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RESEARCH ARTICLE

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Exploring the sequence diversity and surface expression of Factor H-Binding Protein among invasive serogroup B meningococcal strains from selected European countries

Stephen A. Clark[a], Laura Willerton[a], Heike Claus[b], Anna Carannante[c], Paola Stefanelli[c], Raquel Abad[d], Julio A. Vázquez[d], and Ray Borrow[a]

[a]Meningococcal Reference Unit, UK Health Security Agency, Manchester Royal Infirmary, Manchester, UK; [b]National Reference Centre for Meningococci and Haemophilus Influenzae, Institute for Hygiene and Microbiology, Julius-Maximilian University of Wuerzburg, Wuerzburg, Germany; [c]Department Infectious Diseases, Instituto Superiore di Sanità, Rome, Italy; [d]Reference Laboratory for Meningococci, National Centre for Microbiology, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

ABSTRACT

Factor H-Binding Protein (fHbp) is a key component of meningococcal vaccines such as MenB-fHbp, licensed in the EU, UK, and other countries. Sufficient expression of fHbp on the bacterial surface is necessary for vaccine-induced antibodies to bind and exert bactericidal activity. The flow cytometric MEASURE assay quantifies fHbp expression in vitro, and previous studies have shown that strains with a Mean Fluorescence Intensity (MFI) >1000 are likely to be killed by MenB-fHbp-induced antibodies. This study assessed fHbp peptide distribution and expression among 451 invasive group B strains collected in 2016 across England, Wales, and Northern Ireland (EW&NI), Germany, Italy, and Spain. We found that 92% of the strains expressed fHbp above the MFI 1000 threshold. The strain distribution across EW&NI, Germany, and Italy was similar, with coverage ranging from 92.1% to 94.6%, dominated by a small number of clonal complexes and fHbp peptides. Although, the Spanish subset had a higher proportion of lower-expressing strains, particularly clonal complex 213, resulting in a lower predicted coverage for Spain (84%). These results, along with other published MEASURE data, can provide a basis for genotypic MenB-fHbp coverage predictions, however, inclusion of the upstream intergenic sequence of the fHbp gene in the prediction improved its accuracy by distinguishing between low- and high-expressing strains. Future MEASURE analyses of strains with less common fHbp variants would serve to further refine vaccine coverage predictions.

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Introduction

Invasive meningococcal disease (IMD) is a severe clinical condition typically presenting as meningitis and/or septicemia, although more localized infections such as septic arthritis and endocarditis do occur.[1] The causative pathogen, *Neisseria meningitidis*, is well characterized and the past 60 years have seen the development and introduction of increasingly intricate and effective meningococcal vaccines.[2] Polysaccharide conjugate vaccines are available for prevention of IMD caused by the majority of invasive meningococcal serogroups (namely A, C, W, Y and X) and, these vaccines have also been shown to disrupt nasopharyngeal carriage and transmission among high carriage populations (e.g. adolescents) resulting in important herd protective effects.[3,4] The development of vaccines against the most common invasive meningococcal serogroup in Europe, group B (MenB), required a different approach as structural similarities between group B polysaccharide and the neural cell adhesion molecule of human neuronal cells precluded its use as a vaccine antigen.[5] Today, two broadly-protective protein-based MenB vaccines have been licensed in Europe, the US, Australia and Canada- MenB-fHbp (Pfizer Vaccines, US) and 4CMenB (GlaxoSmithKline, UK).[6,7]

Each of these two vaccines has a unique composition of recombinant protein antigens and relies on the presence of a corresponding antigen on the target strain with sufficient peptide sequence similarity and surface expression for bactericidal activity.[8] Factor H-Binding Protein (fHbp) is an antigenic component of both licensed vaccines and so is an important target for meningococcal characterization. As a recruiter of human complement Factor H to the bacterial surface, fHbp provides serum protection for meningococcal strains that find themselves on the wrong side of the nasopharyngeal epithelium.[9] Unique fHbp peptide variants can be broadly divided into two subfamilies (A and B) based on sequence diversity.[10,11] Subfamily B is often referred to as 'variant 1,' whilst subfamily A is commonly divided further into two distinct variant subgroups: 2 and 3. Immunogenicity studies have demonstrated minimal immunological cross-protection between peptides in opposite subfamilies, however, cross protection is observed between peptides within the same

CONTACT Stephen A. Clark stephen.clark@ukhsa.gov.uk Meningococcal Reference Unit, UK Health Security Agency, Manchester Royal Infirmary, Clinical Sciences Building 2, Oxford Road, Manchester M13 9WL, UK.
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subfamily and this is the basis for the broad strain coverage offered by the individual antigenic variants within the fHbp-containing vaccines.[12] MenB-fHbp contains two fHbp peptides- one for each subfamily (fHbp peptides 45 (subfamily A) and 55 (subfamily B)), whilst 4CMenB contains one fHbp peptide (peptide 1, subfamily B), among other recombinant and OMV-derived antigens.[7,12] To July 2024, over 1400 unique fHbp peptide sequences have been uploaded to the PubMLST.org Neisseria database in which each is assigned a unique numeric peptide ID. An alternative, alphanumeric scheme (referred to here as the Pfizer ID) is sometimes used in the published literature.

Most invasive meningococcal strains express fHbp at the cell surface, however, a sufficient density of fHbp is required for antibody cross-linking and subsequent bactericidal killing via classical complement system cascade. In 2016, Biagini and colleagues used mass spectrophotometry and electron microscopy to estimate that at least 757 fHbp molecules per cell, producing a distance of less than 115 nm between fHbp molecules, are required for C1q engagement, complement activation and consequent bactericidal activity.[13] This was the lower limit for killing by sera raised against the homologous variant, but higher expression levels were required for immunological cross-protection by sera raised against heterologous variants.

