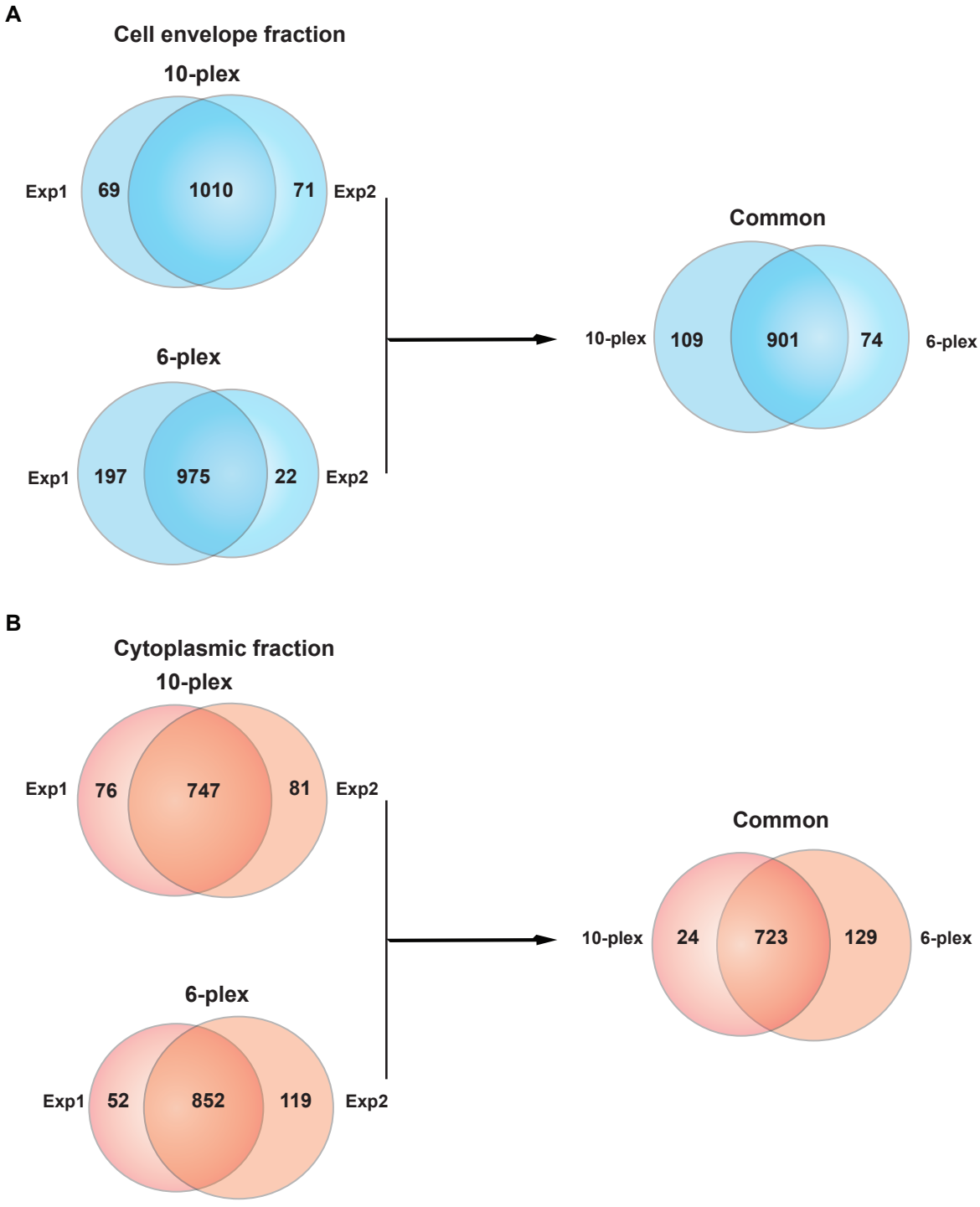


Figure 3

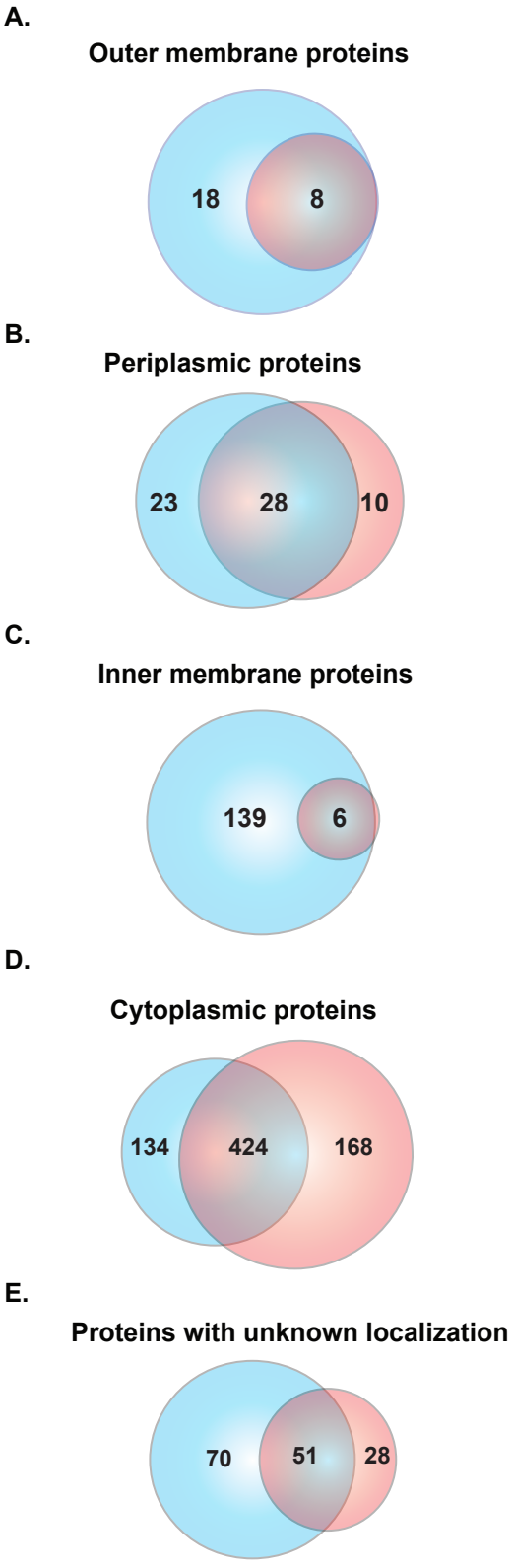


