



<b>Title</b>	Antibody recognition of cathepsin L1-derived peptides in Fasciola hepatica-infected and/or vaccinated cattle and identification of protective linear B-cell epitopes
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<b>Publication date</b>	2018-02-08
<b>Publication information</b>	Garza-Cuartero, Laura, Thomas Geurden, Suman M. Mahan, Grace Mulcahy, and et al. "Antibody Recognition of Cathepsin L1-Derived Peptides in Fasciola Hepatica-Infected and/or Vaccinated Cattle and Identification of Protective Linear B-Cell Epitopes." Elsevier, February 8, 2018. <a href="https://doi.org/10.1016/j.vaccine.2018.01.020">https://doi.org/10.1016/j.vaccine.2018.01.020</a> .
<b>Publisher</b>	Elsevier
<b>Item record/more information</b>	<a href="http://hdl.handle.net/10197/11231">http://hdl.handle.net/10197/11231</a>
<b>Publisher's statement</b>	This is the author's version of a work that was accepted for publication in Vaccine. Changes resulting from the publishing process, such as peer review, editing, corrections, structural formatting, and other quality control mechanisms may not be reflected in this document. Changes may have been made to this work since it was submitted for publication. A definitive version was subsequently published in Vaccine (36, 7, (2018)) <a href="https://doi.org/10.1016/j.vaccine.2018.01.020">https://doi.org/10.1016/j.vaccine.2018.01.020</a>
<b>Publisher's version (DOI)</b>	<a href="https://doi.org/10.1016/j.vaccine.2018.01.020">10.1016/j.vaccine.2018.01.020</a>

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1 Antibody recognition of cathepsin L1-derived peptides  
2 in *Fasciola hepatica*-infected and/or vaccinated cattle  
3 and identification of protective linear epitopes

4

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15

16 **ABSTRACT**

17 *Fasciola hepatica* infection causes important economic losses in livestock and food  
18 industries around the world. In the Republic of Ireland *F. hepatica* infection has an 76%  
19 prevalence in cattle. Due to the increase of anti-helminthic resistance, a vaccine-based  
20 approach to control of Fasciolosis is urgently needed. A recombinant version of the cysteine  
21 protease cathepsin L1(rmFhCL1) from *F. hepatica* has been a vaccine candidate for many  
22 years. We have found that vaccination of cattle with this immunodominant antigen has  
23 provided protection against infection in some experimental trials, but not in others.  
24 Differential epitope recognition between animals could be a source of variable levels of  
25 vaccine protection. Therefore, we have characterised for first time linear B-cell epitopes  
26 recognised within the FhCL1 protein using sera from *F. hepatica*-infected and/or vaccinated  
27 cattle from two independent trials. Results showed that all *F. hepatica* infected animals  
28 recognised the region 19-31 of FhCL1, which is situated in the N-terminal part of the pro-  
29 peptide. Vaccinated animals that showed fluke burden reduction elicited antibodies that  
30 bound to the regions 120-137, 145-155, 161-171 of FhCL1, which were not recognised by

31 non-protected animals. This data, together with the high production of specific IgG2 in  
32 animals showing vaccine efficacy, suggest important targets for vaccine development.

33

34 **Keywords:**

35 *Fasciola hepatica*; Vaccine; Epitope mapping; Cathepsin L1; peptides; cattle.

36

37 **Abbreviations:**

38 rmFhCL1: recombinant *Fasciola hepatica* Cathepsin L1

39 FhCL1: *Fasciola hepatica* Cathepsin L1

40

41 **1. INTRODUCTION**

42 The trematode parasite *Fasciola hepatica* causes fasciolosis in livestock on every  
43 continent of the world. The disease results in important economic losses to the agricultural  
44 community globally, as well as being an highly prevalent food-borne zoonosis, with 180  
45 million of people at risk [1–4]. *F. hepatica* infection has a prevalence of 76% in cattle in the  
46 Republic of Ireland, and an estimated prevalence of 78% in the UK [5,6]. Due to the increase  
47 in anthelmintic resistance in parasite populations, and the inherent difficulties in developing  
48 anthelmintics [7–9], a vaccine-based approach to aid in the control of fasciolosis is urgently  
49 needed.

50 There has been many protein candidates identified as potential vaccines against *F.*  
51 *hepatica*, such as fatty acid-binding proteins (FhFABP) [10–12] and glutathione S-  
52 transferases (FhGST) [13] [14]. Thioredoxin peroxidase (FhPrx) was shown to induce  
53 variable levels of protection in goats [15]. Other antigens, such as leucine aminopeptidase  
54 (FhLAP), have also been demonstrated to induce high levels of protection after vaccination  
55 in sheep [16,17]. Another group of proteases, the cathepsins, have been a major vaccine  
56 target due to their proteolytic actions and potential for immunoregulation [18]. Members  
57 of this family are secreted by the juvenile parasite stage (FhCL3) and adult parasite (FhCL1,  
58 FhCL2, FhCL5) [19]. FhCL1 and FhCL2, in their native state were shown to induce 50–55%  
59 protection in cattle when used alone, and 72.4% reduction in fluke burden when

60 administered with an haem-containing (Hb) fraction in cattle [20–22]. FhCL1 is the major  
61 component found within the excretory and secretory products from adult *F. hepatica* and it  
62 is involved in blood feeding [23,24], as well as acting to suppress pro-inflammatory  
63 cytokines [25,26]. FhCL1 is found as an inactive procathepsin L1 in secretory vesicles in the  
64 parasite gut and only after secretion in the lumen is activated by autocatalytic cleavage of its  
65 propeptide [23,24].

66 A recombinant mutant version of FhCL1 (rmFhCL1), expressed in *Saccharomyces cerevisiae*  
67 or *Pichia pastoris* [23,27], which does not autocatalytically activate, is useful as a reliable  
68 immunodiagnostic tool in *Fasciola hepatica* infections in cattle [5,28]. rmFhCL1 has also  
69 been used as a vaccine antigen that was capable of reducing fluke burdens in cattle by 48.2  
70 % [29]; However, other trials have not shown a similar reduction in fluke burden, although  
71 in a study with goats, a significant decrease in liver pathology was found [30–32].

72 Inconsistency in vaccine efficacy between trials hinders development of a vaccine. These  
73 differences may result from multiple factors, including adjuvant effects, *F. hepatica* strain or  
74 immunological state of the animal. Differential epitope recognition by individual animals  
75 could also be a potential source of variable levels of protection both within and between  
76 trials of *F. hepatica* vaccines. Hence, epitope mapping studies are potentially useful tool in  
77 the quest for a commercialisable vaccine to protect livestock against fasciolosis.

78 To date, B-cell epitope mapping studies on *F. hepatica* antigens have been carried out on  
79 the *F. hepatica* Glutathione S-Transferase (FhGST) in sheep [33], *F. hepatica* saposin-like  
80 protein (Fh-SAP2) in rabbits [34], and, more recently, a range of *F. hepatica* antigens in mice  
81 [35]. In the case of *F. hepatica* cathepsins, Harmsen et al. (2004) described specific regions  
82 of FhCL1 and FhCL3 used to immunize rats which induced 40-64% fluke burden reduction  
83 [36]. Villa-Mancera (2008, 2011 and 2014) developed synthetic peptide mimotopes based  
84 on the FhCL1 protein sequence that could induce fluke burden reduction in sheep [37,38],  
85 mice [39] and goats [40]. In cattle, Cornelissen (1999) described peptides of FhCL1 that  
86 could be used as immunodiagnostics for *F. hepatica* infection [41]. Here, we characterise for  
87 first time linear B-cell epitopes recognised within the FhCL1 protein by antibodies in the  
88 sera from, both, *Fasciola hepatica*-infected only and infected plus vaccinated cattle, in two  
89 independent trials. We identify specific peptides that are the sites of immunodominant  
90 epitopes with potential for future subunit vaccines.

