Pan-genome study underlining the extent of genomic variation of invasive *Streptococcus pneumoniae* in Malawi

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31 Impact Statement

32 Our research applied pan-genomics principles to comprehensively assess diversity within the
33 pneumococcus genome, with the primary objective of identifying pneumococcal virulence genes for
34 advancing vaccine design and drug development. Within this study, we identified Serotypes 1 and 5 as
35 the predominant and highly invasive pneumococcal strains in Malawi, characterized by a short
36 nasopharyngeal colonization period, suggesting their potential for rapid infection of sterile sites within
37 the human body such as blood and the central nervous system. These serotypes exhibited significant
38 genetic divergence from other serotypes in Malawi, notably lacking key genes within the RD8a operon
39 while harboring transporters functioning independently of ATP. It's important to note that these findings
40 are based on computational analysis, and further validation through laboratory experiments is essential
41 to confirm their biological significance and potential clinical applications. The implications of our
42 research offer potential avenues for more effective pneumococcal disease prevention and treatment,
43 not only in Malawi but also in regions facing similar challenges.

44 Abstract

45 *Streptococcus pneumoniae* is a common cause of acute bacterial infections in Malawi. Understanding
46 the molecular mechanisms underlying its invasive behavior is crucial for designing new therapeutic
47 strategies. We conducted a pan-genome analysis to identify potential virulence genes in *S. pneumoniae*
48 by comparing the gene pool of isolates from carriers' nasopharyngeal secretions to isolates from the
49 blood and cerebrospinal fluid of patients. Our analysis involved 1,477 pneumococcal isolates from
50 Malawi, comprising 825 samples from carriers (nasopharyngeal swab) and 652 from patients (368 from
51 blood and 284 from cerebrospinal fluid). We identified 56 serotypes in the cohort. While most serotypes
52 exhibited a similar prevalence in both carriage and disease groups, serotypes 1 and 5, the most
53 abundant serotypes in the entire cohort, were significantly more commonly detected in specimens from

patients compared to the carriage group. This difference is presumably due to their shorter nasopharyngeal colonization period. Furthermore, these serotypes displayed genetic distinctiveness from other serotypes. A magnificent genetic difference was observed in the absence of genes from the RD8a genomic island in serotypes 1 and 5 compared to significantly prevalent serotypes in the nasopharynx. RD8a genes play pivotal roles in binding to epithelial cells and performing aerobic respiration to synthesize ATP through oxidative phosphorylation. The absence of RD8a from serotypes 1 and 5 may be associated with a shorter duration in the nasopharynx, theoretically due to a reduced capacity to bind to epithelial cells and access free oxygen molecules required for aerobic respiration (essential to maintain the carriage state). Serotypes 1 and 5, significantly harbor operons that encode phosphoenolpyruvate phosphotransferase systems, which might relate to transporting carbohydrates, relying on phosphoenolpyruvate as the energy source instead of ATP. In conclusion, serotypes 1 and 5 as the most prevalent invasive pneumococcal strains in Malawi, displayed considerable genetic divergence from other strains, which may offer insights into their invasiveness and potential avenues for further research.

Author summary

Despite introducing the pneumococcal conjugate vaccine in 2011, *Streptococcus pneumoniae* remains a major cause of bacterial infection in Malawi. Whilst some pneumococcal strains harmlessly colonize the nasopharynx, others find their way into normally sterile sites, such as lungs, blood, and nervous system, resulting in serious illness. Our study identified specific pneumococcal serotypes as the most invasive in Malawi, characterized by a short colonization period and significant genetic distinctiveness from other strains. This genetic divergence notably included the absence of several genes associated with aerobic respiration and the presence of genes facilitating ATP-independent carbohydrate transport. The presence or absence of these genes may underlie their heightened invasiveness and shorter colonization period. This hypothesis positions these genes as potential candidates for future therapeutic research.

78 We propose that the specific gene gain and/or loss in invasive versus other serotypes may be linked to
79 the development of invasive pneumococcal diseases.

80 Introduction

81 *Streptococcus pneumoniae,* also known as *pneumococcus*, is a Gram-positive, facultatively anaerobic
82 bacteria and is one of the leading causes of mortality worldwide. Despite reductions in the incidence of
83 pneumococcal disease in countries that introduced pneumococcal conjugate vaccines (PCV), the
84 pneumococcal mortality rate is still high. Pneumococci are estimated to be responsible for 317,300
85 deaths in children aged 1 to 59 months worldwide in 2015 [1]. In the post-PCV era, a high disease
86 burden has still been reported in low-income countries in Africa, such as Malawi [2].

87 Although pneumococcal nasopharyngeal colonization is asymptomatic, it is a prerequisite for
88 transmission and disease development [3][4]. Symptoms appear when isolates from the nasopharynx
89 spread to normally sterile sites such as the lung, blood, and central nervous system. Depending on the
90 infected organ, *S. pneumoniae* can cause two types of infection: (i) non-invasive (mucosal)
91 pneumococcal diseases such as otitis media and sinusitis, and (ii) invasive pneumococcal diseases (IPD)
92 such as bacteremia and meningitis. IPD incidence is highest among infants, the elderly, and
93 immunosuppressed people, most likely due to their less efficient immune systems [5].

94 Pneumococci possess several virulence factors, including the polysaccharide capsule, surface proteins,
95 and enzymes [6][7]. The polysaccharide capsule is the most important virulence factor as it aids the
96 *pneumococcus* in evading the immune response during colonization and invasion [8]. Its biosynthesis is
97 regulated by a cluster of genes in the capsular polysaccharide (*cps*) locus [8][9]. Pneumococcal serotypes
98 are defined by the type and order of monosaccharides that compose the capsule structure. To date, one
99 hundred pneumococcal serotypes have been identified [10]. Each strain has a set of capsular synthesis
100 genes in the *cps* locus that determine its serotype. Immunogenic properties of the capsular
101 polysaccharide were utilized to develop all pneumococcal vaccines in use, including PCV7, PCV10, and

102 PCV13 that cover 7, 10, and 13 serotypes, respectively. PCV13 includes the following serotypes: 1, 3, 4,
103 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, and 23F. Although the introduction of PCVs has significantly reduced
104 the burden of disease caused by vaccine types (VTs), serotype replacement has increased the non-
105 vaccine types (NVTs) carriage rate and IPD incidence [11][12].

106 In November 2011, PCV13 was introduced in Malawi, which markedly decreased the health system
107 burden and rates of severe childhood pneumonia [13]. A case-control study in Malawi showed vaccine
108 effectiveness against VT-IPD of 80·7% [14]. A nasopharyngeal carriage survey conducted in the Karonga
109 district showed that although the vaccine reduced the VT colonization rate, a moderate level of serotype
110 replacement was observed among carriers [15]. Moreover, the emergence of antibiotic-resistant
111 pneumococci due to the overuse of antibiotics is a global concern in the 21st century [16]. To develop
112 new, more effective vaccines against the vaccine-escape clones and design effective drugs against
113 antibiotic-resistant strains, it is critical to understand the functions of genes involved in pneumococcal
114 colonization and pathogenesis. During the past decade, the evolution of high-throughput sequencing
115 technologies has generated enormous amounts of genomic data that have enabled researchers to
116 perform large-scale genomic analysis. A well-known example is pan-genome studies. The pan-genome is
117 the entire gene set in a collection of closely related strains within a specie [17]. Determining the genetic
118 drivers is an active and promising area of research that can provide insights into pneumococcal disease
119 prevention and treatment to reduce mortality rates. The pan-genome is useful for analyzing
120 recombinogenic pathogens such as *pneumococcus* [18]. The recombination level is high in thirteen
121 pneumococcal genomic loci known as regions of diversity (RDs) numbered from RD1 to RD13, some of
122 which are involved in virulence [19][20].

123 In this study, we conducted whole-genome sequencing (WGS) on 1477 pneumococcal samples from
124 residents of Blantyre, Karonga, and Lilongwe in Malawi. Our study aims to: (i) identify serotype
125 distribution, (ii) characterize the pneumococcal population structure, and (iii) identify potential driver
126 genes for invasion and their biological functions.

127 # Materials and methods

128 ## Study design and sample collection

129 The study utilized archived samples maintained by the Malawi-Liverpool Wellcome Trust Clinical
130 Research Programme (MLW). Samples were collected from individuals residing in three distinct regions
131 of Malawi: Blantyre in the south, Karonga in the north, and Lilongwe in the central part of the country.
132 This cohort included isolates obtained from both asymptomatic carriers and symptomatic patients.

133 Carriage samples were collected from the nasopharynx of healthy individuals as part of the Health and
134 Demographic Surveillance System in Karonga and Blantyre. The collection process involved the use of
135 nasopharyngeal swabs. Subsequently, pneumococcal isolates were identified utilizing a previously
136 established protocol [21]. Briefly, the identification method involved culturing isolates on blood agar
137 supplemented with gentamicin, with further confirmation relying on optochin disc-based assays,
138 scrutinizing colony morphology, alpha-hemolysis, and optochin susceptibility, adhering to established
139 norms and practices for pneumococcal isolates. To account for the true diversity of carriages, only a
140 single isolated colony was sequenced and serotyped, therefore no carriage samples included in this
141 study had multiple serotypes.

142 Invasive pneumococcal samples were also sourced from archived bacterial isolates at MLW, which had
143 been collected from the blood and cerebrospinal fluid (CSF) of symptomatic patients attending Queen
144 Elizabeth Central Hospital in Blantyre and Kamuzu Central Hospital in Lilongwe. Notably, the selection of
145 isolates for this group was blind to their serotypes, ensuring an accurate representation of their
146 prevalence in the disease group without any influence from serotype inclusion criteria. These isolates
147 were subsequently streaked onto blood agar plates supplemented with gentamicin, and optochin tests
148 were conducted, according to the procedures outlined in reference [22].

149

150 It's important to note that this study did not involve paired samples. During data collection, each
151 individual contributed only one sample, which was either a nasopharyngeal swab from healthy
152 individuals or a blood or CSF sample from symptomatic patients. In the context of this study, the term
153 'sterile sites' refers to blood and CSF. The term 'invasive samples' specifically refers to those samples
154 obtained from these sterile sites (blood and CSF).

155 **Whole-genome sequencing and quality control**

156 Archived samples were sequenced under the Global Pneumococcal Sequencing project and
157 Pneumococcal African Genomic Consortium at the Wellcome Trust Sanger Institute in the United
158 Kingdom. Bacterial DNA was extracted using the QIAamp DNA mini kit and QIAgen Biorobot by QIAGEN.
159 Whole-genome sequencing was conducted on Illumina Genome Analyzer II and HiSeq platforms,
160 producing 125 nucleotide paired-end reads. Read quality was assessed using Fastqc [23].

161 **In-silico serotyping, sequence typing, and quantification of serotype invasiveness**

162 SeroBA version 1.23.4 was employed to infer the serotype of the samples [24]. SeroBA applies a k-mer
163 method to determine serotypes directly from the paired-end reads in FASTQ format. Any serotype with
164 a relative frequency greater than 5% was categorized as an abundant serotype. To identify serotypes
165 with a significant presence in the nasopharynx and sterile sites, Fisher's exact test was applied. P-values
166 were adjusted using the Benjamini-Hochberg method, and serotypes with an adjusted p-value less than
167 0.01 were considered significant. The odds ratio (OR) was calculated as follows: OR = (ad)/(bc), where 'a'
168 represents the number of invasive serotype k, 'b' is the number of carriage serotype k, 'c' is the number
169 of invasive non-serotype k, and 'd' is the number of carriage non-serotype k. Zero values were replaced
170 by 0.5 in OR calculations. Abundant serotypes with a significant presence in the nasopharynx were
171 considered to have low invasiveness, while abundant serotypes with a significant presence in sterile
172 sites were considered to have high invasiveness. Fisher's exact test was also used to identify serotypes

173 whose frequencies changed significantly after the introduction of PCV13 in 2011 (adjusted p-value <
174 0.01).