Figure 4

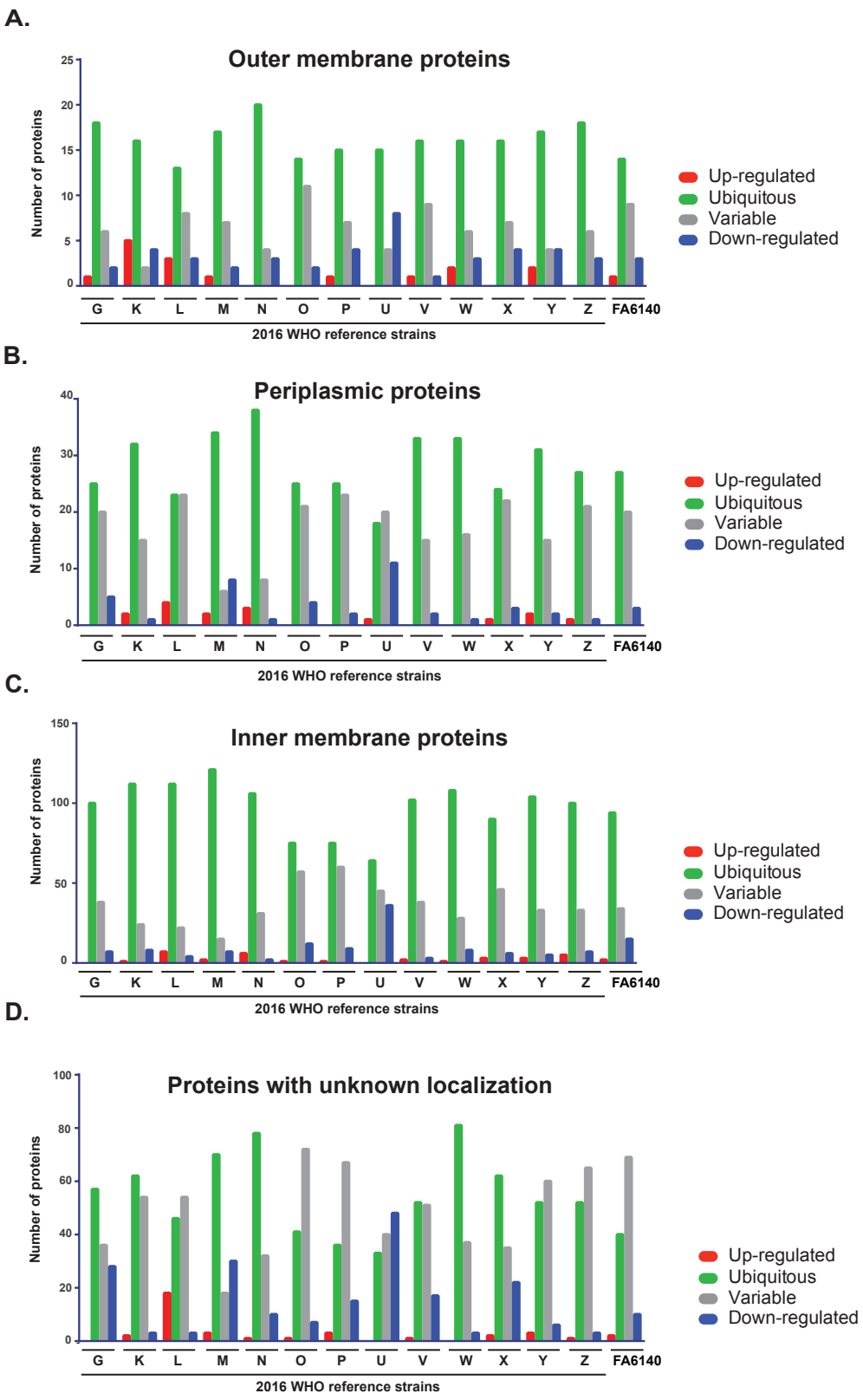


Figure 5

