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An amino acid substitution in *Fasciola hepatica* P-glycoprotein from triclabendazole-resistant and triclabendazole-susceptible populations.

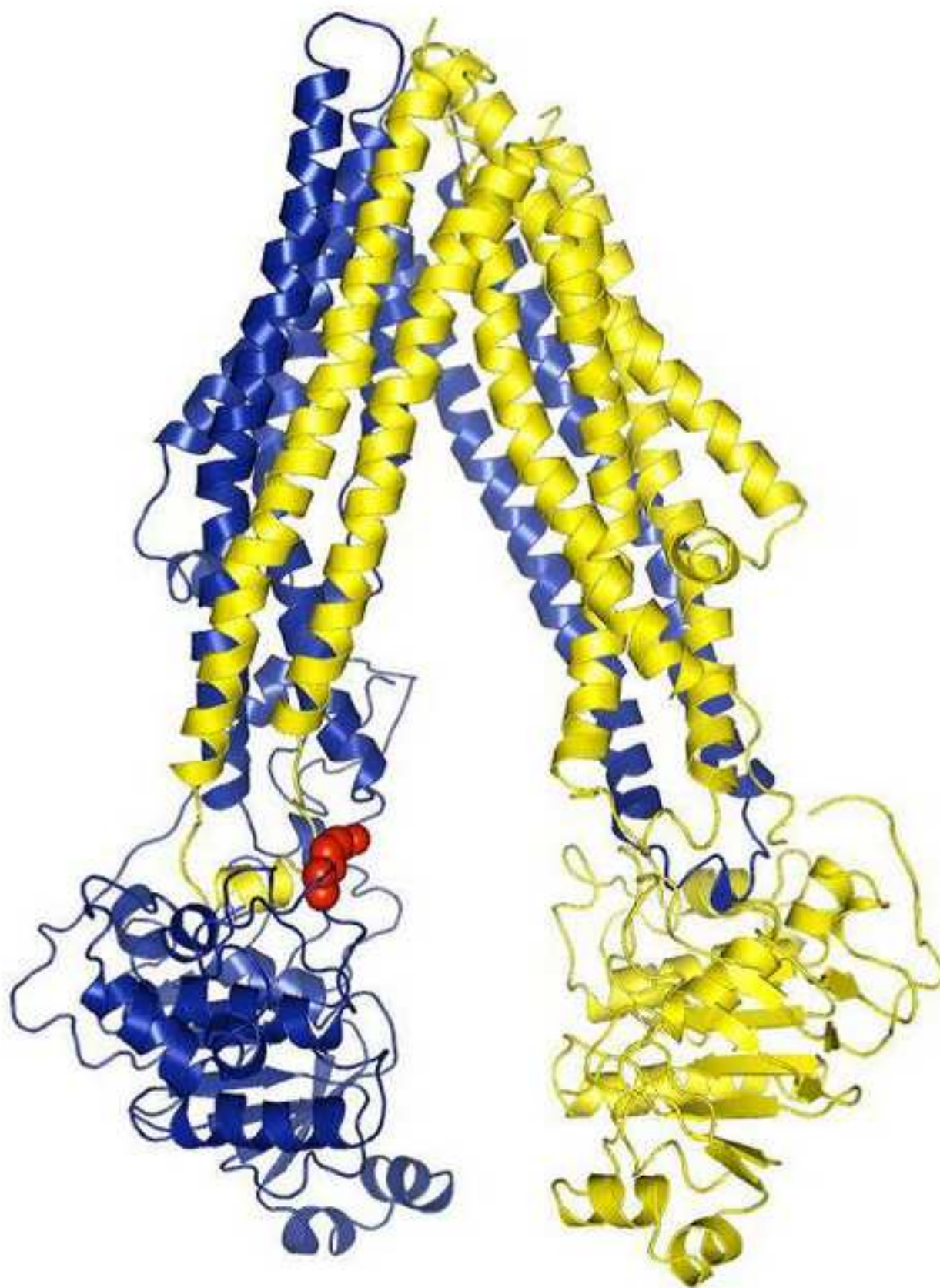
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Key Words: *Fasciola hepatica*; anthelmintic resistance; P-glycoprotein; SNP

Ribbon model of Liver Fluke P-glycoprotein with arginine substitution at residue 1144.



