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1 **Novel biomarkers for the early prediction of pediatric cystic echinococcosis**
2 **post-surgical outcomes**

3 **Running title: Early biomarkers to predict CE post-surgical outcomes**

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42 **Declarations of interest:** none

43 **Summary**

44 **Objective:** This study aims to search for reliable serological biomarkers allowing the
45 early prediction of cystic echinococcosis (CE) post-operative outcomes.

46 **Methods:** We applied immunoprecipitation (IP) of *Echinococcus granulosus*
47 protoscolex antigens with pediatric CE patients' plasma collected at 1-month and 1-
48 year post-surgery, followed by Liquid Chromatography with tandem mass
49 spectrometry (LC-MS/MS). We compared IP proteomic content from relapsed
50 patients within the first-year post-surgery (RCE) to cases with no relapses until 3
51 post-operative years (NRCE). Selected proteins were recombinantly synthesized and
52 assessed for their prognostic performance by Enzyme-linked immunosorbent assay
53 (ELISA).

54 **Results:** A total of 305 immunoreactive parasitic proteins were identified, 59 of which
55 were significantly more abundant in RCE than NRCE for both time-points. Four
56 proteins showed the most promising characteristics for predicting CE outcomes:
57 cytoplasmic malate dehydrogenase (Eg-cMDH), citrate synthase (Eg-CS), Annexin
58 A6 and severin. ELISA-IgG against the four markers were significantly lower at 1-
59 year post-surgery than 1-month in NRCE, in contrast to RCE that displayed either
60 stable or higher levels. The Eg-cMDH and Eg-CS showed the best prognostic
61 performance, with respective probabilities of being “relapse-free” of 83% and 81%, if
62 a decrease of IgG levels occurred between 1-month and 1-year post-surgery.

63 **Conclusion:** The Eg-cMDH and Eg-CS are promising biomarkers to predict early CE
64 post-surgical outcomes.

65 **Keywords:** predictive biomarkers, serological follow-up, cystic echinococcosis, post-
66 surgical outcomes, pediatric patients, proteomics, ELISA.

67 **Introduction**

68 Cystic echinococcosis (CE) is a neglected helminthic zoonosis caused by the larval
69 stage of the tapeworm *Echinococcus granulosus* sensu lato (*E. granulosus*). The
70 parasitic life cycle comprises a definitive host (canids) harboring *E. granulosus* adult
71 tapeworms and an intermediate host (herbi- or omnivores) where the metacestode
72 develops after ingestion of eggs shed in the definitive host's faeces. Humans become
73 accidentally infected following the consumption of food and/or water contaminated
74 with parasite eggs or through close contact with soiled surfaces or animals. Viable
75 eggs hatch in the stomach and small intestine, releasing oncospheres which
76 penetrate the gut wall, travel via blood or lymph to finally reach internal organs and
77 develop into hydatid cyst. Months to years post-infection, these cysts internally
78 produce parasite forms called protoscoleces, which are infectious for new definitive
79 hosts [1]. Most CE cases remain asymptomatic for a long time period, and can be
80 incidentally disclosed upon imaging investigations requested for unrelated complaints
81 [2]. Despite global efforts, CE continues to be a serious public health burden
82 worldwide [3]. One key feature that marks the post-surgical period of CE-treated
83 cases is the high rates of relapses [4]. Up till now, the follow-up of CE primarily relied
84 on imaging [5]. Serology plays a complementary role, but could be extremely useful,
85 particularly in areas where access to imaging facilities is difficult [4]. Serological tests
86 are mainly based on the detection of antibodies against the hydatid fluid, or native
87 and recombinant antigens derived from *E. granulosus* [4], but performance is
88 hampered either by the relatively low number of responsive patients, the long-term
89 persistence of anti-hydatid fluid antibodies after effective clinical cure or limited value
90 for detecting recurrences [6, 7, 8, 9, 10, 11, 12]. Other markers, such as cytokines
91 [13], parasite-derived circulating DNA [14] and Micro-RNAs [15] are promising but

92 pitfalls related to test standardization and potential cross-reactivity were highlighted.
93 Thus, there is an urgent need for the identification of novel parasite-specific
94 biomarkers with enhanced sensitivity for predicting CE post-surgical development
95 and suitable for routine application in the underprivileged endemic areas.

96 Previous studies on the *E. granulosus* proteome have shown that protoscolex
97 proteins are highly immunogenic [16, 17]. The protoscolex is an interesting sample to
98 investigate since it could be studied with minimal host protein contamination and,
99 although not the infective form for humans, it can generate a hydatid cyst when
100 released in a tissue site [18]. Indeed, secondary cysts usually develop after
101 accidental spillage of protoscoleces during the surgical procedure, which is the most
102 frequent reason for CE post-operative relapses [2].

103 The aim of this study was to develop a simple serological test for the early prediction
104 of CE post-surgical outcomes. Hence, we first developed a proteomic approach
105 based on immunoprecipitation (IP) of protoscolex antigens followed by LC-MS/MS
106 analysis, then we (i) compared the protein content of IP-eluates obtained with plasma
107 of pediatric CE patients relapsing within the first-year post-surgery versus patients
108 with no detectable relapses after 3.5 post-operative years (ii); subsequently we
109 selected and produced 4 recombinant proteins particularly interesting to predict the
110 occurrence of CE relapses, and finally (iii) we developed an ELISA to assess its
111 predictive value for human CE post-surgical outcomes.