91

## 2. MATERIALS AND METHODS

### 2.1 Experimental design, vaccination and *F. hepatica* infection

Ten male castrated Holstein-Friesian cattle for each trial, between 6 and 8 months of age in Trial 1 (Kalamazoo) and between 5 and 11 months old for Trial 2 (Dublin), were purchased from areas where *F. hepatica* infection was not reported. Animals were housed under uniform conditions at the experimental research facility (Trial 1) at Kalamazoo (USA) and at University College Dublin (UCD) Lyons Research Farm (Newcastle, County Kildare, Ireland) (Trial 2). In both experiments, to ensure that animals were free from *F. hepatica* infection before starting the study, animals were serologically screened by ELISA using recombinant mutant *F. hepatica* cathepsin L1 (rmFhCL1) and by faecal egg examination, as previously described [29]. Animals from each trial were then randomly divided into two groups, 5 animals in a control group, and 5 in a vaccinated group.

For the vaccine preparation, recombinant *F. hepatica* cathepsin L1 (rmFhCL1) mutant was expressed in *Pichia pastoris* as previously described [23]. Recombinant *F. hepatica* Cathepsin L3 (rmFhCL3) was a purified recombinant protein expressed by Chinese Hamster Ovary (CHO) cells. In both trials, a combination of rmFhCL1 and rmFhCL3 antigens was used to formulate a vaccine containing 200µg of each antigen per dose plus 2ml of adjuvant (ZA1) (Zoetis Adjuvant propriety). For the control group, 2ml of a sterile saline solution was administered as a sham vaccine. The vaccines were kept at 4°C during the storage and transport. In both trials, animals were vaccinated subcutaneously with a 19G needle, two times with a three (Trial 1) and two (Trial 2) weeks-interval.

In Trial 1, animals were infected with a total of 720 *F. hepatica* metacercariae orally administering 40 metacercariae in a gelatine bolus every second day over a period of 6 weeks starting 3 weeks post-2<sup>nd</sup> vaccination. Blood samples were collected by jugular venepuncture at Day 0, 3 weeks post-2<sup>nd</sup> vaccination (pre-infection phase), at 7 weeks post-infection (7wpi) and at 13 weeks post-infection (13wpi). This project was approved by the Kalamazoo Institutional Animal Care and Use Committee.

In Trial 2, animals were infected with a total of 200 *F. hepatica* metacercariae (Baldwin Aquatics, (Oregon) at 2 weeks post-2<sup>nd</sup> vaccination over two consecutive days (100 metacercariae per day). The metacercariae were dispersed in 10ml of dH<sub>2</sub>O and were

123 administered by oral route *via* a 20ml syringe. Blood samples were collected at Day 0, 2  
124 weeks post-2<sup>nd</sup> vaccination (pre-infection), 2 weeks post-infection (2wpi), 6 weeks post-  
125 infection (6wpi), 10 weeks post-infection (10wpi) and 14weeks post-infection (14wpi). This  
126 trial was approved/licenced by the UCD Animal Research Ethics Committee/Health  
127 Products Regulatory Agency (AE18982/P048), University College Dublin, Ireland.

128 Animals were euthanized and the livers collected at 13 (Trial 1) or 14 weeks post-infection  
129 (Trial 2). Flukes in each liver were counted as previously described [42]. In these studies  
130 carried out previously, the vaccinated group in Trial 1 showed a fluke burden reduction of  
131 37.6% in comparison with the non-vaccinated group, whereas in Trial 2 vaccination did not  
132 elicit reduction in fluke burden compared to controls.

133

## 134 **2.2 Measurement of IgG1 and IgG2 anti- rmFhCL1 by** 135 **ELISA**

136 IgG1 and IgG2 levels were measured in serum from all time points in both trials by using an  
137 in-house ELISA. 96 well-plates (Corning) were coated with 5µg/ml of rmFhCL1 in carbonate-  
138 coating buffer at pH 9.6, and incubated overnight at 37°C. Next, plates were washed three  
139 times with Phosphate Buffered Saline plus 0.05% Tween 20 (PBS-T) and then blocked by  
140 adding 100µl of 5% milk in PBS-T for 30min at 37°C. After washing three times, serum  
141 samples diluted 1:20 in PBS-T were added into the wells (in 100µl volume) in duplicate and  
142 then serial dilutions (1:3) were carried out. Plates were incubated for 30min at 37°C and  
143 washed as before, HRP-conjugated monoclonal anti-IgG1 (Prionics) or anti-IgG2 (Bio-rad)  
144 were added at a concentration of 1:100 (anti-IgG1) in PBS-T or 1:1000 (anti-IgG2) in PBS  
145 without Tween. After incubation at the same conditions as previously described, and  
146 washing, 100µl of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) were added for 10min in  
147 the dark. Finally, 50µl of Stop solution (H<sub>2</sub>SO<sub>4</sub>) were added onto each well and absorbance  
148 was measured at 450<sub>nm</sub>. Negative and positive serum controls were included in each plate.  
149 Endpoint titres were calculated for each sample and then transformed into log<sub>10</sub>.

150

151

## 152 **2.3 Linear B-Cell Epitope mapping of FhCL1**

153 For epitope mapping studies, with the purpose of comparing both trials, the following time  
154 points were selected: 3wks post-2<sup>nd</sup> vaccination (pre-infection), 7wpi and 13wpi (Trial 1),  
155 and 2wks post-2<sup>nd</sup> vaccination (pre-infection), 6wpi and 14wpi (Trial 2).

156 A total of 160 overlapping peptides of FhCL1, 9 amino-acids in length, and with an overlap  
157 of 7 amino-acids between successive peptides were synthesised (Mimotopes Pty. Ltd.  
158 Australia), each with a biotin tag (Supplementary data Table S1). The FhCL1 reference  
159 sequence

160 (MRLFVLAFLTVGVLGSNDDLWHQWKRMYNKEYNGADDQHRRNIWEKNVKHIQEHNLRHDLGLVTTYTLGLNQFTD  
161 MTFEEFKAKYLTEMRSASDILSHGVPEANNRAVDPDKIDWRESGYVTEVKDQNGCGSCWAFSTTGTMEGQYMKNR  
162 TSISFSEQQLVDCSRPWGNNCGGGLMENAYQYLKQFLETESYPYTAVEGQCRYNKQLGVAKVTGFYTVHSGSEVE  
163 LKNLVGAEGPAAVAVDVESDFMMYRSGIYQSQTCSPLRVNHAVLAVGYGTQGGTDYWIVKNSWGLSWGERGYIRMV  
164 RNRGNMCGIASLASLPMVARFP) was taken from UniProtKB - Q24940, which included the FhCL1  
165 protein including the signal peptide (positions 1-15), the pro-peptide (activation peptide)  
166 (positions 16-106), and the mature enzyme (107-126). The active site is formed by amino  
167 acids at positions Cys132, His269, Asn289 (Supplementary material. Figure S1)