- Triclabendazole-resistant liver flukes differ in their P-glycoprotein genes.
- Triclabendazole-resistant flukes have greater allelic diversity.
- Triclabendazole resistance correlates with a change in the P-glycoprotein X-loop.
- This may provide the basis for a test for triclabendazole resistance.

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1 Abstract

2 Control of fasciolosis is threatened by the development of anthelmintic resistance. Enhanced
3 triclabendazole (TCBZ) efflux by ABC transporters such as P-glycoprotein (Pgp) has been
4 implicated in this process. A putative full length cDNA coding for a Pgp expressed in adult
5 *Fasciola hepatica* has been constructed and used to design a primer set capable of amplifying
6 a region encoding part of the second nucleotide binding domain of Pgp when genomic DNA
7 was used as a template. Application of this primer set to genomic DNA from TCBZ-resistant
8 and -susceptible field populations has shown a significant difference in the alleles present.
9 Analysis of an allele occurring at a three-fold higher frequency in the “resistant” population
10 revealed that it was characterised by a serine to arginine substitution at residue 1144.
11 Homology modelling studies have been used to locate this site in the Pgp structure and hence
12 assess its potential to modify functional activity.

13
14 The common liver fluke (*Fasciola hepatica*) is a major cause of economic losses to
15 agriculture in temperate regions, with cost estimated at US\$2000 million per annum [1]. In
16 the absence of an effective vaccine against the fluke control is achieved by chemotherapy.
17 However, the effectiveness of the drug of choice, triclabendazole (TCBZ) - a benzimidazole
18 derivative – is threatened by the development of resistant fluke populations; these have been
19 reported from Australia, Europe and South America [reviewed in 2]. Work with TCBZ-
20 resistant isolates [3] has indicated that, in contrast to resistance to benzimidazoles in
21 nematodes [4], TCBZ resistance is not associated with changes in the likely target molecule -
22 tubulin [5]. TCBZ-resistant isolates have been shown to process TCBZ more rapidly and
23 their resistant phenotype can be reversed, under in vitro conditions, by the co-administration
24 of inhibitors of P-glycoprotein (Pgp) drug efflux pumps or drug detoxification pathways [2].

25 *F. hepatica* is known to express a Pgp-like ABC transporter [6], although this protein has
26 been reported to be predominately expressed in juvenile flukes, and, as such, generally will
27 not be involved in TCBZ efflux from adult flukes established in the bile ducts of infected
28 animals. Recent publication of EST libraries for *F. hepatica* using mature flukes [7]
29 (specifically contigs 1473, 14497, 10329 and 15817) in conjunction with work in our
30 laboratory, has allowed the construction of a putative full length sequence encoding a *F.*
31 *hepatica* Pgp expressed in adult flukes (supplementary material 1). A primer set (forward
32 primer 5' ttggtgtgtatcgcaggaa 3', reverse primer 5' agccgaagtagcttcaccca 3'; enclosing
33 residues 1090 to 1175) derived from the region coding for the second nucleotide binding
34 domain of Pgp has been successfully applied to genomic DNA extracted from flukes from
35 infra-populations in cattle raised on farms associated with either TCBZ susceptibility or
36 resistance [8] and the amplicons sequenced. (It should be noted that these are functional
37 designations for the populations present on the two farms – the “resistant” populations
38 contains up to 36% susceptible flukes and conversely, the “susceptible” population may
39 contain a minority (<10%) of resistant flukes) [9]. The primers used were separated by
40 255bp in the constructed coding sequence, but a product of approximately 830bp was
41 routinely obtained. Sequencing of these amplicons revealed that the increased size was due
42 to the presence of a 569bp intron. Characteristic 5' (ATxGTGAG) and 3' (CAGxGGC)
43 splice sites were present at the beginning and end of the non-coding sequence (supplementary
44 material 2). Translation of the partial exons flanking the intron from a putatively TCBZ-
45 susceptible fluke gave a sequence that was identical to the translation of the relevant region
46 of our full length *F. hepatica* sequence and had 77.4% identity with the second ABC
47 transporter region of SMDR2, a *Schistosoma mansoni* putative Pgp (EMBL L26287),
48 confirming that the amplicons produced using this primer set were derived from a gene
49 coding for a liver fluke Pgp. In order to compare the fluke populations present on the