175 **Genome assembly and annotation**

176 Genomes were assembled using Velvet Optimiser version 2.2.5 [25] with settings to generate contigs
177 longer than 500 base pairs, employing a hash range from 61 to 119. The quality of the assembled
178 genomes was assessed using Quast version 5.2 [26], and annotation was performed with Prokka version
179 13.1 [27].

180 **Pan-genome construction**

181 The pan-genome for the samples was generated using Roary version 3.12.0 [28]. Roary was run to
182 perform the core gene alignments with Mafft version 7.313 [29]. Genes in the pan-genome were
183 categorized into three groups based on their abundance among samples: genes present in 100% of
184 samples were designated as core genes, those in more than 95% but not core were termed soft-core
185 genes, and the remaining genes were considered accessory genes.

186 **Analysis of the population structure**

187 Small-scale variations, such as single nucleotide polymorphisms (SNPs) and short indels, within the core
188 genes were analyzed to understand population diversity. A phylogenetic tree, illustrating the genetic
189 separation between samples, was constructed using SNPs and indels in the core gene alignment as
190 phylogeny markers. The core genome alignment served as input for IQ-TREE version 2 [30] to generate a
191 phylogenetic tree, which was visualized using iTol version 3 [31].

192 Diversity in the accessory genome manifests as large-scale gene presence-absence variations. The R
193 package Nonnegative Matrix Factorization [32] was used to create a gene presence-absence heatmap
194 from the pan-genome matrix. To determine the factors influencing gene distribution, including isolation
195 sites (nasopharynx, blood, and CSF), serotypes, geographical locations (Blantyre, Karonga, and

196 Lilongwe), and vaccination eras, a principal component analysis (PCA) of gene distribution was
197 conducted using the R package MixOmics version 6.20.0 [33].

198 **Gene presence-absence statistical analysis**

199 Phenotypic traits of samples were assigned based on population structure and invasiveness. To identify
200 putative virulence factors, a gene presence-absence statistical test was conducted using Scoary version
201 1.6.1643 [34]. This tool scores the components of the pan-genome for associations with observed
202 phenotypic traits while accounting for population stratification. The test was conducted across samples
203 from different sources and serotypes to find putative virulence factors. Genes with a Bonferroni-
204 corrected p-value less than 0.05 were deemed significant.

205 **Functional and gene ontology (GO) enrichment analysis**

206 The list of significant genes was submitted to STRING webtool version 11.5 [35] for functional
207 enrichment analysis. STRING is a network that integrates information from various protein-protein
208 interaction databases, predicting both direct (physical) and indirect (functional) interactions between
209 proteins from five sources, including genomic context predictions, lab experiments, co-expression,
210 automated text mining, and previous knowledge in databases. Functional enrichment analysis in STRING
211 utilizes information from classification systems such as the Kyoto Encyclopedia of Genes and Genomes
212 (KEGG) [36] and the Protein families database (Pfam) [37]. The tool entitled "Multiple Sequences" was
213 selected, and the *S. pneumoniae TIGR4* was chosen as the reference organism. STRING reports the
214 associated enriched pathways with a false discovery rate of less than 0.05.

215 **Results**

216 In total, 825 isolates from the nasopharynx of healthy carriers, 368 isolates from the blood of
217 bacteremia patients, and 284 isolates from the CSF of meningitis patients were sequenced. The
218 demographics of the samples are shown in Table 1.

Table 1. Demographics of 1477 pneumococcal isolates collected from Malawi.

Characteristics	Categories	Nasopharynx	Blood	CSF
Age (in years)	< 5	538	165	141
	5-19	109	42	60
	20-40	60	67	50
	> 40	7	24	7
	Unknown	111	70	26
Sex	Female	401	131	111
	Male	313	122	122
	Unknown	111	115	51
City	Blantyre	169	357	259
	Karonga	656	0	0
	Lilongwe	0	0	23
	Unknown	0	11	2
Sampling period		2009-2014	1997-2015	2000-2015

Serotypes 1, 5, and 12F had the highest invasiveness, likely with a short period of nasopharyngeal colonization.

Altogether, we identified isolates belonging to 56 different serotypes. Irrespective of their isolation sources, serotypes 1 (8.7%), 5 (7.8%), 6B (6.6%), 23F (6.3%), and 19F (5.5%) were the most prevalent, each accounting for over 5% of the entire cohort. Of the samples, 66% were collected prior to the introduction of PCV13 in 2011, while 27% were obtained in the post-PCV13 era (S1 Fig). In the pre-PCV13 era, prominent serotypes with frequencies exceeding 5% included 5 (10.21%), 6B (8.72%), 1 (8.4%), 23F (7.23%), 6A (5.74%), and 16F (5.53%). In the post-PCV13 era, serotypes 1 (11.5%) and 12F (5.4%) predominated. It is worth noting that serotype 1 exhibited sustained dominance, with its frequency increasing following the vaccine rollout, while serotype 12F emerged as an abundant strain after 2011 (S2 Fig). Nevertheless, a more extensive dataset encompassing vaccination information could offer further insights into this phenomenon.

231 Within the carriage isolates, serotypes exhibited abundant frequencies, included 19F (7.88%), 6B
232 (7.27%), 16F (6.79%), and 23F (5.21%), with each surpassing a 5% frequency threshold. The distribution
233 of serotypes among carriers in Blantyre and Karonga largely mirrored each other, with the exception of
234 serotype 13, which displayed higher prevalence in Blantyre, and serotype 6B, which exhibited greater
235 dominance in Karonga (S3 Fig).

236 Among the blood samples, serotype 5 (20.38%) was the most dominant, followed by 1 (16.58%), 23F
237 (8.42%), and 6B (5.98%). In the cerebrospinal fluid (CSF) samples, serotypes 1 (21.13%), 5 (8.1%), 12F
238 (7.04%), 23F (6.69%), 6A (5.63%), and 6B (5.28%) predominated. When considering the combined blood
239 and CSF groups, the most frequently observed serotypes were 1 (18.56%), 5 (15.03%), 23F (7.67%), 6B
240 (5.67%), and 6A (5%). It's noteworthy that the majority of invasive samples were collected in Blantyre
241 (96.5%). The invasive samples from Lilongwe were exclusively CSF samples, with serotypes 1 and 12F
242 being dominant (S4 Fig).

243 As depicted in Fig 1 and detailed in Supplementary Table 1 (S1 Table), among the serotypes that were
244 abundant in either the blood or CSF, serotypes 1 ($p = 1.96E-34$), 5 ($p = 3.97E-19$), and 12F ($p = 5.29E-06$)
245 exhibited a significant presence among patients, with a low frequency of occurrence in the nasopharynx.
246 Given that the colonization phase is a prerequisite for infection, it is plausible that serotypes 1, 5, and
247 12F may have a short period of nasopharyngeal colonization before infecting sterile sites. Considering
248 that serotypes 1 and 5 were also the most prevalent across the entire cohort, they could be regarded as
249 the most common serotypes with the highest invasiveness. In this study, we categorize serotypes 1, 5,
250 and 12F as 'significant invasive serotypes' or 'hyper-invasive serotypes'.

251 In contrast, abundant serotypes 16F and 19F in the nasopharynx were significantly prevalent among
252 carriers, suggesting that they may have a lower potential to cause invasive disease. Other frequently
253 detected serotypes, such as 6A, 6B, and 23F, were both abundant and evenly distributed among carriers
254 and patients. It is conceivable that they might require a longer period of nasopharyngeal colonization

255 compared to the hyper-invasive serotypes (1, 5, and 12F) before causing infections at sterile sites.

256 Serotypes 6A, 6B, and 23F have previously been reported as common and abundant serotypes among

257 both non-invasive carriers and those with invasive infections [38] [39].

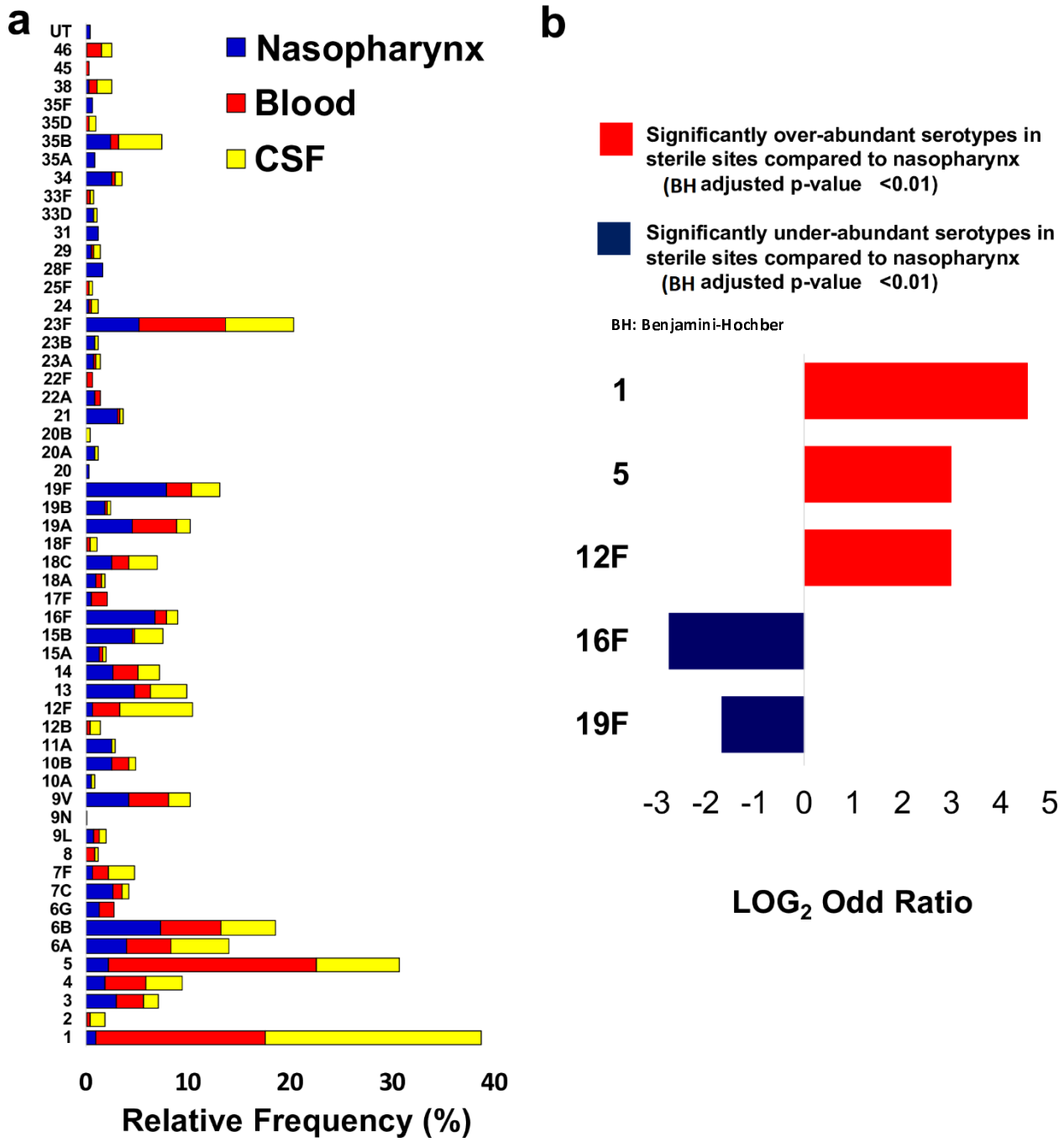

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Fig 1. The distribution of the 56 pneumococcal serotypes assigned to 1477 samples from Malawi. (a) The relative frequency of each serotype in the nasopharynx of carriers, the blood of bacteremia patients, and the CSF of meningitis patients is shown in blue, red, and yellow, respectively (UT: Un-Typeable). (b) The log-transformed odds ratio of the significantly over- and under-abundant serotypes in

the sterile sites (blood and CSF). Fisher's exact test was applied to identify serotypes with a significant differential abundance among carriers and patients (nasopharynx and sterile sites) at the significance level of the Benjamini-Hochberg adjusted p-value < 0.01 (BH: Benjamini-Hochberg).

