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

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Running title

Group A Streptococcus in meningitis

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32 **Abstract**

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

42 **Introduction**

43 *Streptococcus pyogenes* or group A *Streptococcus* (GAS) asymptomatically colonizes the
44 human throat and skin. In addition to asymptomatic colonization, GAS causes mild to life-
45 threatening infections, such as necrotizing fasciitis and meningitis. GAS meningitis cases are
46 rare and represent only 1-4% of all invasive GAS cases detected across Europe and the
47 United States [1-3]. GAS meningitis is a fulminant disease associated with high morbidity,
48 including neurologic sequelae, and mortality. It occurs mainly in patients with predisposing
49 factors such as otitis, sinusitis, recent head injury or neurosurgery, presence of a
50 neurosurgical device, cerebrospinal fluid leak, or alcoholism [4, 5]. This suggests extension
51 *per continuitatem* from a neighboring infection such as otitis media, resulting in otogenic
52 meningitis. However, in a third of cases there are no predisposing factors present. The
53 prevalence of the predisposing factors for GAS meningitis in children are acute otitis media
54 and acute mastoiditis (2-3% [6, 7] and 11% [8], respectively). GAS is also responsible for 41%
55 of acute mastoiditis and 14% of acute otitis cases that require hospitalization in children [9,
56 10] and for 19% of acute otitis and mastoiditis cases requiring hospitalization in adults [11].
57 The most common host-associated risk factors for invasive GAS infections in adults are
58 crowding, diabetes, cardiac disease, cancer and corticosteroid use [12]. Other risk factors are
59 breaching of the physical barriers skin and mucosa by trauma or preceding viral infections
60 such as varicella [13] and influenza [14, 15].

61 On the pathogen's side, GAS components such as the streptococcal virulence factors
62 Streptolysin O (SLO), streptococcal DNases, the IL-8 protease SpyCEP and the M-protein, play
63 key roles in the invasiveness and ability of the pathogen to successfully invade and survive
64 within the host environment [16]. SLO is a pore forming toxin that can lead to inhibition of
65 phagocytosis and cell lysis of target cells [17], the DNase sda1 was shown to degrade

66 neutrophils extracellular traps and to prevent TLR9-dependent recognition, leading to
67 enhanced pathogen spread in the host [18-20], while SpyCEP is involved in degradation of
68 the cytokine IL-8, impairing neutrophils recruitment to the site of infection [21].

69 The M-protein is a GAS surface protein promoting GAS survival in the host by increasing
70 adherence to host cells [22], escape from opsonophagocytosis [23] and neutrophil
71 activation, leading to inflammation [24]. To date, more than 200 different M-protein types
72 (*emm*-types) have been described, based on the sequence of the variable region of the M-
73 protein. Genotyping methods used for the classification of GAS strains include serotyping of
74 the M-protein as well as Multilocus Sequence Typing (MLST), based on the sequences of
75 seven housekeeping genes [25]. MLST is normally used to study the epidemiology and
76 population genetics of a specific set of isolates, proving particularly useful for the detection
77 and classification of outbreaks. Strains of *emm* type 1 (*emm*-1) and MLST-28 (ST28) or emm-
78 28 ST52 are highly prevalent among GAS meningitis isolates [26].

79 The aim of this study focused on assessing whether specific pathogen traits distinguish
80 GAS meningitis isolates from colonizing GAS isolates or GAS causing otitis and whether these
81 specific traits could be associated with disease severity. We investigated five patients
82 suffering from GAS meningitis, analyzing both host and pathogen characteristics. On the host
83 side, various clinical parameters were analyzed. On the pathogen side, we phenotypically
84 and genetically compared five GAS isolates derived from the abovementioned meningitis
85 cases with ten GAS isolates derived either from otitis patients or asymptomatic colonization.
86 To our knowledge, this is the first study comparing the virulence traits of meningitis isolates
87 with otitis or colonizing ones.

88 **Material and methods**

89 **Patients and ethics**

90 Five patients diagnosed between 2013 and 2017 with clinically confirmed community-
91 acquired GAS meningitis in Switzerland were retrospectively included in this study (Tab.1).
92 No specific surveillance program for GAS meningitis exists in Switzerland and the cases were
93 selected using a convenience sampling strategy. Otitis isolates were also selected using a
94 convenience sampling strategy while GAS colonizing isolates were selected to reflect the
95 population present in the meningitis and otitis groups (*emm*-1 and *emm*-28 types). This
96 study was approved by the regional committee for medical research ethics (BASEC-ID 2016-
97 00145 and 2017-02225).