50 “susceptible” and “resistant” farms, genomic DNA from 10 flukes from each population was
51 prepared and subjected to a PCR reaction using the second nucleotide binding site primers.
52 After sequencing of the amplicons in both directions, an alignment was constructed and
53 edited to give sequences of equal length. The Phase 2.1 algorithm [10] incorporated in
54 DnaSP [11] was used to reconstruct haplotypes where consistent evidence of heterozygosity
55 (double peaks on sequencing chromatograms) was seen in order to allow the derivation of
56 parameters of genetic diversity. The susceptible population contained five haplotypes with a
57 nucleotide diversity (π) of 0.00228. Four of the ten flukes were heterozygous. The resistant
58 population contained seven haplotypes with a nucleotide diversity (π) of 0.00358 and three of
59 these were heterozygous. The presence of heterozygotes indicated that sexual reproduction
60 with cross-fertilisation (with the possibility of recombination) had occurred in previous
61 generations in each population. A consideration of the mitochondrial haplotypes of the
62 individual heterozygous flukes [8] revealed that there was no association between Pgp alleles
63 and maternal lineage, again indicating cross-fertilisation. There was significantly greater
64 diversity (0.768 versus 0.442, $P = 0.0002$ by a one tailed t-test) in Pgp haplotypes seen in the
65 fluke population from the farm exhibiting TCBZ resistance. Ten unique haplotypes were
66 observed when the resistant and susceptible populations were taken *in toto*. The distribution
67 of these haplotypes over the two populations and the Single Nucleotide Polymorphisms
68 (SNPs) that define them are shown in Table 1. Haplotype 1 was the most common, being
69 present in all of the flukes from the susceptible population and eight of the flukes from the
70 resistant population. The remainder of the haplotypes were segregated between the
71 susceptible and resistant populations. As the two farms are approximately 100 km apart these
72 differences could reflect geographical separation, however, with regard to TCBZ resistance,
73 the mitochondrial haplotypes have been shown to be acting as neutral markers with the most
74 common haplotype being present in approximately one third of the flukes from both farms

75 [8]. We consider that the differences in frequency of alleles observed in this study are more
76 likely to be due to different selective pressures having operated, directly or indirectly, on the
77 expressed products of the Pgp genes present in the ancestors of the flukes presently at the two
78 locations.

79 There were 11 individual SNPs, of which 3 were transitions and the rest transversions. Three
80 of the changes were within the partial exons and also resulted in an amino acid change. They
81 were T28A, resulting in a valine to glutamic acid substitution at residue 1112; G54A,
82 resulting in an alanine to threonine substitution at residue 1121; and T687G, resulting in a
83 serine to arginine change at residue 1144. The first of these changes (T28A) was seen in 3
84 out of 10 TCBZ “susceptible” flukes and in 2 out of 10 TCBZ “resistant” flukes populations
85 and the second (G54A) was exclusively (4 out of 10) in the TCBZ “susceptible” population.
86 The third change (T687G giving S1144R) was seen more frequently in the TCBZ “resistant”
87 population (3 out of 10 for the “resistant” flukes versus 1 out of 10 for the “susceptible”
88 flukes) and was located four residues before the signature motif (LSGGQ) which interacts
89 with adenosine triphosphate (ATP), suggesting that it may have functional significance.

