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

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ABSTRACT

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

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

Keywords:

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

Abbreviations:

rmFhCL1: recombinant *Fasciola hepatica* Cathepsin L1

FhCL1: *Fasciola hepatica* Cathepsin L1

1. INTRODUCTION

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

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

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

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

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

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

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$_{o}$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$_{nd}$ vaccination. Blood samples were collected by jugular venepuncture at Day 0, 3 weeks post-2$_{nd}$ 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$_{nd}$ vaccination over two consecutive days (100 metacercariae per day). The metacercariae were dispersed in 10ml of dH_2O and were

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

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

2.2 Measurement of IgG1 and IgG2 anti- rmFhCL1 by ELISA

IgG1 and IgG2 levels were measured in serum from all time points in both trials by using an in-house ELISA. 96 well-plates (Corning) were coated with 5µg/ml of rmFhCL1 in carbonate-coating buffer at pH 9.6, and incubated overnight at 37oC. Next, plates were washed three times with Phosphate Buffered Saline plus 0.05% Tween 20 (PBS-T) and then blocked by adding 100µl of 5% milk in PBS-T for 30min at 37oC. After washing three times, serum samples diluted 1:20 in PBS-T were added into the wells (in 100µl volume) in duplicate and then serial dilutions (1:3) were carried out. Plates were incubated for 30min at 37oC and washed as before, HRP-conjugated monoclonal anti-IgG1 (Prionics) or anti-IgG2 (Bio-rad) were added at a concentration of 1:100 (anti-IgG1) in PBS-T or 1:1000 (anti-IgG2) in PBS without Tween. After incubation at the same conditions as previously described, and washing, 100µl of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) were added for 10min in the dark. Finally, 50µl of Stop solution (H_2SO_4) were added onto each well and absorbance was measured at 450nm. Negative and positive serum controls were included in each plate. Endpoint titres were calculated for each sample and then transformed into log10.

2.3 Linear B-Cell Epitope mapping of FhCL1

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

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

(MRLFVLAVLTVGVLGSNDDLWHQWKRMYNKEYNGADDQHRRNIWEKNVKHIQEHNLRHDLGLVTYTLGLNQFTD
MTFEEFKAKYLTEMSRASDILSHGVPYEANNRAVPDKIDWRESGYVTEVKDQGNCGSCWAFSTTGTMEGQYMKNER
TSISFSEQQLVDCSRPWGNNGCGGGLMENAYQYLKQFGLETESSYPYTAVEGQCRYNKQLGVAKVTGFYTVHSGSEVE
LKNLVGAEGPAAVAVDVESDFMMYRSGIYQSQTCSPLRVNHAVLAVGYGTQGGTDYWIVKNSWGLSWGERGYIRMV
RNRGNMCGIASLASLPMVARFP) was taken from UniProtKB - Q24940, which included the FhCL1 protein including the signal peptide (positions 1-15), the pro-peptide (activation peptide) (positions 16-106), and the mature enzyme (107-126). The active site is formed by amino acids at positions Cys132, His269, Asn289 (Supplementary material. Figure S1)

Peptides were used as the solid-phase antigen in ELISA-based assays. Lyophilised peptides were solubilised in 50% Acetonitrile (Fisher Chemical) in H_2O (HyClone GE Life Sciences) and then further diluted (1/20) in 0.1% Sodium Azide (Sigma) plus 0.1% in BSA (Sigma) in PBS. Next, 96-well plates (Nunc. high binding) were coated with 5µg/ml of Streptavidin (Sigma) overnight at 4oC and washed four times with PBS-T. Biotinylated peptides were added at a final concentration of 50µg/ml (in 0.1% Sodium Azide, 0.1% BSA in PBS). After incubation overnight at 4oC, and blocking with BSA (2% BSA in PBS-T) for 1.5h, each serum sample was diluted to 1/50 in 2% BSA PBS-T and added to the wells. The plates were then incubated for 1.5h at room temperature with shaking, and washed as before. Next, peroxidase-conjugated secondary antibody (anti-bovine total anti-IgG (cat: A5295 Sigma)) was added at a dilution of 1:3000 in blocking buffer and incubated for another 1.5h. After washing plates as previously, TMB (Sigma) was added and incubated for 15min in the dark. All the volumes were 100µl. Absorbance was measured at 450nm. Control sera, along with blank wells without peptide and with peptide but without serum, were included in each plate. Background (OD from blank wells) was subtracted from the wells containing peptides in each plate.