98

99 **Bacterial strains and cell lines**

100 All meningitis, otitis and colonizing GAS clinical isolates are listed in Tab.S1 and were
101 cultured in THY [Todd-Hewitt broth (BD) plus 2% yeast extract (Oxoid)] at 37°C. Immortalized
102 human brain microvascular endothelial cells (HBMECs) [27] were cultured in Roswell Park
103 Memorial Institute (RPMI) medium supplemented with 10% FBS and L-glutamine, unless
104 specified otherwise.

105

106 **Adherence and invasion assays**

107 Adherence and invasion assays were performed as previously described, with minor
108 modifications [28]. HBMECs were seeded in 24 well plates to $2.2*10^4$ cells/well in RPMI
109 medium supplemented with 10% FBS and L-glutamine and incubated at 37ºC in the presence
110 of 5% CO_2 for 48h. Overnight (ON) cultures of the GAS strains were grown at 37ºC in a static
111 incubator in 5ml THY. Bacterial cultures were diluted 1:10 in fresh THY medium and grown

112 to an optical density at 600nm (OD_{600}) of 0.4, washed once with PBS and resuspended in
113 infection medium (RPMI+L-glutamine+0.4% bovine serum albumin). Prior to infection,
114 HBMECs were washed once with PBS and bacteria, resuspended in 500 ul of infection
115 medium, were added to reach a multiplicity of infection (MOI) of 1 or 10 for adherence and
116 invasion assays respectively. The plates were spun down at 1200g for 5 minutes to and
117 subsequently incubated 37ºC in the presence of 5% CO_2. To assess adherence, infected
118 HBMECs were incubated for 30 minutes, washed six times with PBS to remove unbound
119 bacteria and subsequently lysed using 500 ul of deionized sterile water (dH2O). To assess
120 invasion, infected HBMECs were incubated for 2 hours and washed three times with PBS to
121 remove excess bacteria. 500 ul of infection medium supplemented with penicillin and
122 gentamycin, at a final concentration of 10 ug/ml and 100 ug/ml respectively, were added to
123 each well to kill extracellular bacteria. The plates were then incubated for 2 hours at 37ºC in
124 the presence of 5% CO_2 and the cells were subsequently lysed with 500 ul of dH2O after
125 three washes with PBS. Ten-fold serial dilution of the cell lysates were plated on THY plates
126 to enumerate bacterial colonies after ON incubation at 37ºC. The percentage of adherence
127 and invasion were calculated relatively to the initial inoculum.
128
129 **Viability assay**
130 Cell viability was assessed as follows. HBMECs and bacteria were prepared for adherence
131 and invasion assays, as described above. After 30 minutes infection followed by six washes
132 with PBS or after the two hours incubation with antibiotics followed by three washes with
133 PBS, HBMECs were harvested using 100 µl of trypsin (Gibco), washed and resuspended in
134 500 µl PBS. Three wells/condition were pooled and the experiment was carried out in
135 duplicate.

136 250 µL of the cell suspension were transferred to the wells of a conical 96well plate, spun
137 down at 470g for 5min, washed with Annexin V binding buffer (ThermoFisher) and spun
138 down at 470g for 5min. Dead cells were stained using 40 µl/well of a cocktail of Annexin V –
139 FITC (#640906; Biolegend, 1:50 dilution) and 7 AAD (#420404; Biolegend 1:25 dilution)
140 diluted in Annexin V binding buffer, for the detection of apoptotic and late apoptotic cells.
141 The cells were incubated for 20min at room temperature, 160 µL of Annexin V binding buffer
142 were added and data were acquired using the AttuneNxT flow cytometer (ThermoFisher).
143

144 **Virulence factors activity**

145 Activity of the virulence factors SLO, streptococcal DNases and SpyCEP was assessed in
146 the supernatants of exponentially growing GAS as previously described [29]. ON cultures of
147 the various strains were statically grown at 37ºC in THY medium. Bacterial cultures were
148 diluted 1:10 in fresh THY medium and grown to an optical density at 600nm (OD_{600}) of OD
149 0.4. After centrifugation, the supernatants were removed and filter sterilized using 0.22 µm
150 filtration membranes.