90 To allow us to investigate the potential significance of the S144R substitution we constructed
91 a homology model of *F. hepatica* Pgp. The amino acid sequence of the constructed *F.*
92 *hepatica* Pgp sequence was used to search against the NCBI (National Center for
93 Biotechnology Information) nonredundant protein sequence database with the programme
94 PSI-BLAST [12] and identified mouse ABCB1, a multidrug transporter [13], as the closest
95 relative of known structure. The backbone co-ordinates for the core of the homology model
96 were built based on the crystal structure of ABCB1a (PDB ID: 3G5U), in the inward-facing,
97 nucleotide-free conformation as the template. Alignment of the reconstructed *F. hepatica*
98 and *M. musculus* primary sequences revealed a 41% identity between the two proteins. The
99 N-terminus (residues 1-33) of the template crystal structure was not resolved and electron

100 density for the flexible linker region (residues 627-683) that connects the two halves of the
101 transporter via nucleotide binding domain 1 (NBD1) and transmembrane domain 2 (TMD2)
102 was missing [13]. Therefore, the N-terminus (residues 1-16) and linker region (residues 631
103 to 674) were not built into our model of the TCBZ-resistant *F. hepatica* transporter. The final
104 model corresponded to residues V17-H630 and V675-E1278 and consisted of 12 α -helices
105 arranged in two 6-helical bundle transmembrane domains (TMD1 and TMD2) and two
106 cytoplasmic nucleotide-binding domains (NBD1 and NBD2). The protein takes on an overall
107 V-shaped conformation in which NBD1 is associated with helices 1, 2, 3 and 6 of TMD1 and
108 helices 10 and 11 of TMD2, and NBD2 is associated with helices 4 and 5 of TMD1 and
109 helices 7, 8, 9 and 12 of TMD2 (Fig. 1). The TMDs form two arms that straddle a substrate
110 binding and translocation pore that is closed towards the extracellular side. The NBDs both
111 contain the Walker A and B motifs and the conserved LSGGQ ABC transporter signature
112 sequence, or C-motif. As shown in Figs. 1 and 2, the arginine residue (R1144) is located at
113 the interface of NBD2 and its associated transmembrane domain, where it protrudes and
114 introduces a positive charge into the space between the two domains. It is also positioned to
115 participate in formation of the NBD dimer interface that occurs upon ATP binding.

116 The stereochemical properties of the model as determined by PROCHECK revealed that 93%
117 and 6.5% of residues were in the allowed and generously allowed regions, respectively, of the
118 Ramachandran plot. This compares favourably with the Ramachandran plot of the
119 coordinates of the template structure in which 92.5% of residues were in the allowed regions,
120 and 7.5% in the generously allowed. The modelling of the *F. hepatica* Pgp demonstrated that
121 residue 1144 is located in the region termed the X-loop, a part of the molecule which is
122 thought to be involved in the cross-linking of the long intracellular loops (ICLs) in response
123 to ATP binding and may transmit conformational changes to the ICLs [14]. Molecular
124 dynamic simulations [15] have indicated that the regions of the molecule with which the X-

125 loop interacts potentially change during the cycle of nucleotide attachment and release,
126 emphasising its probable importance in the functional activity of efflux pumps. The S1144R
127 substitution thus has the potential to modulate these conformational changes. In a recent
128 study of cattle ticks resistant to the macrocyclic lactone ivermectin, it was shown that there
129 was an up-regulation of mRNA coding for an ABC transporter in resistant isolates [16]. This
130 transcript also had an arginine in the position corresponding to residue 1144 in our assembled
131 liver fluke sequence whereas two other cattle tick ABC transporters which had a glycine
132 rather than arginine at this position were not up-regulated in the resistant tick isolates,
133 supporting the hypothesis that this substitution may have functional consequences.

134 The observation that the TCBZ-resistant population does not contain flukes homozygous for
135 this allele may be due to the sample size; alternatively it may indicate that the S1144R
136 substitution, whilst advantageous in the presence of TCBZ, is less appropriate for the other
137 functions of Pgp required by the fluke. It is intended to investigate this issue using *F.*
138 *hepatica* Pgp expressed *in vitro*.