2.4 Crystal 3D model construction

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

2.5 Statistical analysis

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

3 RESULTS

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

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

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

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

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

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

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

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

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

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

3.3 Localization of the epitopes recognised by cattle in the linear sequence and 3D molecule of FhCL1 and comparison among groups

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

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

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

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

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

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

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

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

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

3.3.3 Localization of epitopes recognised non-specifically

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

4 DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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Conflicts of interest

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

Authors' contributions

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

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Supplementary material

Figure S1. Secondary and Tertiary model of FhCL1

Table S1. Overlapping peptides of FhCL1

Table S2. Level of statistically significance of peptides recognised in each group

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Figure Legends

Figure 1. IgG1 and IgG2 levels specific to rmFhCL1induced after *F. hepatica* infection in CL1 CL3 ZA1-vaccinated and controls (non-vaccinated) cattle. IgG1 (a, b) and IgG2 (c, d) specific to rmFhCL1 were measured by end-point titration in Trial 1 (a, c) and Trial 2 (b, d). Control group = dashed green line; CL1/CL3/ZA1= purple line. n=5, **^** indicates 1st and 2nd vaccinations; Red arrow indicates *F. hepatica* infection. *= p<0.05, **=p<0.01; ***=p<0.001; *****=p<0.0001.

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

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

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

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

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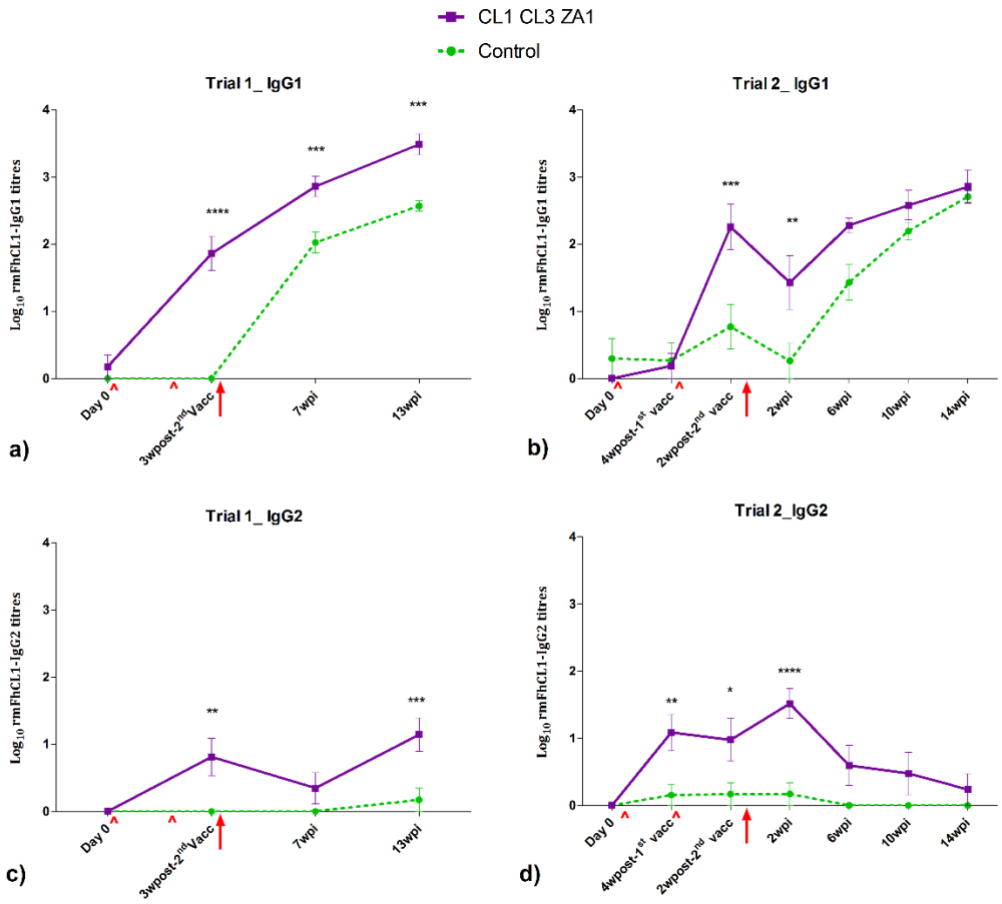
Figure 6. Comparison of FhCL1-epitopes recognised by partially protected and non-protected groups. All the colours except white represent epitopes recognised by vaccinated but not controls. White = "switched –off" epitopes recognised in control groups but not in vaccinated groups. Red = regions recognised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