151 SLO activity was assessed as follows. 4 mM DTT and 0.0004% Trypan-blue were added to
152 the supernatants, the samples were incubated for ten minutes at room temperature (RT)
153 and two-fold dilutions were made in PBS in a 96 well plate. PBS and dH_2O alone were used
154 respectively as negative and positive controls. Erythrocytes were diluted to 2% v/v in PBS
155 and added to the diluted supernatants (1 part erythrocytes, 4 parts supernatant) or PBS or
156 water for no lysis and full lysis controls, respectively. Samples were incubated at 37ºC for 30
157 minutes and spun down at 3000g for five minutes. The OD_{541} of the supernatants, directly
158 proportional to the activity of SLO, was used as a readout.

159 Streptococcal DNase activity was assessed as follows. 5 ul of bacterial supernatant or 5 ul
160 of THY (negative control) were mixed with 5 ul of 10X DNase buffer (50 mM CaCl2, 500 mM
161 Tris, pH 7.9) and 5ul of calf thymus DNA (1mg/ml, Sigma) in a total volume of 50 ul and
162 incubated at 37ºC for five minutes. The reaction was stopped by adding 8 ul of 0.5M EDTA
163 and the samples were loaded on a 0.8% agarose gel to assess DNA degradation.
164 Streptococcal DNase activity was evaluated with a score corresponding to no activity (0=no
165 degradation of calf thymus DNA), intermediate activity (1=partial degradation of calf thymus
166 DNA) and high activity (2=complete degradation of calf thymus DNA).

167 SpyCEP activity was assessed by measuring the degradation of the cytokine IL-8. Bacterial
168 supernatants were incubated with 1ng/ul IL-8 for 16 hours at 37ºC after which an IL-8 ELISA
169 was carried out according to the manufacturer's instruction (R&D). THY was used as a
170 negative control. SpyCEP activity is depicted as the percentage of IL-8 cleavage compared to
171 the negative control (medium only).

172

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

174 ECIS® was performed with the ECIS® Z-Theta instrument (Applied Biophysics) to monitor
175 the effects of the GAS strains on human brain microvascular endothelial cells (HBMECs) [27]
176 barrier integrity. Before seeding, the wells of the arrays (ECIS 8W10E+, IBIDI) were washed
177 twice with sterile, nuclease-free dH_2O, coated with 5 µg/ml collagen IV for 1h at room
178 temperature (RT), washed three times with dH_2O and incubated with 10 mM L-cysteine for
179 2h at RT. The wells were washed two times with dH_2O before 200 µl of culture medium were
180 added and the assay started (37ºC, 5% CO2). After 30 min, 200µl culture medium containing
181 10^5 HBMECs were seeded per well. After 22 h incubation 50 µl of medium were removed
182 and 2h later HBMECs were infected with log phase GAS strains resuspended in

183 DMEM+10%FCS at an MOI of 10. DMEM+10%FCS alone was used as a control. After 1.5h
184 penicillin (10 µg/ml) and gentamycin (100 µg/ml) were added. Barrier integrity was
185 monitored at 4000 Hz from seeding to 70h post infection. Resistance data were normalized
186 to the time of infection.
187
188 **Bacterial genomics**
189 Whole genome sequencing and genomic data analysis were performed as previously
190 described [30]. Briefly, from 150 bp paired end reads, *de novo* assemblies were generated
191 with SPAdes v.3.10 [30] and annotated with Prokka v.1.13 [31]. A pangenome was
192 constructed with Roary v.3.12 [32] and the resulting core-gene alignment was used to build
193 a maximum likelihood phylogenetic tree with Fasttree v.2.1.10 [33]. *Emm*-typing was
194 performed *in-silico* by querying assemblies against the curated CDC database [34, 35] with
195 blastn v.2.9.0. Virulence genes were identified by querying the assemblies against the full
196 VFDB database, i.e. VFDB_setB_nt.fasta, downloaded on the 04.08.23 [36], using ABRIcate
197 v.0.5 (minimum identity and coverage: 85%). ENA accession number: PRJEB57816.
198
199 **Statistics**
200 Three biological replicates were carried out for each strain, except for HBMECs viability and
201 ECIS assessment where two biological replicates were carried out for each strain. The
202 average of the biological replicates for each strain is represented on the graphs as a single
203 data point. The average of these values represents the final value depicted on the graphs.
204 Error bars represent standard deviations. The Mann-Whitney test was used to assess
205 statistical significance for all assays apart from HBMECs viability data that were analyzed
206 using the Kruskal-Wallis test.