259 As mentioned earlier, the temporal distribution of the hyper-invasive serotypes (1, 5, and 12F)
260 concerning the vaccine rollout timeline was noteworthy. The relative frequency of serotype 1 exhibited
261 a significant increase (pre-PCV13=8.6% and post-PCV13=11.3%), while serotype 5 displayed a significant
262 decrease (pre-PCV13=11% and post-PCV13=1.5%) following the introduction of the vaccination program
263 in Malawi. This suggests that vaccination may be effective against serotype 5 but did not alleviate the
264 burden of invasive pneumococcal diseases (IPDs) caused by serotype 1. Additionally, serotype 12F,
265 which is not included in PCV13, showed a significant increase (pre-PCV13=1.1% and post-PCV13=5.7%)
266 in the post-PCV13 era, indicating its potential emergence as an invasive strain. Nevertheless, a more
267 extensive dataset, containing more recent samples, is essential to comprehensively characterize the
268 long-term effects of PCV13.

269 **High diversity in the pneumococcal pan-genome**

270 The genome assembly produced an average optimized assembly hash value of 96 and an average N50 of
271 113,986. The mean assembled genome size was estimated to be 2,116,779 nucleotides, with a standard
272 deviation of 106,481. This length aligns within the range of previously reported *S. pneumoniae* genome
273 sizes [40].

274 The pan-genome spanned 5,178,167 base pairs and encompassed 6,803 genes, comprising 729 core
275 genes (10.7%), 820 soft-core genes (12.1%), and 5,254 accessory genes (77.2%). The pan-genome
276 remained open, demonstrating a continuous increase in the number of genes as the sample size
277 expanded (S5 Fig). The gene presence-absence heatmap in Fig 2 illustrates the pan-genome, revealing
278 serotypes as the primary factor influencing gene distribution. Notably, distinct clustering was observed
279 for hyper-invasive serotypes 1, 5, and 12F, forming unique clades. Specific sets of genes present in
280 different serotypes were represented as distinctive blue blocks within the heatmap.

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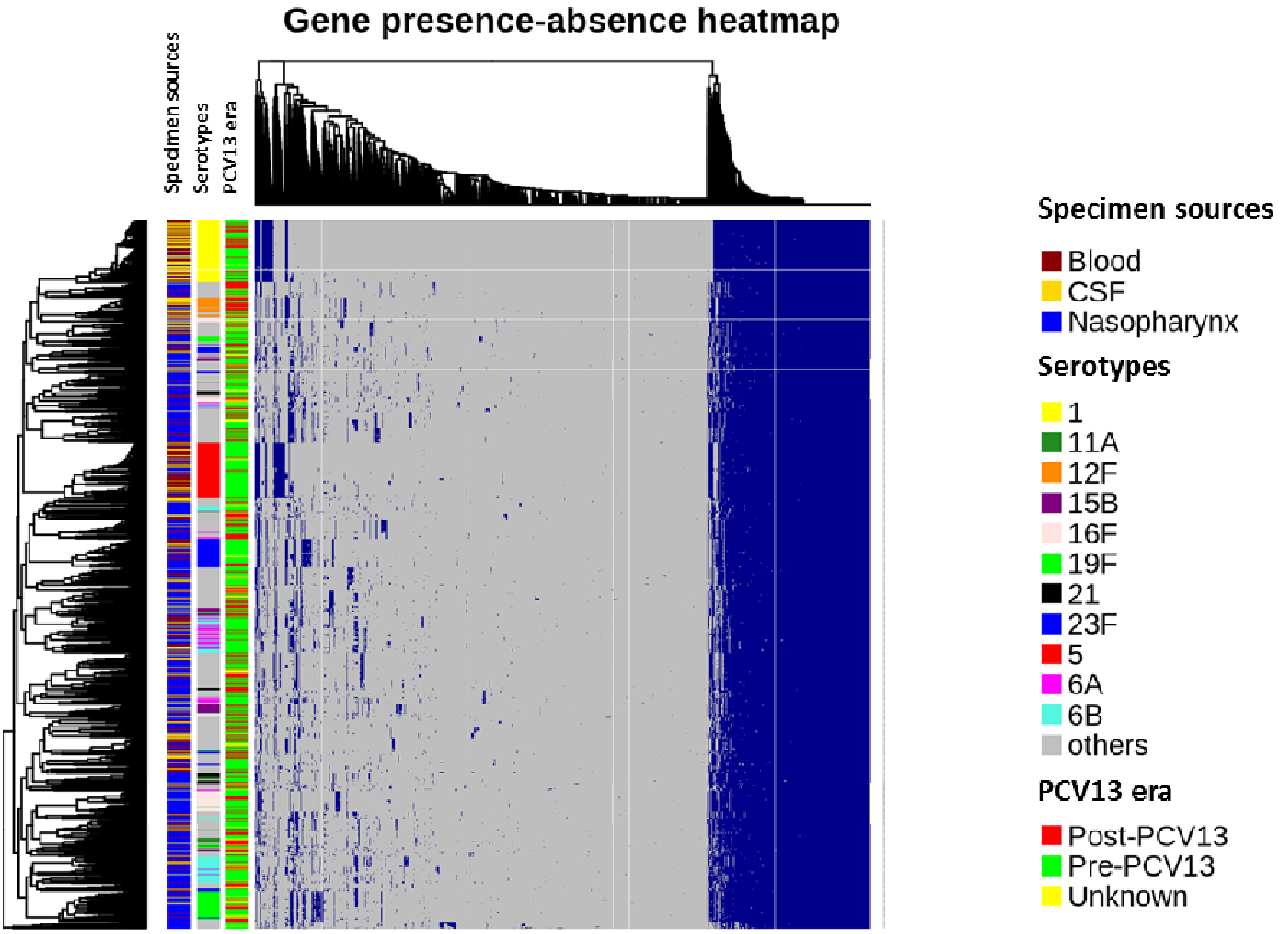

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Fig 2. The pan-genome matrix of 1477 pneumococcal isolates from Malawi. The pan-genome is visualized as a gene presence-absence heatmap representing the hierarchical unsupervised clustering of samples based on the distribution of genes in the pan-genome. Each row is a sample, and each column is a gene. A blue dot denotes the presence of each gene. On the right side of the heatmap, the large blue block shows core genes present in all samples. The left side of the heatmap represents the accessory genome along with the clustering bands. In addition to the significant serotypes 1, 5, 12F, 16F, and 19F, other abundant serotypes, including 6A, 6B, and 23F, as well as serotypes with source-based p-value < 0.05, including 21, 11A, and 15B, are also highlighted on the heatmap.

283 **The significant invasive serotypes (1, 5, and 12F) showed the highest distinction in the core-**

284 **and accessory-genome**

285 The maximum-likelihood tree, depicting the distribution of small-scale variants (SNPs and indels) within

286 the core-genome, highlighted the hyper-invasive serotypes 1, 5, and 12F as monophyletic clusters (Fig

287 3). These serotypes (1, 5, and 12F) were distinct, forming individual clusters that were more prominently

288 separated compared to other abundant serotypes, such as 6B, 19F, and 23F, which appeared as multiple

289 clusters on the phylogenetic tree (see Fig 3)

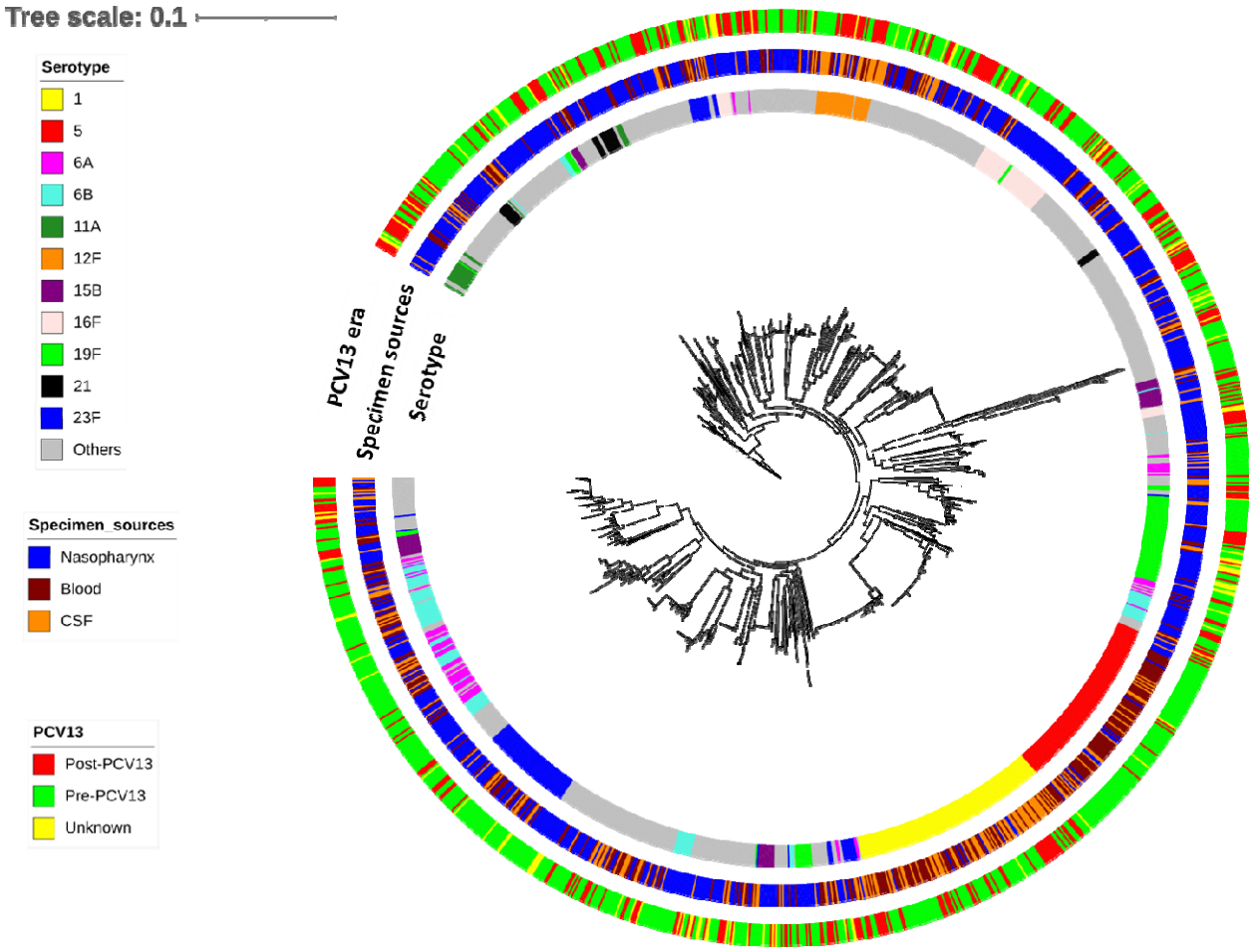

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Fig 3. The phylogenetic population structure of 1477 pneumococcal samples from Malawi. The phylogenetic tree was built based on the multiple sequence alignment of the core genome using the maximum likelihood method. Colors on the loops show the serotypes, specimen sources (isolation sites), and PCV13 eras. In addition to the significant serotypes 1, 5, 12F, 16F, and 19F, other abundant serotypes, including 6A, 6B, and 23F, as well as serotypes with source-based p-value < 0.05, including 21, 11A, and 15B, are also highlighted on the tree.

291 The PCA of the large-scale variants in the accessory-genome (gene presence/absence) displayed

292 serotypes 1 and 5 as distantly clustered from other strains (Fig 4). Additionally, a moderate level of

293 separation was evident for serotypes 12F, 19F, and 23F. This distinct clustering of serotypes 1 and 5 is

294 aligned with profiles demonstrated by the phylogenetic tree.