112 **Materials and Methods**

113 ***Echinococcus granulosus* proteins**

114 *E. granulosus* protoscolex soluble and membrane antigenic extracts (PSMAs) were
115 obtained after collection of protoscoleces from infected livers of freshly slaughtered
116 sheep (G1 genotype), as previously described [10].

117 **Plasma specimens**

118 For proteomic analyses, 20 plasma samples were collected from 10 Tunisian
119 pediatric CE-confirmed patients aged from 6 to 16 years (thus 2 temporally different
120 plasma samples per patient). All cases had CE1 active cysts, according to the current
121 WHO-IWGE (Informal Working Group on Echinococcosis) classification system, and
122 at least one pulmonary location [19]. All cysts were surgically removed from all
123 patients. Blood specimens were obtained from each patient at 1-month (1M) and 1-
124 year (1Y) post-surgery. Based on the respective post-operative clinical development,
125 patients were clustered into two groups: patients with no relapses detectable after 3.5
126 years post-surgery (Non-relapsed (NRCE); n = 5). The 5 other patients showed
127 radiological evidence of relapse within the first post-surgical year (Relapsed (RCE); n
128 = 5). An additional post-operative albendazole medication was prescribed for 3/5 and
129 2/5 of NRCE and RCE, respectively.

130 For assessing the ELISA predictive performance, the same plasma collection was
131 used as described in our two previous studies [9, 10]. Briefly, 153 plasma samples
132 were collected from 51 CE-surgically treated young patients who were categorized to
133 NRCE (n = 34) and RCE (n = 17) based on the above-mentioned criteria (15/34 and
134 2/17 NRCE and RCE patients, respectively, received a post-operative prophylactic
135 medication with albendazole). Specimens were collected at 3 time-points: pre-
136 operatively (D0), 1M, and 1Y post-surgery. With regards to cyst stages, CE1 was the
137 most representative, detected in 27 and 16 NRCE and RCE patients, respectively, 3
138 NRCE had a unique CE2 cyst and 4 had CE3a cysts. The latter stage was observed

139 only in one RCE patient. Diagnosis, surgical treatment, and follow-up of young
140 patients were carried out at the Pediatric Surgery Department of Fattouma-Bourguiba
141 Hospital, Monastir, Tunisia. The negative control consisted of a human plasma pool
142 collected from 18 healthy blood donors. This study was approved by the Ethics
143 committee of the Faculty of Medicine, Monastir, Tunisia (Acceptance Report IORG
144 0009738 N°22/OMB 0990-0279).

145 **Immunoprecipitation (IP)**

146 IP was performed using the Dynabeads™ Protein G Kit (ThermoFisher Scientific)
147 according to the manufacturer's instructions. A total of 21 IP reactions were
148 performed, 20 reactions with plasma from the 10 CE pediatric patients (as described
149 in the previous section) and 1 reaction with the pool of controls. Briefly, magnetic
150 beads were incubated with plasma samples to coat antibodies. After 3 washings,
151 PSMAs sample was incubated to catch only immunoreactive proteins. A final elution
152 step permitted the obtention of the target antibody-antigen complex (see
153 Supplementary Methods). IP eluates were treated under reducing conditions and
154 separated by SDS-PAGE. Gel bands were then excised for LC-MS/MS analyses.

155 **Mass spectrometry and proteins' selection strategy**

156 Briefly, the gel pieces were destained, subjected to standard tryptic "in-gel digestion"
157 and then analyzed by LC-MS/MS. Proteins identification was performed on XTandem
158 Pipeline [20]. The False Discovery Rate (FDR) levels were estimated to 0.6% for
159 peptides and 0.0% for proteins using Xtandem "include reverse" option. The protein
160 quantification was determined after analysis of the total number of tandem mass
161 spectra (SN). The mass spectrometry proteomics data have been deposited to the
162 ProteomeXchange Consortium via the PRIDE [21] partner repository with the dataset
163 identifier PXD028301. More details are provided in supplementary methods.

164 We applied a selection strategy to search for candidate proteins most useful for the
165 prediction of CE outcomes as follows: Firstly, after eliminating proteins identified with
166 less than 5 spectra and with a foldchange less than 1.5, heat was performed using
167 heatmap.2 from ggplots with Euclidean distance. Secondly, clustering was performed
168 using sota from ciValid [22]. The proteins were quantified after analyzing the total
169 number of tandem mass spectra per sample. This spectral counting (SC) has been
170 based on a comparative approach between NRCE versus RCE groups and 1M
171 samples versus 1Y specimens. To identify proteins showing a significant modification
172 of abundance, we performed a generalized linear model and selected proteins with
173 P-Value adjusted less than 0.05 [23]. The protein cluster revealing the best
174 discrimination between the groups for both dates was retained. Out of this cluster,
175 non-specific proteins identified with SN > 5 in the control's sample and proteins with
176 non-significative p-value, as shown with the generalized linear model, were
177 eliminated. Subsequently, we considered together a high average of SN calculated
178 on the 1Y post-surgery from RCE patients in comparison with NRCE and the fact that
179 most elevated SN was only obtained for NRCE samples. Then, among the remaining
180 proteins, function of each protein was considered to estimate non-deleterious effect
181 on *Escherichia coli* strains during stages of recombinant antigens production.

182 **Production of recombinant antigens**

183 Recombinant vectors synthesized with encoding sequence of the selected proteins
184 were transfected into *Escherichia coli* BL21 strains. Transformants were grown in a
185 large volume of LB medium and induced by Isopropyl β -D-1-thiogalactopyranoside
186 (IPTG). Recombinant proteins were purified using a chromatography method (see
187 Supplementary Methods).

