Group A *Streptococcus* strains causing meningitis without distinct invasive phenotype

Laura Marquardt[a*], Federica Andreoni[a*], Mathilde Boumasmoud[a], Tiziano A. Schweizer[a], Dorothea M. Heuberger[b], Elena Parietti[a], Jana Epprecht[a], Dario Mattle[a], Anna K. Raez[a], Ewerton Marques-Maggio[c]; Reto A. Schuepbach[b], Barbara Hasse[a], Srikanth Mairpady-Shambat[a], Silvio D. Brugger[a], Annelies S. Zinkernagel[a**]

[a]Departement of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, Zurich, Switzerland

[b]Institute for Intensive Care Medicine, University Hospital Zurich, University of Zurich, Zurich, Switzerland

[c]Division of Clinical Pathology, University Hospital Zurich, University of Zurich, Zurich, Switzerland.

*These authors contributed equally to this manuscript

**Corresponding author

Keywords

Group A Streptococcus; meningitis; virulence determinants

Running title

Group A Streptococcus in meningitis

25 **Corresponding author**

26 **Prof. Dr. Dr. Med. Annelies Zinkernagel**

27 University Hospital Zurich

28 Raemistrasse 100

29 8091, Zurich

30 Tel. +41 44 255 12 59

31 annelies.zinkernagel@usz.ch

32

33 **Alternative corresponding author**

34 **Dr. Federica Andreoni**

35 University Hospital Zurich

36 Raemistrasse 100

37 8091, Zurich

38 Tel. +41 44 255 12 58

Abstract

Group A streptococcal (GAS) meningitis is a fulminant disease associated with high morbidity and mortality. To elucidate the mechanisms underlying the invasiveness of GAS in meningitis, we compared GAS isolates derived from five cases of meningitis, to otitis and colonizing isolates. We did not observe differences in adherence to and invasion of human brain microvascular endothelial cells, virulence factors activity or barrier disruption. Whole genome sequencing did not reveal particular invasiveness traits. Most patients previously suffered from otitis media suggesting that meningitis likely resulted from a continuous spread of the infection rather than being attributable to changes in pathogen's virulence.

48 **Introduction**

49 *Streptococcus pyogenes* or group A *Streptococcus* (GAS) asymptomatically colonizes the
50 human throat and skin. In addition to asymptomatic colonization, GAS causes mild to life-
51 threatening infections, such as necrotizing fasciitis and meningitis. GAS meningitis cases are
52 rare and represent only 1-4% of all invasive GAS cases detected across Europe and the
53 United States [1-3]. GAS meningitis is a fulminant disease associated with high morbidity,
54 including neurologic sequelae, and mortality. It occurs mainly in patients with predisposing
55 factors such as otitis, sinusitis, recent head injury or neurosurgery, presence of a
56 neurosurgical device, cerebrospinal fluid leak, or alcoholism [4, 5]. This suggests extension
57 *per continuitatem* from a neighboring infection such as otitis media, resulting in otogenic
58 meningitis. However, in a third of cases there are no predisposing factors present.

59 Pathogen components such as streptococcal virulence factors Streptolysin O (SLO), DNase
60 Sda1, protease SpyCEP and the M-protein, play a key role in the invasiveness and ability of
61 the pathogen to survive within the host environment [6].

62 In this study, we investigated five patients suffering from GAS meningitis, analyzing both
63 the host's and pathogen's characteristics. On the host side, various clinical parameters were
64 analyzed. On the pathogen side, aiming to identify particular pathogenic traits, we compared
65 phenotypically and genetically five GAS isolates derived from meningitis cases with ten GAS
66 isolates derived either from otitis or asymptomatic colonization.

67 **Material and methods**

68 **Patients and ethics**

69 Five patients diagnosed with clinically confirmed community-acquired GAS meningitis
70 between 2013 and 2017 in Switzerland were retrospectively included in this study (Tab.S1).
71 No specific surveillance program for GAS meningitis exists in Switzerland and the cases were
72 selected using a convenience sampling strategy. This study was approved by the regional
73 committee for medical research ethics (BASEC-ID 2016-00145 and 2017-02225).

74

75 **Bacterial strains and cell lines**

76 All meningitis, otitis and colonizing GAS clinical isolates are listed in Tab.S2 and were
77 cultured in THY (Todd-Hewitt broth (BD) plus 2% yeast extract (Oxoid)) at 37°C. Immortalized
78 human brain microvascular endothelial cells (HBMECs) were cultured as previously described
79 [7].

80

81 **Adherence, invasion and viability assays**

82 Adherence and invasion assays were performed as previously described [8], except lysis
83 was carried out using 500 µl deionized water (dH_2O). Cell viability was assessed as previously
84 described [9].