207

208 **Results**

209

210 **Cases description**

211 Five GAS meningitis cases, occurring between 2013 and 2017 in Switzerland, were
212 included in this study and the corresponding GAS isolates were retrospectively retrieved for
213 analysis. The median age of the patients was 64 years (42 to 79 years) and 60% (n=3) were
214 female. The mortality rate was 20% (n=1) while 2 out of 6 patients (33%) experienced
215 neurological sequelae (patients 3 and 4), which included slight hearing loss, vertigo and
216 slight paresis of the limbs. The median duration of the hospital stay was 17 days (3 to 29
217 days) and 4 patients needed intensive care treatment with endotracheal intubation.
218 Relevant clinical and laboratory findings are depicted in table 1. Mastoiditis was the
219 underlying diagnosis in three cases. During the course of the disease one patient had septic
220 thrombosis and one developed a brain abscess (Fig.1A-1B). Surgical treatment such as
221 antrotomy, mastoidectomy, or burr-hole trepanation was required in three patients. In
222 patient 4, histological analysis from the surgically-removed mastoid tissue showed necrotic
223 tissue and individual mucosal fragments covered by simple cuboid epithelium with severe
224 acute and chronic inflammation and showing extracellular accumulation of bacterial cells
225 (Fig.1C-1D).

226

227 **Bacterial virulence**

228 The assessment of adherence to and invasion of HBMECs, the activity of the streptococcal
229 virulence factors DNases, SLO and SpyCEP as well as barrier integrity of HBMECs upon GAS
230 challenge was carried out on meningitis, otitis and colonizing clinical isolates. No significant
231 difference between adherence and invasion behavior to HBMECs upon infection with

232 meningitis, otitis or colonizing GAS isolates was found (Fig.2A-2B) and the toxicity of the
233 isolates toward HBMECs during infection did not vary according to their sampling location
234 (Fig.S1A-S1B). Furthermore, no differences in virulence factors activity or the isolates
235 potential to disrupt the barrier formed by HBMECs among the three groups were observed
236 (Fig.2C-2F). A trend, albeit not significant, towards reduced SpyCEP activity was observed in
237 the meningitis group (Fig.2D).
238
239 **Genotyping**
240 Typing of meningitis and otitis isolates showed a high prevalence of emm-1/ST28 (n=4/5 and
241 n=5/5, respectively, Tab.S1). Since one meningitis clinical isolate belonged to the emm-
242 28/ST52, we chose to include four emm-1/ST28 and one emm-28/ST52 in the colonizing
243 isolates group. Only one meningitis clinical isolate belonged to the *emm*-28/ST52 and one
244 colonizing isolate to the *emm*-28/ST458. A core genome phylogeny revealed that, within the
245 M1 clade, the meningitis isolates did not form a monophyletic group. (Fig.S2A-S2B). The
246 presence of genes encoding virulence factors was analyzed and this showed no specific
247 pattern for meningitis isolates when compared to otitis or colonizing ones (Fig. S2C). We
248 additionally checked for the presence of mutations in the GAS CovRS regulatory genes that
249 may lead to upregulated virulence factors activity (Tab.S2) [37]. No mutations were found in
250 CovR, while we found CovS mutations in two of the meningitis isolates (CI1224, *emm*-1 and
251 CI407, *emm*-28) and in one of the colonizing ones (CI571, *emm*-28). An in-frame deletion of
252 the proline in position 16 was found in CI1224. CI407 presented a deletion of the first 46
253 amino acids as well as non-synonymous mutations in position 47 (Leu-Met), 226 (Glu-Gly)
254 and 332 (Val-Glu). Isolate CI571 had the same non-synonymous mutations as CI407 in
255 position 226 (Glu-Gly) and 332 (Val-Glu), common to the emm-28 lineage [38]. However,

256 these mutations did not result in a reduced CovS function or increased virulence factors

257 activity.

258 **Discussion**

259 We found no particular phenotypic or genotypic characteristics in the five GAS isolates
260 causing meningitis as compared to otitis or colonizing isolates. The only common
261 denominator was preceding otitis and mastoiditis on the host's side. GAS meningitis is an
262 extremely rare condition, representing only the 0.2%-0.9% of cases of bacterial meningitis in
263 the United States and Brazil [39-41] and 1-4% of invasive GAS cases in Europe and the United
264 States [1-3]. A report from Switzerland described nine cases of GAS meningitis registered
265 between 1983 and 1999 across all major Swiss hospitals, again emphasizing the rarity of this
266 disease [42]. A recent report showed an increase in the number of GAS meningitis case in
267 the Netherlands in 2022-2023, compared with the previous 40 years, with emm-1 being the
268 most represented M-protein variant [43].