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

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

Figures Paper



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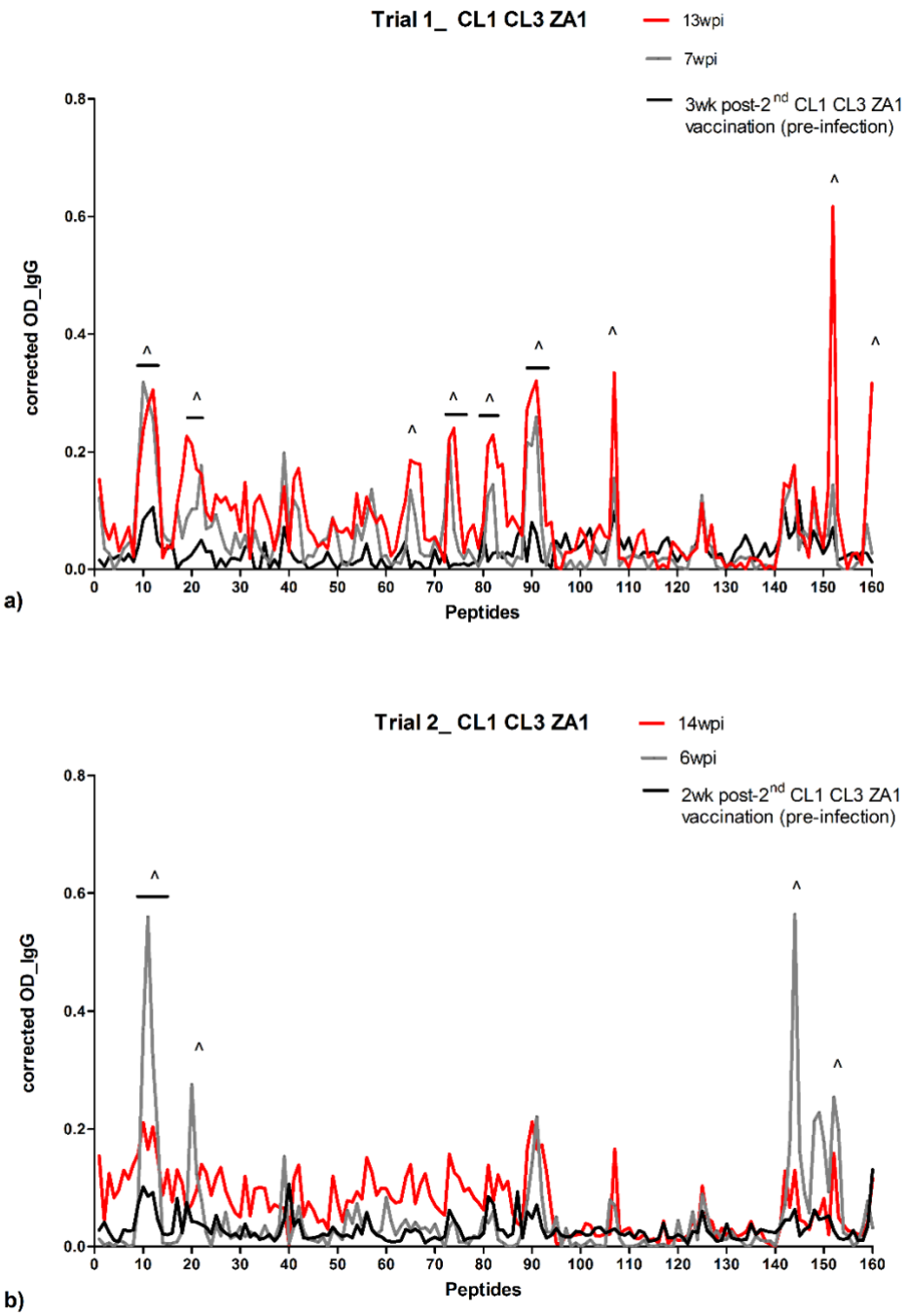