The expression of *fHbp* is primarily controlled from an intergenic region immediately upstream of the gene (*fHbp* IGR). In the PubMLST.org Neisseria database, the *fHbp* IGR is indexed and curated, and over 270 unique alleles for this sequence have been identified (^{igr_up}NEIS0349).[14,15] In a minority of strains (mainly cc32 strains), *fHbp* is also transcribed in a bicistronic fashion from the promoter of the preceding gene, fructose-bisphosphate aldolase (*fba*), due to inefficient Rho-independent transcription termination in the *fHbp* IGR.[16] The expression of *fHbp* can be regulated by temperature via an RNA thermosensor in the *fHbp* IGR and signal peptide, and influenced by oxygen concentration through a putative fumarate and nitrate reduction (FNR) regulator.[16–18] Iron availability has also been shown to influence *fHbp* expression, an effect enhanced by the presence of an 186bp insertional element in a minority of strains (primarily cc1, cc4, cc5 and cc41/44), but repressed in cc32 strains with bicistronic transcription.[17]

Previous studies have sought to characterize the features of the IGR, highlight important expression determinants, and identify correlations between specific *fHbp* IGR, peptide sequences and expression. Cayrou *et al.* (2018) characterized the *fHbp* and *fba* upstream promoter sequences and grouped fHbp IGR sequences into expression clusters with similar *fHbp* mRNA transcription levels.[15] In a more recent study of UK isolates, *fHbp* promoter sequences were grouped into clades based on sequence diversity. Significant variation in *fHbp* expression was demonstrated between different promoter sequences in an isogenic strain background.[19] These results supported earlier data which suggest strain background (e.g. clonal complex) has limited influence of fHbp expression.[13] They also investigated differences in translated protein levels of different variants when expressed from the same promoter. Even with similar mRNA transcript expression levels, there were significant differences in the amount of translated protein, with subfamily B peptides present at higher levels than subfamily A.[19] This finding is consistent with earlier data suggesting many subfamily A peptides (particularly variant 2) are less stable and more susceptible to proteolysis than subfamily B.[20]

The variation in fHbp peptide sequence and surface expression among invasive strains makes it challenging to assess the susceptibility of the strains against antibodies induced by fHbp-containing vaccines. In vaccine trials, the bactericidal activity of vaccine sera against a small number of indicator strains, each with different antigenic repertoires, has been assessed using hSBA assays.[8] Unfortunately, difficulties in sourcing suitable exogenous human complement precludes the use of human serum bactericidal antibody (hSBA) assay for testing larger panels of isolates representative of circulating strains. To overcome this limitation, the ELISA-based Meningococcal Antigen Typing System (MATS) was developed to estimate strain coverage of 4CMenB.[21] The MATS allows high throughput *in vitro* quantification of expression and immunological cross-reactivity of fHbp, and other 4CMenB antigens, among invasive MenB isolates. The output is a relative potency (RP) value for each recombinant antigen per strain, which can be compared to a defined positive bactericidal threshold (PBT) above which a high probability of bactericidal activity (by 4CMenB-derived antibodies) is predicted.[21] A similar approach has also been used for strain coverage prediction of MenB-fHbp. The Meningococcal Antigen Surface Expression (MEASURE) assay is a flow cytometric assay which utilizes an fHbp-specific monoclonal (MN86-994-11-1) to quantify *in vitro* fHbp expression among fixed meningococcal cells.[22] Earlier work demonstrated a positive correlation between the mean fluorescence intensity (MFI) produced using the MEASURE and the likelihood of serum bactericidal activity of MenB-fHbp-induced sera in serum bactericidal assays.[10,23] In 2018, McNeil and colleagues compared MEASURE assay and hSBA results (using anti-MenB-fHbp sera from young adults) from over 100 invasive meningococcal strains from the US and Europe.[23] Using a weighted analysis, they calculated a 91.2% probably of hSBA killing for isolates producing a MEASURE assay MFI readout of > 1000, regardless of fHbp variant/subfamily.

These *in vitro* strain coverage assays provide useful data on strain susceptibility; however, they are reliant on a meningococcal culture, which isn't available for all disease cases. To allow a culture-independent meningococcal strain coverage prediction, several genotypic prediction schemes have been developed. The genetic MATS (gMATS) scheme uses historic MATS data collected from diverse clinical isolates across many countries and years to predict the likelihood of MATS-positivity for strains harboring specific 4CMenB antigenic variants (including fHbp).[24] A similar scheme, the Meningococcal Deduced Vaccine Antigen Reactivity (MenDeVAR) Index, developed by the University of Oxford, utilizes published strain characterization data to predict coverage.[25] MenDeVAR is built-in to the PubMLST.org Neisseria database and automatically predicts strain coverage of both licensed fHbp-containing vaccines based on the antigenic profiles of isolates uploaded to the database. The 4CMenB coverage prediction is based on

published MATS data, whilst, for MenB-fHbp coverage, it relies on publicly available MEASURE and SBA results to make its prediction.

These genotypic prediction schemes may support outbreak response given that not all cases yield a viable isolate (~50% of IMD cases in England are confirmed by PCR alone), and these specialist *in vitro* assays are not performed in most reference laboratories.[26] Although, the accuracy of these predictions is dependent on the representativeness of the underlying data and there is a dearth of MEASURE assay data available in the literature. The largest published MEASURE assay dataset includes 1,814 isolates from the US and Europe, however, they were collected over 18 years ago (2000 to 2006) and a detailed line listing of the MEASURE MFIs wasn't provided so it is impossible to infer the coverage of specific fHbp peptide variants. Despite subsequent publication of MEASURE results on smaller panels of strains for specific countries (e.g. Greece[27] and Canada),[28] data on larger and more representative strain panels are lacking.

Here, we describe fHbp peptide and surface expression data for 451 invasive MenB strains isolated in 2016 across four European countries. These data illustrate fHbp diversity and variation in expression levels, as well as providing an indication of the strain coverage of the MenB-fHbp vaccine.

Materials and methods

Strain panel

The panel included strains from four European countries: England, Wales and Northern Ireland (EW&NI), Germany, Italy and Spain. The panel included the available viable invasive MenB strains received by the respective national reference labs for the calendar year 2016 (January to December, inclusive).

Genomic and antigenic characterisation

DNA extraction, whole-genome sequencing and genomic assembly were performed in accordance with local protocols for the respective reference laboratories. All assemblies were uploaded to the PubMLST.org Neisseria database for allelic indexing.[14] Multi-locus sequence typing (MLST) and fHbp locus data (fHbp peptide IDs and igr_upNEIS0349 alleles) were then extracted from PubMLST. The numeric fHbp peptide IDs used in this study are those assigned and curated within the PubMLST Neisseria database, although to aid comparisons, the alternative Pfizer scheme IDs for the predominant fHbp peptides are specified in Table 1.

MEASURE assay testing and analysis

Surface expression of fHbp was quantified using the MEASURE assay at the UKHSA Meningococcal Reference Unit (MRU), Manchester UK, following the protocol previously described by Loschko et al.[22] Each bacterial strain was tested in duplicate for MFI, and the recorded MEASURE result for each strain was the geometric mean of the two MFI readouts. For each fHbp peptide/IGR combination, the median MFI and interquartile ranges (IQR) were calculated, excluding the median in the IQR calculation. The percentage of isolates with MFI values greater than 1000 was calculated with 95% confidence intervals for these proportions using the Wilson Score Interval. The significance of the difference in expression values between fHbp subfamilies was evaluated using the Mann-Whitney U test.