188 **ELISA**

189 We performed ELISA for the detection of IgG against the 4 recombinant *E.*
190 *granulosus* antigens that were produced. ELISA plates were incubated overnight at
191 4°C with 5 µg/ml of the recombinant protein per well, diluted in the carbonate-
192 bicarbonate buffer, pH 9.6. The plasma samples, as well as negative and positive
193 controls were tested by IgG-ELISA and results were expressed as previously
194 described [9].

195 **Statistical analyses**

196 Protein quantification was performed and illustrated by the heat-map tool. More
197 details are described in the supplementary methods. The Wilcoxon test was used to
198 assess IgG levels in NRCE and RCE patients. In addition, a Mann-Whitney *U* test
199 was performed to compare IgG levels regarding each monitoring time-point in NRCE
200 versus RCE. IgG levels were further assessed in NRCE patients treated exclusively
201 by surgery (n =19) versus NRCE with adjuvant albendazole chemotherapy (n=15)
202 using the Mann-Whitney *U* test. However, we could not assess the IgG antibody
203 response in the RCE (n= 2) versus NRCE (n =15) patients treated with adjuvant
204 albendazole chemotherapy due to the unbalanced constitution of the two sub-groups.

205 The receiver operating characteristic (ROC) curve was used to determine a cut-off
206 value to distinguish between NRCE and RCE patients. We further assessed the
207 ELISA using each of the recombinant antigens for the prediction of relapse/no
208 relapse at 1Y post-surgery by evaluating the evolution of the antibody levels between
209 1M and 1Y post-surgery as a decrease, an increase, or a stability as follows: a factor
210 of ± 2 coefficient of variation (CV)% was applied on the first time point (D030) level
211 and compared to the second one (D365). If the second level was higher than the
212 previous one by +2CV%, the level was considered as increased. If it was lower than
213 the previous level by -2CV%, we considered it as a decrease in level, and if it was

214 between high and low ranges, it was considered stable. The chi-square test was
215 applied to compare the number of NRCE versus RCE patients presenting either an
216 increase, a decrease, or a stability of IgG levels between 1M and 1Y. All tests were
217 two-sided, and a probability value of $p < 0.05$ was judged to be significant. Statistical
218 analysis was performed using GraphPad Prism (Version 8.3.0.) and R (Version 3.6.1)
219 Software.

220 **Results**

221 **Immunoreactive proteins**

222 Our proteomic analysis led to the identification of 305 proteins derived from *E.*
223 *granulosus* protoscolex. The RCE group yielded significantly higher abundant
224 proteins for both time-points of follow-up period as compared to NRCE patients (**Fig.**
225 **1**). Based on a comparative approach between NRCE versus RCE groups, and 1M
226 versus 1Y samples, the analysis of SN allowed the selection of 140 among the 305
227 immunoreactive proteins which were classified into 5 clusters (**Fig. 2**). Higher
228 differences in protein abundance between NRCE and RCE for both follow-up time
229 points were observed in cluster 2 that included 59 proteins. Therefore, we focused
230 our ensuing proteomic analysis on cluster 2.

231 **Selection of candidate proteins**

232 Applying the criteria described above in the Methods, we retained a total of 10
233 proteins after the first screening (Cytoplasmic malate dehydrogenase (Eg-cMDH),
234 Putative citrate synthase (Eg-CS), Annexin A6 (Eg-ANX A6), Severin (Eg-SVN),
235 Calpain-A, Myoferlin, Spectrin beta chain, Alpha actinin sarcomeric, Paramyosin, and
236 Glycogen phosphorylase), for which, the SN was significantly lower in NRCE than
237 RCE for both time-points (**Fig. 3, supplementary Fig. S1**). Finally, for production of

238 recombinant antigens, we applied the above-mentioned selection criteria (SN > 3 in
239 1Y-eluates of all RCE patients). This led us to keep 4 proteins: Eg-cMDH, Eg-CS, Eg-
240 ANX A6 and Eg-SVN.

241 **ELISA with recombinant proteins**

242 Significantly lower levels of IgG were observed at 1Y as compared to 1M in NRCE for
243 the 4 antigens (approximately 0.8 times lower in terms of group median values). Anti-
244 Eg-cMDH and Anti-Eg-CS IgG were significantly higher at 1Y as compared to pre-
245 operative levels in RCE (approximately 2 and 1.5 times higher in terms of group
246 median values, respectively) (**Fig. 4**).

247 The RCE group displayed significantly higher anti-Eg-cMDH, -CS and -ANX A6 IgG
248 levels as compared to NRCE at 1Y (Mann-Whitney *U* test < 0.01). Based on this
249 observation, we speculated that determining a cut-off value could be helpful in
250 predicting whether CE patients will likely develop post-surgical relapses or not. The
251 area under the ROC curve (AUC) values for discriminating RCE from NRCE were the
252 best for Eg-cMDH, Eg-ANX A6 and Eg-CS (0.77, 0.74 and 0.72) respectively
253 (**Supplementray Fig. S2**).

254 The best performance to predict the probability of being “relapse-free” when a
255 decrease in specific antibodies occurred between 1M and 1Y was observed with the
256 Eg-cMDH (83%) and Eg-CS (81%) antigens (**Table 1**). For the four tested antigens,
257 we perceived no significant differences of IgG levels between NRCE treated solely by
258 surgery and NRCE with adjuvant albendazole chemotherapy when compared with
259 respect to each time point of the follow-up.