85

86 **Virulence factors activity**

87 Activity of the virulence factors SLO, Sda1 and SpyCEP was assessed as previously
88 described [10].

89

90 **Electric cell-substrate impedance sensing (ECIS®) assay**

91 ECIS® was performed with the ECIS® Z-Theta instrument (Applied Biophysics) to monitor
92 the effects of the GAS strains on human brain microvascular endothelial cells (HBMECs) [7]
93 barrier integrity. Before seeding, the wells of the arrays (ECIS 8W10E+, IBIDI) were washed
94 twice with sterile, nuclease-free dH_2O, coated with 5 µg/ml collagen IV for 1h at room
95 temperature (RT), washed three times with dH_2O and incubated with 10 mM L-cysteine for
96 2h at RT. The wells were washed two times with dH_2O before 200 µl of culture medium were
97 added and the assay started (37ºC, 5% CO2). After 30 min, 200µl culture medium containing
98 10^5 HBMECs were seeded per well. After 22 h incubation 50 µl of medium were removed
99 and 2h later HBMECs were infected with log phase GAS strains resuspended in
100 DMEM+10%FCS at an MOI of 10. After 1.5h penicillin (10 µg/ml) and gentamycin (100 µg/ml)
101 were added. Barrier integrity was monitored at 4000 Hz from seeding to 70h post infection.
102 Resistance data were normalized to the time of infection.
103
104 **Bacterial genomics**
105 Whole genome sequencing and genomic data analysis were performed as previously
106 described [11]. Briefly, from 150 bp paired end reads*, de novo* assemblies were generated
107 with SPAdes v.3.10[11] and annotated with Prokka v.1.13 [12]. A pangenome was
108 constructed with Roary v.3.12 [13] and the resulting core-gene alignment was used to build
109 a maximum likelihood phylogenetic tree with Fasttree v.2.1.10 [14]. *Emm*-typing was
110 performed *in-silico* by querying assemblies against the curated CDC database[15] with blastn
111 v.2.9.0. Virulence genes were identified by querying the assemblies against the core
112 sequences of the VFDB [16], using ABRIcate v.0.5 with the parameter -minid 85. ENA
113 accession number: PRJEB57816.
114

115 **Results**

116 **Cases description**

117 Five GAS meningitis cases, occurring between 2013 and 2017 in Switzerland, were
118 included in this study and the corresponding GAS isolates were retrospectively retrieved for
119 analysis. The median age of the patients was 64 years (42 to 79 years) and 60% (n=3) were
120 female. The mortality rate was 20% (n=1), neurological sequelae included slight hearing loss
121 and vertigo. The median duration of the hospital stay was 17 days (3 to 29 days) and 4
122 patients needed intensive care treatment with endotracheal intubation. Relevant clinical and
123 laboratory findings are depicted in table 1. Mastoiditis was the underlying diagnosis in three
124 cases. During the course of the disease one patient had a septic thrombosis and one
125 developed a brain abscess (Fig.1A-1B). Surgical treatment such as antrotomy,
126 mastoidectomy, or burr-hole trepanation was required in three patients. In patient 4,
127 histological analysis from the surgically removed mastoid tissue showed necrotic tissue and
128 individual mucosal fragments covered by simple cuboid epithelium with severe acute and
129 chronic inflammation and showing extracellular accumulation of bacterial cells (Fig.1C-1D).

130

131 **Bacterial virulence**

132 A detailed analysis of streptococcal virulence factors, adherence to and invasion of
133 HBMECs as well as barrier integrity of HBMECs upon GAS challenge was carried out on
134 meningitis, otitis and colonizing clinical isolates. We found no significant difference between
135 adherence and invasion behavior to HBMECs upon infection with meningitis, otitis or
136 colonizing GAS isolates (Fig.2A-2B). Furthermore, no differences in virulence factors activity
137 or the isolates potential to disrupt the barrier formed by HBMECs among the three groups
138 were observed (Fig.2C-2F). Typing showed a high prevalence of *emm*-1/ST28 (n=4/5, Tab.S1).

139 This was also the most common genotype among otitis and colonizing isolates (n=5/5 and
140 4/5 respectively, Tab.S1). Only one meningitis clinical isolate belonged to the *emm*-28/ST52
141 and one colonizing isolate to the *emm*-28/ST458. The meningitis isolates did not form a
142 monophyletic group (Fig.S1A-S1B). The presence of genes for virulence factors determinants
143 was analyzed and this showed no specific pattern for meningitis isolates when compared to
144 otitis or colonizing ones (Fig. S1C).

145 **Discussion**

146 We found no particular pathogenic characteristics in the five GAS isolates causing
147 meningitis. The only common denominator was preceding otitis and mastoiditis on the
148 host's side. GAS meningitis is an extremely rare condition, representing only the 0.2%-0.9%
149 of cases of bacterial meningitis in the United States and Brazil [17-19] and 1-4% of invasive
150 GAS cases in Europe and the United States [1-3]. A report from Switzerland described nine
151 cases of GAS meningitis registered between 1983 and 1999 across all major Swiss hospitals,
152 again emphasizing the rarity of this disease [20].