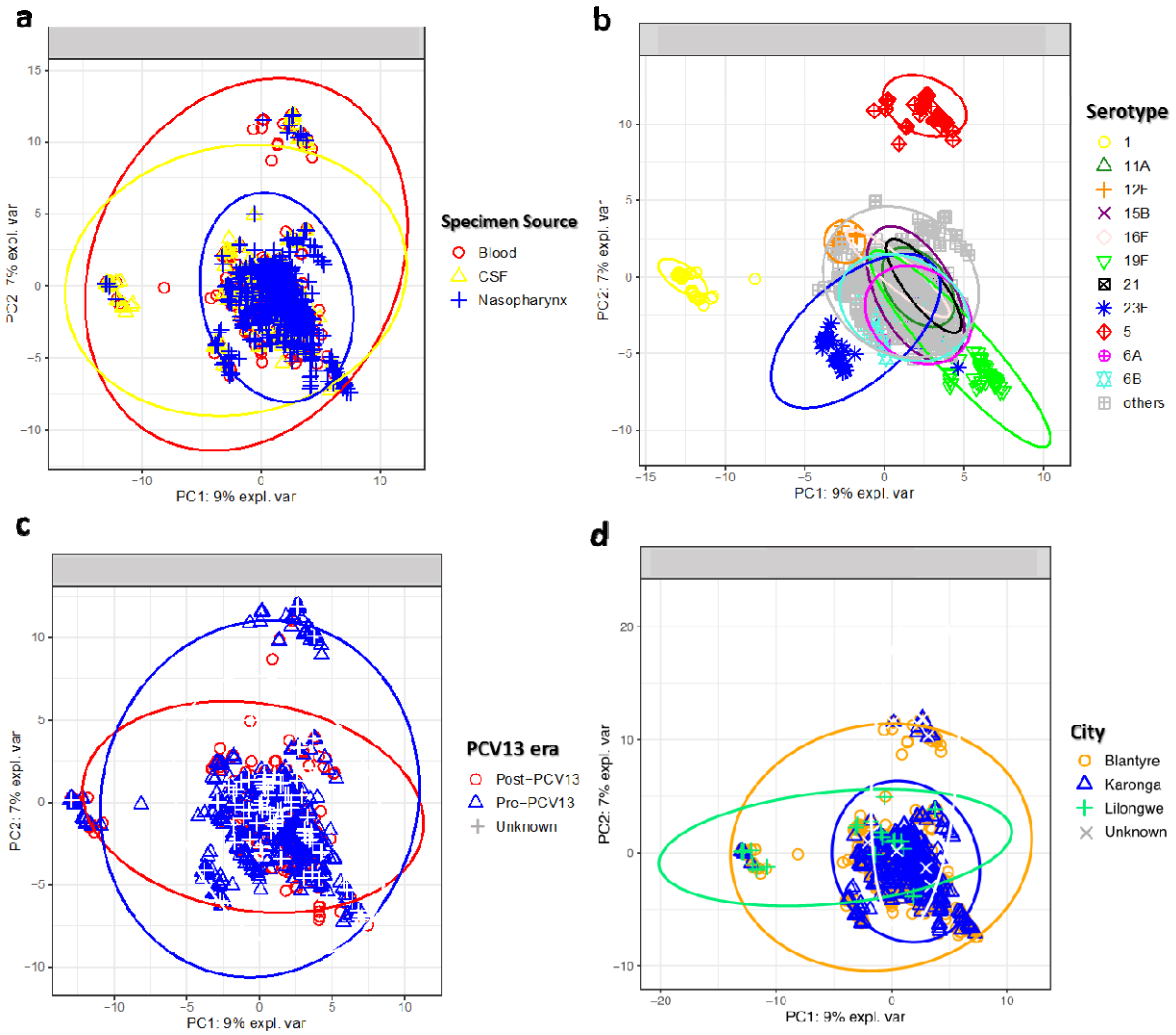


Fig 4. The PCA of the gene distribution in the pan-genome of pneumococcal isolates from 1477 Malawians. The PCA of variants (gene presence-absence) in the accessory-genome indicates the influence of (a) specimen sources (isolation sites), (b) serotypes, (c) PCV13 (vaccination) era, and (d) geographical locations on the gene presence-absence profile of pneumococcal isolates in Malawi. Serotypes 1 and 5 were clearly separated from other samples.

Figures 3 and 4 indicate that factors such as time, locations, and isolation sites (specimen sources) failed to sufficiently explain the small- and large-scale genetic variants in the pan-genome. Instead, the serotype of the samples emerged as the primary driver of the population structure. The hyper-invasive serotypes (1, 5, and 12F) exhibited the most significant core and accessory distances from other strains, signaling their genetic distinctiveness among disease-associated serotypes. This heightened genetic dissimilarity might be linked to their invasive potential.

302 It's crucial to thoroughly consider population structure and assess any associations with disease across
303 the population. An important observation is the absence of the same level of genetic distinction in
304 serotype 12F, potentially due to its smaller sample count (n=35) compared to serotypes 1 (n=129) and 5
305 (n=116). Additionally, the near-complete separation of serotypes 1 and 5 from the others may primarily
306 reflect their infrequent presence in the carriage group, suggesting that these patterns might stem more
307 from sampling biases than genuine genetic variations. To address these concerns, ten samples from
308 each hyper-invasive serotype and the PCV13 vaccine types were randomly selected from the
309 nasopharynx, blood, and CSF. A PCA of the gene distribution was performed on the downsampled
310 dataset, reiterated a noticeable pattern of clustering evident for serotypes 1, 5, and 12F each positioned
311 far from other strains (S6 Fig).

312 Another aspect to consider revolves around the differentiation of serotypes. Theoretically, serotypes are
313 differentiated due to their distinct serotype-defining capsule genes. However, a question emerges
314 regarding the notable distinction in clustering observed among hyper-invasive serotypes (1, 5, and 12F)
315 compared to other serotypes. Our investigation focused on the hypothesis that serotypes 1, 5, and 12F
316 might have undergone gene acquisitions or losses, potentially contributing to their invasiveness. While
317 there are other prevalent serotypes in blood and CSF, such as 6B and 23F, their similar prevalence in the
318 nasopharynx suggests they might persist in the nasopharynx for extended durations compared to the
319 hyper-invasive serotypes (1, 5, and 12F).

320 **The gene presence-absence statistical analysis**

321 The following issues could skew the gene presence-absence analysis:

322 a) **The batch effect introduced by geographical locations:** 85% of carriage samples were from
323 Karonga, whereas 95% of disease samples were from Blantyre (Table 1). A comparison between
324 carriage and disease groups may only identify the difference between pneumococcal genomes
325 from two geographical locations rather than between the non-invasive and invasive groups.

326 b) **Study limitation:** The likely presence of invasive serotypes in the carriage group made it unclear
327 which nasopharyngeal samples progressed to disease after collection. Indeed, the carriage
328 population likely contained invasive serotypes that could bias the test between carriage and
329 disease samples to identify potential virulence genes.
330 c) **Population structure:** The significant abundance of the hyper-invasive serotypes (1, 5, and 12F)
331 in the patient group and their highest genetic distinction would skew the test between the
332 carriage and patient groups. The difference between carriage and disease groups would actually
333 be the difference between the carriage and hyper-invasive serotypes (and not all strains in the
334 blood and CSF).

335 To assess the geographical batch effect, carriage isolates from Karonga were compared with those from
336 Blantyre. The gene presence-absence statistical test did not identify any significant genes differing
337 between the Blantyre and Karonga groups, indicating a similarity in gene content within the carriage
338 samples from both locations. Additionally, as previously observed, the serotype distributions in the
339 carriage groups from Karonga and Blantyre displayed similarities (S3 Fig). Consequently, the impact of
340 geographical location on the pneumococcal genomes was not substantial. This outcome aligns with
341 expectations considering that Karonga and Blantyre are approximately 830 kilometers apart, and the
342 demographic similarities between the populations in these cities.

343 While serotypes like 6A, 6B, and 23F, potentially associated with invasive traits, were found in both
344 carriers and patients, comparing the entirety of the carriage and patient groups remains important. This
345 is because serotypes identified as potential invasive in the nasopharynx might undergo genomic
346 alterations before reaching sterile sites. During the colonization phase in the nasopharynx, these
347 serotypes likely engage in genetic exchange via recombination and horizontal gene transfer with other
348 pneumococci or bacterial species. Therefore, the genomic profile of an invasive serotype in the
349 nasopharynx might differ from that in the blood and CSF.

350 To account for these complexities, our analysis involved an association test between the entire carriage
351 and disease groups, excluding the hyper-invasive serotypes 1, 5, and 12F (a location-based analysis). This
352 exclusion aimed to prevent these hyper-invasive serotypes from introducing biases when comparing the
353 carriage and patient groups. The location-based analysis identified 27 significant genes, including 11
354 genes significantly present in the blood and CSF and 16 genes in the nasopharynx (Table 2, Table 3, and
355 S2 Table for further details)

356 The most significant genes identified in both blood and CSF belonged to the cps locus (RD3), suggesting
357 a potentially increased level of encapsulation during disease. Specifically, genes SP_0357, SP_0358, and
358 SP_0360 encode epimerases involved in the biosynthesis of complex lipopolysaccharides, which are
359 essential components of the pneumococcal capsule. SP_0351 encodes a membrane protein
360 glycosyltransferase responsible for catalyzing glycosyl group transfer during capsule synthesis, and
361 SP_0359 encodes UDP-2-acetamido-2,6-beta-L-arabino-hexul-4-ose reductase, a crucial protein involved
362 in capsular polysaccharide biosynthesis. Other significant genes in the blood and CSF, including SP_1953,
363 SP_0535, SP_1037, and SP_1056, are involved in toxic secretion and recombination. SP_1056 is part of
364 the pneumococcal pathogenicity island 1 (PPI1) located within RD6. This gene encodes a mobilization
365 protein necessary for the horizontal transfer of genes and plasmids via bacterial conjugation. SP_1056
366 plays a role in forming the relaxation complex or relaxosome by interacting with other enzymes [41].

Table 2. Significant genes (p-value < 0.05) present in pneumococci in the blood and CSF (hyper-invasive serotypes 1, 5, and 12F were excluded) compared to the nasopharyngeal pneumococci.

ID	Annotation	P-value	Odds ratio
SP_0360	Capsular polysaccharide biosynthesis protein (from RD3)	8.17E-05	4.897059
SP_0358	Capsular polysaccharide biosynthesis protein (from RD3)	8.17E-05	4.897059
SP_0351	Capsular polysaccharide biosynthesis protein (from RD3)	8.17E-05	4.897059
SP_0359	Capsular polysaccharide biosynthesis protein (from RD3)	8.17E-05	4.897059
SP_0357	Capsular polysaccharide biosynthesis protein	0.000175	4.758421
SP_1037	Type II restriction endonuclease BcgI	0.001074	2.878307
SP_1953	Bacteriocin/lantibiotic secretion ABC transporter permease protein	0.001864	4.631892
SP_0535	Putative immunity protein	0.004674	1.988523
SP_1056	Relaxase/Mobilisation nuclease domain (From RD6)	0.012604	2.932153
SP_1656	Hypothetical protein	0.014050	1.963212
SP_0347	Capsular polysaccharide biosynthesis protein (from RD3)	0.020560	1.808997

367 Significant genes identified in the nasopharynx (absent in samples from blood and CSF) originated from
368 RD10, recognized as the SecY2A2 island responsible for the secretion of pneumococcal serine-rich
369 repeat protein (PsrP) (S7 Fig) [42]. RD10 contains several glycosyltransferases and secretory components
370 that were significantly missing from the genome of samples obtained from blood and CSF.

Table 3. Significant genes (p-value < 0.05) present in the nasopharyngeal pneumococci compared to pneumococci in the blood and CSF (hyper-invasive serotypes 1,5, and 12F were excluded).