168 Peptides were used as the solid-phase antigen in ELISA-based assays. Lyophilised peptides  
169 were solubilised in 50% Acetonitrile (Fisher Chemical) in H<sub>2</sub>O (HyClone GE Life Sciences)  
170 and then further diluted (1/20) in 0.1% Sodium Azide (Sigma) plus 0.1% in BSA (Sigma) in  
171 PBS. Next, 96-well plates (Nunc. high binding) were coated with 5µg/ml of Streptavidin  
172 (Sigma) overnight at 4°C and washed four times with PBS-T. Biotinylated peptides were  
173 added at a final concentration of 50µg/ml (in 0.1% Sodium Azide, 0.1% BSA in PBS). After  
174 incubation overnight at 4°C, and blocking with BSA (2% BSA in PBS-T) for 1.5h, each serum  
175 sample was diluted to 1/50 in 2% BSA PBS-T and added to the wells. The plates were then  
176 incubated for 1.5h at room temperature with shaking, and washed as before. Next,  
177 peroxidase-conjugated secondary antibody (anti-bovine total anti-IgG (cat: A5295 Sigma))  
178 was added at a dilution of 1:3000 in blocking buffer and incubated for another 1.5h. After  
179 washing plates as previously, TMB (Sigma) was added and incubated for 15min in the dark.  
180 All the volumes were 100µl. Absorbance was measured at 450nm. Control sera, along with  
181 blank wells without peptide and with peptide but without serum, were included in each  
182 plate. Background (OD from blank wells) was subtracted from the wells containing peptides  
183 in each plate.

184

## 185 **2.4 Crystal 3D model construction**

186 A 3D model of FhCL1 was built based on the published crystal structure [24]. The models  
187 are extracted from Uniprot, accession number [Q24940](#) and RCSB PDB:2O6X. The 3D  
188 diagrams were generated using the programme PROSAT (Figure S1).

189

## 190 **2.5 Statistical analysis**

191 For IgG1/IgG2 and epitope mapping studies, 2-way ANOVA and Bonferroni post-test was  
192 used to compare differences between groups and time points.

193

## 194 **3 RESULTS**

### 195 **3.1 Antibody levels after vaccination and *F. hepatica* infection**

196 At 3wks and 2wks post-2<sup>nd</sup> vaccination, in both Trial 1 and Trial 2, the vaccinated group had  
197 a higher anti-CL1 IgG1 response in comparison with the control group ( $p < 0.001$ ) (Figure 1  
198 a, b). These differences between vaccinated and control groups persisted until 7 and 6wpi,  
199 respectively, in both trials. However, at the latest time point examined, weeks 13 or 14 post-  
200 infection, in Trials 1 and 2, respectively, the vaccinated group in Trial 1 had a greater IgG1  
201 response than controls ( $P < 0.001$ ) whereas in Trial 2 (14wpi) the difference between the  
202 groups was no longer significant (Figure 1 a, b). When comparing control groups at 2wks  
203 post-2<sup>nd</sup> vaccination with saline, Trial 2 showed some IgG1 production (Figure 1 b) which is  
204 not present in the control group from Trial 1 at 3wks post-2<sup>nd</sup> vaccination (Figure 1 a).

205 Anti-rmFhCL1 IgG2 production was also higher at 3wks and 2wks post-2<sup>nd</sup> vaccination  
206 ( $p < 0.01$ - $p < 0.05$ ) in the vaccinated groups in comparison to the controls in both trials.  
207 However, at 13wpi this higher IgG2 production was maintained in vaccinated animals from  
208 Trial 1 ( $p < 0.001$ ), but not in Trial 2 at 14wpi (Figure 1 c, d).

209

### 210 **3.2 Epitope mapping analysis of FhCL1 recognised by *F. hepatica*** 211 **infected and/or vaccinated-cattle**

212

213           **3.2.1 Peptides from FhCL1 recognised by cattle infected with *F.***  
214 ***hepatica* and vaccinated with CL1/CL3/ZA1 formulation**

215 In Trial 1, serum from vaccinated animals specifically recognised peptides 10-12, 65, 73-74,  
216 81-82 and 89 at 7wpi and 13wpi (Figure 2 a). Peptides 152 and 160 also showed higher  
217 serum binding at 13wpi than at 7wpi ( $p<0.0001$ ) (Table S2) for Trial 1.

218 In Trial 2, peptides 10-12 were also recognised by vaccinated animals, but in this case, a  
219 higher binding was found at 6wpi than at 14wpi (Figure 2 b). These peptides bound to sera  
220 from all groups from both trials, and from both, controls and vaccinated animals. In addition,  
221 in this Trial 2, peptides 20, 145 and 152 were specifically recognised by vaccinated animals  
222 at 6wpi ( $p<0.0001$ ), however this reactivity declined to non-significant levels by 14wpi  
223 (Figure 2 b and Table S2).

224 When comparing peptide recognition between vaccinated and control groups in Trial 1,  
225 peptides 145, 152 and 160 were more highly recognised by the vaccinated group than the  
226 control group at 3wk post-2<sup>nd</sup> vaccination (for 145) and at 13wpi (for 152, 160). However,  
227 these peptides were recognised by both control and vaccinated animals in Trial 2 (Figures 2  
228 and 3, Table S2).

229

230           **3.2.2 Peptides from FhCL1 recognised by cattle infected with *F.***  
231 ***hepatica* but not vaccinated**

232 Serum from cattle infected with *F. hepatica* (control-unvaccinated group) recognised linear  
233 epitopes from FhCL1 that were not recognised by the same animals pre-infection (Figure 3  
234 a, b). Specifically, peptides 9-13, 20-22, 90-91, 107, 144, 148-149, and 151 -152 were  
235 recognised ( $p<0.05$  –  $p<0.0001$ ) by non-vaccinated animals after infection, at 7wpi or 13wpi  
236 in Trial 1 (Figure 3 a and Table S2). In Trial 2, peptides 10 to 12 were also recognised after  
237 infection. In this Trial, peptide 160 showed increased specific binding to serum at 14wpi in  
238 comparison to both pre-infection and to 6wpi ( $p<0.001$ - $p<0.0001$ ) (Figure 3 b and Table S2).

239

240

### 241 **3.3 Localization of the epitopes recognised by cattle in the linear** 242 **sequence and 3D molecule of FhCL1 and comparison among** 243 **groups**

244 Next, selected amino acid regions corresponding to the overlapping peptides that were  
245 specifically recognised ( $p < 0.05$ - $p < 0.0001$ , Figure 2 and Figure 3) were localised in the linear  
246 sequence (Figure 4) (see Table S2 to link peptide number, with aa positions and with aa  
247 sequence). In parallel, these highly-recognised epitopes were localised in the FhCL1 3D  
248 structure [24]. The schematic representation of FhCL1 is shown in Figure S1. The active site  
249 is situated in the centre of the 3D molecule, formed by amino-acids at positions Cys132,  
250 His269 and Asn289.

251

#### 252 **3.3.1 Localization of epitopes recognised by vaccinated animals and** 253 **comparison between partially protected and non-protected groups**

254 In Trial 1 where vaccination induced partial protection, the regions spanning positions 120-  
255 137, 145-155, 161-171 (CGSCWAFST, YMKNERTSISF, VDCSRPGWNGG) (Figure 4) were  
256 specifically recognised by vaccinated animals 13wpi in comparison to pre-infection ( $p < 0.05$ -  
257  $p < 0.001$ ) (Figure 5 a) and in comparison, to control groups (Figure 6 a). Those peptides were  
258 not recognised at 7wpi, showing a different 3D binding profile with the stage of the infection.