153 Meningitis occurs either by hematogenous spread or by direct spread of bacteria from a
154 neighboring infection. Hematogenous spread normally occurs when colonizing bacteria
155 breach the nasopharyngeal mucosa, reach the blood [21] and subsequently cross the blood-
156 brain barrier. Spread from the upper respiratory tract's mucosa may be due to an altered
157 balance in the host microbe population, possibly favoring GAS number increase [5]. On the
158 other hand, direct spread to the brain occurs *per continuitatem* from infected adjacent
159 anatomic structures, such as the ear or the mastoid bone [21].

160 We searched for common traits in the clinical presentation of our patients and
161 characteristics shared by the clinical isolates to find possible explanations for the spreading
162 of GAS to the brain. In our patients' cohort, the presence of mastoiditis in four patients and
163 thrombosis in one supports the idea of direct spread through bone erosion from coalescent
164 oto-mastoiditis and osteo-thrombophlebitis. Moreover otitis, a condition often associated
165 with GAS meningitis [5], was present in the majority of cases suggesting continuous spread
166 as a likely cause of infection. In contrast, hematogenous spreading was less common and
167 only observed in one patient (patient 3). The presence of a local focus of infection may
168 explain the high surgery rate in our GAS meningitis cohort, which has been reported

169 previously. In a nationwide cohort study in the Netherlands, 46% of GAS meningitis patients
170 required surgical intervention, while this remains an absolute rarity only described in case
171 reports for the more common meningitis pathogens (pneumococcus, meningococcus) [4,
172 22].

173 On the pathogen's side, no common features explaining bacterial spread to the brain
174 were found among the meningitis isolates. This points to host rather than pathogen factors
175 playing a role in facilitating bacterial invasion, as previously described for *S. aureus* [11].
176 Adherence and invasion assays towards HBMECs confirmed the absence of a particular
177 tropism towards brain microvascular tissue for the meningitis isolates, excluding an
178 advantage in crossing or damaging the blood brain barrier for these isolates, as compared to
179 otitis and colonizing ones, in the tested settings. Moreover, virulence factors activity did not
180 significantly vary among the three groups of isolates. Isolates from the *emm*-1 and *emm*-28
181 lineage represented both in the meningitis and colonizing groups, behaved very similarly in
182 terms of virulence factors activity.

183 In accordance with previous studies stating an association of *emm*-1/ST28 with GAS
184 meningitis [23], 4/5 meningitis isolates belonged to the *emm*-1/ST28 lineage. The fifth
185 isolate belonged to the *emm*-28 lineage, which is another prevalent *emm*-type previously
186 described in GAS meningitis [23] and, together with *emm*-1, among the most representative
187 *emm*-types for invasive GAS infections across Europe and Nord America [24]. All otitis and
188 colonizing isolates belonged either to the *emm*-1 or the *emm*-28 lineage as well. The
189 presence or absence of GAS virulence determinants did not reveal a specific pattern for
190 meningitis isolates when compared to otitis and colonizing ones.

191 GAS meningitis occurs very rarely, leading to the availability of a small number of cases
192 that could be included in this study. Nevertheless, despite the small simple size, our data

193 suggest that the most common pathophysiological cause in this patients' cohort was local
194 spread from a site of bacterial infection in neighboring tissues, with hematogenous spread
195 found in only one case. The invasive GAS strains did not exhibit particular virulence traits
196 when compared to colonizing or otitis strains, suggesting that host factors such as breach of
197 the mucosa or local infections play the main role in transition of a bacterial strain from
198 colonization to invasion, without distinct virulence determinants.

199 **Author contributions**

200 LM: Experimental design, acquisition, analysis and interpretation of data, writing of the
201 manuscript.

202 FA: Experimental design, acquisition, analysis and interpretation of data, writing of the
203 manuscript.

204 MB: Acquisition, analysis and interpretation of data, writing of the manuscript

205 TAS: Experimental design, data acquisition, data analysis and critical reading of the
206 manuscript

207 DMH: Experimental design, data acquisition, data analysis

208 EP: Experimental design, data acquisition, data analysis

209 JE: Data acquisition, critical reading of the manuscript

210 DM: Data acquisition

211 AKR: Data acquisition

212 EMM: Histological analysis

213 RAS: Funding

214 BH: Funding

215 SMS: Experimental design, funding, critical reading of the manuscript

216 SDB: Conceptualization, funding, critical reading of the manuscript

217 ASZ: Conceptualization, funding, critical reading of the manuscript

218

219 **Funding**

220 This work was supported by the "Schweizerischer Nationalfonds (SNF)" [grants
221 31003A_169962 and 310030_204343 (to A.S.Z.)], by the "University of Zürich CRPP
222 Personalized medicine of persisting bacterial infections aiming to optimize treatment and

223 outcome" (to S.D.B, B.H. and A.S.Z.), by the "Promedica Foundation" [grant 1449/M] (to
224 S.D.B.), by the "Swedish Society for Medical Research (SSMF) foundation" [grant P17-0179
225 (to S.M.S)] and by the Vontobel stiftung (to R.A.S.).