MenDeVAR MenB-fHbp strain coverage prediction

The current MenDeVAR scheme within the PubMLST.org Neisseria database performs MenB-fHbp strain coverage prediction on the fHbp peptide variant of the strain of interest and is based on associated MEASURE and SBA data in the published literature (only for fHbp peptides represented by ≥ 5 strains in the literature).[25] Strains are given one of four predictions: 'Exact match' (fHbp is one of the two vaccine peptides (45 or 55)), 'Cross-reactive' (≥3/4 of the results in the literature indicate cross-reactivity to vaccine antigen), 'Insufficient data' (<5 strains tested or inconclusive results in the literature, therefore a prediction cannot be made) and 'Not reactive' (<1/4 of results in the literature indicate coverage). The MenDeVAR prediction for all strains was downloaded directly from PubMLST.org. Neisseria database. The prediction is calculated for all strains automatically based on the fHbp peptide ID of each strain according to the published criteria.[25]

Results

Strain panel characteristics

The panel included a total of 451 MenB strains (Table S1). These were from England, Wales and Northern Ireland (n = 204), Germany (n = 126), Spain (n = 92) and Italy (n = 29). Table 1 contains the clonal complex and fHbp peptide distribution for each country. Eighty percent of the strains were represented by six common hyperinvasive clonal complexes (cc), namely cc41/44, cc269, cc213, cc32, cc162 and cc461. Another 12% of strains were not assigned to a clonal complex according to PubMLST at the time of the analysis. The remaining 36 isolates (8%) were represented by 11 different clonal complexes (Table 1). EW&NI and Germany were similar in terms of cc distribution with approximately three-quarters of each dataset belonging to four major cc's: cc41/44, cc269, cc213, and cc32 (75.5% and 77.0%, respectively). Although the number of strains was much smaller, the Italian isolate panel was more evenly distributed among the clonal complexes with only one cc269 isolate and with cc162 predominating (n = 9/29, 31.0%). Relative to the other countries, cc213 was the most prevalent in the Spanish isolate panel with 31.5% of isolates belonging to that cc (Table 1).

In terms of fHbp peptide distribution, three isolates (0.67%) lacked the *fHbp* gene (Table 1). Two of these strains were isolated in EW&NI and were both ST-1867 (unassigned to cc). The third *fHbp*-null isolate was from Italy and was ST-6349 (cc41/44). Among the 448 isolates that possessed *fHbp*, there were 84 unique fHbp peptide sequences. The majority of isolates (n = 296, 65.6%)

Table 1. Clonal complex and fHbp peptide distribution among European invasive MenB strains in the study panel (total n = 451).

	Country of origin				
	EW&NI	Germany	Italy	Spain	Grand Total
Clonal complex					
ST-41/44 complex	61	38	5	7	111
ST-269 complex	48	19	1	8	76
ST-213 complex	20	18	2	29	69
ST-32 complex	25	22	5	5	57
not assigned	23	18	2	12	55
ST-162 complex	6	5	9	5	25
ST-461 complex	8	1	1	12	22
ST-35 complex	2	2	0	3	7
ST-60 complex	3	1	0	3	7
ST-1572 complex	0	1	2	2	5
ST-18 complex	1	1	0	2	4
ST-103 complex	1	0	0	2	3
ST-9316 complex	2	0	0	0	2
ST-865 complex	0	0	1	1	2
ST-11 complex	2	0	0	0	2
ST-1157 complex	1	0	0	1	2
ST-167 complex	0	0	1	0	1
ST-22 complex	1	0	0	0	1
fHbp peptide (Pfizer ID)					
Negative/fHbp null	2	0	1	0	3
Subfamily A					
45 (A05)	6	13	1	15	35
19 (A22)	10	10	2	8	30
47 (A06)	4	1	1	12	18
16 (A19)	4	1	2	5	12
21 (A07)	3	1	2	2	8
Other A	18	11	4	14	47
All Subfamily A	45	37	12	56	150
Subfamily A/B hybrid					
207	0	0	0	2	2
Subfamily B					
4 (B16)	44	10	0	3	57
13 (B09)	31	9	0	5	45
14 (B03)	15	20	1	4	40
1 (B24)	17	15	2	3	37
15 (B44)	18	12	0	2	32
510 (B133)	3	5	1	1	10
110 (B23)	3	4	1	0	8
390 (NK*)	0	1	7	0	8
Other B	26	13	4	16	59
All Subfamily B	157	89	16	34	296
Grand Total	**204**	**126**	**29**	**92**	**451**

*The Pfizer scheme ID for fHbp peptide 390 is not known.

harbored a subfamily B variant, and 150 isolates (33.5%) harbored peptide variants belonging to subfamily A. Spain was the only country of the four within a majority of strains expressing subfamily A peptides (56/92, 60.9%), caused largely by a relatively high proportion of strains with fHbp peptide 45 and 47. Two isolates (both from Spain) had fHbp peptide 270 which is a subfamily A/B hybrid variant. Regarding the fHbp peptides contained in the licensed MenB vaccines, 37 isolates (8%) harbored fHbp peptide 1 (4CMenB vaccine peptide) and 35 isolates (7%) fHbp peptide 45 (MenB-fHbp vaccine peptide). The other MenB-fHbp peptide (peptide 55) was not found in the isolate panel.

Figure 1 shows the fHbp peptide distribution among the major clonal complexes for the MenB study panel. There were strong associations between fHbp peptide and clonal complex with the majority of isolates within each of the major clonal complexes harboring one of two or three predominant fHbp variants (Figure 1). The thirteen most common fHbp variants (each represented by at least 5 isolates) represented three-quarters of the panel (n = 340/451, 75.4%).

fHbp surface expression

The results of the fHbp surface expression quantification are illustrated in Figure 2 and Table 2. On average, isolates expressing subfamily A fHbp peptide variants expressed significantly lower levels of fHbp compared to those expressing subfamily B variants (Mann Whitney U test, p = .001). Ninety two percent of all isolates with fHbp expressed the antigen above the proposed bactericidal MFI threshold of 1000. Overall, 18% of isolates harboring subfamily A variants exhibited expression <1000 MFI, compared with only 2.7% subfamily-B expressing isolates. Among the thirteen predominant fHbp peptide variants, the median MFIs ranged from 1686 (peptide 21) to 17,230 (peptide 15, Table 2, Figure 3). Only five of the thirteen were represented