259 In contrast, these regions recognised by the vaccinated partially protected animals (Trial 1)  
260 were not bound by antibodies in Trial 2 by the vaccinated (non-protected) group (Figure 5  
261 b and Figure 6 b).

262 In Trial 2, two regions (39-47 HRRNIWEKN and 310-311 MVRNRGNMC) (Figure 4) were  
263 recognised by the vaccinated group at 6wpi (but not at 14wpi) in comparison to pre-  
264 infection (Figure 5 b) and in comparison to controls (Figure 6 b). However, those peptides,  
265 additionally, were reactive in both the vaccinated and control groups from Trial 1 at 13wpi  
266 in comparison to pre-infection (Figure 5 a and Figure 6 a).

267 Recognition of some regions of the molecule was found to be switched off in vaccinated  
268 animals (Trial 1), such as the region 283-300 (DYWIVKNSWGLSWGGERGY) that was  
269 recognised by the control group but not in the vaccinated group at 7 or 13wpi of trial

270 1(Figure 4 and Figure 6 a), or region SLPMVARFP which was reactive in the control group  
271 from Trial 2 but not in the vaccinated group (Figure 4 and Figure 6 b).

272

### 273 **3.3.2 Epitopes from FhCL1 recognised by all groups after *F. hepatica*** 274 **infection**

275 The region centred on residue numbers 21-27 (WHQWCRM), which corresponds to peptides  
276 10-12, was consistently recognised by vaccinated and control animals in both trials at all  
277 time points post-infection (Figure 4). This region forms part of a larger domain  
278 (DLWHQWKRMYNKE) which contains peptides that also react at various time points of  
279 infection. This region is situated at the N-terminal of the pro-peptide of the molecule and  
280 positioned away from the main body of the 3D structure (Figure 7). In addition epitope  
281 recognition profile was modified after *F. hepatica* infection at the early and late time points  
282 in Trial 1 (Figure 8 a) and Trial 2 (Figure 8 b).

283

### 284 **3.3.3 Localization of epitopes recognised non-specifically**

285 In Trial 2, the peptides 144 and 145, were reactive with antibodies in the control  
286 group pre-infection (Figure 3 b), which corresponds to position 287-299 VKNSWGLSWGGE  
287 in the protein (Figure 4). However, this non-specific recognition was not observed in Trial  
288 1 (Figure 2 a, Figure 3 a). This peptide region includes part of the active site of FhCL1 at  
289 position Asn289, situated at the centre of the 3D molecule (Figure 4).

290

291

292

## 293 **4 DISCUSSION**

294 For many years, researchers have strived to develop a vaccine capable of consistently  
295 protecting ruminants against *F. hepatica* infection. In various studies, vaccination with  
296 cathepsins from *F. hepatica*, reductions in fluke burden ranging from 30 to 72% have been  
297 reported. Specifically, vaccination with the native FhCL1 or FhCL2 antigens in combination  
298 with fluke haemoglobin induced protection of up to 72% in cattle [20–22]. A more stable

299 recombinant antigen (rmFhCL1), induced a reduction in fluke burdens in cattle of 48.2 %  
300 [29]. However, other trials have not shown significant levels of protection with these  
301 antigens [30–32]. In this study, the antibody kinetics and linear B-cell epitope recognition of  
302 FhCL1 by sera from vaccinated and control animals was compared from two separate  
303 experiments where significant partial protection was observed in the first but not in the  
304 second.

305 Firstly, elevated levels of anti-FhCL1 IgG1 induced after *F. hepatica* infection and higher  
306 levels post-vaccination indicated successful vaccination and infection in both trials.  
307 Interestingly, in Trial 2, control animals at pre-infection induced some IgG1, potentially from  
308 maternal antibodies or other exposure to similar pathogens. Those cross-reactive antibodies  
309 could be present also in the immunised animals from the same trial, however, they would be  
310 masked by the effect of the vaccine.

311 Secondly, vaccinated animals from both trials showed anti-CL1 IgG2 production after  
312 immunisation. However, in Trial 1 only, where vaccination provided partial protection, IgG2  
313 levels were maintained until 13wpi. In contrast, in Trial 2, specific IgG2 production by  
314 vaccinated animals peaked at 2wpi and disappeared by 14wpi. This finding confirms  
315 previous studies indicating that an elevated IgG2 response is an indicator of protection  
316 [21,22].

317 Our epitope mapping analysis show that in Trial 1, the vaccinated, but not the control  
318 group, specifically recognised the regions spanning amino-acids 120-137, 145-155, 161-171  
319 (CGSCWAFST, YMKNERTSISF, VDCSRPGWNG) at 14wpi. These regions were not highly  
320 recognised by either group in Trial 2. To note as above, that the first of these 3 peptides  
321 (120-137) and partially the last one (161-163), are found within the sequence of FhCL3.  
322 Previous studies have shown that the peptide 153-167 (overlapping with regions described  
323 above (145-155 and 161-171)) was immunogenic in vaccinated mice [35]. Additionally,  
324 Villa-Mancera (2014), showed that mimotopes containing aa 164-165 induced 46.9% fluke  
325 burden reduction in goats and up to 79.5% when combined with Quil A as adjuvant [40].

326  
327 In Trial 2, two regions specifically reacted with serum from the vaccinated group at 6wpi  
328 but not from the control group (39-47 HRRNIWEKN and 310-311 MVRNRGNMC). However,  
329 these regions were also reactive in both the vaccinated and control groups from Trial 1, at  
330 13wpi. This latter region aligns with some mimotopes shown to reduce fluke burden up to  
331 33.9% in sheep and 45.83% in mice [37,39].

332

333 Another region (181-190) recognised after infection in Trial 1, by control and  
334 vaccinated groups, coincides with mimotopes shown to reduce fluke burden by 47.6% in  
335 sheep and 66.6% in mice [37,39].

336

337 The sequence WHQWKRM (aa 21-27) is consistently and highly recognised within 6 or 7  
338 weeks after the start of the experimental infection, and that this reactivity is maintained as  
339 the infection moves into the chronic stage. This sequence, found in the pro-peptide of FhCL1  
340 is also found in the pro-peptide of other members of the cathepsin L family, including FhCL3  
341 which is exclusively secreted by the early infective parasitic stages (UniprotKB-Q9GRW4).  
342 In a previous study in rats, vaccination with a synthetic peptide from FhCL1 (15-33,  
343 GSNDDLWHQWKRMYNKEYN), which included the above mentioned sequence, induced a  
344 fluke burden reduction of 63% [36]. This region is exposed in the periphery of the 3D  
345 molecule, and is therefore accessible for antibody recognition. Antibodies to this region are  
346 also likely to account for the usefulness of FhCL1 as a diagnostic antigen [41]. Significantly,  
347 the mutant form of the molecule (rmFhCL1), in which the active site has a single mutation  
348 [23] and which therefore does not auto process, retaining the pro-peptide, unlike the native  
349 CL1 molecule, is as a consequence superior for use in diagnostic assays [5,28].

350

351 Surprisingly, in Trial 2, a peptide at position 287-299 (VKNSWGLSWGE) was non-  
352 specifically bound by antibodies during pre-infection in the control group.  
353 Individual animals recognising these peptides pre-infection also showed some anti-FhCL1  
354 IgG1 in standard ELISA at this time point. This reactivity could be due to the presence of  
355 maternal antibodies (although these would be more likely to bind to the major  
356 immunodominant epitopes) or, perhaps, this linear epitope may present a structural  
357 conformation that happens to cross-react with antibodies induced by some underlying but  
358 unknown infection.