ID	Annotation	P-value	Odds ratio
SP_1770	Glycosyl transferase, glyB (from RD10)	0.000132	0.502513
SP_1771	Glycosyl transferase, family 2/family 8 (from RD10)	0.012770	0.560236
SP_1763	Preprotein translocase secY family protein (from RD10)	0.017254	0.563256
SP_1765	Glycosyl transferase, glyF (from RD10)	0.018150	0.564542
SP_0939	Hypothetical protein	0.020740	0.483266
SP_1766	Glycosyl transferase, glyE (from RD10)	0.020740	0.483266
SP_1767	Glycosyl transferase, glyD (from RD10)	0.023196	0.568879
SP_1762	Accessory secretory protein asp1 (from RD10)	0.023196	0.568879
SP_1761	Accessory secretory protein asp2 (from RD10)	0.023196	0.568879
SP_1760	Accessory secretory protein asp3 (from RD10)	0.023196	0.568879
SP_1755	Hypothetical protein	0.031054	0.558554
SP_1757	Glycosyl transferase, glyB (from RD10)	0.031054	0.558554
SP_1764	Glycosyl transferase, glyG (from RD10)	0.023196	0.568879
SP_1768	Conserved hypothetical protein (from RD10)	0.031054	0.558553
SP_1758	Poly(glycerol-phosphate) alpha-glucosyltransferase, tagE (from RD10)	0.031122	0.571941
SP_1759	Preprotein translocase, secA subunit (from RD10)	0.031321	0.574557

371 To explore the divergence of the hyper-invasive serotypes (1, 5, and 12F), they were compared to
372 serotypes 16F and 19F, which were significantly present in the nasopharynx (serotype-based analysis).
373 Serotypes 16F and 19F could represent non-invasive strains better than the whole nasopharyngeal
374 population that potentially contained some invasive serotypes. Indeed, it was a test between serotypes
375 with the highest and lowest invasiveness to characterize the genomes of serotypes 1, 5, and 12F. The
376 gene gain and loss profiles in the hyper-invasive serotypes may include components contributing to their
377 virulence and short colonization period.

378 The serotype-based analysis identified 184, 157, and 186 significant genes (present/absent) in serotypes
379 1, 5, and 12F, respectively (S3 Table, S4 Table, and S5 Table) that were much larger than the number of
380 significant genes identified by the location-based analysis. The functional enrichment analysis identified
381 the phosphotransferase system (PTS, KEGG ID: spn02060) as over-represented and oxidative
382 phosphorylation (KEGG ID: spn00190) as under-represented pathways in the hyper-invasive serotypes
383 1, 5, and 12F (p-value < 0.05).

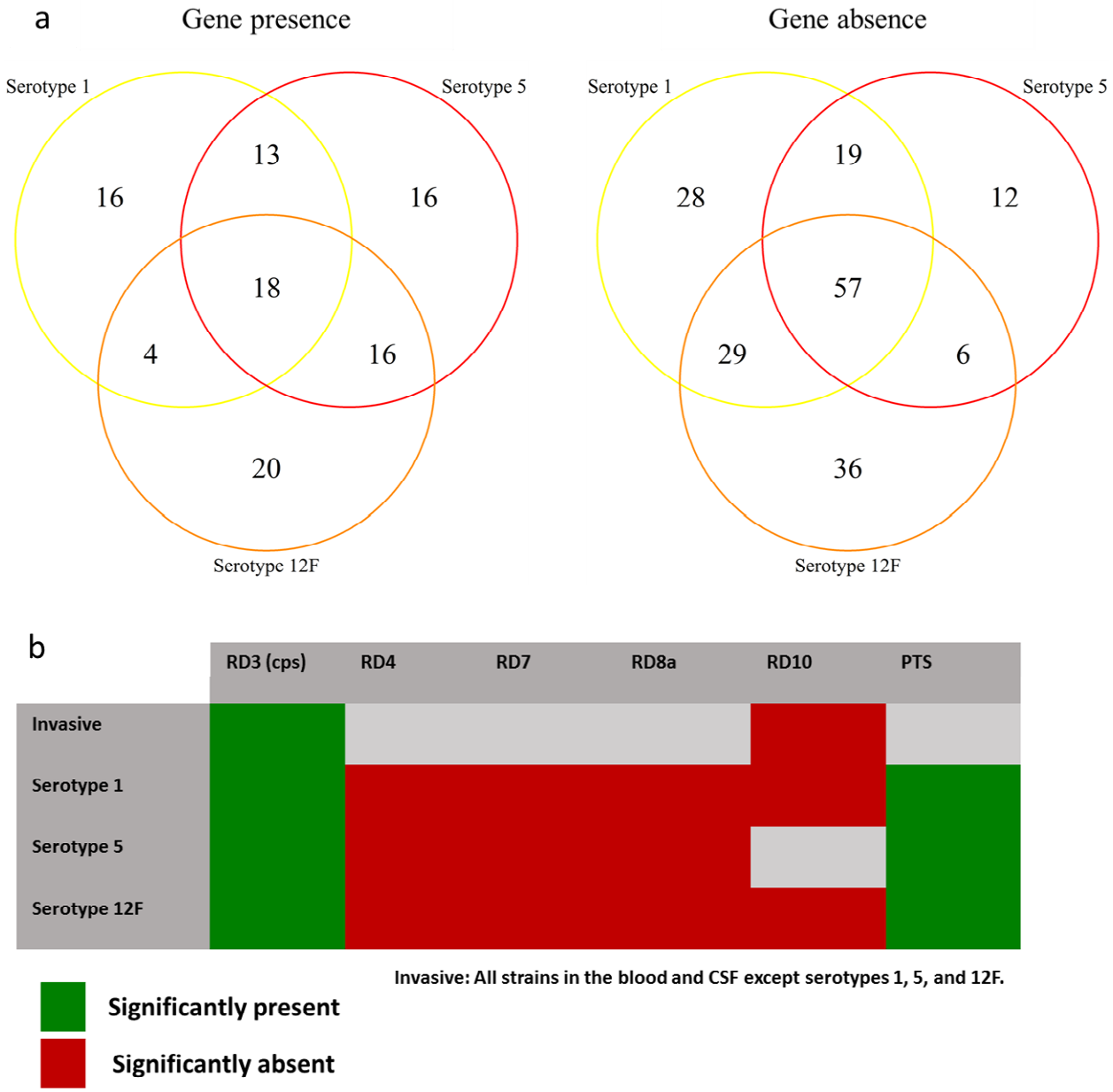
384 In total, there were 18 significant genes jointly present in the hyper-invasive serotypes (Fig 5.a),
385 including elements of the PTSs that transport sucrose and lactose across the membrane (SP_0302,
386 SP_0303, SP_0304, SP_0305, SP_0306, SP_0308, SP_0309, and SP_0310), bacteriocins (SP_0544 and
387 SP_1051) and a permease protein (SP_1527). The over-represented pathway (spn02060) was associated
388 with significant genes that code for PTS transporters involved in carbohydrate metabolism. Seven genes
389 were unannotated. The PTS transporters genes were also present in a high proportion of abundant
390 serotypes in sterile sites such as 6B (67%) and 23F (86%).

391 A total of 57 significant genes were absent in serotypes 1, 5, and 12F (Fig 5.a). The most significant
392 absences were observed within RD8a, consisting of two operons, RD8a1 (SP_1315-1324) and RD8a2
393 (SP_1325-SP_1331) (S8 Fig). RD8a1 harbors eight *ntp* genes, that code *V-type proton/sodium ATP*
394 *synthase complex* that produces ATP via oxidative phosphorylation in the presence of a Na+ gradient
395 across the membrane[43]. RD8a2 includes *neuraminidase*, *N-acetylneuraminate lyase (nanA),* and *N-*
396 *acetylmannosamine-6-phosphate epimerase (nanE).* These genes cleave carbohydrates from the
397 glycoproteins on the surface of epithelial cells. Other genes in RD8a2 encode the Sodium/solute
398 symporter subunits that use Na^+ gradient to import the carbohydrates [44]. Symporter refers to a
399 channel that transports the solute (carbohydrates) and co-solute (Na+) in the same direction by utilizing
400 the energy stored in an inwardly directed sodium gradient. Fundamentally, the genes within RD8a
401 operons collaborate to generate ATP, cleave carbohydrates from the host epithelial cells, and import
402 them into the bacterial cell. RD8a was absent in all samples associated with serotypes 1, 5, and 12F,

403 while it was present in other prevalent serotypes like 6A, 6B, 16F, and 19F. The pathway sp00190, which
404 was underrepresented in hyper-invasive serotypes, was associated with genes within RD8a. The absence
405 of RD8a in hyper-invasive serotypes (1,5, and 12F) may be linked to their rapid invasion into the blood
406 and CSF, where the availability of free oxygen molecules necessary for oxidative phosphorylation is
407 limited [45].

408 Other significant genes absent from the hyper-invasive serotypes were from RD4 and RD7. RD4 consists
409 of a cluster of sortase enzymes responsible for the assembly of pilins into pili and anchoring these
410 structures and other surface proteins to the cell wall [46][47]. The pilus is a hair-like structure associated
411 with bacterial adhesion and colonization [48]. Owing to the hypothesized short colonization period of
412 hyper-invasive serotypes, they may not harness the benefits of RD4 genes involved in pilus assembly.
413 RD7 genes remain uncharacterized as of the present date.

414 It is worth mentioning that there were similarities between hyper-invasive serotypes (1,5, and 12F) and
415 other strains in the blood and CSF. As observed for samples in the blood and CSF (Table 2), capsule
416 genes were also significantly present in the hyper-invasive serotypes (1,5, and 12F). Moreover, RD10
417 (previously found to be significantly absent from blood and CSF as described in Table 3) was also absent
418 from serotypes 1 and 12F. However, RD10 was fully conserved in serotype 5. RD10 was also conserved
419 in 100% of serotypes 16F and 19F. The summary of the gene present-absent analysis is illustrated in Fig
420 5.b.



421

Fig 5. The summary of the gene presence-absence analysis. (a) The number of significant genes present-absent in the hyper-invasive serotypes. The gene presence-absence analysis was applied using Scoary to compare the gene pools of the hyper-invasive serotypes and serotypes 16F and 19F. P-values were corrected by the Bonferroni method, and significant genes had an adjusted p-value of less than 0.05. (b) The significant presence and absence of RDs in samples from blood and CSF is shown as a presence-absence heatmap.

422 Finally and as mentioned, serotypes 6B and 23F were abundant in both carrier and patient groups, the

423 intra gene presence/absence statistical test for serotype 6B between the nasopharynx (n=60) and sterile

424 sites (n=37), and for serotype 23F between the nasopharynx (n=43) and sterile sites (n=50) did not

425 identify any significant gene. The gene content of these two serotypes in the nasopharynx and sterile
426 sites was similar.

427 To highlight genes that might assist pneumococci in crossing the blood-brain barrier, a test was also
428 conducted between the samples from blood and CSF. The analysis between whole blood (n=368) and
429 CSF (n=284), serotype 1 samples from blood (n=60) and CSF (n=61), and serotype 5 samples from blood
430 (n=75) and CSF (n=23) did not identify any significant genes.

431 Discussion

432 The *S. pneumoniae* genome is highly diverse, with only a small portion of genes conserved across all
433 strains. In this species, the pan-genome is open, allowing for an extensive gene repertoire due to the
434 highly recombinogenic nature of pneumococci. Changes in the *S. pneumoniae* habitat may lead to the
435 utilization of various gene combinations, enabling organisms to diversify their genome and respond
436 effectively to environmental stresses. This study found a high genetic diversity, with merely 10.7% of
437 genes classified as core. These core genes have been conserved across all samples for an extensive
438 period, at least from 1997 to 2015. Their presence may be crucial for cell survival, making them
439 potential targets for drug design and vaccine development. Specifically, core genes without any SNPs in
440 their structure are of particular interest. Notable conserved core genes identified in this study included
441 SP_1961 (rpoB, DNA-dependent RNA polymerase), SP_0251 (formate acetyltransferase), SP_1891 (amiA,
442 Oligopeptide binding protein), and SP_0855 (parC, Topoisomerase IV). These conserved core genes play
443 integral roles in DNA transcription and translation.