260 We further speculated that combining Eg-cMDH and Eg-CS together, and with the
261 two other recombinant proteins would increase the prognostic performance.

262 Nonetheless, we found that patients with a decrease in anti-Eg-SVN and -ANX A6
263 antibody level (between 1M and 1Y post-surgery) were the same as those with a
264 decrease in Eg-cMDH and Eg-CS IgG antibodies, hence, the combination did not add
265 any further prognostic advantage.

266 **Discussion**

267 The key objective of the present study was the search of reliable antigens of *E.*
268 *granulosus* to improve post- surgical monitoring of CE, using a simple and
269 standardized ELISA. The monitoring was carried out during 3.5 years for all patients,
270 patients who relapsed within the first year after surgery were clustered in the RCE
271 group, and patients without relapses during the whole period of follow-up were
272 classified as NRCE group. The comparative proteomic analysis of plasma from CE
273 patients with different post-surgical developments enabled the identification of 59
274 proteins that were notably more abundant in IP eluates of patients who relapsed than
275 in patients without relapses for 1M and 1Y follow-up time-points. Based on selective
276 and feasibility criteria, we retained 4 proteins (cytoplasmic malate dehydrogenase,
277 Putative citrate synthase, Annexin A6 and Severin) which were produced as
278 recombinant antigens for a subsequent evaluation by ELISA. Our findings indicated
279 that a decrease of anti-Eg-cMDH or anti-Eg-CS IgG levels between 1 month and 1
280 year post-surgery appears as the best antigenic tool - among all tested candidates -
281 to predict post-surgical outcomes (probability of being “relapse-free” at 1Y above
282 80%). We already highlighted the importance of rigorous time-based serum sampling
283 after surgery to implement a strategy based on the comparison of individual
284 serological parameters between two time-points of follow-up and these findings are in
285 line with our previous investigations [9, 10].

286 Previous proteomic studies reporting *E. granulosus*' antigenic proteins have, often,
287 assessed a pooled serum collected from patients with Echinococcosis [16, 24]. The
288 originality of our study is based on the search for immunoreactive proteins of *E.*
289 *granulosus* protoscolex with regards to patients' post-operative clinical development,
290 which allowed to identify proteins that could be considered as viability biomarkers.
291 Indeed, Eg-cMDH, previously assessed as a diagnostic antigen for human CE [25,
292 26] and Eg-CS evaluated for the diagnosis of ovine CE [27], are major actors in the
293 metabolic process of *E. granulosus*. Thus, cMDH plays a critical role in the reduction
294 of oxaloacetate to malate, thus generating energy to protoscolex who have
295 predominantly a fermentative metabolism [28]. Eg-CS was reported as ubiquitously
296 expressed in all developmental stages of *E. granulosus* [27, 29] and as the key
297 enzyme of the tricarboxylic acid cycle [27].

298 We hypothesize that decrease in antibody levels in non-relapsed patients could be
299 related to a reduction or discontinuation of metabolic activity of the parasite, due to
300 either complete surgical removal or inactivation via medication. In the same way, high
301 levels of antibodies against Eg-cMDH and Eg-CS at 1Y post-surgery in patients who
302 experienced relapses could be related to a persistent metabolic activity of the
303 parasite, likely, linked to residual cysts or to the development of new lesions after
304 accidental spillage of protoscoleces.

305 The ELISA using Eg-ANX A6 and Eg-SVN antigens exhibited lower performance for
306 the prediction of surgical treatment efficacy. Some annexins were reported to
307 maintain the parasite's structural integrity and to promote its survival through
308 regulating host's immune responses. However, there is a paucity of information
309 regarding the role of annexin A6 in the physiopathology of CE. Up until now, only *E.*
310 *granulosus* B3, B33 and B38 annexins were investigated [30, 31]. The severin, also

311 known as gelsolin, is an actin-binding protein involved in actin assembly and
312 disassembly. Little is known about Eg-SVN, yet it could play a key role in the
313 parasite development, since the *E. granulosus* life cycle involves several
314 morphological and physiological changes according to the different parasitic stages
315 [32].

316 In our previous investigation, as well as, in the present study, we could not analyze
317 the immune response regarding CE stages because of the enrollment of children
318 mainly presenting active cysts (inactive and transitional cysts being less common in
319 the pediatric population) [10]. The prognostic accuracy of the recombinant proteins
320 should be, therefore, further assessed in larger groups of patients (especially adults)
321 with transitional/ inactive CE cysts.

322 Summarized, our results suggest that Eg-cMDH and Eg-CS are promising
323 biomarkers to predict early CE post-surgical outcomes. An ELISA using these 2
324 antigens could be easily applicable for routine follow-up of CE cases. As next, these
325 findings should be confirmed in larger multicenter cohort studies, in a longer follow-up
326 period to confirm the significative loss of antibodies, and also in adult CE patients
327 with cysts more frequently located in liver than in pediatric CE cases, and finally also
328 with CE patients being affected with other *Echinococcus* species causing CE, e.g. *E.*
329 *ortleppi* and a few others.

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341 **Color for figures**

342 We included this statement to indicate that all figures, the graphical abstract and the
343 two supplementary figures in the “supplementary material” should be color printed.