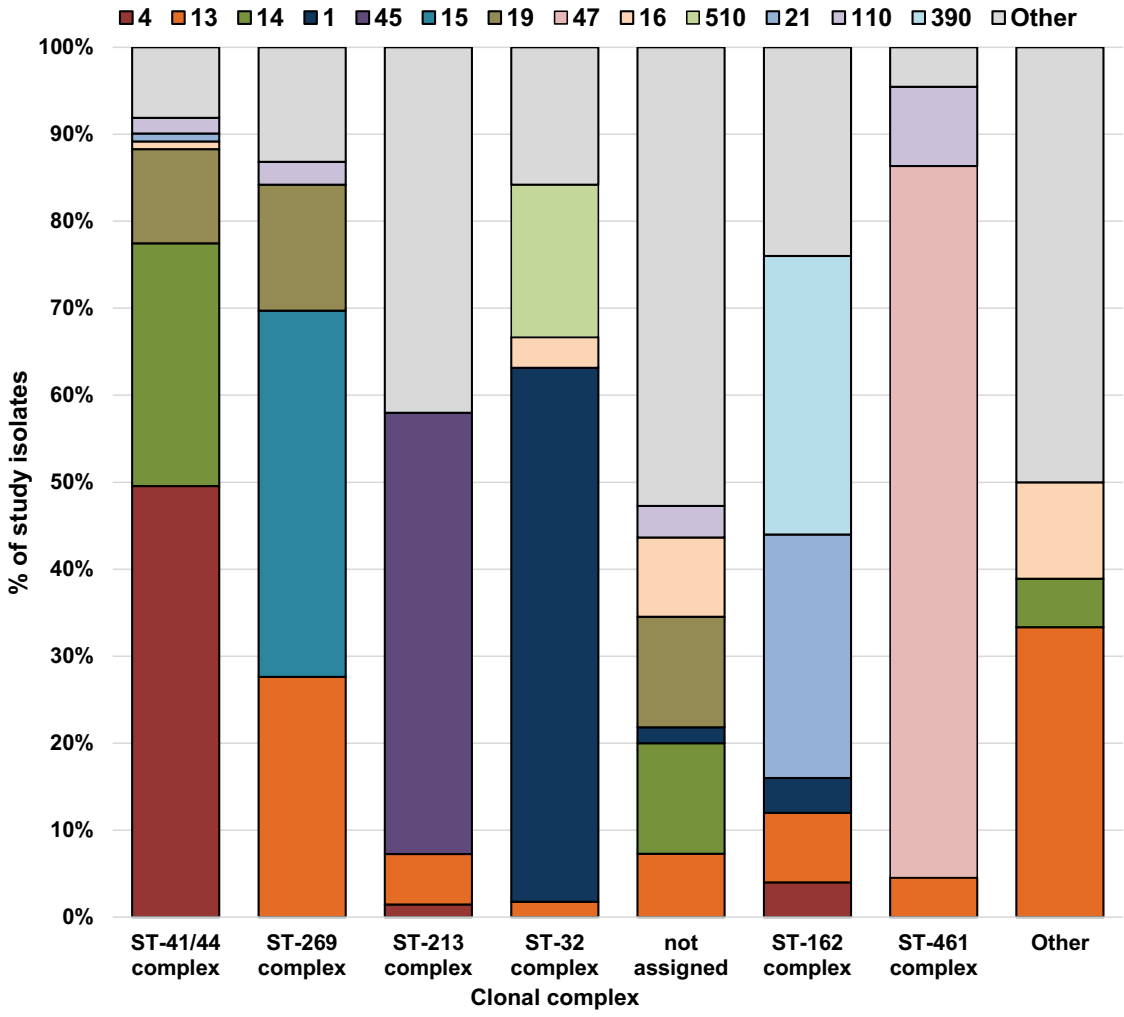

Figure 1. fHbp peptide distribution within the major clonal complexes in the European MenB study panel (all countries combined, $n = 451$).

by isolates expressing <1000; four of which were subfamily A. Interestingly, only 62.9% of isolates with peptide 45 expressed MFI > 1000 (95% CI: 44.9–78.5), despite the median MFI being 4264 (IQR: 2271–3758), indicating a segregated population.

The strain panel contained 65 unique *fHbp* IGR alleles (^{igr_up}NEIS0349, Table S1). The contribution of the *fHbp* IGR to expression is explored in Figure 4 which shows MEASURE assay results from isolates with the most common fHbp peptide and *fHbp* IGR allele combinations (each combination harbored by at least three isolates, total isolates = 323, 72.1% of strain panel). Five predominant fHbp peptides (4, 14, 15, 45 and 110) were combined with more than one *fHbp* IGR allele (among combinations represented by ≥3 isolates). Isolates with peptide 4 all expressed fHbp >1000 MFI regardless of IGR (alleles 1 or 2). Peptide 14 was consistently expressed above >1000 when under control of IGR allele 7 with a median MFI of 5418, however, under IGR alleles 1 or 271, 15.8% (3/19) of isolates expressed below the threshold (median MFI 2358 and 1288, respectively). All isolates with peptide 15 expressed well above the threshold regardless of the IGR allele (4 or 10). Peptide 45 was combined with five different *fHbp* IGR alleles (among combinations represented by ≥3 isolates). Isolates with peptide 45 and IGR alleles 14, 33, 61 or 91 (n = 18) all had MFI >1000. Those with peptide 45 and IGR allele 8, however, all exhibited an MFI < 1000 ($n = 13$). All peptide 45 harboring strains belonged to cc213 regardless of the IGR allele. In the wider strain panel, two additional isolates that harbored IGR allele 8 but uncommon fHbp peptides (952 and 1140), also exhibited an MFI below the threshold (data not shown). Finally, isolates with peptide 110 expressed >1000 regardless of IGR allele (21 or 32).

The highest MFI value in the panel was 148,194. This was observed in a German strain (strain ID: DE13715) belonging to ST-32 (cc32) with fHbp peptide 510 and *fHbp* IGR allele 267. This IGR allele possesses a T to C point mutation in the putative −35 box (position 82) which has been previously shown to increase *fHbp* expression ten times.[19] Three other *fHbp* IGR alleles in the strain panel had this mutation (alleles 18, 72 and 270). Interestingly, the three highest expressing isolates in the panel harbored one of these four *fHbp* IGR alleles (18, 72 and 267).