359

360

361 In conclusion, higher anti-rmFhCL1 IgG2 levels were induced at the chronic infection by  
362 the vaccinated group that showed some degree of protection, as demonstrated in other  
363 studies. The vaccinated group from Trial 1, in which a reduction of fluke burden was seen,  
364 strongly recognised the aa regions 120-137, 145-155, 161-171 (CGSCWAFST,

365 YMKNERTSISF, VDCSRPGWNG). However, recognition of these peptides in Trial 2, where  
366 no protection was demonstrated, was not so strong. The epitope WHQWKRM (aa 21-27)  
367 that is contained in the pro-peptide of FhCL1 (and FhCL3), previously shown to be protective  
368 as a vaccine antigen in rats, was highly immunogenic following *F. hepatica* infection in both  
369 vaccinated and control animals in two separate trials. Hence, these two regions of the  
370 protein, together with the induction of specific IgG2, could potentially be useful targets for  
371 improving vaccine strategies.

372

### 373 **Financial support**

374 The project carried out in Kalamazoo, USA, was funded by Zoetis Inc.

375 The project carried out in University College Dublin, the Republic of Ireland, was funded by  
376 PARAGONE Horizon2020. Grant number R14863.

377

### 378 **Conflicts of interest**

379 The authors have submitted patent applications related to the work. These interests do not  
380 alter the author's adherence to policies on sharing data and materials.

381

### 382 **Authors' contributions**

383 LGC conducted Trial 2 and designed, carried out, analysed and interpreted the epitope  
384 mapping study. TG designed and supervised both trials. SMM and JMH designed, carried out  
385 and coordinated Trial 1. JPD provided materials and contributed to the design of the trials  
386 and experiments. GM designed and supervised Trial 2. All the authors participated in the  
387 interpretation of results and reviewed the manuscript.

388

### 389 **Acknowledgments**

390 We want to thank Lyons experimental farm at University College Dublin (UCD) and the  
391 laboratory teams at UCD and Queens University as well as all Zoetis members for their  
392 support.

393

394

395 **Supplementary material**

396 Figure S1. Secondary and Tertiary model of FhCL1

397 Table S1. Overlapping peptides of FhCL1

398 Table S2. Level of statistical significance of peptides recognised in each group

399

400

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551

## 552 Figure Legends

553 **Figure 1. IgG1 and IgG2 levels specific to rmFhCL1 induced after *F. hepatica* infection in CL1**  
554 **CL3 ZA1-vaccinated and controls (non-vaccinated) cattle.** IgG1 (a, b) and IgG2 (c, d) specific to  
555 rmFhCL1 were measured by end-point titration in Trial 1 (a, c) and Trial 2 (b, d). Control group =  
556 dashed green line; CL1/CL3/ZA1= purple line. n=5, ^ indicates 1<sup>st</sup> and 2<sup>nd</sup> vaccinations; Red arrow  
557 indicates *F. hepatica* infection. \* = p<0.05, \*\*=p<0.01; \*\*\*=p<0.001; \*\*\*\*=p<0.0001.

558

559 **Figure 2.- FhCL1-epitope mapping profile in sera from cattle vaccinated with CL1/CL3/ZA1**  
560 **formulation and then infected with *F. hepatica*.** In Trials 1 (a) and 2 (b), serum was examined at  
561 one timepoint pre-infection (at 3wks or 2wks post-2<sup>nd</sup> vaccination with CL1/CL3/ZA1, for Trial 1 (a)  
562 or Trial 2 (b), respectively), and at 2 timepoints post-infection (7wpi, 13wpi (Trial 1) and 6wpi,  
563 14wpi (Trial 2)). ^=single peptide and  $\hat{=}$  consecutive peptides that are recognised significantly  
564 different at post-infection (p<0.05- p<0.0001). n=5 for each group – See Table S2 for details.

565

566 **Figure 3.- FhCL1-Epitope mapping profile in sera from the control group.** In Trials 1 (a) and 2  
567 (b), serum was examined at one timepoint pre-infection, and two timepoints post-infection in each  
568 case; at the early infection (7wpi and 6wpi) and late infection (13wpi and 14wpi). ^=single peptide  
569 and  $\hat{=}$  consecutive peptides that are recognised significantly different at post-infection (p<0.05-  
570 p<0.0001). n=5 for each group – See Table S2 for details.

571

572 **Figure 4: Localization of the peptides recognised in the linear FhCL1 sequence and**  
573 **comparison between both trials.** The highlighted regions represent the peptide binding regions  
574 that are significantly recognised post-infection (See Table S2). 1 Con = Trial 1 Control group; 2 Con=  
575 Trial 2 Control group; 1 Vac= Trial 1 CL1/CL3/ZA1-vaccinated group; 2 Vac= Trial 2, CL1/CL3/ZA1-  
576 vaccinated group. Grey=epitopes binding only at the early time points (7wpi for Trial 1 or 6wpi for  
577 Trial 2). Red= epitopes binding at the late time points only (13wpi for Trial 1 or 14wpi for Trial 2;  
578 Blue= epitopes binding at both time points (early and late). Dark green= non-specific binding of  
579 epitopes at pre-infection in the control group of Trial 2. Purple = active sites of the protein

580

581 **Figure 5. Epitope recognition after *F. hepatica* infection in vaccinated groups in comparison**  
582 **to pre-infection and localisation in the 3D FhCL1 structure.** In Trial 1 (a) and Trial 2 (b) epitopes  
583 significantly recognised after *F. hepatica* infection in vaccinated animals, in comparison to pre-  
584 infection were localized in the CL1-3D structure. Grey=epitopes recognised only at early infection at  
585 7wpi (Trial 1) and 6wpi (Trial 2), red = epitopes recognised only in late infection at 13wpi (Trial 1)

586 and 14wpi (Trial 2) and blue = epitopes recognised at both, early and late, time points. Purple shows  
587 the active site. The region of the active site that is recognised by serum during early infection is  
588 shown in pink and at late infection, in orange.

589

590 **Figure 6. Comparison of FhCL1-epitopes recognised by partially protected and non-protected**  
591 **groups.** All the colours except white represent epitopes recognised by vaccinated but not controls.  
592 White = “switched –off” epitopes recognised in control groups but not in vaccinated groups. Red =  
593 regions recognised only at late infection (120-137, 145-155, 161-171 and 318-326 (CGSCWAFST,  
594 YMKNERTSISF, VDCSRPGWNGG, SLPMVARFP)). Blue = region (ME 177-178) recognised at both,  
595 early (7wpi) and late (13wpi) infection. Grey= region (39-47 HRRNIWEKN and 310-311  
596 MVRNRGNMC) only recognised in early infection (6wpi). Purple= active site and orange the part of  
597 the active site that is recognised in late infection (13wpi).