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441

1 **Figure captions**

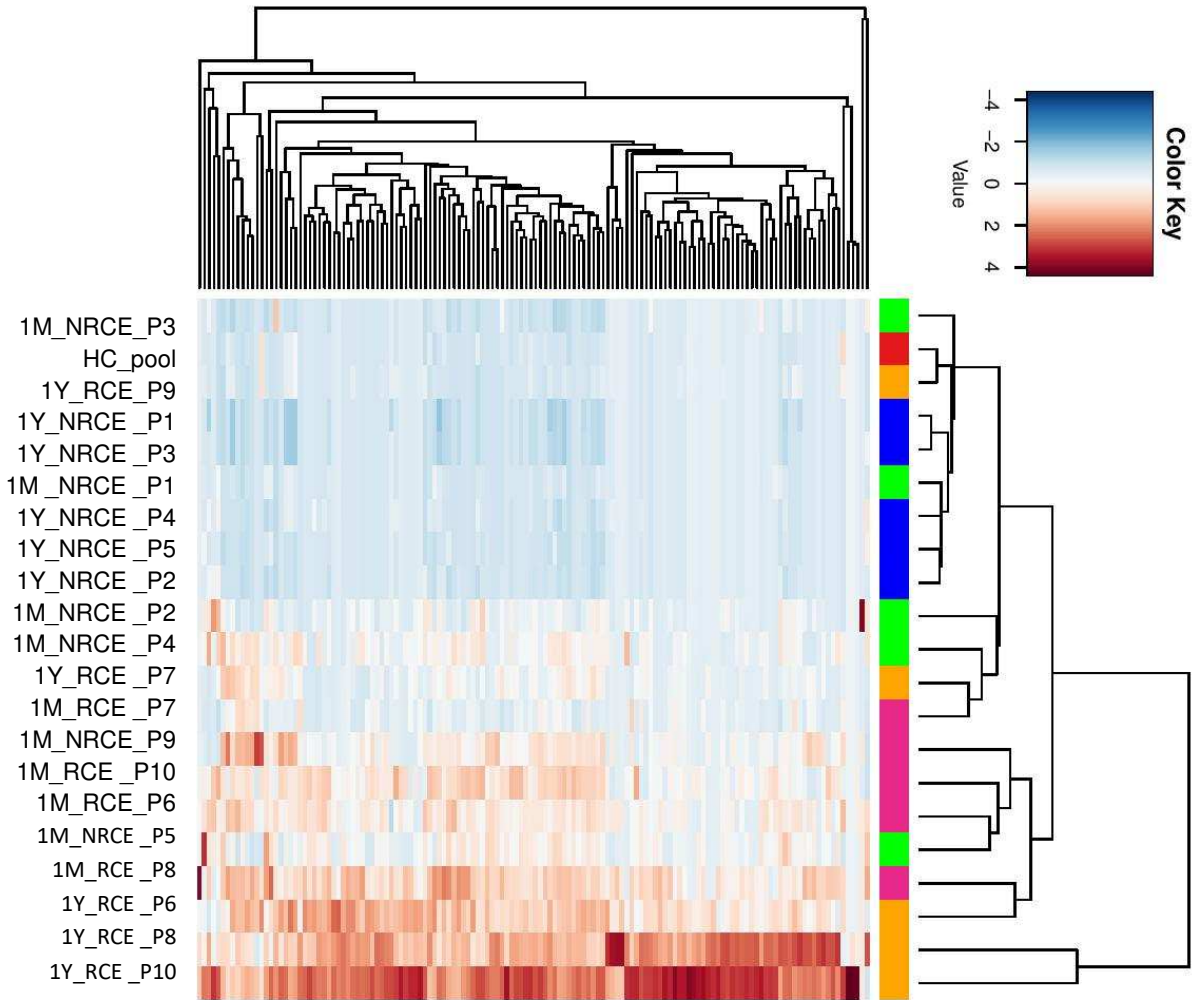
2 **Figure 1.** Quantitive comparison of protoscoleces proteins after immunoprecipitation
3 (IP) with plasma of CE patients illustrated by heatmap analysis. As the color key
4 indicates, the normalised intensity of proteins obtained in the IP of each sample is
5 illustrated by a colored rectangle with a colorimetry gradient from low (blue) to high
6 (red) intensity. White rectangles indicate that proteins could not be identified with IP
7 analysis. Each line corresponds to a protein.