444 Serotypes 1, 5, and 12F exhibited a high prevalence among patients but were rarely found in the carrier
445 group. This observation indicates their increased invasiveness, likely due to a short duration of
446 nasopharyngeal colonization. Conversely, serotypes 16F and 19F were significantly more frequent
447 among carriers, suggesting their dominance in the nasopharynx but with lower invasiveness. Most other
448 serotypes were common among both carriers and patients. Knowing that pneumococcal virulence

449 strongly depends on the serotype of isolates, we sought to address why several serotypes were shared
450 across both nasopharynx and sterile sites. Here, we discuss two possible scenarios that could justify the
451 ubiquitous presence of some serotypes in both nasopharynx and sterile sites.

452 The first scenario is related to the colonization of *S. pneumoniae*, which is known as a prerequisite for
453 virulence[4]. Several samples from the carriage group may be actually the invasive serotypes collected
454 during their colonization phase. Abundant serotypes such as 6B and 23F that had a similar frequency
455 amongst carriers and patients need to colonize the upper respiratory tract longer than the hyper-
456 invasive serotypes before entering the sterile organs. In contrast, the hyper-invasive serotypes 1, 5, and
457 12F colonize the nasopharynx for a short period and quickly enter the sterile sites.

458 The second possible scenario relates to the differential gene expression pattern of shared genes in the
459 ubiquitous serotypes. Although the type-specific *cps* genes were identified in the isolates of both
460 nasopharynx and sterile sites, the expression pattern of these genes could vary within each strain, which
461 would contribute to the invasiveness of some strains. Several studies described a cycle of encapsulation
462 and un-encapsulation amongst pneumococcal strains. Isolates benefit from mutations in the *cps* locus to
463 either cease or re-start the capsule expression [49]. The lack of a capsule at the epithelial surface
464 enables the bacterium to expose its surface proteins on the cell wall underneath the capsule and
465 promote adherence to the host epithelial cells. It has been estimated that 15% of isolates in the upper
466 respiratory tract are unencapsulated and adhere to the respiratory epithelial cells more efficiently than
467 encapsulated isolates [50][51]. Lack of capsule also facilitates acquiring virulence and resistance genes
468 from other isolates. The thick capsule prevents immunoglobulins from interacting with the pathogen
469 surface proteins during disease. Meanwhile, the negatively charged CPS interferes with the function of
470 the host phagocytes [52][53]. Taken together, the presence of the *cps* locus in the genome of isolates
471 assigned to the same serotype does not necessarily reflect the encapsulation of all cells.

472 Serotypes 1 and 5 can infect all age groups and cause severe IPDs [54]. Serotype 1 is genetically distinct
473 between different geographical regions [55] and is known as the leading cause of pneumococcal
474 meningitis in Africa [56][57]. Our findings support previous research showing that serotypes 1, 5, and
475 12F are the major cause of IPDs in Malawi [58]. Serotype 1 was persistently dominant in pre- and post-
476 PCV13 eras, serotype 5 was only predominant in pre-PCV3, and serotype 12F emerged after vaccination.
477 The study also characterized the high genetic distinction of the hyper-invasive serotypes in Malawi by
478 identifying significant genes that are present or absent in their genome structure compared to other
479 serotypes. Many of the significant genes present in the nasopharynx and sterile sites were homologous
480 and had the same function, and there were significant genes with an unknown function (hypothetical
481 proteins). However, of greatest interest, we did find several significant genes with specific functions that
482 could explain the difference between the biology of the hyper-invasive serotypes and nasopharyngeal
483 samples.

484 Genes within RD8a were significantly absent in the genome of serotypes 1, 5, and 12F. RD8a is known as
485 a region previously linked to the virulence of serotypes 6B and 14 in the United States [59]. Our study
486 identified this region's conservation in serotypes 13, 14, 16F, and 19F in Malawi. This observation
487 prompts the hypothesis that RD8a may be essential for the prolonged colonization of serotypes
488 contrasting with the quicker colonization of serotypes 1, 5, and 12F. The functions of genes in RD8a
489 strengthen the assumption to some extent that RD8a may be essential for long nasopharyngeal
490 colonization. The genes within RD8a, such as neuraminidase, *nanA*, and *nanE*, are involved in the
491 cleaving of terminal sialic acid residues from mucoglycans and epithelial glycoconjugates. This activity
492 aids the pathogen in breaching the mucus layer and adhering to the epithelial cells in the nasopharynx.
493 Additionally, since free carbohydrates are limited in the upper respiratory tract [60], cleaved sialic acid
494 can be used as the carbon source for metabolism. Moreover, during colonization, secretion of the
495 pneumococcal toxins elevates the level of sodium ions (Na^+) in the nasopharynx [61], which enables the
496 sodium-solute symporter in RD8a to import a wide variety of substrates with the sodium ions into the

497 cell [62]. Most importantly, the *ntp* gene cluster in RD8a encodes the *V-type sodium ATP synthase* that
498 pumps the extra sodium ions out of the cell [43] and uses the sodium-motive force for oxidative
499 phosphorylation and ATP synthesis [63]. Oxidative phosphorylation is the final step of aerobic
500 respiration that requires free oxygen molecules for ATP synthesis. Pneumococci are facultative
501 anaerobes that can either perform aerobic or anaerobic respiration with or without oxygen. In the upper
502 respiratory tract, they access atmospheric oxygen molecules that can be used by *ntp* genes to perform
503 oxidative phosphorylation. However, genes in RD8a may not be beneficial for hyper-invasive serotypes
504 that supposedly do not stay in the nasopharynx for long. Thus, the level of aerobic ATP synthesis is
505 presumably higher in serotypes 13, 14, 16F, and 19F in contrast with serotypes 1, 5, and 12F, which lack
506 RD8a.

507 Pneumococci can ferment up to 30 types of carbohydrates, imported mainly by two types of membrane
508 transporters, including ATP-binding cassette (ABC) transporters and PTS transporters [64]. The major
509 differences between ABC and PTS transporters are: (i) ABC transporters use energy from ATP, but PTS
510 transporters use energy from phosphoenolpyruvate, and (ii) ABC transporters do not modify the
511 imported substrate, but PTS transporters phosphorylate the incoming sugar upon transport. Generally,
512 ABC transporters require more energy than PTS transporters, albeit they can transport longer and more
513 complicated carbohydrates [65]. Unlike isolates in the nasopharynx, serotypes 1, 5, and 12F have access
514 to more simple and free host dietary carbohydrates in the blood and the central nervous system. Due to
515 a potential lower ATP synthesis level in serotypes 1, 5, and 12F (due to the lack of RD8a), they may
516 prefer to use PTS transporters to uptake sugars such as fructose and lactose, and that is why genes that
517 encode PTS transporters are significantly more present in the hyper-invasive serotypes (1, 5, and 12F). In
518 addition to the sugar uptake, PTS transporters regulate several pathways in bacteria, such as gene
519 expression and communication between cells. Thus, the phenotypic effects of the PTS transporters
520 should not be limited just to their ability to import carbohydrates [66].

521 Genes within RD10 were absent from serotypes 1 and 12F. However, they were conserved in serotypes
522 5, 16F, and 19F. Moreover, the location-based gene presence-absence analysis showed that RD10 was
523 significantly present in nasopharyngeal samples in comparison to samples collected from sterile sites.
524 Operon RD10 in pneumococcus shares homology with the general secretion pathway protein B
525 sceA2/Y2 system components in Streptococcus gordonii, which are involved in secreting the general
526 secretion pathway protein B linked to infective endocarditis [67]. In the *S. pneumoniae* genome, the
527 homolog of general secretion pathway protein B is PsrP, which is transported to the bacterial cell
528 surface by the SecA2/Y2 system encoded by genes in RD10. Research on *Streptococcus gordonii* has
529 indicated that the presence of SecA2/Y2 facilitates adhesion to both epithelial cells in the nasopharynx
530 and erythrocytes in the blood. [68][69]. This may explain why SecA/Y2 is significantly present in
531 nasopharyngeal samples and serotype 5 (abundant in the blood). The presence of the secA2/Y2-like
532 component should also facilitate the export of pneumolysin, which enhances adhesion to the host cell
533 and contribute to survival in the blood [70][71].

534 In conclusion, specific genes present or absent in the hyper-invasive serotypes (1, 5, and 12F) may play a
535 role in their invasiveness and lower colonization rate. Nonetheless, experimental validation is necessary
536 to confirm the computational findings from this study. While the serotype is the primary determinant of
537 the pneumococcal population structure, this research has highlighted the substantial genetic divergence
538 of serotypes 1, 5, and 12F compared to other serotypes. Their substantial presence in the blood and CSF
539 accounted for the most pronounced genomic and functional differences observed between the
540 nasopharynx and sterile sites. The lower frequency of serotypes 1, 5, and 12F among carriers could be
541 attributed to their shorter colonization duration before entering sterile sites. These invasive serotypes
542 possess elements of PTS transporters but lack genes from RD8a. Interestingly, RD10 is highly conserved
543 in serotype 5, while it is absent in serotypes 1 and 12F. Notably, this study demonstrates that isolation
544 sites do not significantly influence the genomic structure of pneumococcal isolates. Although a few
545 genes were linked to the virulence of commonly present serotypes in both the nasopharynx and sterile

546 sites, it is suggested that other high-throughput techniques like gene expression analysis may reveal the
547 differences between these isolates more comprehensively. In summary, this research sheds light on the
548 pneumococcal population structure and serotypes in Malawi. The unique cluster of significant genes in
549 the hyper-invasive serotypes, along with highly conserved core genes, could serve as potential
550 therapeutic targets.

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Conflicts of interest

The author(s) declare that there are no conflicts of interest.

Ethical approval

Not required as the research delas with bacterial samples.

References

[1] T. D. Swarthout *et al.*, 'High residual carriage of vaccine-serotype Streptococcus pneumoniae after introduction of pneumococcal conjugate vaccine in Malawi', *Nature Communications 2020 11:1*, vol. 11, no. 1, pp. 1–12, May 2020, doi: 10.1038/s41467-020-15786-9.

[2] P. Kamthunzi, 'Impact of PCV13 vaccination in Blantyre, Malawi', *Lancet Glob Health*, vol. 9, no. 7, pp. e893–e894, Jul. 2021, doi: 10.1016/S2214-109X(21)00258-8.

[3] L. Paixão *et al.*, 'Host glycan sugar-specific pathways in streptococcus pneumonia: Galactose as a key sugar in colonisation and infection', *PLoS One*, 2015, doi: 10.1371/journal.pone.0121042.

586 [4] D. Bogaert, R. de Groot, and P. W. M. Hermans, 'Streptococcus pneumoniae colonisation: The key
587 to pneumococcal disease', *Lancet Infectious Diseases*. 2004. doi: 10.1016/S1473-3099(04)00938-
588 7.

589 [5] E. Backhaus *et al.*, 'Epidemiology of invasive pneumococcal infections: Manifestations, incidence
590 and case fatality rate correlated to age, gender and risk factors', *BMC Infect Dis*, 2016, doi:
591 10.1186/s12879-016-1648-2.

592 [6] A. Kadioglu, J. N. Weiser, J. C. Paton, and P. W. Andrew, 'The role of Streptococcus pneumoniae
593 virulence factors in host respiratory colonization and disease', *Nature Reviews Microbiology*.
594 2008. doi: 10.1038/nrmicro1871.

595 [7] J. N. Weiser, D. M. Ferreira, and J. C. Paton, 'Streptococcus pneumoniae: Transmission,
596 colonization and invasion', *Nature Reviews Microbiology*. 2018. doi: 10.1038/s41579-018-0001-8.