598

599

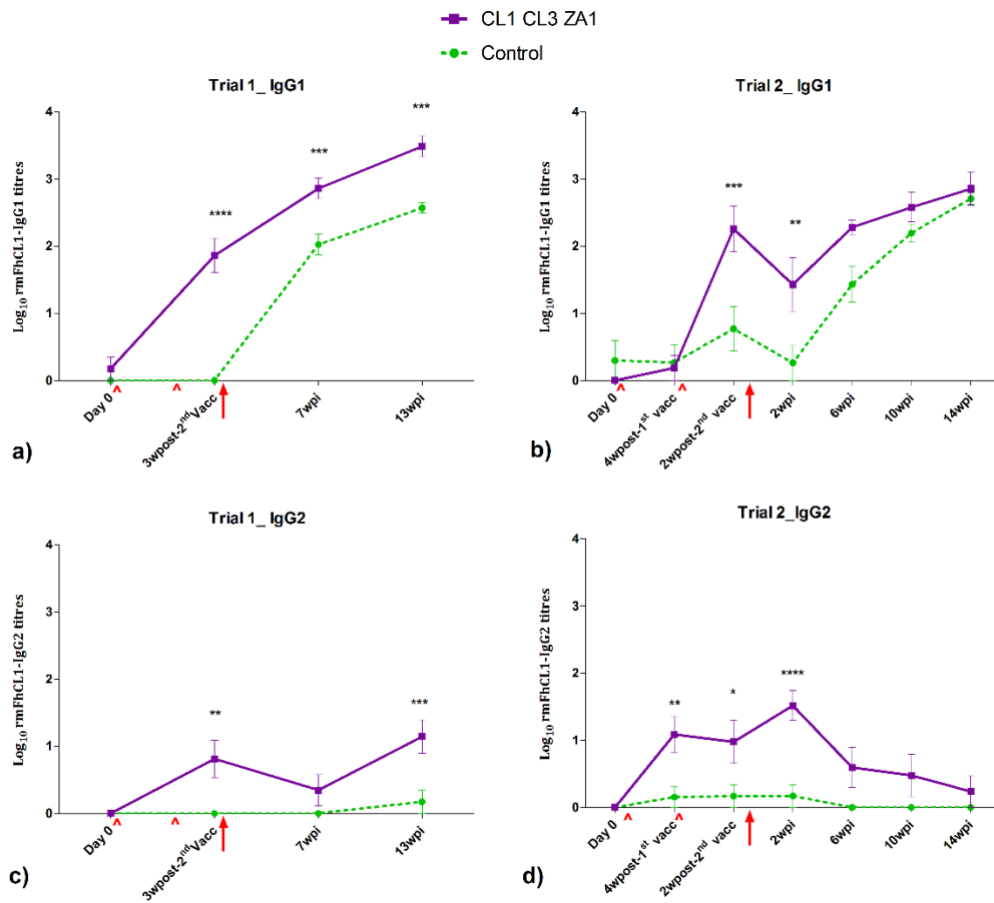
600 **Figure 7. Epitopes localised in the 3D FhCL1 structure that are bound by antibodies from all**  
601 **groups in both trials after *F. hepatica* infection.** The figure shows localisation of the peptides 21-  
602 31 (DLWHQWKRMYNKE) that were consistently recognised after *F. hepatica* infection at both, early  
603 or late time points, in the FhCL1 3D structure. This region (blue) is found at the N-terminal region of  
604 the pro-peptide of the molecule (region 16-106, shown in yellow), and faces outwards from the main  
605 body of the mature enzyme (shown as grey backbone). (a) and (b) represent the same model with a  
606 turn of 180°C. Purple coloured residues represent the active site.

607

608 **Figure 8. Epitope recognition after *F. hepatica* infection in non-vaccinated groups in**  
609 **comparison to pre-infection and the localization in the FhCL1 3D structure.** In Trial 1 (a) and  
610 Trial 2 (b) epitopes significantly recognised after *F. hepatica* infection in control groups, in  
611 comparison to pre-infection were localized in the CL1-3D structure. Grey=epitopes recognised only  
612 at early infection at 7wpi (Trial 1) and 6wpi (Trial 2), red = epitopes recognised only in late infection  
613 at 13wpi (Trial 1) and 14wpi (Trial 2) and blue = epitopes recognised at both, early and late, time  
614 points. Purple shows the active site. Pink shows a region of the active site that is recognised  
615 specifically during early infection.

## 616 **Figures Paper**

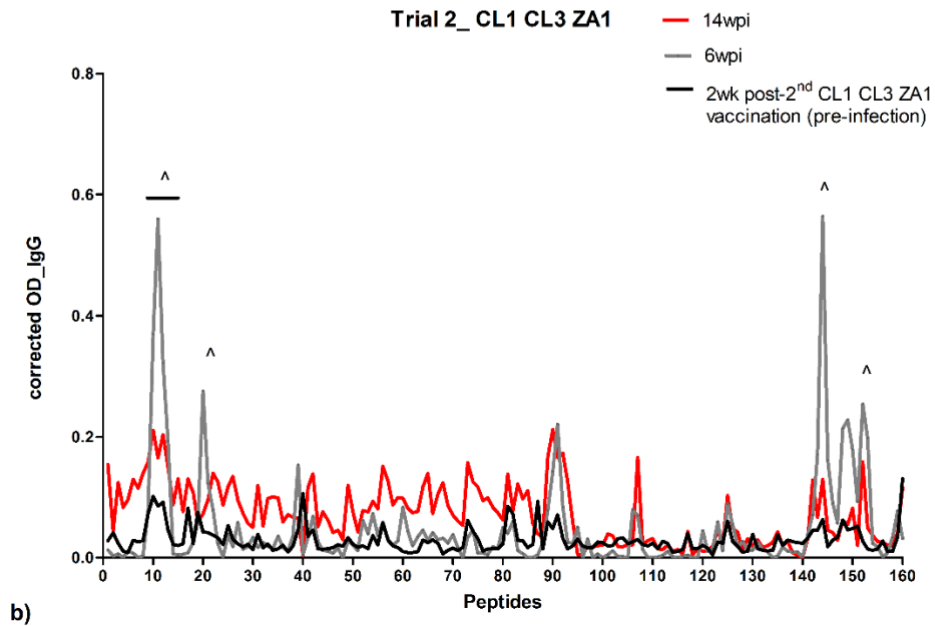
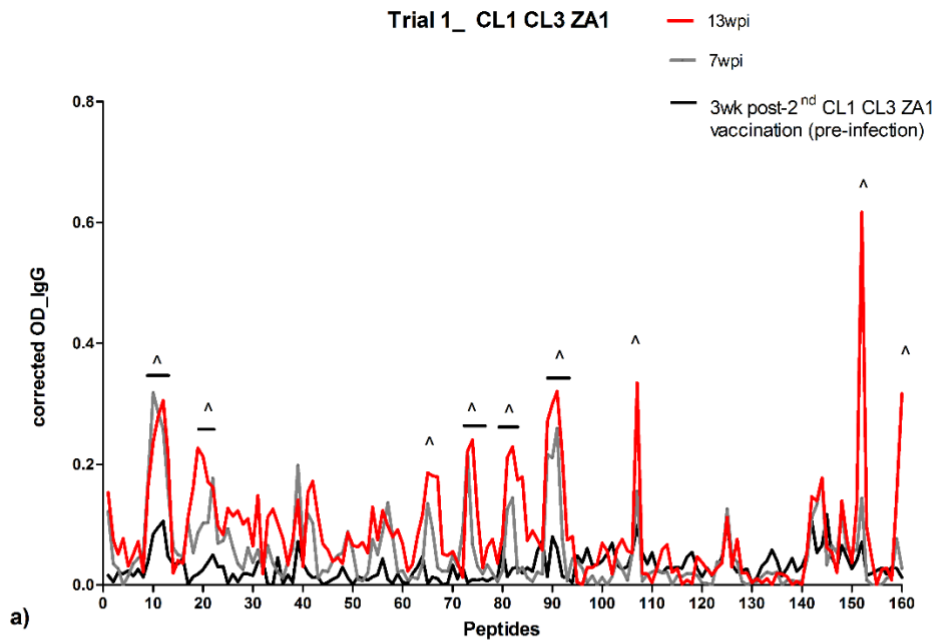
617