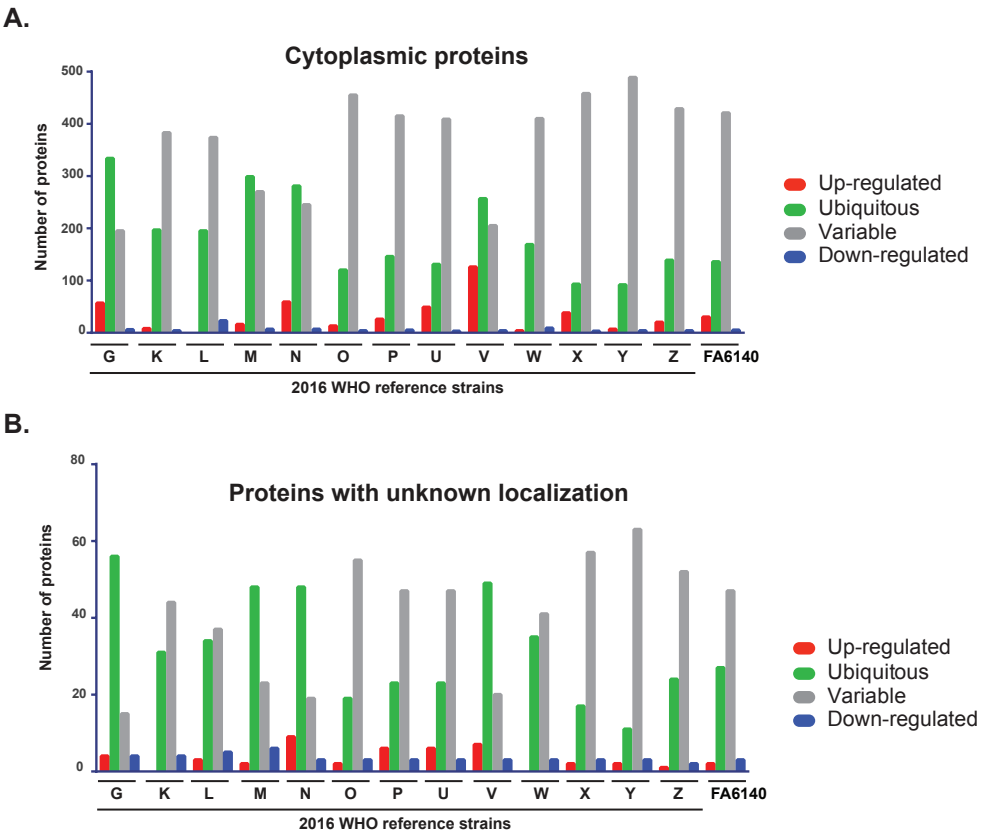
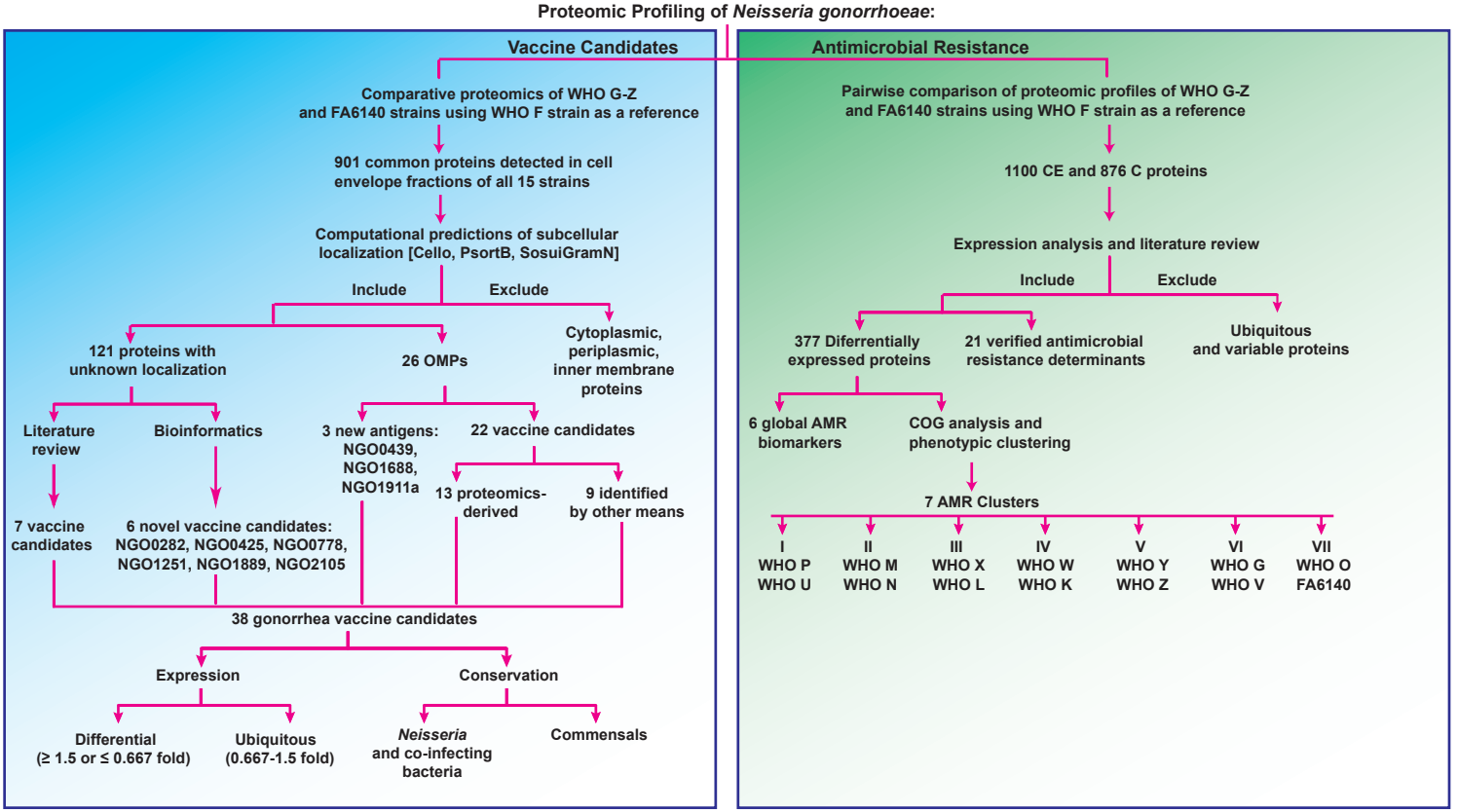