Overall, when using the putative MFI > 1000 cutoff, the proportion of isolates predicted to be susceptible to MenB-fHbp-induced antibodies was similar between EW&NI (94.6%, 95% CI: 90.6–97.0), Italy (93.1%, 95% CI: 78.0–98.1) and Germany (92.1%, 95% CI: 86.0–95.6). The corresponding value among the Spanish isolates was lower at 83.7% (95 CI:

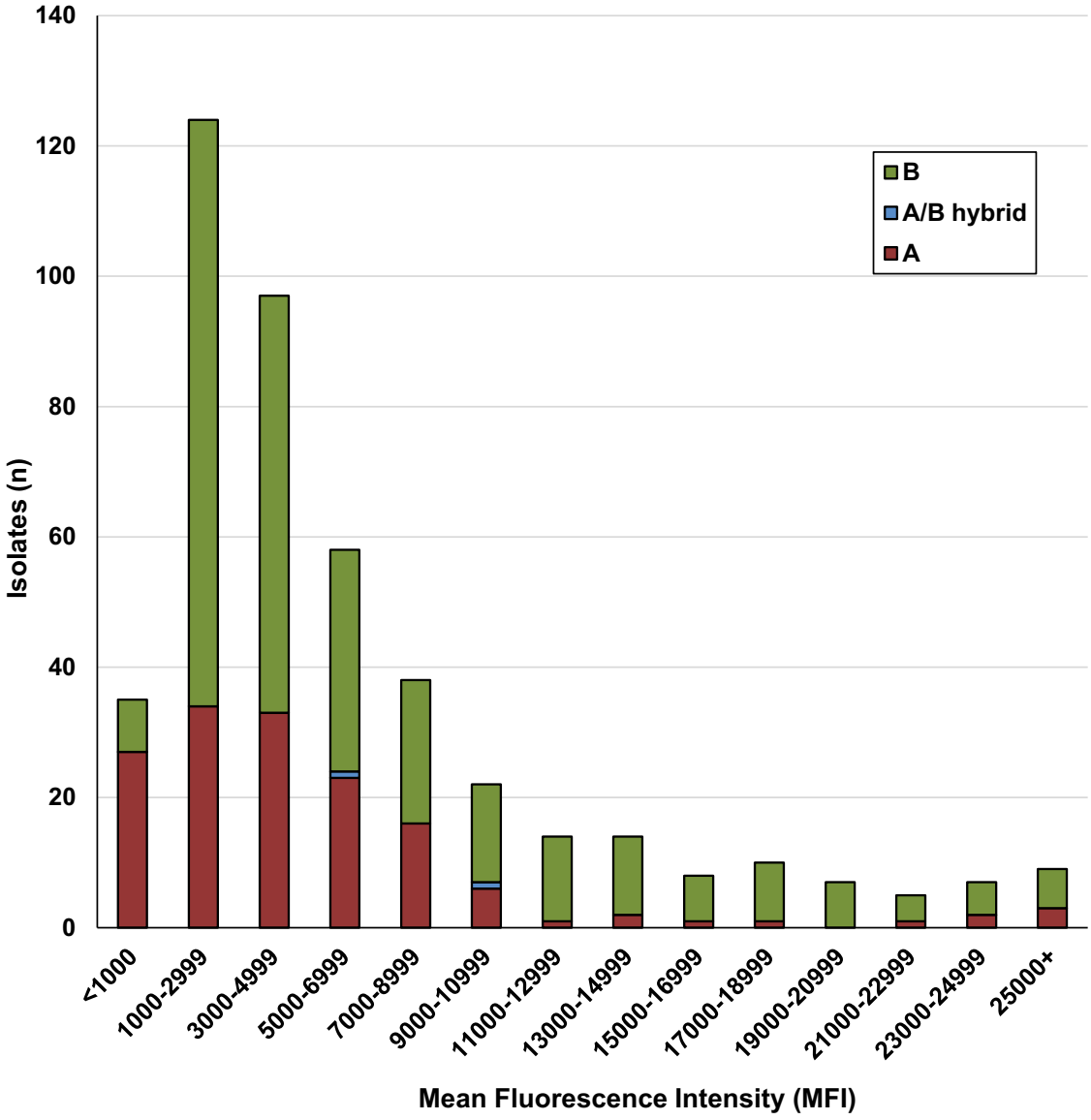

Figure 2. MFI ranges from MEASURE assay results of fHbp expression in European MenB isolates ($n = 448$). The subfamily of the fHbp peptide expressed is indicated in the stacked columns.

Table 2. MEASURE MFI results: Median, interquartile range and proportion of isolates exceeding the putative threshold (MFI >1000).

fHbp peptide	fHbp Subfamily	Isolates (n)	Median MFI (IQR)	% of isolates with MFI > 1000 (95% CI)
4	B	57	2738 (2122–3346)	100.0 (93.7–100.0)
13	B	45	2911 (2271–3758)	100.0 (92.1–100.0)
14	B	40	4293 (2081–5448)	92.5 (80.1–97.4)
1	B	37	9417 (7475 –12,745)	100.0 (90.6–100.0)
45	A	35	4264 (2271–3758)	62.9 (46.3–76.8)
15	B	32	17230 (14796 –21,602)	100.0 (89.3–100.0)
19	A	30	4412 (2276–5298)	93.3 (78.7–98.1)
47	A	18	3705 (2299–6234)	100.0 (82.4–100.0)
16	A	12	1843 (1136–2190)	83.3 (55.2–95.3)
510	B	10	6063 (3923–7914)	100.0 (72.2–100.0)
21	A	8	1686 (233–2222)	62.5 (30.6–86.3)
110	B	8	7441 (6119 –13,284)	100.0 (67.6–100.0)
390	B	8	2569 (2059–3380)	100.0 (67.6–100.0)
All subfamily A	A	150	4031 (1635–6375)	82.0 (75.1–87.3)
All subfamily B	B	296	4338 (2622–9368)	97.3 (94.8–98.6)
All isolates*	All isolates	448	4266 (2370–7831)	92.2 (89.3–94.3)

*excluding three fHbp-negative isolates.