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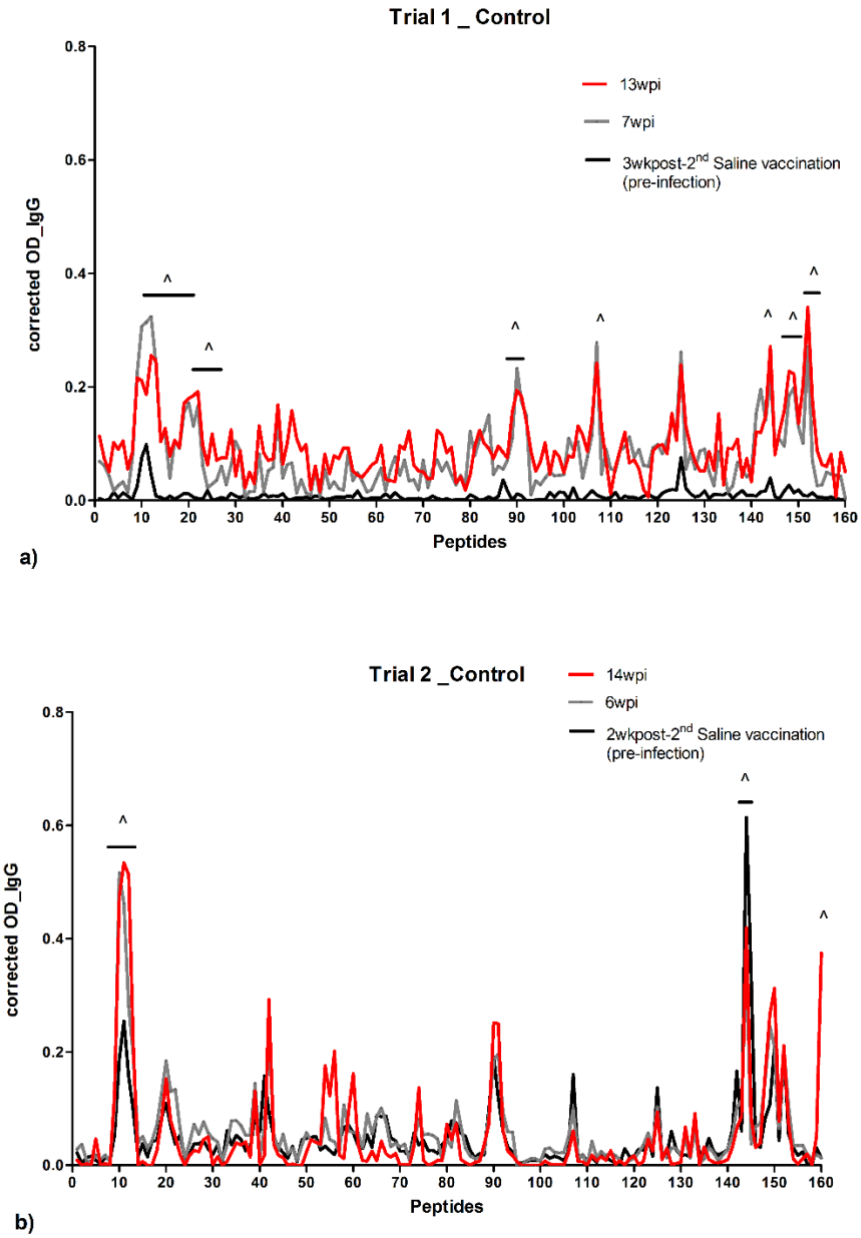