Figure 6


Proteomic Profiling of *Neisseria gonorrhoeae*:
Vaccine Candidates
Comparative proteomics of WHO G-Z and FA6140 strains using WHO F strain as a reference
901 common proteins detected in cell envelope fractions of all 15 strains
Computational predictions of subcellular localization [Cello, PsortB, SosuiGramN]
Include
Exclude
121 proteins with unknown localization
26 OMPs
Cytoplasmic, periplasmic, inner membrane proteins
Literature review
Bioinformatics
3 new antigens: NGO0439, NGO1688, NGO1911a
22 vaccine candidates
7 vaccine candidates
6 novel vaccine candidates: NGO0282, NGO0425, NGO0778, NGO1251, NGO1889, NGO2105
13 proteomics-derived
9 identified by other means
38 gonorrhea vaccine candidates
Expression
Conservation
Differential (≥ 1.5 or ≤ 0.667 fold)
Ubiquitous (0.667-1.5 fold)
Neisseria and co-infecting bacteria
Commensals
Antimicrobial Resistance
Pairwise comparison of proteomic profiles of WHO G-Z and FA6140 strains using WHO F strain as a reference
1100 CE and 876 C proteins
Expression analysis and literature review
Include
Exclude
377 Diferrentially expressed proteins
21 verified antimicrobial resistance determinants
Ubiquitous and variable proteins
6 global AMR biomarkers
COG analysis and phenotypic clustering
7 AMR Clusters
I WHO P WHO U
II WHO M WHO N
III WHO X WHO L
IV WHO W WHO K
V WHO Y WHO Z
VI WHO G WHO V
VII WHO O FA6140