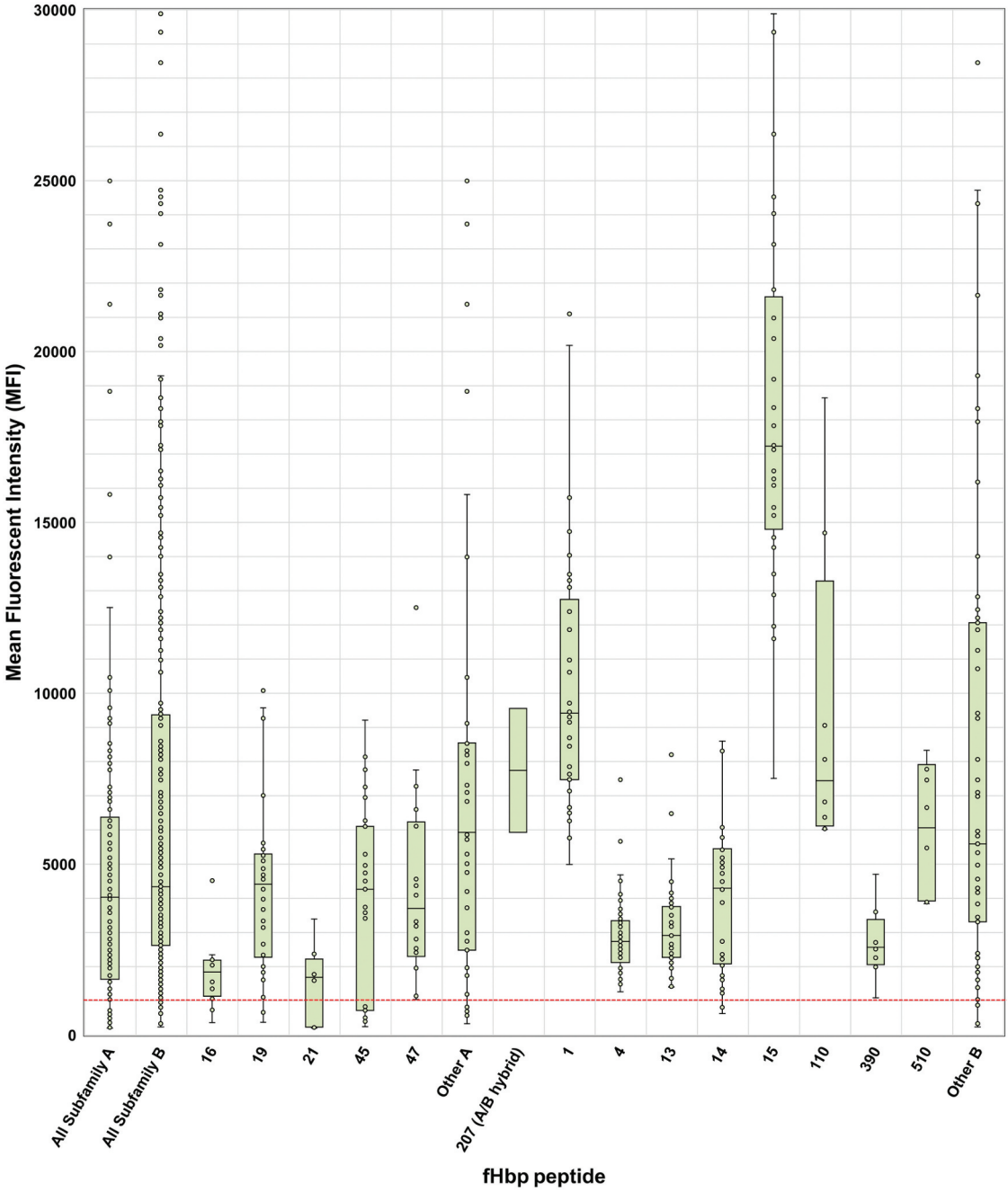

Figure 3. Box plot of the MEASURE MFI values of meningococcal isolates by fHbp peptide. The horizontal line inside the boxes represents the median MFI, the lower and upper limits of the boxes represent the 25th and 75th quartiles, respectively. The whiskers represent the lowest and highest values within 1.5 times of the interquartile range. The red line indicates the 1000 MFI putative bactericidal threshold. Individual values are plotted as circles; however, isolates with identical or very similar MFI values are grouped into a single circle for clarity, so not all data points are individually visible on the plot. Isolates expressing above 30,000 MFI were excluded from the graph but were included in the median/iqr calculations ($n = 5$).

74.8–89.9). This was primarily due to the higher prevalence of cc213 isolates with peptide 45 and *fHbp* IGR 8 (7/92, 7.6% of Spanish isolates), whilst only six other such isolates were seen between the other three countries panels (four in Germany and one each in EW&NI and Italy).

Comparison to MenDeVAR

To compare the results of the current MenDeVAR scheme with our MEASURE assay outcomes (MFI > 1000), we downloaded the automated MenDeVAR prediction for all fHbp-harboring strains in our panel ($n = 448$) (Table 3). Approximately a quarter of the strains ($n = 115$, 25.7%) were given an 'Insufficient data' designation indicating a prediction could not be made. Thirty-five isolates had an exact match to a vaccine variant (all peptide 45). The remaining isolates ($n = 298$, 66.5%) were given a 'cross-reactive' prediction. None of the isolates were considered 'Not reactive,' however, as under the current MenDeVAR MenB-fHbp scheme, no fHbp peptide is considered not reactive.

Table 3 shows a comparison of MenDeVAR and MEASURE results for isolates with predominant fHbp