269 Meningitis occurs either by hematogenous spread or by direct spread of bacteria from a
270 neighboring infection. Hematogenous spread normally occurs when colonizing bacteria
271 breach the nasopharyngeal mucosa, reach the blood [44] and subsequently cross the blood-
272 brain barrier. It was suggested that spread from the upper respiratory tract's mucosa may be
273 due to an altered balance in the host microbe population, possibly favoring GAS number
274 increase and subsequent spread [5]. This interesting hypothesis was however never tested
275 and remains a speculation. On the other hand, direct spread to the brain can also occur *per*
276 *continuitatem* from infected adjacent anatomic structures, such as the ear or the mastoid
277 bone [44].

278 We searched for common traits in the clinical presentation of our patients and
279 characteristics shared by the meningitis clinical isolates, as opposed to otitis and colonizing
280 ones, to find possible explanations for the spreading of GAS to the brain. In our patients'
281 cohort, the presence of mastoiditis in four patients and thrombosis in one supports the idea

282 of direct spread through bone erosion from neighboring oto-mastoiditis and osteo-
283 thrombophlebitis. Moreover otitis, a condition often associated with GAS meningitis [5], was
284 present in the majority of cases suggesting continuous spread as a likely cause of infection.
285 In contrast, hematogenous spread was less common and a plausible cause of infection for
286 patient 3 only. There is no way to determine whether the presence of bacteria in the blood
287 in patients 1, 2 and 5 occurred before or after spread to the central nervous system.
288 However, given the infection focus and the evidence pointing to continuous spread from the
289 ear and mastoid bone as one of the main causes of GAS meningitis, we postulate that
290 hematogenous spread, In the case of patients 1, 2 and 5, was not the cause of meningitis.

291 The presence of a local focus of infection may explain the high surgery rate in our GAS
292 meningitis cohort, which has been reported previously. In a nationwide cohort study in the
293 Netherlands, 46% of GAS meningitis patients required surgical intervention, while this
294 remains an absolute rarity only described in case reports for the more common meningitis
295 pathogens (Pneumococcus, Meningococcus) [4, 45].

296 On the pathogen side, no common features explaining bacterial spread to the brain were
297 found among the meningitis isolates, as compared to otitis and colonizing ones. This points
298 to host rather than pathogen factors playing a role in facilitating bacterial invasion, as
299 previously described for *S. aureus* [30]. Adherence and invasion assays towards HBMECs
300 confirmed the absence of a particular tropism towards brain microvascular tissue for the
301 meningitis isolates, excluding an advantage in crossing or damaging the blood brain barrier,
302 as compared to otitis and colonizing ones, in the tested settings. Moreover, virulence factors
303 activity did not significantly vary among the three groups of isolates. The *emm*-type of the
304 strains did not generally seem to influence adherence, invasion or virulence factors activity,
305 although the number of strains and *emm*-types tested is too little to be able to make a

306 conclusive statement. A trend towards decreased SpyCEp activity was observed in the
307 meningitis group with the M28 strain CI407 acting as an outlier and presenting a level of
308 activity remarkably higher than the other meningitis isolates. CI407 was characterized by a
309 pronounced deletion of the first portion of the CovS protein, a key regulator of virulence
310 factors activity in GAS, as well as by the presence of three non-synonymous mutations
311 (Tab.S2), as compared to the sequence of the CovS protein of GAS M1T1 5448 [37]. Normally
312 a complete loss of CovS function, as for the GAS M1T1 5448 AP strain, would lead to a
313 complete IL-8 degradation leading to 100% cleavage [29], which we did not observe. SLO and
314 DNase activities were also identical to the non-mutated strains. A partial loss of CovS
315 functionality could be postulated in this case [46], although the mutations in CI407 mainly
316 affect the N-terminal portion of the CovS protein where no functional domains are present
317 [18, 47]. Further investigations involving a larger group of clinical isolates, including different
318 *emm*-types, is needed to address the question whether a decreased IL-8 degradation
319 potential might facilitate invasion of the blood brain barrier. Isolates from the *emm*-1 and
320 *emm*-28 lineages represented both in the meningitis and colonizing groups, behaved very
321 similarly in terms of virulence factors activity.