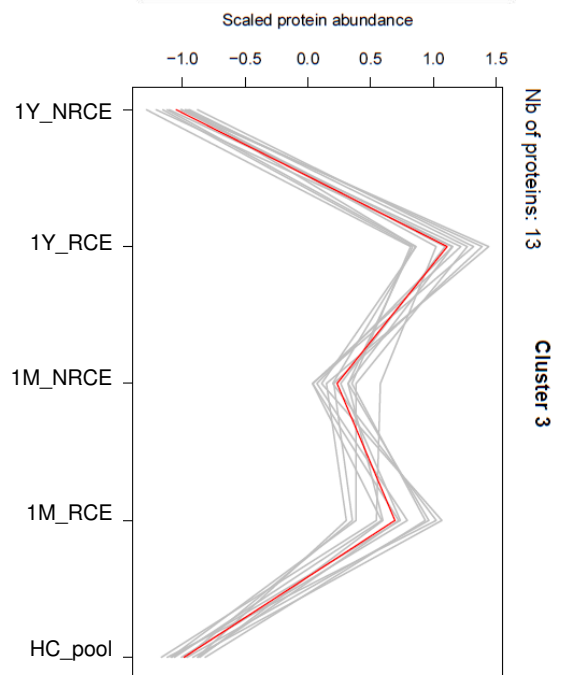
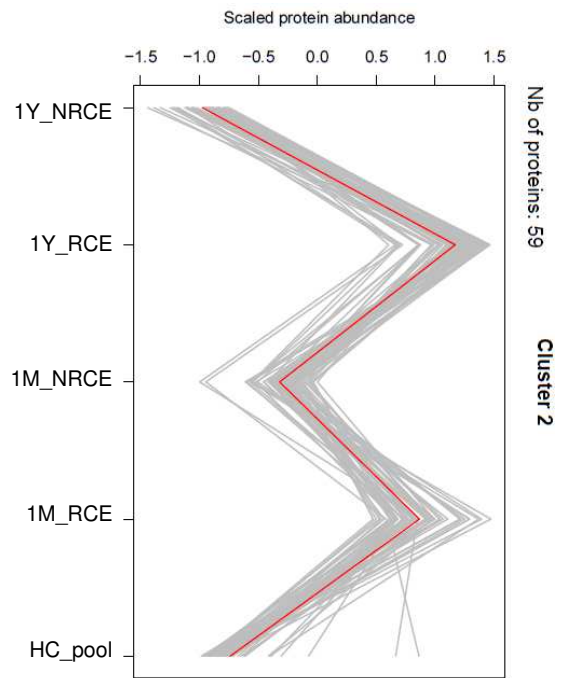
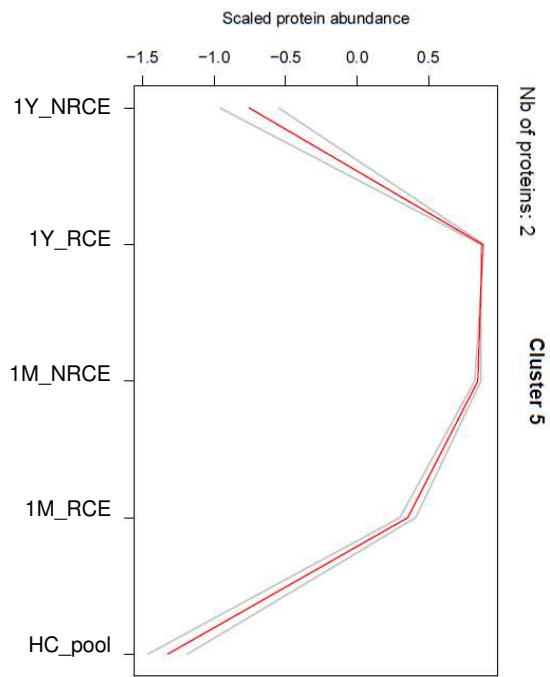
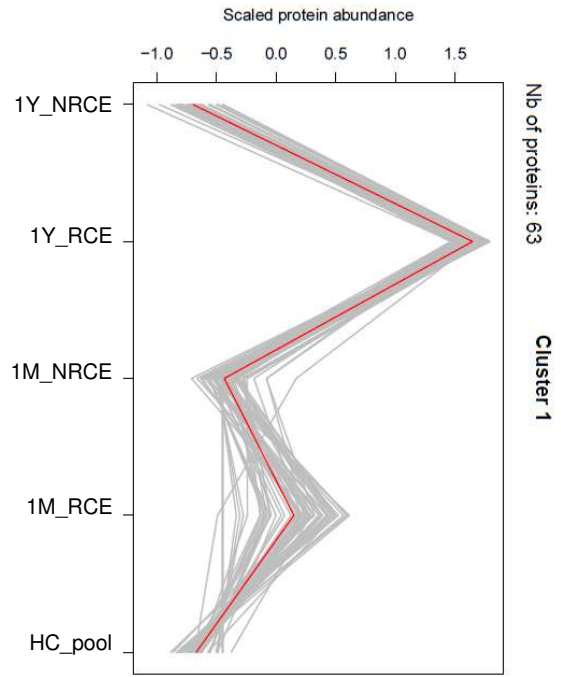
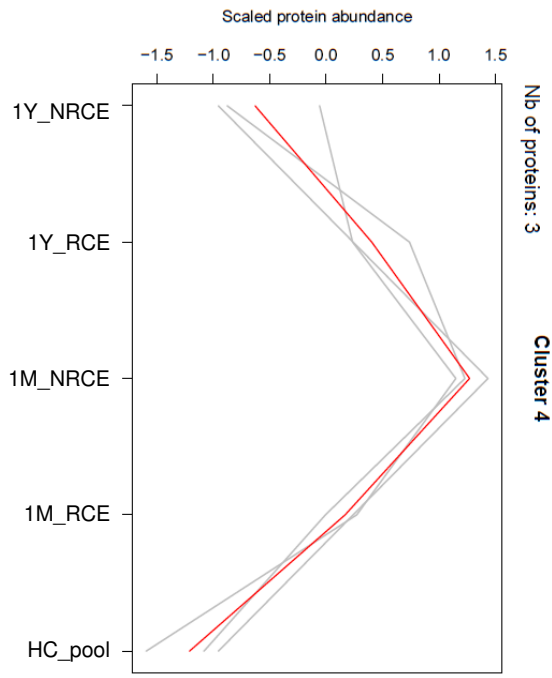
8 P1 to P10 designates the patients used for IP analyses. NRCE. « Non-relapsed » CE
9 patient, RCE. « Relapsed » CE patient, 1M. 1 Month after surgical treatment, 1Y. 1
10 Year post-surgery, HC_pool. Pool of plasma of 18 healthy blood donors.