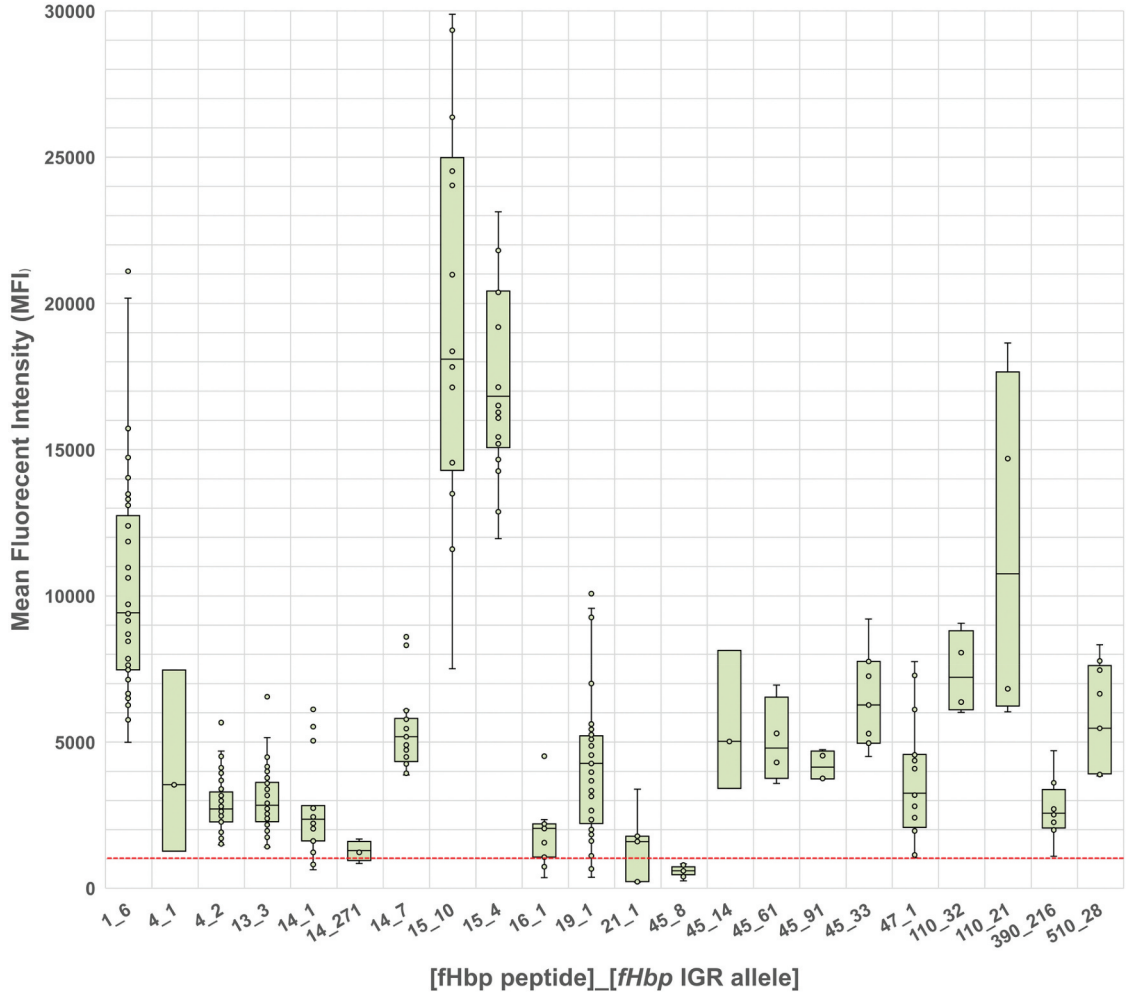

Figure 4. Box plot of the MEASURE MFI values among predominant fHbp peptide and *fHbp* IGR combinations (each represented by ≥3 isolates). The horizontal line inside the boxes represents the median MFI, the lower and upper limits of the boxes represent the 25th and 75th quartiles, respectively. The whiskers represent the lowest and highest values within 1.5 times of the interquartile range. The red line indicates the 1000 MFI putative bactericidal threshold. Individual values are plotted as circles; however, isolates with identical or very similar MFI values are grouped into a single circle for clarity, so not all data points are individually visible on the plot. None of the isolates with the indicated combinations expressed fHbp at an MFI > 30,000.

Table 3. Comparison of MenDeVAR designation/prediction and European MenB panel MEASURE assay outcome- including predominant fHbp peptides with either a conclusive MenDeVAR designation (exact match, cross-reactive or not reactive) and/or >5 representative isolates in the European panel.

Predominant fHbp peptide	MenDeVAR designation	Number of isolates in panel	% of isolates with MFI > 1000 in European panel
1	Cross reactive	37	100.0
4	Cross reactive	57	100.0
13	Cross reactive	45	100.0
14	Cross reactive	40	92.5
15	Cross reactive	32	100.0
16	Cross reactive	12	83.3
19	Cross reactive	30	93.3
21	Cross reactive	8	62.5
23	Cross reactive	1	100*
25	Cross reactive	4	25.0*
30	Cross reactive	2	100*
45	Exact match	35	62.9
47	Cross reactive	18	100.0
110	Insufficient data	8	100.0
276	Cross reactive	2	100*
390	Insufficient data	8	100.0
510	Cross reactive	10	100.0
Others	Insufficient data	99	91.0

*Fewer than 5 isolates in panel.

variants. Among the 14 peptides considered to be cross-reactive by MenDeVAR, nine were consistently expressed above MFI = 1000 by the relevant isolates in the MEASURE database. Three additional fHbp peptides (14, 16 and 19) were expressed by more than three quarters of isolates, thus consistent with the MenDeVAR criteria. Peptide 21 was only expressed sufficiently by 5 of 8 (62.5%) isolates, and peptide 25 was only expressed above the threshold in 25% of cases (<5 isolates tested) despite a cross-reactive MenDeVAR prediction for these peptides (Table 3).

As a vaccine variant, peptide 45 is given an 'Exact match' designation by MenDeVAR. This is highlighted by a green color in the scheme's traffic light system strongly implying the peptide is automatically covered by the vaccine. In our panel, only 62.9% of isolates with peptide 45 expressed above the threshold. Furthermore, two peptides that were predominant in the European panel (≥5 isolates), peptides 110 and 390, were consistently sufficiently expressed in the MEASURE but given an Insufficient data MenDeVAR designation (Table 3).

Discussion

In this study, we used genomic sequencing and the flow cytometric MEASURE assay to assess the sequence diversity and surface expression of fHbp among 451 MenB strains isolated across four European countries in 2016. This represents the largest published dataset of MEASURE assay results since the initial validation was performed on an older panel of 1,814 isolates by McNeil et al in 2018.[23] More than 99% of all isolates in the panel possessed *fHbp,* and thirteen fHbp peptide variants and six clonal complexes represented around three quarters of the strain panel. Subfamily B variants were most prevalent in EW&NI, Germany and Italy, although, in the Spanish isolate subset, subfamily A fHbp peptides, fHbp peptide 45 in particular, were more prevalent, a finding noted in a previous study of Spanish strains.[29] This difference in epidemiology resulted in a slightly lower MenB-fHbp strain coverage prediction for Spain (83.7%) compared to the other countries (92.1–94.6%). In 2016, the only widespread use of an fHbp-containing vaccine was in EW&NI where 4CMenB had been introduced into the national infant immunization program the year before. Although, only a small proportion of eligible infants had received a full regimen of the vaccine at this time and so the expected impact on strain distribution is minimal.[30] Despite differences in the predominance of specific strains across these four countries, the relatively limited strain diversity simplifies coverage assessments. Characterising a small number of predominant peptide variants allows for the prediction of strain susceptibility in most invasive strains within these countries, and potentially across other European countries.

Expression of fHbp was assessed using the MEASURE assay. A key benefit of the assay is its quantification of surface-localized fHbp in its native state, as opposed to *fHbp* mRNA transcripts or total fHbp from cellular lysates.[22] Surface expression is critical for antibody recognition and complement-mediated bacterial killing, so the assay provides a more appropriate metric for predicting strain susceptibility. Another strength of the assay is its use of a monoclonal antibody that has been shown to bind diverse fHbp peptides at similar affinities.[23] Unlike the MATS assay, which utilizes ELISA plates coated with antibodies raised against specific 4CMenB antigenic variants, the MEASURE assay provides a relatively objective quantification of fHbp expression independent of any particular vaccine variants. Although, conversely, this is also a limitation as the MEASURE assay cannot be used to confirm cross-reactivity between MenB-fHbp-derived antibodies and the fHbp peptides expressed by the test strains. A presumption of MenB-fHbp cross-reactivity across all fHbp peptides is made based on immunological data demonstrating broad coverage against diverse fHbp variants.[12,31]

Our MEASURE assay expression data was consistent with previous observations showing a higher level of expression among isolates with subfamily B fHbp variants compared to subfamily A. This finding has been attributed to a lower stability in the structure of subfamily A peptides,[20] and an strong association between subfamily A peptides and *fHbp* promoter sequences that result in lower *fHbp* transcription.[19] Whilst MFI values varied greatly from 202 to 148,194, most isolates expressed at an MFI between 1000 and 9000- a finding also observed in the initial MEASURE validation study on US and European strains.[23] Three of the highest expressing isolates harbored *fHbp* IGR alleles with mutations in the −35 box previously associated with substantially enhanced expression.[19] Whilst the serum protection afforded by fHbp is well understood, the implications of hyper-expression on clinical outcomes is not yet clear. Haralambous *et al.* (2006) demonstrated associations between mutations in the human *fH* gene, serum fH concentrations and the risk of IMD.[32] Future analysis of clinical data in association with fHbp expression data may help to reveal the influence of expression levels on disease progression.