322 In accordance with previous studies stating an association of *emm*-1/ST28 with GAS
323 meningitis [26], 4/5 meningitis isolates belonged to the *emm*-1/ST28 lineage. The fifth
324 isolate belonged to the *emm*-28 lineage, which is another prevalent *emm*-type previously
325 described in GAS meningitis [26] and, together with *emm*-1, among the most representative
326 *emm*-types for invasive GAS infections across Europe and nord America [48]. All otitis and
327 colonizing isolates belonged either to the *emm*-1 or the *emm*-28 lineages as well. A
328 prevalence of the emm-1 linage in acute otitis media in children (around 30%) was
329 previously reported [49]. The serotype of colonizing isolates was however specifically

330 selected to reflect the serotypes present in the meningitis and otitis groups. It cannot be
331 excluded that the overrepresentation of *emm*-1 serotypes in meningitis and otitis might
332 reflect the global success and dominance of *emm*-1 strains causing GAS invasive infections
333 [48]. The presence or absence of GAS virulence determinants did not reveal a specific
334 pattern for meningitis isolates when compared to otitis and colonizing ones.

335 GAS meningitis occurs very rarely, leading to the availability of a small number of cases
336 that could be included in this study. Nevertheless, despite the small sample size, our data
337 suggest that the most common pathophysiological cause in this patients' cohort was local
338 spread from a site of bacterial infection in neighboring tissues, with hematogenous spread a
339 possible explanation in one case.

340 In the absence of distinct virulence determinants characterizing the meningitis isolates,
341 we conclude that, in this particular cohort, host factors such as breach of the mucosa or local
342 infections play a preponderant role in the transition from colonization to invasion. These
343 results call for future studies including a higher number of cases and clinical isolates and a
344 more comprehensive phenotypic and genotypic investigation to support our conclusion.

Author contributions

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

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

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

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

DMH: Experimental design, data acquisition, data analysis

EP: Experimental design, data acquisition, data analysis

SH: Experimental design, data acquisition, data analysis

JE: Data acquisition, critical reading of the manuscript

DM: Data acquisition

AKR: Data acquisition

EMM: Histological analysis

RAS: Funding

BH: Funding

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

SDB: Conceptualization, funding, critical reading of the manuscript

ASZ: Conceptualization, funding, critical reading of the manuscript

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373

374 **Conflict of interest**

375 The authors have no conflict of interest to declare.

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503

504 **Figure legend**

505 **Figure 1 - Patients scans and histology results**

506 **A)** CT-Scan: septic thrombosis with mastoiditis and otitis media on the right side. The red
507 arrow indicates thrombosis **B)** MRI-Scan: T2 sequence showing penetration of lower
508 temporal lobe with abscess formation. The red arrow indicates an abscess **C)** HE Staining of
509 mastoid tissue: the black arrow indicates necrotic mastoid tissue, the dashed black arrow
510 indicates mixed neutrocytic and lymphocytic infiltration, foam cells and cholesterol crystals
511 (not depicted) indicating chronic inflammation. **D)** Brown-Brenn staining of mastoid tissue:
512 The arrows indicates accumulation of extracellular bacteria in chains.

513

514 **Figure 2 - Bacterial strains virulence determinants**

515 **A)** Adherence to and **B)** invasion of human brain vascular endothelial cells (HBMECs) were
516 assessed after 30 minutes (MOI 1) or two hours (MOI 10) of infection, respectively.
517 Adherence and invasion percentages were calculated based on the initial bacterial inoculum
518 used to infect HBMECs. Bacterial adherence above 100% occurred in one case and could be
519 explained by increased initial bacterial growth or by disruption of long bacterial chains or
520 clumps due to sheer pipetting forces during the eukaryotic cells lysis step. Bacterial virulence
521 factors activity of **C)** Streptococcal DNases, **D)** the IL-8 protease SpyCEP and **E)** the pore-
522 forming toxin SLO were assessed in supernatants of exponentially growing GAS. **F)** HBMECs
523 barrier function disruption was carried with the ECIS® Z-Theta instrument (Applied
524 Biophysics), measuring the impedance generated by the cell barrier in the presence or
525 absence of bacteria. Control experiments were carried out using medium only. **A, B, C, D)**
526 The average of the biological replicates for each strain is depicted as a single data point on
527 the graphs, so that each data point represents a single strain. The two strains indicated in