139 In conclusion, the data presented in this study, although preliminary, support the suggestion
140 that the development of TCBZ resistance in liver flukes is a multifactorial process involving
141 changes in drug uptake, efflux and metabolism [2] and indicate that selection for variant Pgps
142 may be part of this process. The S1144R substitution is located in a region of the protein
143 which is likely to be associated with its transporter functions. If an enhanced ability to
144 transport TCBZ and its metabolites is confirmed by expression studies with a multidrug
145 efflux protein bearing this substitution this would strengthen its association with the
146 development of TCBZ resistance in the liver fluke. Analysis of isolates characterised with
147 regard to TCBZ susceptibility [3] and field populations will be necessary to establish the
148 importance of the S1144R substitution in the development of the TCBZ-resistant phenotype
149 in locations other than the Netherlands. If changes in this region of Pgp are widely associated

150 with TCBZ resistance it may be possible to develop an allele-specific molecular test
151 analogous to that used for nematodes [17] for potentially TCBZ-resistant populations based
152 upon genomic DNA extracted from fluke eggs. These are a source of *F. hepatica* genomic
153 DNA readily available for epidemiological surveys.

154

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159 or the decision to submit this article for publication.

160

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214

215 **Legends to figures**

216 **Fig 1.** Homology model of the *F. hepatica* P-glycoprotein – front and rear views. The
217 position of residue R1144 (represented as a red space-fill) is indicated by the circle and is
218 located at the interface of NBD2 and its associated transmembrane domain, TM4. TM,
219 transmembrane domain; NBD, nucleotide binding domain. Homology modelling was
220 performed using MolIDE 1.7 [18]. PSIPRED was used to perform initial secondary structure
221 prediction of the target, and the target-template sequence alignment was adjusted manually
222 using the predicted secondary structure of the target and the experimental secondary structure
223 of the template as a guide for gap placement. During building of the backbone core of the
224 model, only those side-chains that are conserved between target and template were preserved.
225 The remaining, non-conserved side chains were built onto the backbone with SCWRL4 [19],
226 and loops modelled with Loopy [20]. The model was visualised using The PyMOL
227 Molecular Graphics System, Version 1.3 (Schrödinger, LLC).