The primary purpose of the MEASURE assay is to predict strain susceptibility against MenB-fHbp-induced antibodies. In the MEASURE validation study, a subset of 109 strains were also tested in the hSBA and a high probability of bactericidal activity (91.2%) was calculated in isolates expressing fHbp at an MFI of ≥1000, regardless of fHbp peptide.[23] In the current study, 92% of all isolates expressed fHbp above this MFI = 1000 cut off. Whilst eight predominant fHbp peptide variants were consistently expressed over the threshold, five fHbp peptides (14, 16, 19, 21 and 45) were not. The incorporation of *fHbp* IGR allele (^{igr_up}NEIS0349) into the analysis showed that, in certain instances, low expression is strongly associated with specific IGR alleles. For example, all the isolates expressing fHbp peptide 45 by at an MFI < 1000 had IGR allele 8, whilst higher expression was observed when in conjunction with other IGR alleles. Peptide 45 is associated with cc213 and the high proportion of strains with IGR allele 8 was a key driver behind a lower estimated strain coverage of MenB-fHbp in Spain. Similarly, among isolates with peptide 14, those with IGR allele 7 exhibited consistently high expression as opposed to those with other IGR alleles. This study can thus provide a useful reference dataset for predicting MenB-fHbp strain susceptibility in scenarios where decisions on vaccine selection need to be made (e.g. IMD outbreaks). This is particularly critical in cases where no isolate was obtained, for example, the 40–60% of all English

IMD cases that are confirmed by PCR alone. To facilitate the characterization of *fHbp* in these cases, a nested PCR sequencing assay was developed in 2013.[26]

In 2020, this aim of predicting MenB-fHbp coverage of new strains using genotypic data led to the development of the MenDeVAR scheme by the University of Oxford.[25] MenDeVAR provides an easily accessible and interpretable prediction and is built into the widely-used Neisseria database at PubMLST.org.[14] Although, the prediction for MenB-fHbp is primarily based on published SBA data (due to a lack of MEASURE data in the literature), which means the number of unique strains tested for each fHbp peptide variant tested is low. Across the fifteen MenB-fHbp trials and studies from which the SBA data was gathered, only 57 unique strains expressing 23 different fHbp peptides were tested. Almost all these strains have been shown to express fHbp above the MFI > 1000 threshold. So, whilst these studies clearly demonstrate the ability of MenB-fHbp-induced antibodies to cross-react with diverse fHbp peptide variants, the lack of published MEASURE data on larger, more representative panels means the variability in expression levels among invasive strains is not considered in the current MenDeVAR MenB-fHbp prediction. For example, MenDeVAR gives strains with peptide 45 an 'Exact match' designation (with a green light in the traffic light scheme). This is primarily based on SBA data showing killing against two isolates expressing peptide 45 above the 1000 MFI threshold, although, as our data show, a significant proportion of invasive strains with peptide 45 (those with IGR allele 8) express the antigen below this threshold, so in many cases this prediction may be incorrect. Future revision of the MenDeVAR scheme to include the IGR allele and more recent MEASURE data would serve to improve this prediction.

Whilst the MEASURE assay provides a prediction of vaccine-derived bactericidal activity, it is, important to note that an MFI of < 1000 doesn't necessarily preclude bactericidal activity, especially in strains expressing fHbp peptides with high amino acid similarity to the vaccine variant. In the initial MEASURE validation study, bacterial killing was observed in five strains (out of 17) that expressed fHbp below the threshold, although the peptide variants expressed by these strains wasn't specified.[23] Thus, publication of additional SBA data on specific strains for which there is questionable coverage (e.g. an outbreak strain) may be required for a conclusive prediction.

The limitations of this analysis include the testing for strains over only one year which may mean the panel is not necessarily representative of strains causing IMD over the last decade. Furthermore, since the panel was collected, the world experienced a global pandemic which led to significant disruption in meningococcal transmission and disease rates in Europe and beyond, although recent data suggest there was little change in overall MenB strain distribution.[33,34]

In summary, our data suggests that >90% of invasive MenB strains isolated across these four European countries in 2016 expressed fHbp at a sufficient level to be susceptible to MenB-fHbp vaccine-derived antibodies. Strains harboring five predominant fHbp variants exhibited variable expression that straddled the defined threshold, so coverage could not be predicted based on peptide alone, however, in some cases, knowledge of the *fHbp* IGR allele allowed differentiation of the low and high expressor strains. Unfortunately, the less common fHbp peptides in the dataset were not sufficiently represented to make a reasonable assessment. Future work to test more strains with these minor variants in the MEASURE assay would be valuable and would expand the proportion of strains with characterized fHbp expression levels and a MenB-fHbp coverage prediction. This may require testing of strains from multiple years/countries in order to generate sufficient data on rare antigenic variants. The MenDeVAR scheme, hosted by the PubMLST.org Neisseria database, is an informative and easy-to-use tool for genotypic prediction of MenB-fHbp coverage. Although, reevaluation and adjustment of the predictions in the light of new strain characterization data, as well as inclusion of the *fHbp* IGR allele in the scheme, would improve the accuracy of the prediction and better support clinicians and public health professionals in making vaccination decisions in response to IMD cases and outbreaks.

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Disclosure statement

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Notes on contributor

Dr Stephen Clark is a Senior Scientist at the UKHSA Meningococcal Reference Unit in Manchester, UK. He specialises in characterisation and strain surveillance of *Neisseria meningitidis* with a particular interest in vaccine strain coverage.

Data availability statement

The genomic data for all isolates are publicly available on PubMLST.org/Neisseria. The PubMLST IDs for the isolates can be found in the supplementary table.

Ethics statement

This study does not involve human participants and so no ethical approval was required.

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