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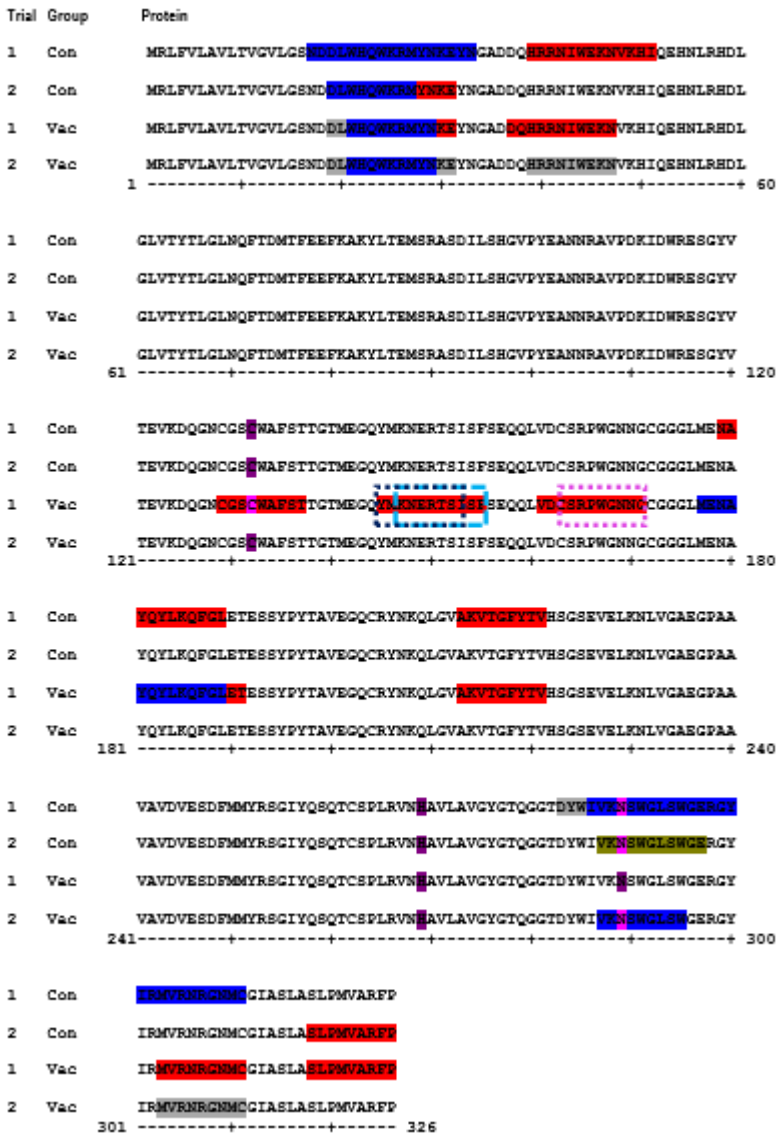

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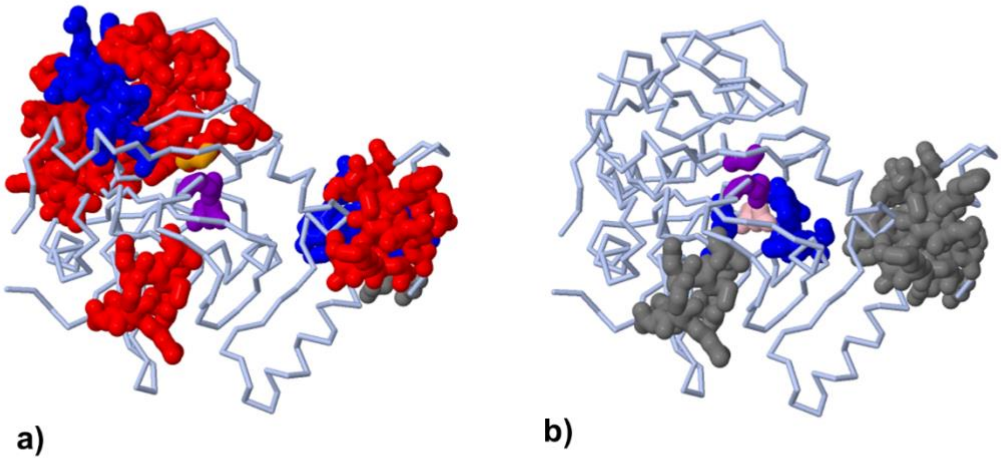