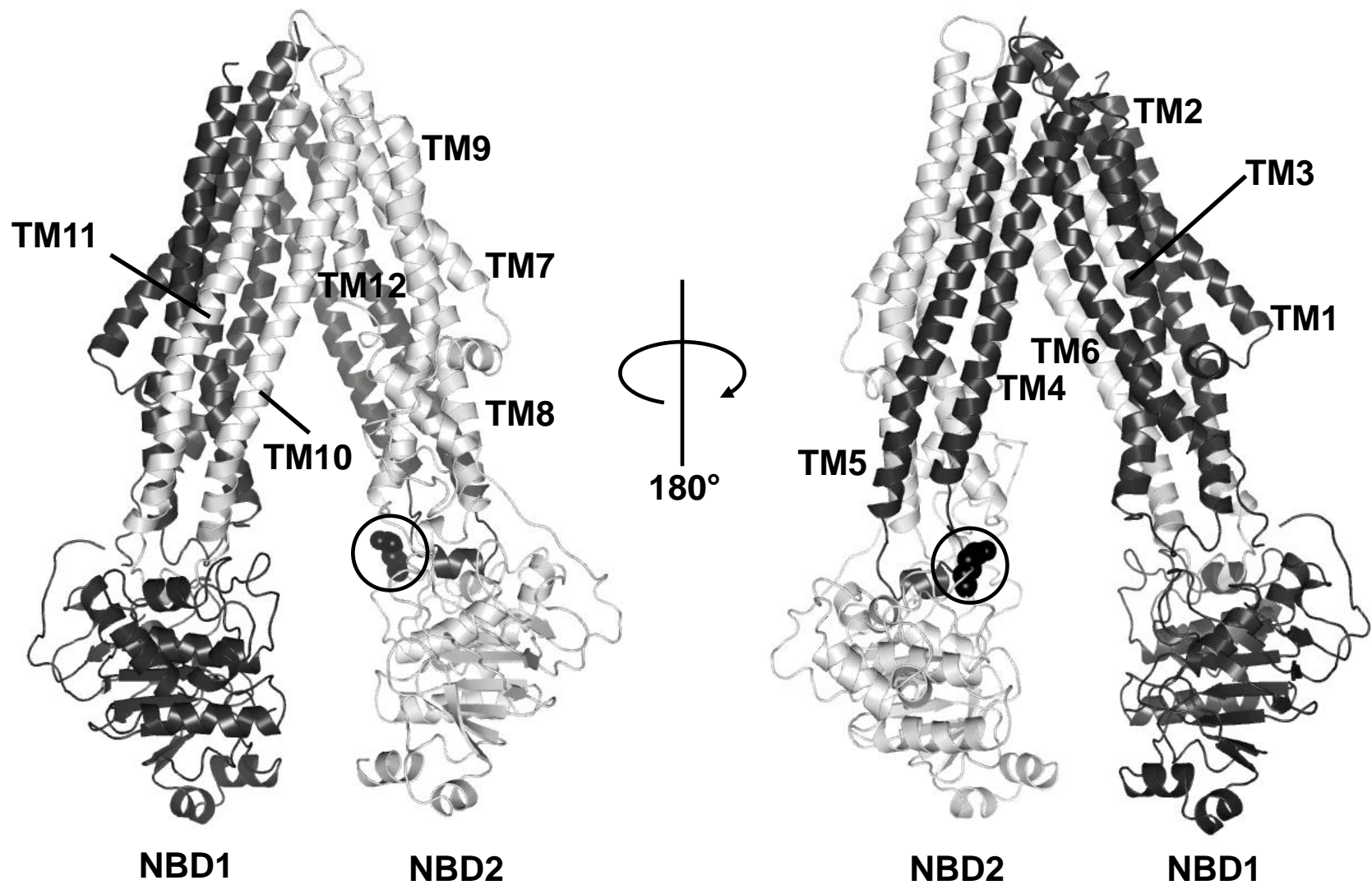
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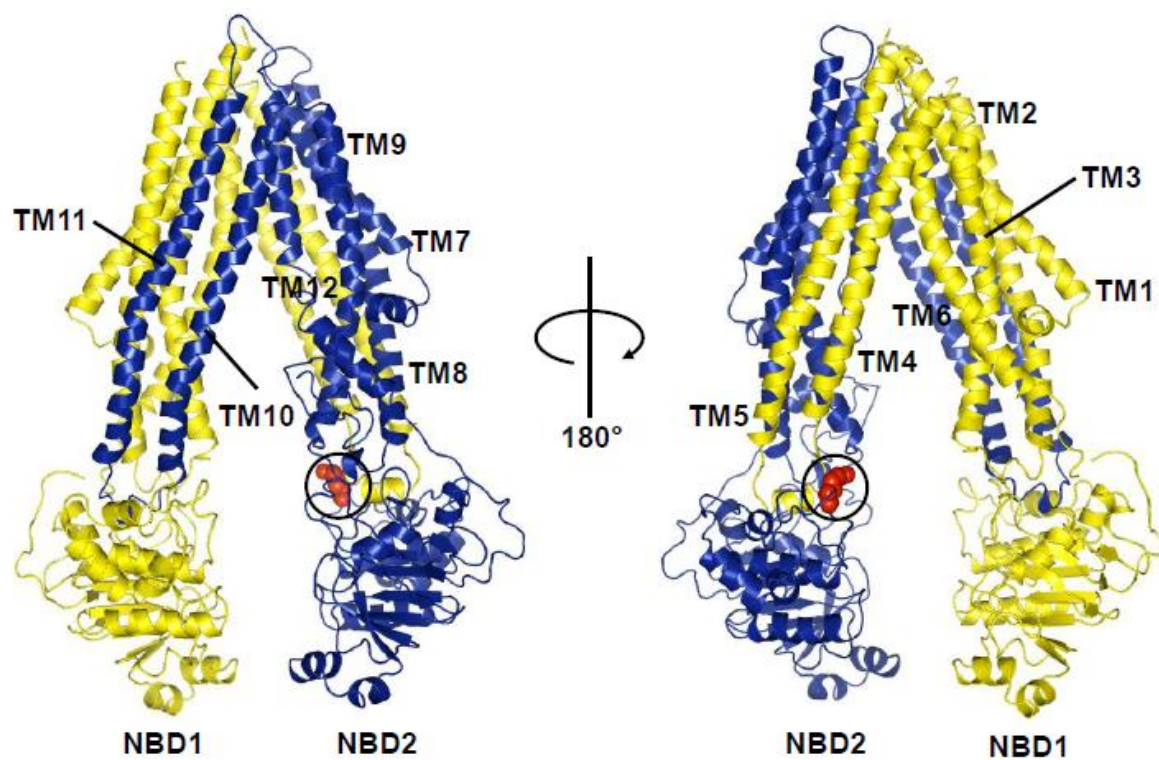
229 **Fig 2.** Close-up of the NBD2 region of the homology model showing residue 1144. A –
230 modelled with arginine as residue 1144, B – modelled with serine as residue 1144.