226

227 **Conflict of interest**

228 The authors have no conflict of interest to declare.

229 **References**

230 1. Imöhl M, Reinert RR, Ocklenburg C, van der Linden M. Epidemiology of invasive
231 Streptococcus pyogenes disease in Germany during 2003-2007. FEMS immunology and
232 medical microbiology **2010**; 58:389-96.
233 2. Lamagni TL, Darenberg J, Luca-Harari B, et al. Epidemiology of severe Streptococcus
234 pyogenes disease in Europe. Journal of clinical microbiology **2008**; 46:2359-67.
235 3. O'Loughlin RE, Roberson A, Cieslak PR, et al. The epidemiology of invasive group A
236 streptococcal infection and potential vaccine implications: United States, 2000-2004. Clinical
237 infectious diseases : an official publication of the Infectious Diseases Society of America
238 **2007**; 45:853-62.
239 4. Lucas MJ, Brouwer MC, Bovenkerk S, Man WK, van der Ende A, van de Beek D. Group A
240 Streptococcal meningitis in adults. The Journal of infection **2015**; 71:37-42.
241 5. van de Beek D, de Gans J, Spanjaard L, Sela S, Vermeulen M, Dankert J. Group a
242 streptococcal meningitis in adults: report of 41 cases and a review of the literature. Clinical
243 infectious diseases : an official publication of the Infectious Diseases Society of America
244 **2002**; 34:e32-6.
245 6. Cole JN, Barnett TC, Nizet V, Walker MJ. Molecular insight into invasive group A
246 streptococcal disease. Nature reviews Microbiology **2011**; 9:724-36.
247 7. Nizet V, Kim KS, Stins M, et al. Invasion of brain microvascular endothelial cells by group B
248 streptococci. Infection and immunity **1997**; 65:5074-81.
249 8. Andreoni F, Ogawa T, Ogawa M, et al. The IL-8 protease SpyCEP is detrimental for Group A
250 Streptococcus host-cells interaction and biofilm formation. Frontiers in microbiology **2014**;
251 5:339.

252 9. Seidl K, Zinkernagel AS. The MTT assay is a rapid and reliable quantitative method to
253 assess Staphylococcus aureus induced endothelial cell damage. Journal of microbiological
254 methods **2013**; 92:307-9.
255 10. Andreoni F, Zürcher C, Tarnutzer A, et al. Clindamycin Affects Group A Streptococcus
256 Virulence Factors and Improves Clinical Outcome. J Infect Dis **2017**; 215:269-77.
257 11. Räz AK, Andreoni F, Boumasmoud M, et al. Limited adaptive evolution of
258 Staphylococcus aureus during transition from colonization to invasive
259 infection. bioRxiv **2021**:2021.10.25.465068.
260 12. Seemann T. Prokka: rapid prokaryotic genome annotation. Bioinformatics **2014**; 30:2068-
261 9.
262 13. Page AJ, Cummins CA, Hunt M, et al. Roary: rapid large-scale prokaryote pan genome
263 analysis. Bioinformatics **2015**; 31:3691-3.
264 14. Price MN, Dehal PS, Arkin AP. FastTree: Computing Large Minimum Evolution Trees with
265 Profiles instead of a Distance Matrix. Molecular Biology and Evolution **2009**; 26:1641-50.
266 15. Control CoD. Available at: ftp://ftp.cdc.gov/pub/infectious_diseases/biotech/tsemm/.
267 16. VFDB. Available at: http://www.mgc.ac.cn/VFs/download.htm.
268 17. Schlech WF, 3rd, Ward JI, Band JD, Hightower A, Fraser DW, Broome CV. Bacterial
269 meningitis in the United States, 1978 through 1981. The National Bacterial Meningitis
270 Surveillance Study. Jama **1985**; 253:1749-54.
271 18. Santos MS, Ribeiro GS, Oliveira TQ, et al. Burden of group A streptococcal meningitis in
272 Salvador, Brazil: report of 11 years of population-based surveillance. Int J Infect Dis **2009**;
273 13:456-61.
274 19. Randhawa E, Woytanowski J, Sibliss K, Sheffer I. Streptococcus pyogenes and invasive
275 central nervous system infection. SAGE Open Med Case Rep **2018**; 6:2050313x18775584.