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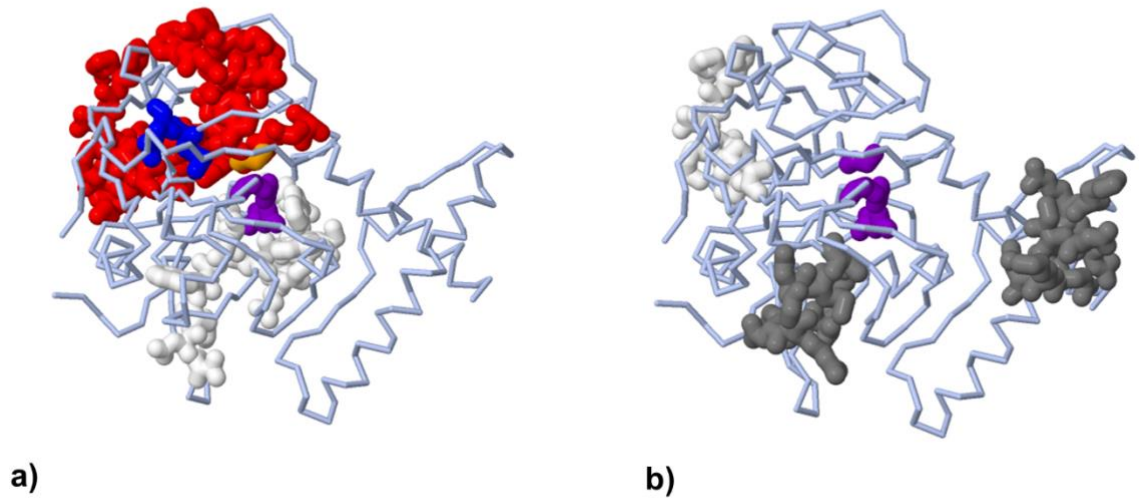

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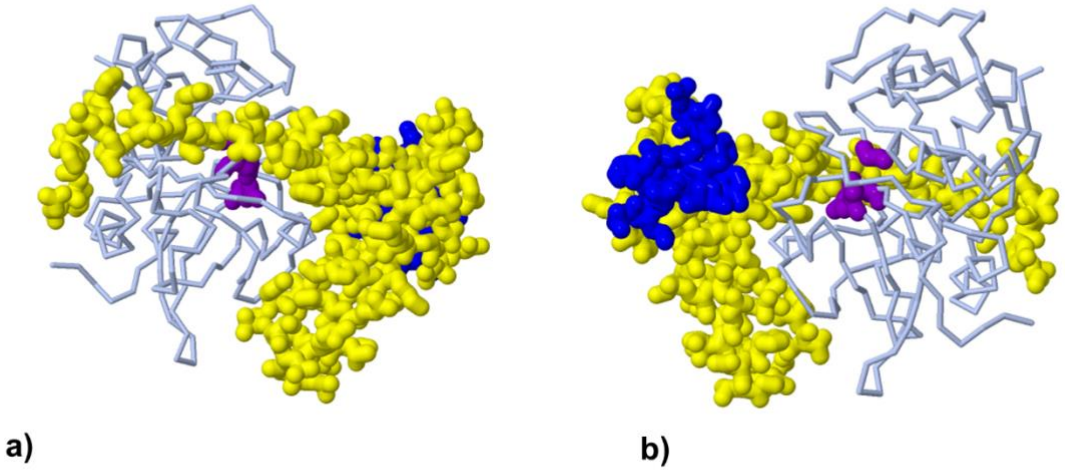

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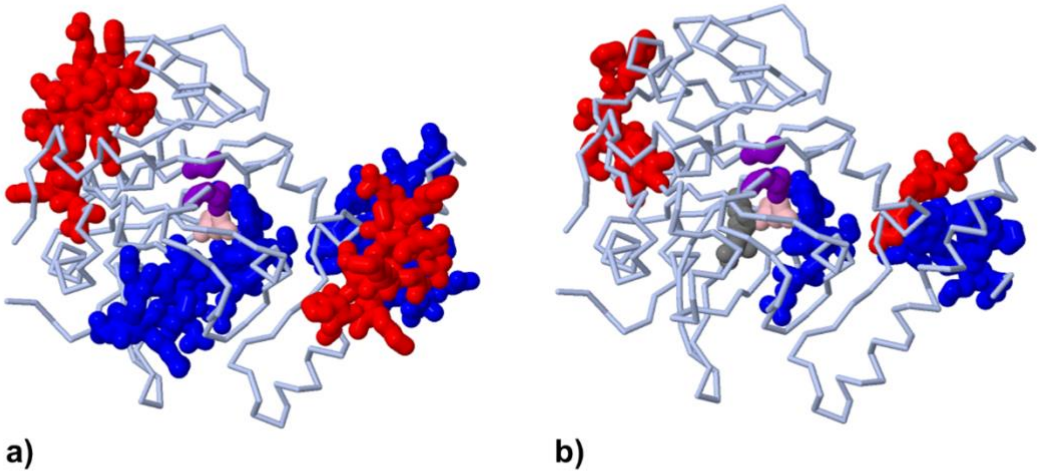

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