618

619 **Figure 1. IgG1 and IgG2 levels specific to rmFhCL1 induced after *F. hepatica* infection in CL1**  
 620 **CL3 ZA1-vaccinated and controls (non-vaccinated) cattle.**

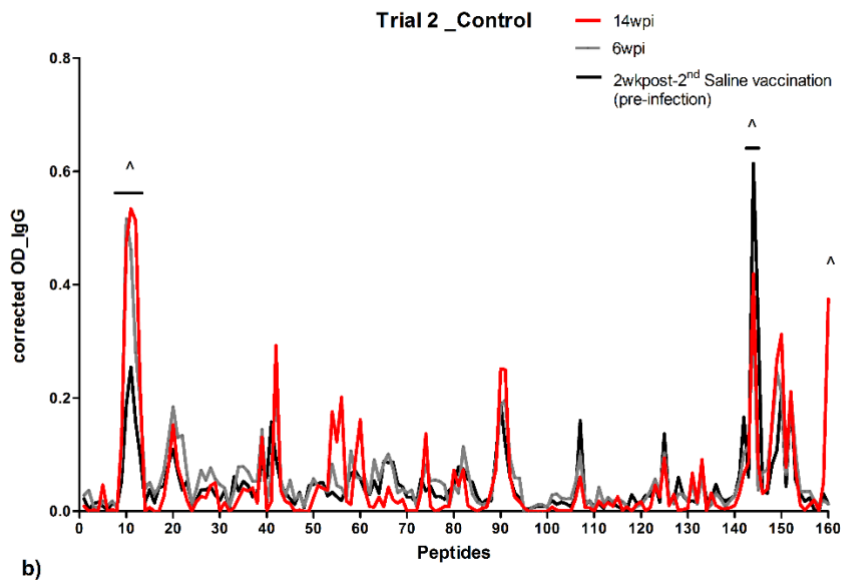
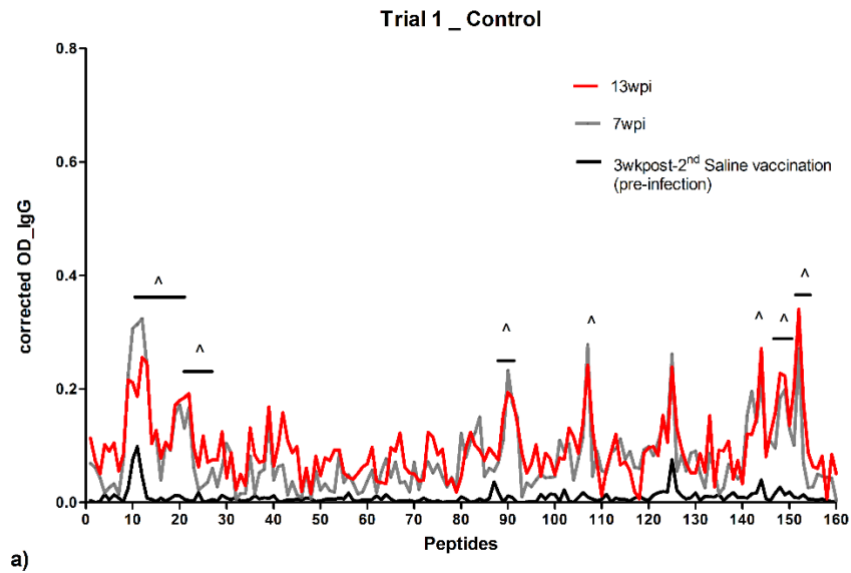
621 IgG1 (a, b) and IgG2 (c, d) specific to rmFhCL1 were measured by end-point titration in Trial 1 (a, c)  
 622 and Trial 2 (b, d). Control group = dashed green line; CL1/CL3/ZA1= purple line. n=5, ^ indicates 1<sup>st</sup>  
 623 and 2<sup>nd</sup> vaccinations; Red arrow indicates *F. hepatica* infection. \*= p<0.05, \*\*=p<0.01; \*\*\*=p<0.001;  
 624 \*\*\*\*\*=p<0.0001.



625

626 **Figure 2.- FhCL1-epitope mapping profile in sera from cattle vaccinated with CL1/CL3/ZA1**  
 627 **formulation and then infected with *F. hepatica*.** In Trials 1 (a) and 2 (b), serum was examined at  
 628 one timepoint pre-infection (at 3wks or 2wks post-2<sup>nd</sup> vaccination with CL1/CL3/ZA1, for Trial 1 (a)  
 629 or Trial 2 (b), respectively), and at 2 timepoints post-infection (7wpi, 13wpi (Trial 1) and 6wpi,  
 630 14wpi (Trial 2)). ^=single peptide and ^ = consecutive peptides that are recognised significantly  
 631 different at post-infection ( $p < 0.05$ -  $p < 0.0001$ ). n=5 for each group – See Table S2 for details.

632

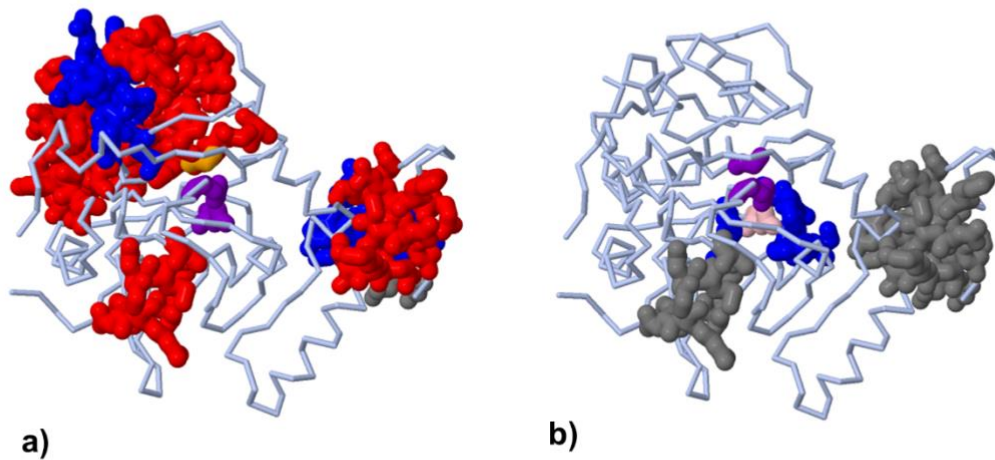


633

634 **Figure 3.- FhCL1-Epitope mapping profile in sera from the control group.** In Trials 1 (a) and 2  
 635 (b), serum was examined at one timepoint pre-infection, and two timepoints post-infection in each  
 636 case; at the early infection (7wpi and 6wpi) and late infection (13wpi and 14wpi). ^=single peptide  
 637 and  $\wedge$  = consecutive peptides that are recognised significantly different at post-infection ( $p < 0.05$ -  
 638  $p < 0.0001$ ). n=5 for each group – See Table S2 for details.