276 20. Sommer R, Rohner P, Garbino J, et al. Group A beta-hemolytic streptococcus meningitis:
277 clinical and microbiological features of nine cases. Clinical infectious diseases : an official
278 publication of the Infectious Diseases Society of America **1999**; 29:929-31.
279 21. Hersi K GF, Kondamudi NP. Meningitis. **2021**.
280 22. Perin A, Nascimben E, Longatti P. Decompressive craniectomy in a case of intractable
281 intracranial hypertension due to pneumococcal meningitis. Acta Neurochir (Wien) **2008**;
282 150:837-42; discussion 42.
283 23. Plainvert C, Doloy A, Joubrel C, et al. Characterization of Streptococcus pyogenes isolates
284 responsible for adult meningitis in France from 2003 to 2013. Diagnostic microbiology and
285 infectious disease **2016**; 84:350-2.
286 24. Gherardi G, Vitali LA, Creti R. Prevalent emm Types among Invasive GAS in Europe and
287 North America since Year 2000. Front Public Health **2018**; 6:59-.
288

289 **Figure legend**

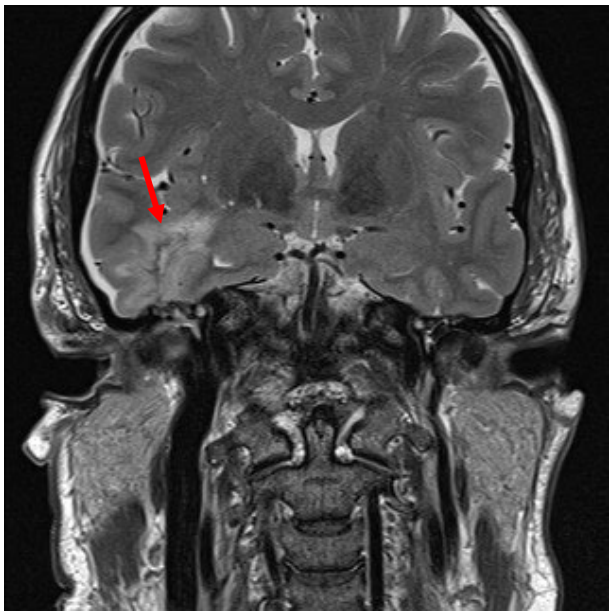
290 **Figure 1 - Bacterial strains virulence determinants**. **A)** Adherence and **B)** invasion to HBMEC
291 cells shows no difference between isolates groups. Bacterial virulence factors activity of **C)**
292 the nuclease Sda1, **D)** the IL-8 protease SpyCEP and **E)** SLO as well as **F)** HBMEC cells barrier
293 function disruption did not vary among isolates groups. For adherence, invasion as well as
294 Sda1 and SpyCEP activity, each point on the graph represents the average of at least three
295 biological replicates for one strain. Excluding barrier disruption experiments, which were
296 carried out in duplicate, all experiment were conducted at least in triplicate. Control
297 experiments were carried out using medium only.