597 [8] K. A. Geno *et al.*, 'Pneumococcal capsules and their types: Past, present, and future', *Clin*
598 *Microbiol Rev*, 2015, doi: 10.1128/CMR.00024-15.

599 [9] A. L. Nelson, A. M. Roche, J. M. Gould, K. Chim, A. J. Ratner, and J. N. Weiser, 'Capsule enhances
600 pneumococcal colonization by limiting mucus-mediated clearance', *Infect Immun*, 2007, doi:
601 10.1128/IAI.01475-06.

602 [10] J. Brown, S. Hammerschmidt, and C. Orihuela, *Streptococcus Pneumoniae: Molecular*
603 *Mechanisms of Host-Pathogen Interactions*. 2015. doi: 10.1016/C2012-0-00722-3.

604 [11] F. Ganaie *et al.*, 'A new pneumococcal capsule type, 10D, is the 100th serotype and has a large
605 cps fragment from an oral streptococcus', *mBio*, 2020, doi: 10.1128/mBio.00937-20.

606 [12] D. M. Weinberger, R. Malley, and M. Lipsitch, 'Serotype replacement in disease after
607 pneumococcal vaccination', *The Lancet*. 2011. doi: 10.5455/apd.239006.

608 [13] D. R. Feikin *et al.*, 'Serotype-Specific Changes in Invasive Pneumococcal Disease after
609 Pneumococcal Conjugate Vaccine Introduction: A Pooled Analysis of Multiple Surveillance Sites',
610 *PLoS Med*, 2013, doi: 10.1371/journal.pmed.1001517.

611 [14] E. D. McCollum *et al.*, 'Impact of the 13-valent pneumococcal conjugate vaccine on clinical and
612 hypoxemic childhood pneumonia over three years in central Malawi: An observational study',
613 *PLoS One*, 2017, doi: 10.1371/journal.pone.0168209.

614 [15] N. Bar-Zeev *et al.*, 'Impact and Effectiveness of 13-Valent Pneumococcal Conjugate Vaccine on
615 Population Incidence of Vaccine and Non-Vaccine Serotype Invasive Pneumococcal Disease in
616 Blantyre, Malawi, 2006-2018: Prospective Observational Time-Series and Case-Control Studies',
617 *SSRN Electronic Journal*, Dec. 2020, doi: 10.2139/SSRN.3745169.

618 [16] E. Heinsbroek *et al.*, 'Pneumococcal carriage in households in Karonga District, Malawi, before
619 and after introduction of 13-valent pneumococcal conjugate vaccination', *Vaccine*, 2018, doi:
620 10.1016/j.vaccine.2018.10.021.

621 [17] C. Cillóniz, C. Garcia-Vidal, A. Ceccato, and A. Torres, 'Antimicrobial Resistance Among
622 Streptococcus pneumoniae', in *Antimicrobial Resistance in the 21st Century*, Springer, 2018, pp.
623 13–38.

624 [18] A. Iranzadeh and N. J. Mulder, 'Bacterial Pan-Genomics', in *Microbial Genomics in Sustainable*
625 *Agroecosystems*, Springer, 2019, pp. 21–38.

626 [19] A. Embry, E. Hinojosa, and C. J. Orihuela, 'Regions of Diversity 8, 9 and 13 contribute to
627 Streptococcus pneumoniae virulence', *BMC Microbiol*, 2007, doi: 10.1186/1471-2180-7-80.

628 [20] R. Brückner, M. Nuhn, P. Reichmann, B. Weber, and R. Hakenbeck, 'Mosaic genes and mosaic
629 chromosomes-genomic variation in Streptococcus pneumoniae', *International Journal of Medical*
630 *Microbiology*. 2004. doi: 10.1016/j.ijmm.2004.06.019.

631 [21] A. W. Kamng'ona *et al.*, 'High multiple carriage and emergence of Streptococcus pneumoniae
632 vaccine serotype variants in Malawian children', *BMC Infect Dis*, vol. 15, no. 1, pp. 1–11, Jun.
633 2015, doi: 10.1186/S12879-015-0980-2/FIGURES/7.

634 [22] C. Chaguza *et al.*, 'Population genetic structure, antibiotic resistance, capsule switching and
635 evolution of invasive pneumococci before conjugate vaccination in Malawi', *Vaccine*, vol. 35, no.
636 35 Pt B, pp. 4594–4602, Aug. 2017, doi: 10.1016/J.VACCINE.2017.07.009.

637 [23] S. Andrews, 'FastQC: A quality control tool for high throughput sequence data.', *Babraham*
638 *Bioinformatics*, p. http://www.bioinformatics.babraham.ac.uk/projects/, 2010, doi: citeulike-
639 article-id:11583827.

640 [24] L. Epping, A. J. van Tonder, R. A. Gladstone, S. D. Bentley, A. J. Page, and J. A. Keane, 'SeroBA:
641 rapid high-throughput serotyping of Streptococcus pneumoniae from whole genome sequence
642 data', *Microb Genom*, vol. 4, no. 7, Jul. 2018, doi: 10.1099/MGEN.0.000186.

643 [25] S. Gladman and T. Seemann, 'VelvetOptimiser', *Free Software Foundation*. 2008. doi:
644 10.1016/S0925-8574(99)00040-3.

645 [26] A. Gurevich, V. Saveliev, N. Vyahhi, and G. Tesler, 'QUAST: Quality assessment tool for genome
646 assemblies', *Bioinformatics*, 2013, doi: 10.1093/bioinformatics/btt086.

647 [27] T. Seemann, 'Prokka: Rapid prokaryotic genome annotation', *Bioinformatics*, vol. 30, no. 14, pp.
648 2068–2069, 2014, doi: 10.1093/bioinformatics/btu153.

649 [28] A. J. Page *et al.*, 'Roary: Rapid large-scale prokaryote pan genome analysis', *Bioinformatics*, vol.
650 31, no. 22, pp. 3691–3693, May 2015, doi: 10.1093/bioinformatics/btv421.

651 [29] K. Katoh and D. M. Standley, 'MAFFT multiple sequence alignment software version 7:
652 Improvements in performance and usability', *Mol Biol Evol*, 2013, doi: 10.1093/molbev/mst010.

653 [30] B. Q. Minh *et al.*, 'IQ-TREE 2: New Models and Efficient Methods for Phylogenetic Inference in the
654 Genomic Era', *Mol Biol Evol*, vol. 37, no. 5, pp. 1530–1534, May 2020, doi:
655 10.1093/MOLBEV/MSAA015.

656 [31] I. Letunic and P. Bork, 'Interactive tree of life (iTOL) v3: an online tool for the display and
657 annotation of phylogenetic and other trees', *Nucleic Acids Res*, 2016, doi: 10.1093/nar/gkw290.

658 [32] R. Gaujoux, 'An introduction to NMF package', *BMC Bioinformatics*, 2010, doi: 10.1186/1471-
659 2105-11-367.

660 [33] F. Rohart, B. Gautier, A. Singh, and K. A. Lê Cao, 'mixOmics: An R package for 'omics feature
661 selection and multiple data integration', *PLoS Comput Biol*, 2017, doi:
662 10.1371/journal.pcbi.1005752.

663 [34] O. Brynildsrud, J. Bohlin, L. Scheffer, and V. Eldholm, 'Rapid scoring of genes in microbial pan-
664 genome-wide association studies with Scoary', *Genome Biol*, vol. 17, no. 1, p. 238, 2016, doi:
665 10.1186/s13059-016-1108-8.

666 [35] D. Szklarczyk *et al.*, 'STRING v11: Protein-protein association networks with increased coverage,
667 supporting functional discovery in genome-wide experimental datasets', *Nucleic Acids Res*, 2019,
668 doi: 10.1093/nar/gky1131.

669 [36] H. Ogata, S. Goto, K. Sato, W. Fujibuchi, H. Bono, and M. Kanehisa, 'KEGG: Kyoto encyclopedia of
670 genes and genomes', *Nucleic Acids Research*, vol. 27, no. 1. pp. 29–34, 1999. doi:
671 10.1093/nar/27.1.29.

672 [37] A. Bateman *et al.*, 'The Pfam protein families database', *Nucleic Acids Res*, vol. 32, no. suppl_1,
673 pp. D138–D141, Jan. 2004, doi: 10.1093/NAR/GKH121.

674 [38] Q. Wang *et al.*, 'Serotype distribution of Streptococcus pneumoniae isolated from children
675 hospitalized in Beijing children's hospital (2013–2019)', *Vaccine*, vol. 38, no. 49, pp. 7858–7864,
676 2020, doi: 10.1016/j.vaccine.2020.10.005.

677 [39] M. Alizadeh Chamkhaleh *et al.*, 'Serotype distribution of Streptococcus pneumoniae among
678 healthy carriers and clinical patients: a systematic review from Iran', *European Journal of Clinical*
679 *Microbiology and Infectious Diseases*. 2020. doi: 10.1007/s10096-020-03963-z.

680 [40] N. L. Hiller *et al.*, 'Comparative genomic analyses of seventeen Streptococcus pneumoniae
681 strains: Insights into the pneumococcal supragenome', *J Bacteriol*, vol. 189, no. 22, pp. 8186–
682 8195, Nov. 2007, doi: 10.1128/JB.00690-07/SUPPL_FILE/SUPPLEMENTARY_TABLE_3.ZIP.

683 [41] S. Zhang and R. Meyer, 'The relaxosome protein MobC promotes conjugal plasmid mobilization
684 by extending DNA strand separation to the nick site at the origin of transfer', *Mol Microbiol*,
685 1997, doi: 10.1046/j.1365-2958.1997.4861849.x.

686 [42] C. Obert *et al.*, 'Identification of a candidate Streptococcus pneumoniae core genome and
687 regions of diversity correlated with invasive pneumococcal disease', *Infect Immun*, vol. 74, no. 8,
688 pp. 4766–4777, 2006, doi: 10.1128/IAI.00316-06.

689 [43] K. Yokoyama and H. Imamura, 'Rotation, structure, and classification of prokaryotic V-ATPase',
690 *Journal of Bioenergetics and Biomembranes*. 2005. doi: 10.1007/s10863-005-9480-1.

691 [44] J. Reizer, A. Reizer, and M. H. Saier, 'A functional superfamily of sodium/solute symporters',
692 *Biochim Biophys Acta*, vol. 1197, no. 2, pp. 133–166, Jun. 1994, doi: 10.1016/0304-
693 4157(94)90003-5.

694 [45] P. Gaudu, 'Aerobic respiration metabolism in lactic acid bacteria and uses in biotechnology', doi:
695 10.1146/annurev-food-022811-101255.

696 [46] C. Manzano *et al.*, 'Sortase-Mediated Pilus Fiber Biogenesis in Streptococcus pneumoniae',
697 *Structure*, 2008, doi: 10.1016/j.str.2008.10.007.

698 [47] J. Lemieux, S. Woody, and A. Camilli, 'Roles of the sortases of Streptococcus pneumoniae in
699 assembly of the RlrA pilus', *J Bacteriol*, 2008, doi: 10.1128/JB.00379-08.

700 [48] L. Williams, F. Stapleton, and N. Carnt, 'Microbiology, lens care and maintenance', *Contact*
701 *Lenses*, pp. 65–96, Jan. 2019, doi: 10.1016/B978-0-7020-7168-3.00004-0.

702 [49] J. D. Langereis and M. I. de Jonge, ' Non-encapsulated Streptococcus pneumoniae , vaccination as
703 a measure to interfere with horizontal gene transfer ', *Virulence*, 2017, doi:
704 10.1080/21505594.2017.1309492.

705 [50] D. van der Windt, H. J. Bootsma, P. Burghout, C. E. van der Gaast-de Jongh, P. W. M. Hermans,
706 and M. van der Flier, 'Nonencapsulated Streptococcus pneumoniae resists extracellular human
707 neutrophil elastase- and cathepsin G-mediated killing', *FEMS Immunol Med Microbiol*, 2012, doi:
708 10.1111/j.1574-695X.2012.01028.x.