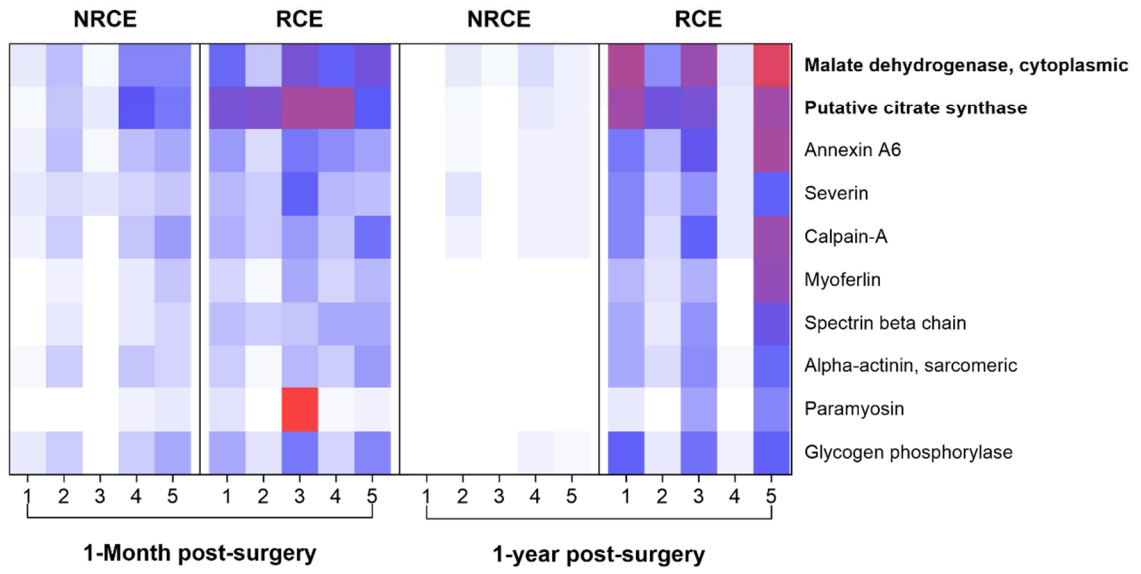
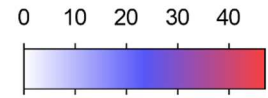
11 **Figure 2.** Classification of the abundant immunoreactive proteins derived from
12 *Echiococcus granulosus* into 5 clusters according to the total number of tandem
13 mass spectra per sample (SN) and on a double comparative basis NRCE versus
14 RCE and 1M versus 1Y monitoring dates. NRCE. « Non-relapsed » CE patients,
15 RCE. « Relapsed » CE patients, 1M. 1 Month after surgical treatment, 1Y. 1 Year
16 post-surgery.

17 **Figure 3.** Differential expression of the selected immunoreactive proteins in NRCE
18 versus RCE patients in 1M versus 1Y post-surgery. Quantification represented by the
19 heat-map tool was based on the spectral counting of each protein. NRCE. « Non-
20 relapsed » CE patients, RCE. « Relapsed » CE patients.

21 **Figure 4.** Anti-Eg-cMDH (A), anti-Eg-CS (B), anti-Eg-SVN (C) and anti-Eg-ANX A6
22 (D) median IgG levels (SI) in NRCE and RCE patients. D0, the time of diagnosis. 1M
23 and 1Y; 1-month and 1-year post-surgery, respectively. NRCE. « Non-relapsed » CE
24 patients, RCE. « Relapsed » CE patients. * p <0.5, ** p <0.01, **** p <0.0001.