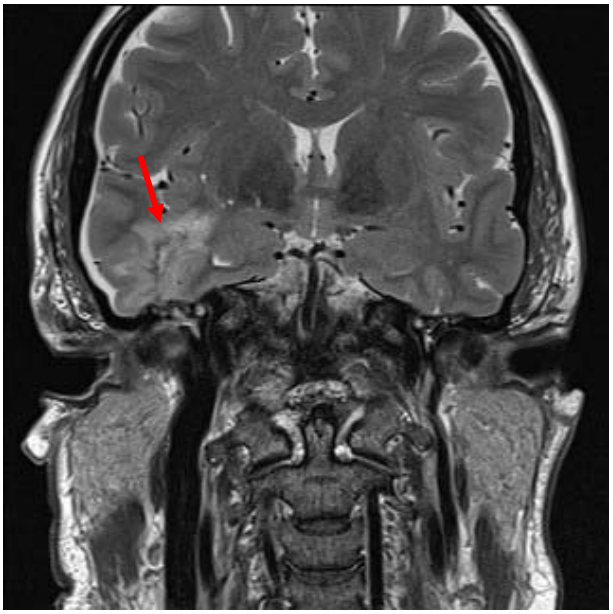
528 the figure legend with a black symbol (CI407 and CI571) are the only strains of *emm*-type 28
529 (M28), as opposed to all other strains that are *emm*-type 1 (M1). **A, B, C, D, E)** Three
530 biological replicates were carried out for each strain. The average values for strains
531 belonging to a single group of isolates (meningitis, otitis or colonizing) is depicted in the
532 graphs and the error bars represent the standard deviation. **F)** Two biological replicates were
533 carried out for each strain and the average of the strains belonging to the same group is
534 depicted on the graph. Control=medium only. No significant differences in adherence,
535 invasion, virulence factors activity or barrier disruption was observed among the different
536 groups of isolates. A significant difference was found between the respective controls and
537 meningitis, otitis or colonizing isolates for DNase (p=0.0159, 0.0159 and 0.0159, respectively)
538 and SpyCEP (p=0.0179, 0.0357 and 0.0179, respectively) activity. Statistical significance was
539 assessed using the Mann-Whitney test. Men=meningitis, Col=colonizing, Ctrl=control.