709 [51] U. M. Talbot, A. W. Paton, and J. C. Paton, 'Uptake of Streptococcus pneumoniae by respiratory
710 epithelial cells', *Infect Immun*, 1996.

711 [52] C. J. Lee, S. D. Banks, and J. P. Li, 'Virulence, immunity, and vaccine related to streptococcus
712 pneumoniae', *Crit Rev Microbiol*, 1991, doi: 10.3109/10408419109113510.

713 [53] L. E. Keller, D. A. Robinson, and L. S. McDaniel, ' Nonencapsulated Streptococcus pneumoniae: 714 Emergence and Pathogenesis ', *mBio*, 2016, doi: 10.1128/mbio.01792-15.

715 [54] W. P. Hausdorff, 'The roles of pneumococcal serotypes 1 and 5 in paediatric invasive disease', 716 *Vaccine*, 2007, doi: 10.1016/j.vaccine.2006.09.009.

717 [55] J. E. Cornick *et al.*, 'Region-specific diversification of the highly virulent serotype 1 Streptococcus 718 pneumoniae', *Microb Genom*, vol. 1, no. 2, 2015.

719 [56] J. Leimkugel *et al.*, 'An outbreak of serotype 1 Streptococcus pneumoniae meningitis in northern 720 Ghana with features that are characteristic of Neisseria meningitidis meningitis epidemics', *J* 721 *Infect Dis*, vol. 192, no. 2, pp. 192–199, 2005.

722 [57] B. D. Gessner, J. E. Mueller, and S. Yaro, 'African meningitis belt pneumococcal disease 723 epidemiology indicates a need for an effective serotype 1 containing vaccine, including for older 724 children and adults', *BMC Infect Dis*, 2010, doi: 10.1186/1471-2334-10-22.

725 [58] J. E. Cornick *et al.*, 'Invasive streptococcus pneumoniae in children, Malawi, 2004-2006', *Emerg* 726 *Infect Dis*, 2011, doi: 10.3201/eid1706.101404.

727 [59] C. Obert *et al.*, 'Identification of a Candidate Streptococcus pneumoniae core genome and 728 regions of diversity correlated with invasive pneumococcal disease', *Infect Immun*, vol. 74, no. 8, 729 pp. 4766–4777, Aug. 2006, doi: 10.1128/IAI.00316-06.

730 [60] D. M. Walters, V. L. Stirewalt, and S. B. Melville, 'Cloning, sequence, and transcriptional 731 regulation of the operon encoding a putative N-acetylmannosamine-6-phosphate epimerase 732 (nanE) and sialic acid lyase (nanA) in Clostridium perfringens', *J Bacteriol*, 1999.

733 [61] L. E. Bakeeva, K. M. Chumakov, A. L. Drachev, A. L. Metlina, and V. P. Skulachev, 'The sodium 734 cycle. III. Vibrio alginolyticus resembles Vibrio cholerae and some other vibriones by flagellar

735 motor and ribosomal 5S-RNA structures', *BBA - Bioenergetics*, 1986, doi: 10.1016/0005-
736 2728(86)90115-5.

737 [62] M. H. Saier, 'Families of transmembrane sugar transport proteins', *Molecular Microbiology*. 2000.
738 doi: 10.1046/j.1365-2958.2000.01759.x.

739 [63] P. D. Boyer, 'THE ATP SYNTHASE—A SPLENDID MOLECULAR MACHINE', *Annu Rev Biochem*, 2002,
740 doi: 10.1146/annurev.biochem.66.1.717.

741 [64] A. Bidossi *et al.*, 'A functional genomics approach to establish the complement of carbohydrate
742 transporters in Streptococcus pneumoniae', *PLoS One*, 2012, doi: 10.1371/journal.pone.0033320.

743 [65] C. M. Buckwalter and S. J. King, 'Pneumococcal carbohydrate transport: Food for thought', *Trends*
744 *in Microbiology*. 2012. doi: 10.1016/j.tim.2012.08.008.

745 [66] M. H. Saier, 'The Bacterial Phosphotransferase System: New Frontiers 50 Years after Its
746 Discovery', *Journal of Molecular Microbiology and Biotechnology*. 2015. doi: 10.1159/000381215.

747 [67] B. A. Bensing, B. W. Gibson, and P. M. Sullam, 'The Streptococcus gordonii Platelet Binding
748 Protein GspB Undergoes Glycosylation Independently of Export', *J Bacteriol*, 2004, doi:
749 10.1128/JB.186.3.638-645.2004.

750 [68] B. A. Bensing and P. M. Sullam, 'Transport of preproteins by the accessory Sec system requires a
751 specific domain adjacent to the signal peptide', *J Bacteriol*, 2010, doi: 10.1128/JB.00373-10.

752 [69] M. Yamaguchi *et al.*, 'Streptococcus pneumoniae Invades Erythrocytes and Utilizes Them to
753 Evade Human Innate Immunity', *PLoS One*, 2013, doi: 10.1371/journal.pone.0077282.

754 [70] M. Bandara *et al.*, 'The accessory Sec system (SecY2A2) in Streptococcus pneumoniae is involved
755 in export of pneumolysin toxin, adhesion and biofilm formation', *Microbes Infect*, 2017, doi:
756 10.1016/j.micinf.2017.04.003.

757 [71] R. Wu and H. Wu, 'A molecular chaperone mediates a two-protein enzyme complex and
758 glycosylation of serine-rich streptococcal adhesins', *Journal of Biological Chemistry*, 2011,
759 doi: 10.1074/jbc.M111.239350.

760 Supporting information

761 **S1 Fig. Characteristics of the 1477 pneumococcal isolates used in the study.** (a) The relative frequency
762 of serotypes in the entire cohort, samples were assigned to 56 serotypes. For each sample, the in-silico
763 serotyping was accomplished by SeroBA. (b) Frequency of isolates in the pre- and post-PCV13 eras in
764 Malawi. (c) Frequency of isolates obtained from each specimen source.

765 **S2 Fig. Distribution of the abundant serotypes (frequency > 5%) before and after the vaccination**
766 **rollout in Malawi in 2011.** Serotype 1 persistently dominated both the pre- and post-vaccination eras.

767 **S3 Fig. The serotype distribution among carriers in Karonga and Blantyre.** Distributions were similar,
768 except for serotype 6B, which was more dominant in Karonga, and serotype 13, which was more
769 prevalent in Blantyre.

770 **S4 Fig. Serotype distribution among meningitis patients in Lilongwe and Blantyre.** Only 3.5% of disease
771 samples (23 out of 652, i.e., 3.5%) were collected from Lilongwe. Serotypes 1 and 12F were predominant
772 in both regions; however, a larger dataset from Lilongwe is needed to accurately reflect the true
773 serotype distribution in this area.

774 **S5 Fig. The pan-genome of 1477 pneumococcal samples isolated in Malawi was obtained from 1997 to**
775 **2015.** The pan-genome is an open pan-genome, which means the number of total genes increases
776 unlimitedly when the sample size grows. The dashed line represents the number of total genes, and the
777 solid line represents the number of conserved genes in the pan-genome.

778 **S6 Fig. The three-dimensional PCA of the gene distribution in the vaccine types.** For each serotype and
779 for downsampling, 10 samples were randomly selected from the nasopharynx, blood, and CSF. The PCA
780 was conducted using the R package MixOmics. Hyper-invasive serotypes 1, 5, and 12F clustered
781 separately from other strains.

782 **S7 Fig. Genes in RD10 are absent from serotypes 1 and 12F but conserved in serotype 5, 16F, and 19F.**
783 Genes from RD10 encode the components of the secretory system SecA2/Y2 that transports
784 glycoproteins to the bacterial cell surface, which are required for binding to the human proteins on the
785 surface of epithelial cells and erythrocytes.

786 **S8 Fig. RD8a consists of two operons RD8a1 (SP_1315-1324) and RD8a2 (SP_1325-1331).** This region is
787 not detected in the significant invasive serotypes 1, 5, and 12F, but it is present in more than 80% of
788 serotype 16F and 19F that significantly dominates the nasopharynx. The important biological processes
789 carried out by these genes are the transport of ions across the membrane and the synthesis of ATP
790 molecules.

791 **S1 Table. Statistical analysis of serotypes' prevalence across specimen sources.**

792 **S2 Table. Gene presence-absence analysis (Invasive vs Nasopharyngeal, serotypes 1, 5, and 12F were**
793 **excluded).**

794 **S3 Table. Gene presence-absence analysis (Serotype 1 vs 16F & 19F).**

795 **S4 Table. Gene presence-absence analysis (Serotype 5 vs 16F & 19F).**

796 **S5 Table. Gene presence-absence analysis (Serotype 12F vs 16F & 19F).**

797 Figure captions

798 **Fig 1. The distribution of the 56 pneumococcal serotypes assigned to 1477 samples from Malawi.** (a)
799 The relative frequency of each serotype in the nasopharynx of carriers, the blood of bacteremia
800 patients, and the CSF of meningitis patients is shown in blue, red, and yellow, respectively (UT: Un-
801 Typeable). (b) The log-transformed odds ratio of the significantly over- and under-abundant serotypes in

802 the sterile sites (blood and CSF). Fisher's exact test was applied to identify serotypes with a significant
803 differential abundance among carriers and patients (nasopharynx and sterile sites) at the significance
804 level of the Benjamini-Hochberg adjusted p-value < 0.01 (BH: Benjamini-Hochberg).

805 **Fig 2. The pan-genome matrix of 1477 pneumococcal isolates from Malawi.** The pan-genome is
806 visualized as a gene presence-absence heatmap representing the hierarchical unsupervised clustering of
807 samples based on the distribution of genes in the pan-genome. Each row is a sample, and each column
808 is a gene. A blue dot denotes the presence of each gene. On the right side of the heatmap, the large blue
809 block shows core genes present in all samples. The left side of the heatmap represents the accessory
810 genome along with the clustering bands. In addition to the significant serotypes 1, 5, 12F, 16F, and 19F,
811 other abundant serotypes, including 6A, 6B, and 23F, as well as serotypes with source-based p-value <
812 0.05, including 21, 11A, and 15B, are also highlighted on the heatmap.

813 **Fig 3. The phylogenetic population structure of 1477 pneumococcal samples from Malawi.** The
814 phylogenetic tree was built based on the multiple sequence alignment of the core genome using the
815 maximum likelihood method. Colors on the loops show the serotypes, specimen sources (isolation sites),
816 and PCV13 eras. In addition to the significant serotypes 1, 5, 12F, 16F, and 19F, other abundant
817 serotypes, including 6A, 6B, and 23F, as well as serotypes with source-based p-value < 0.05, including 21,
818 11A, and 15B, are also highlighted on the tree.

819 **Fig 4. The PCA of the gene distribution in the pan-genome of pneumococcal isolates from 1477**
820 **Malawians.** The PCA of variants (gene presence-absence) in the accessory-genome indicates the
821 influence of (a) specimen sources (isolation sites), (b) serotypes, (c) PCV13 (vaccination) era, and (d)
822 geographical locations on the gene presence-absence profile of pneumococcal isolates in Malawi.
823 Serotypes 1 and 5 were clearly separated from other samples.

824

825 **Fig 5. The summary of the gene presence-absence analysis.** (a) The number of significant genes
826 present-absent in the hyper-invasive serotypes. The gene presence-absence analysis was applied using
827 Scoary to compare the gene pools of the hyper-invasive serotypes and serotypes 16F and 19F. P-values
828 were corrected by the Bonferroni method, and significant genes had an adjusted p-value of less than
829 0.05. (b) The significant presence and absence of RDs in samples from blood and CSF is shown as a
830 presence-absence heatmap.

831 Data summary

832 **S6 Table. Samples IDs on European Nucleotide Archive (ENA)**