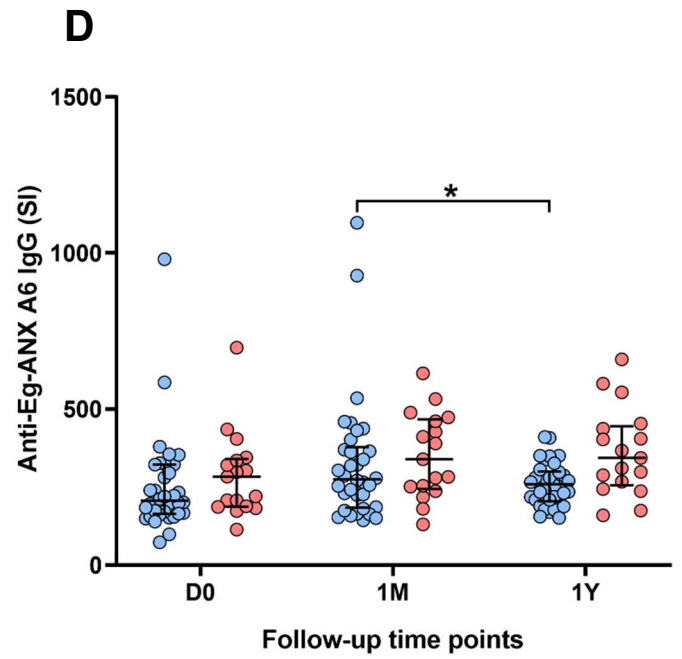
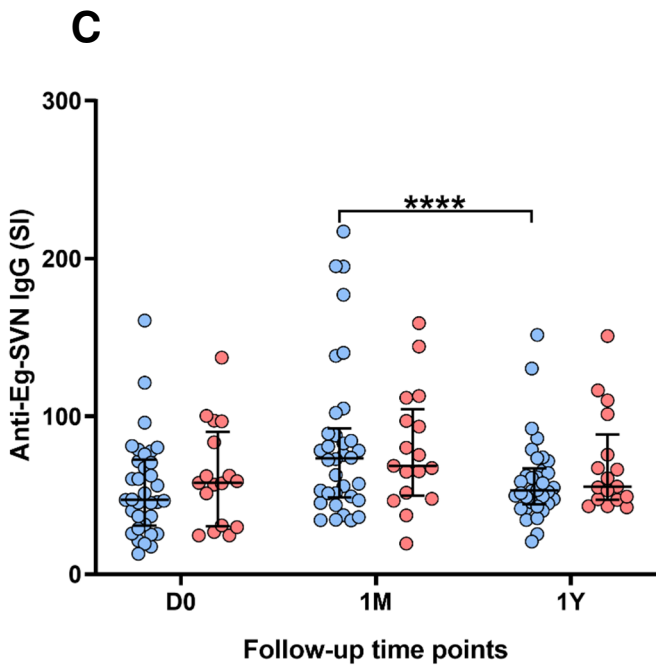
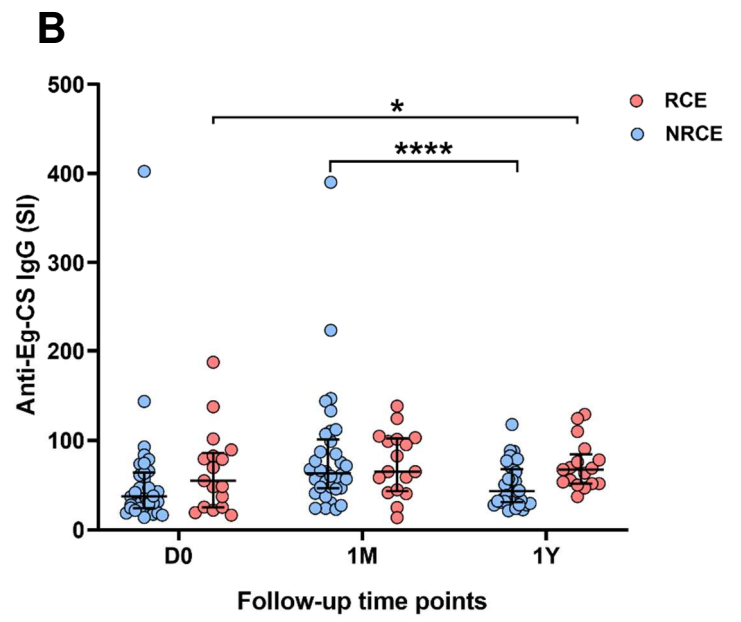
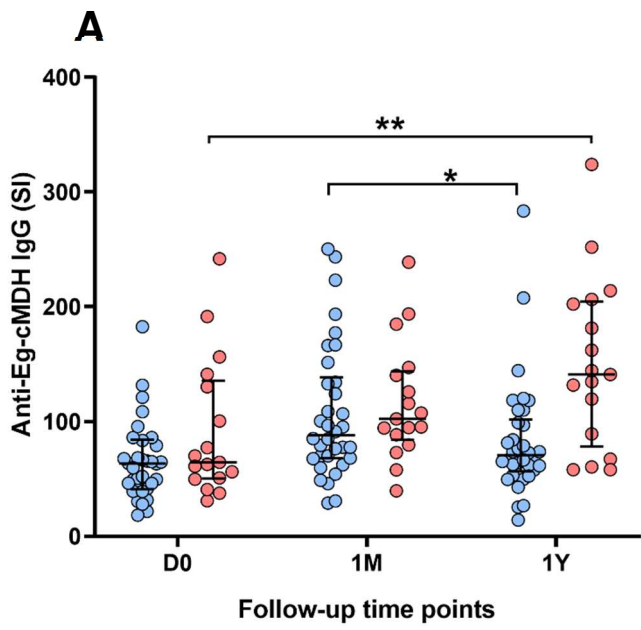


Table 1. Evolution of IgG levels between 1M and 1Y post-surgery in NRCE and RCE patients

IgG antibody response against	Evolution	NRCE; n (%)	RCE; n (%)	Chi-square test	Probability of being “relapse free” at 1Y ^a	Probability of being “relapsed” at 1Y ^b
Eg-cMDH	Decrease	25 (56)	6 (23)	p = 0.02	83%	
	Increase	5 (15)	8 (54)	p = 0.01		64%
	Stability	9 (29)	6 (23)	Ns		
Eg-CS	Decrease	22 (65)	5 (29)	p = 0.01	81%	
	Increase	3 (9)	4 (24)	Ns		57%
	Stability	9 (26)	8 (47)	Ns		
Eg-ANX A6	Decrease	15 (44)	6 (35)	Ns	71%	
	Increase	6 (18)	7 (41)	Ns		54%
	Stability	13 (38)	4 (24)	Ns		
Eg-SVN	Decrease	18 (53)	7 (41)	Ns	72%	
	Increase	4 (12)	2 (12)	Ns		33%
	Stability	12 (35)	8 (47)	Ns		

^a The probability of being “relapse-free” was calculated as follows: number of NRCE with a decreased level between 1M and 1Y/number of all patients (NRCE & RCE) with the same status, ^b The probability of being “relapsed” was calculated as follows: number of RCE with an increased level between 1M and 1Y/number of all patients (NRCE & RCE) with the same status