A

CT scan

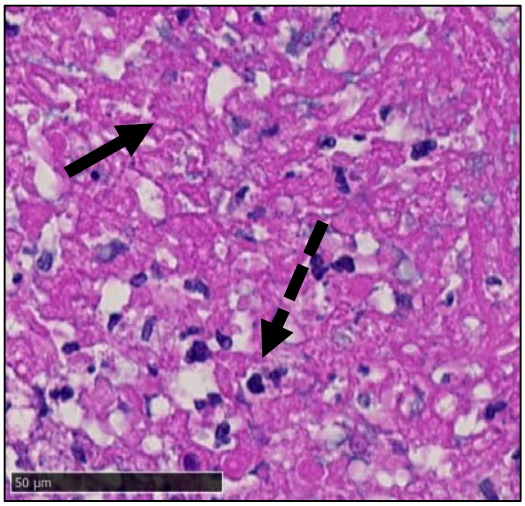
B

MRI



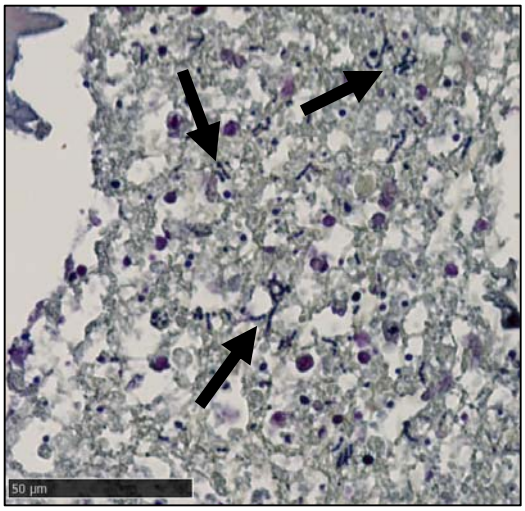
C

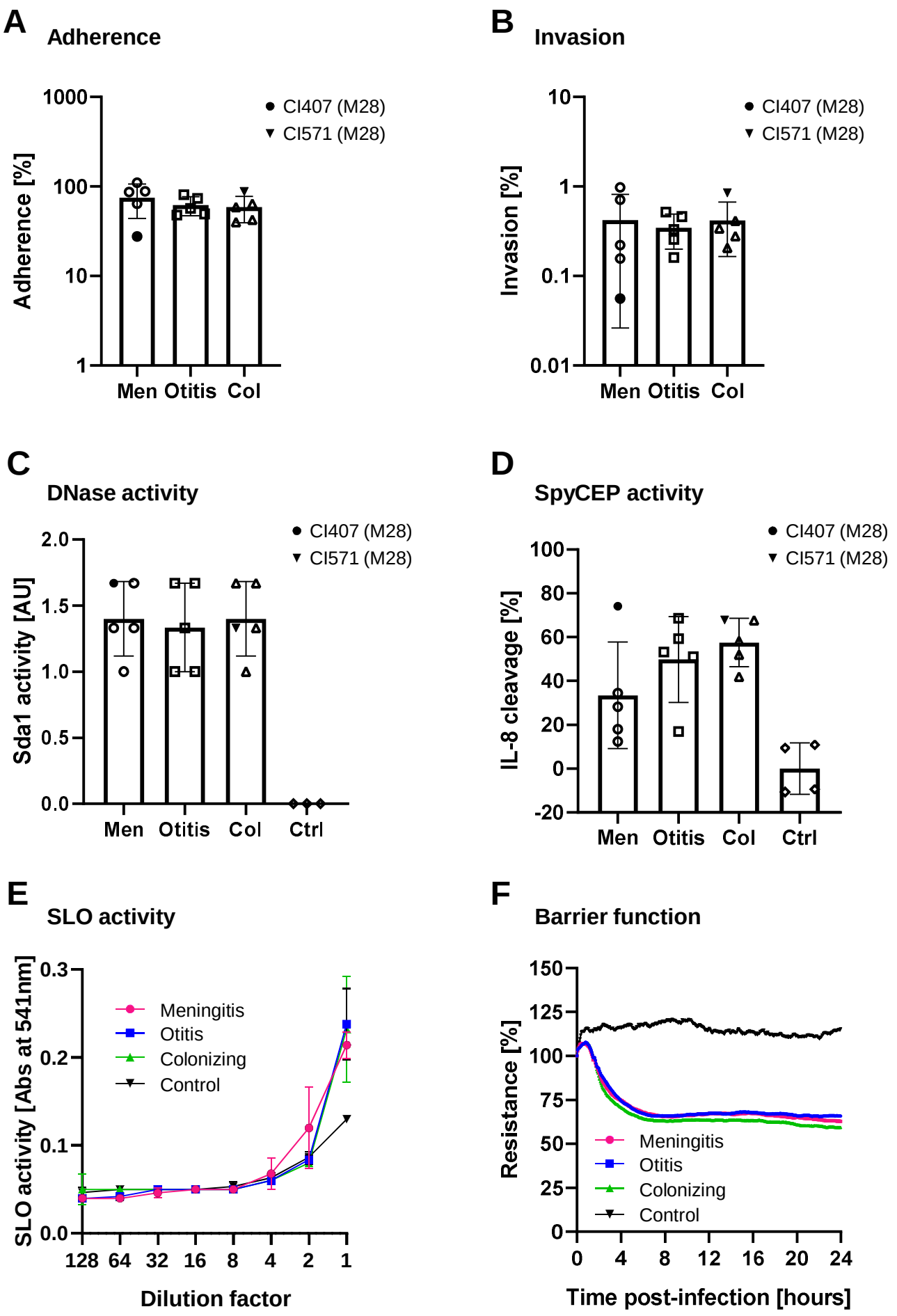
H&E stain



D

Brown-Brenn stain




A
Adherence
Adherence [%]
1000
100
10
1
Men
Otitis
Col
CI407 (M28)
CI571 (M28)
B
Invasion
Invasion [%]
10
1
0.1
0.01
Men
Otitis
Col
CI407 (M28)
CI571 (M28)
C
DNase activity
Sda1 activity [AU]
2.0
1.5
1.0
0.5
0.0
Men
Otitis
Col
Ctrl
CI407 (M28)
CI571 (M28)
D
SpyCEP activity
IL-8 cleavage [%]
100
80
60
40
20
0
-20
Men
Otitis
Col
Ctrl
CI407 (M28)
CI571 (M28)
E
SLO activity
SLO activity [Abs at 541nm]
0.3
0.2
0.1
0.0
128
64
32
16
8
4
2
1
Dilution factor
Meningitis
Otitis
Colonizing
Control
F
Barrier function
Resistance [%]
150
125
100
75
50
25
0
0
4
8
12
16
20
24
Time post-infection [hours]
Meningitis
Otitis
Colonizing
Control

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.