A

CT scan

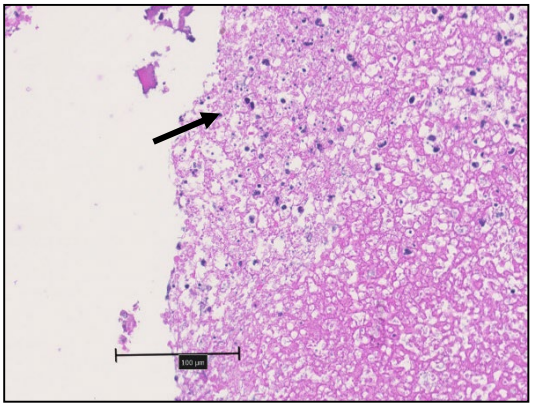
B

MRI



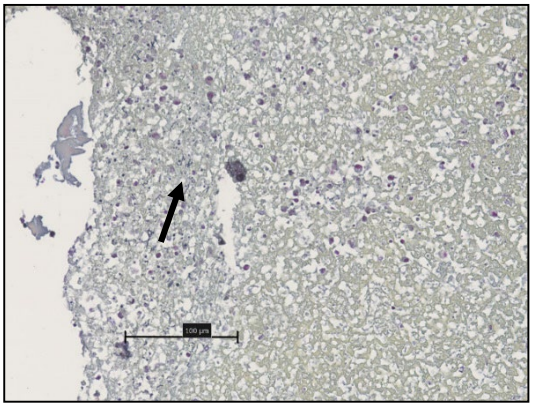
C

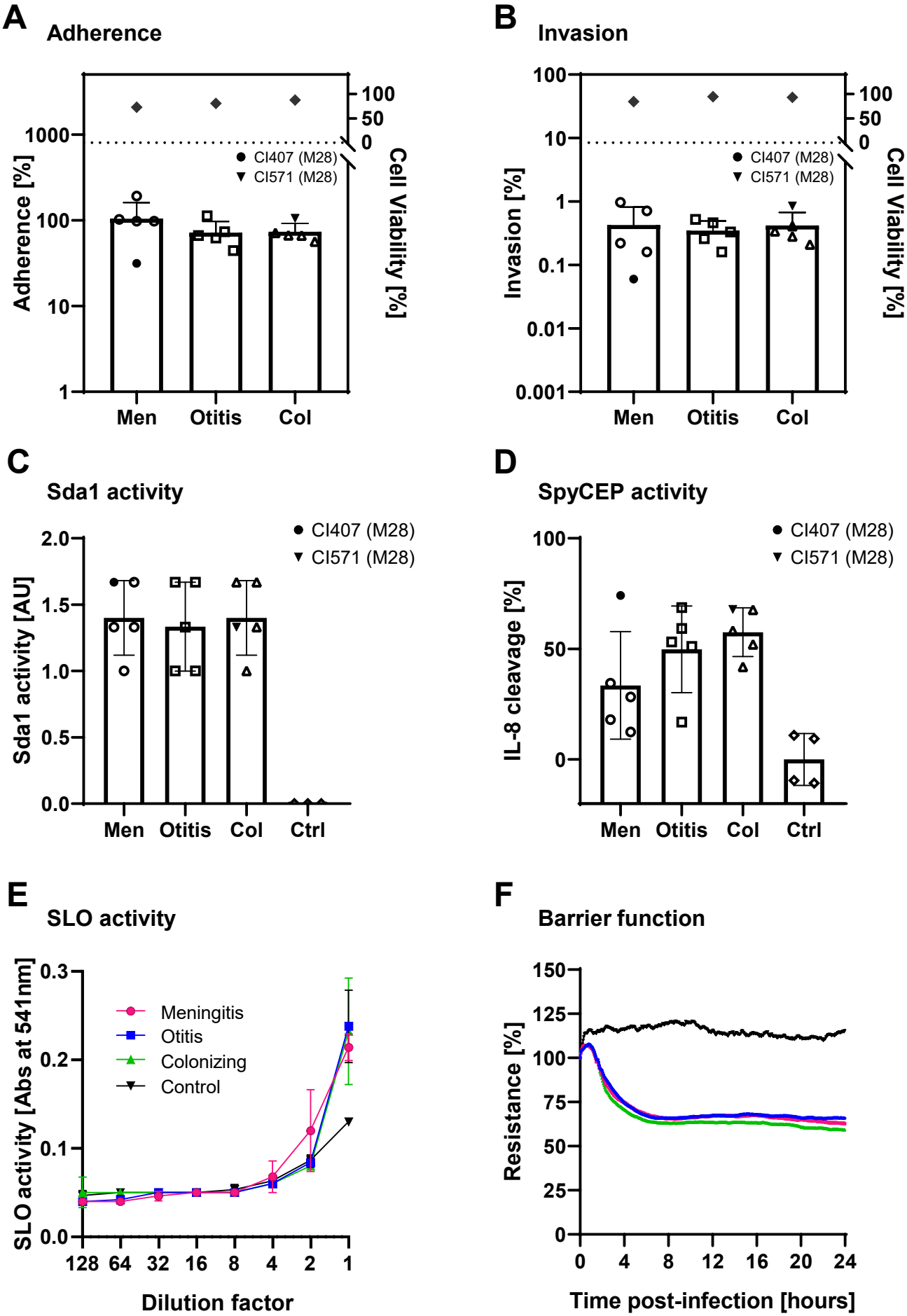
H&E stain



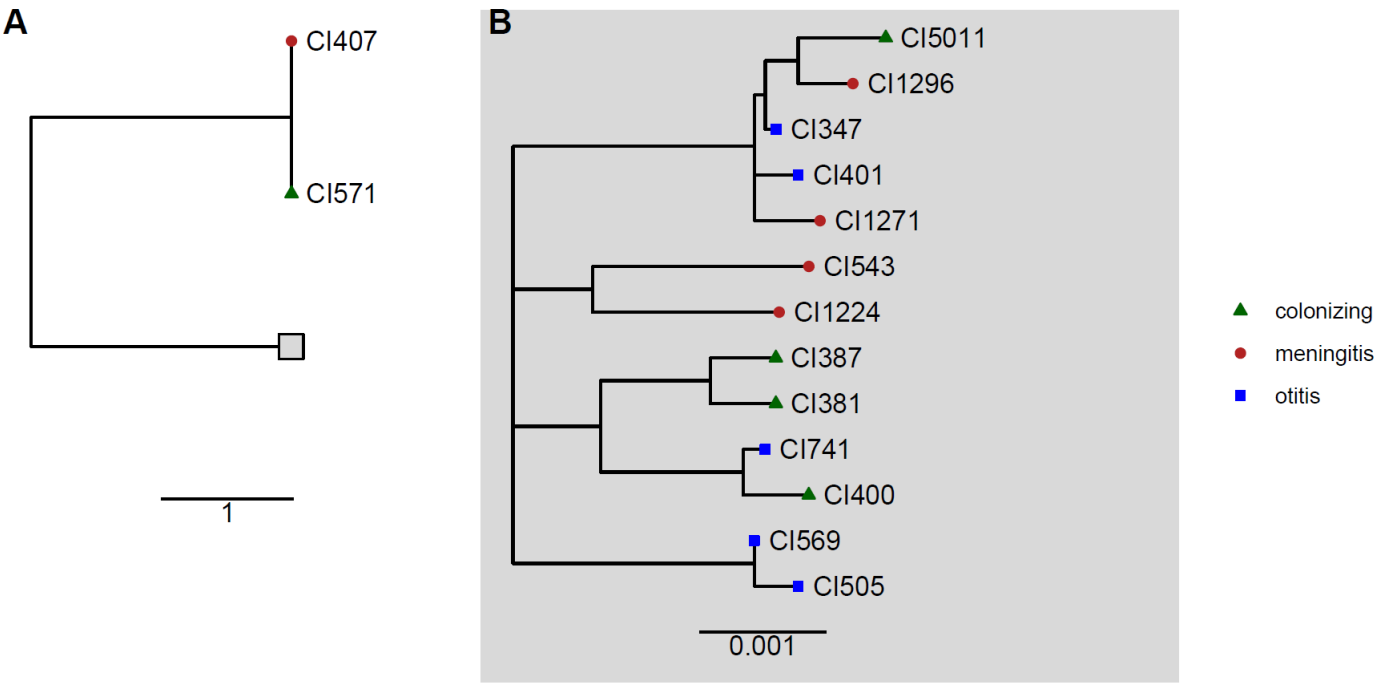
D

Brown-Brenn stain

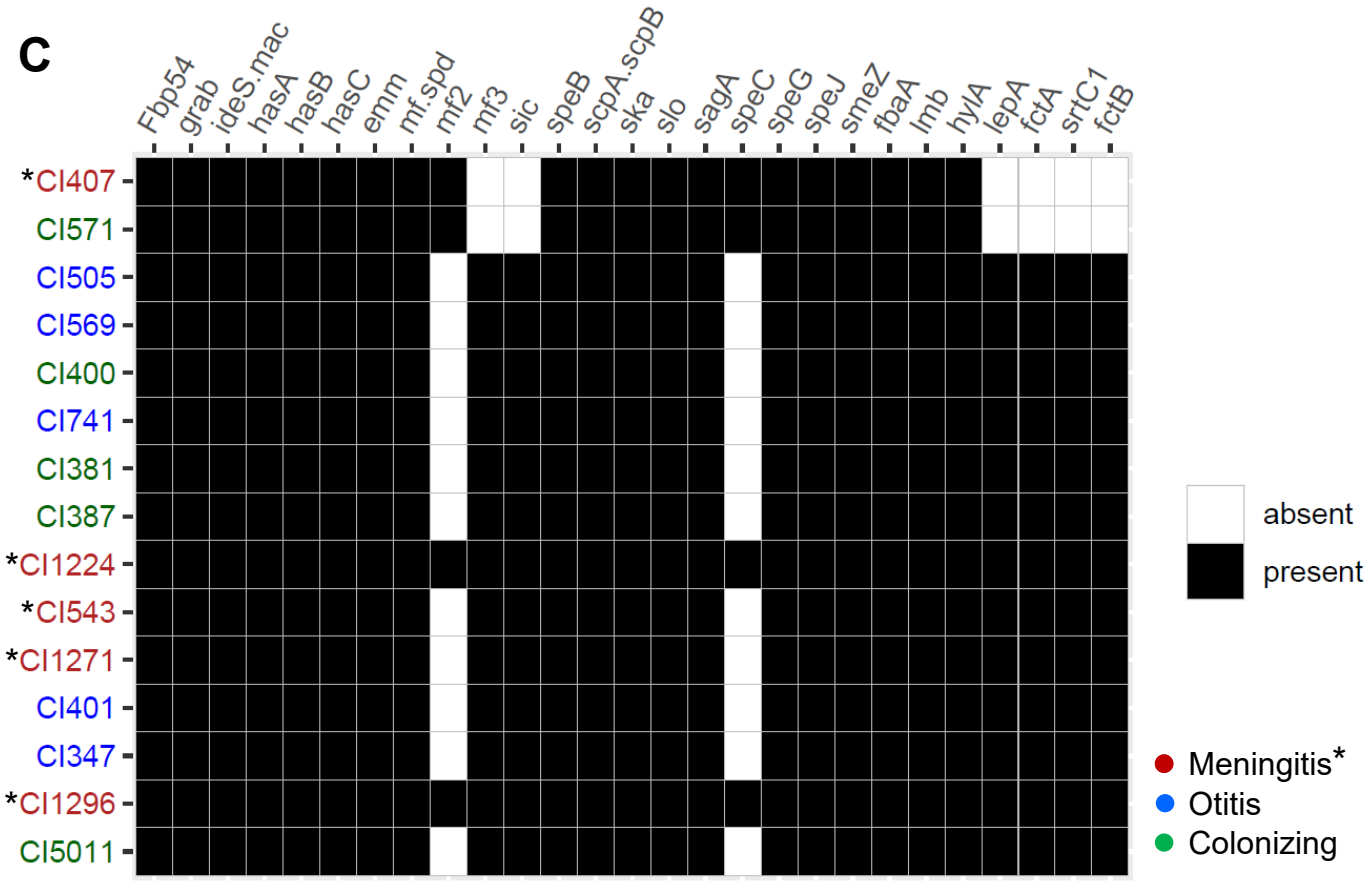



A
Adherence
Adherence [%]
Cell Viability [%]
CI407 (M28)
CI571 (M28)
Men
Otitis
Col
B
Invasion
Invasion [%]
Cell Viability [%]
C
Sda1 activity
Sda1 activity [AU]
Ctrl
D
SpyCEP activity
IL-8 cleavage [%]
E
SLO activity
SLO activity [Abs at 541nm]
Meningitis
Otitis
Colonizing
Control
Dilution factor
F
Barrier function
Resistance [%]
Time post-infection [hours]

Phylogenetic tree



Genetic determinants



Patient ID Sex Age(y) Isolate Nr.	Associated health conditions	Clinical presentation	Complications	Site of GAS identification	Antibiotic treatment regimen	Death
1 F 73 CI1224	• Perforated otitis media • Diabetes • Hypothyreosis • Depression	• Altered vigilance and mental state • Vertigo • Hypothermia • Hypotension • Acute abdomen	• Partial septic sinus transversus thrombosis • Coma • Irreversible hypoxic brain damage • ICU involvement	CSF Blood Ear swab	3d ceftriaxon 3d amoxicillin 3d clindamycin	yes