639



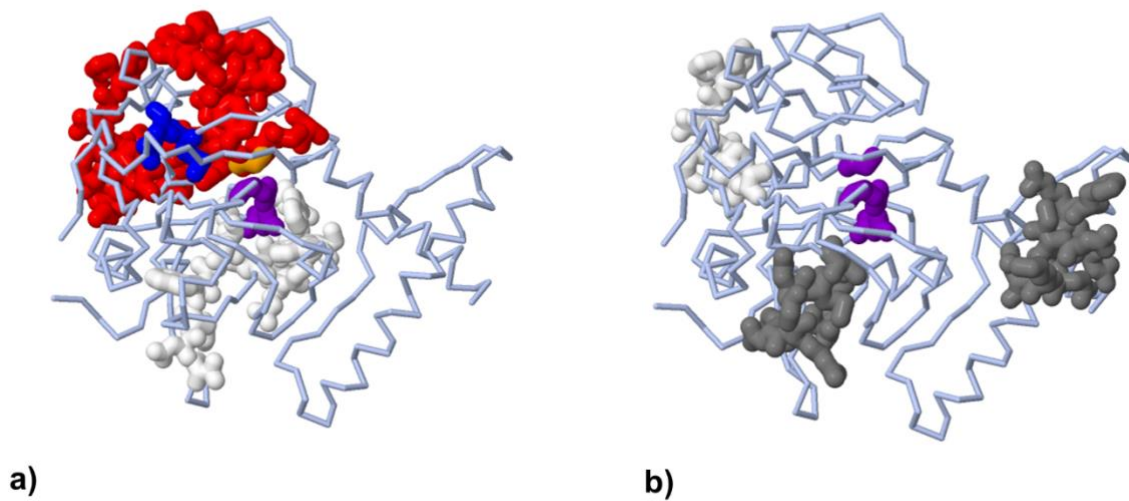


649

650

651 **Figure 5. Epitope recognition after *F. hepatica* infection in vaccinated groups in comparison**  
 652 **to pre-infection and localisation in the 3D FhCL1 structure.** In Trial 1 (a) and Trial 2 (b) epitopes  
 653 significantly recognised after *F. hepatica* infection in vaccinated animals, in comparison to pre-  
 654 infection were localized in the CL1-3D structure. Grey=epitopes recognised only at early infection at  
 655 7wpi (Trial 1) and 6wpi (Trial 2), red = epitopes recognised only in late infection at 13wpi (Trial 1)  
 656 and 14wpi (Trial 2) and blue = epitopes recognised at both, early and late, time points. Purple shows  
 657 the active site. The region of the active site that is recognised by serum during early infection is  
 658 shown in pink and at late infection, in orange.

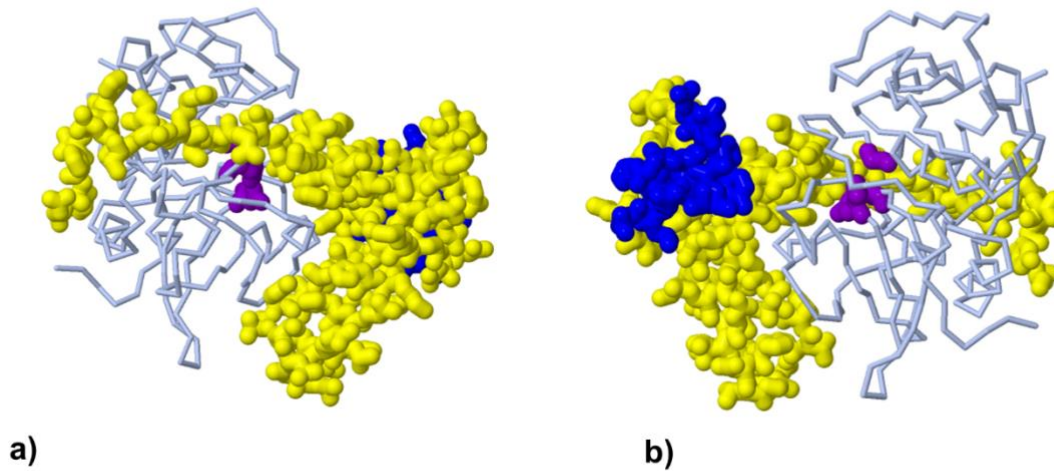
659



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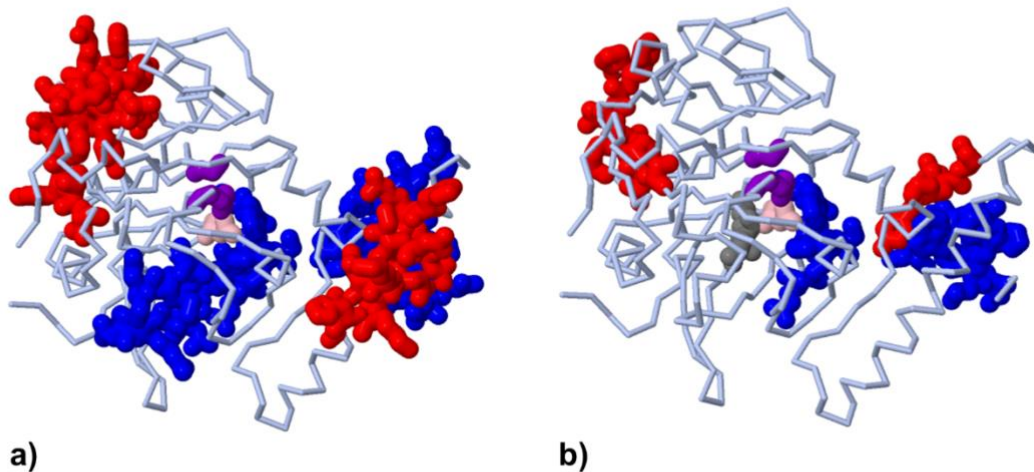
661 **Figure 6. Comparison of FhCL1-epitopes recognised by partially protected and non-protected**  
 662 **groups.** All the colours except white represent epitopes recognised by vaccinated but not controls.  
 663 White = “switched -off” epitopes recognised in control groups but not in vaccinated groups. Red =  
 664 regions recognised only at late infection (120-137, 145-155, 161-171 and 318-326 (CGSCWAFST,  
 665 YMKNERTSISF, VDCSRPGWNGG, SLPMVARFP)). Blue = region (ME 177-178) recognised at both,  
 666 early (7wpi) and late (13wpi) infection. Grey= region (39-47 HRRNIWEKN and 310-311  
 667 MVRNRGNMC) only recognised in early infection (6wpi). Purple= active site and orange the part of  
 668 the active site that is recognised in late infection (13wpi).

669



670

671 **Figure 7. Epitopes localised in the 3D FhCL1 structure that are bound by antibodies from all**  
 672 **groups in both trials after *F. hepatica* infection.** The figure shows localisation of the peptides 21-  
 673 31 (DLWHQWKRMYNKE) that were consistently recognised after *F. hepatica* infection at both, early  
 674 or late time points, in the FhCL1 3D structure. This region (blue) is found at the N-terminal region of  
 675 the pro-peptide of the molecule (region 16-106, shown in yellow), and faces outwards from the main  
 676 body of the mature enzyme (shown as grey backbone). (a) and (b) represent the same model with a  
 677 turn of 180°C. Purple coloured residues represent the active site.



678

679 **Figure 8. Epitope recognition after *F. hepatica* infection in non-vaccinated groups in**  
680 **comparison to pre-infection the localization in the FhCL1 3D structure .** In Trial 1 (a) and Trial  
681 2 (b) epitopes significantly recognised after *F. hepatica* infection in control groups, in comparison to  
682 pre-infection were localized in the CL1-3D structure. Grey=epitopes recognised only at early  
683 infection at 7wpi (Trial 1) and 6wpi (Trial 2), red = epitopes recognised only in late infection at 13wpi  
684 (Trial 1) and 14wpi (Trial 2) and blue = epitopes recognised at both, early and late, time points.  
685 Purple shows the active site. Pink shows a region of the active site that is recognised specifically  
686 during early infection.

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