2 M 55 CI1271	• Otitis media • Depression	• Altered vigilance • Meningism • Slight one-sided arm paralysis • Otalgia • Diarrhea • Nausea	• Coma • Mastoiditis • Cerebral abscess • ICU involvement	Blood Mastoid tissue	2d ceftriaxon 1d amoxicillin Escalation to: 18d penicillin 5d metronidazole 5d clindamycin + Surgical intervention	no
3 F 64 CI1293	• Bicuspid aortic valve	• Acute reduction of general condition • Meningism • Right sided paresis of upper and lower limbs • Right sided tongue deviation • Anisocoria • Petechial bleeds	• Thrombocytopenia • Acute kidney failure • Aortitis • Nosocomial Influenza infection	CSF Blood	Initially ceftriaxon then change to penicillin due to elevated transaminases, total 18d 3d gentamycin 15d steroids 10d vancomycin 10d clindamycin	no
4 F 79 CI407	• Otitis media (perforated) • Coronary heart disease • Pre-diabetic metabolism	• High temperatures • Vertigo • Altered mental state and vigilance	• Tegmen tympani penetration • Mastoiditis • Labyrinthitis • Coma • Long-term one sided deafness • ICU involvement	Mastoid tissue	Initially 1d ceftriaxon, then escalation to meropeneme for 2d, then back to ceftriaxone for 20 days Initially 2d acyclovir 1d amoxicillin 4d mephamesone + Surgical intervention	no
5 M 42 CI543	• Otitis media • Bullous • Myringitis with insertion of tympanostomy tube	• One day after insertion of tympanostomy tube acute reduction of vigilance • Altered mental state and meningism	• Mastoiditis • Subdural empyema • Cerebritis • Ventrikulitis • Coma • ICU involvement	Blood CSF Mastoid tissue	9d ceftriaxon, then escalation to 18d ceftazidime and de-escalation back to ceftriaxone for another 35d 2d amoxicillin + clavulanic acid 2d acyclovir 2d metronidazol 2d, pause 3d, then another 2d 9d intrathecal gentamycin 9d intrathecal vancomycin + Surgical intervention	no

Table 1 – Patients description. Clinical findings, complications and treatment regimen of the patients included in this study. Abbreviations: CSF=cerebrospinal fluid; d=day; ICU=intensive care unit.