Haplotype	Single Nucleotide Polymorphisms	Number (Resistant/ Susceptible)	Change in Protein
1	-	23 (Resistant and Susceptible)	-
2	A564G, T687G	1 (Susceptible)	S1144R
3	T28A, G54A, G181T, T267G, G323T, A564G,	1 (Susceptible)	V1112E, A1121T,
4	T28A, G54A, C60T,	2 (Susceptible)	V1112E, A1121T
5	G54A	1 (Susceptible)	A1121T
6	G181T, T267G, A617T	1 (Resistant)	
7	G181T, T267G, G323T, A617T, T687G	2 (Resistant)	S1144R
8	T267G, A617T,	6 (Resistant)	
9	T28A, G181T, T307A	1 (Resistant)	V1112E
10	G20C, T28A, T307A, T687G	1 (Resistant)	V1112E, S1144R

Table 2 Constructed haplotypes, their distribution in the two populations and non-synonymous changes in exons.

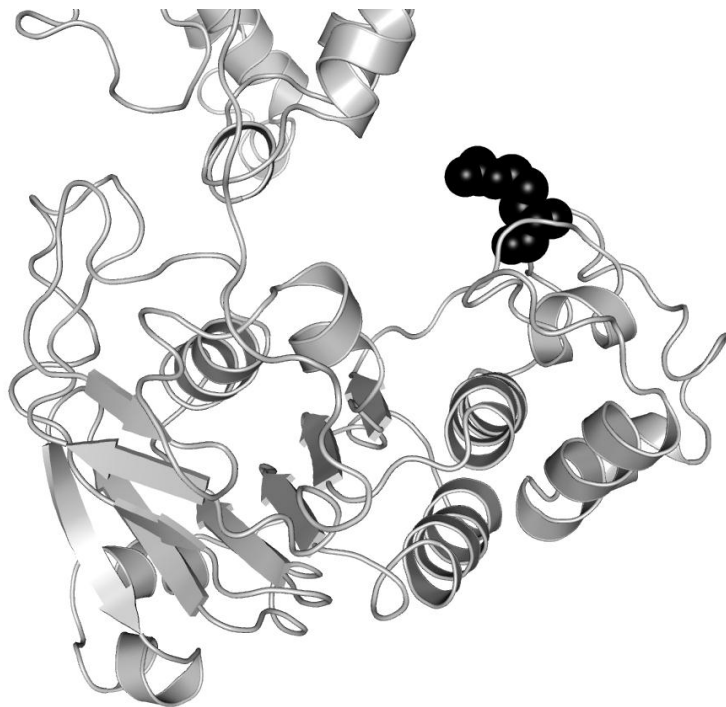
Figure(s)



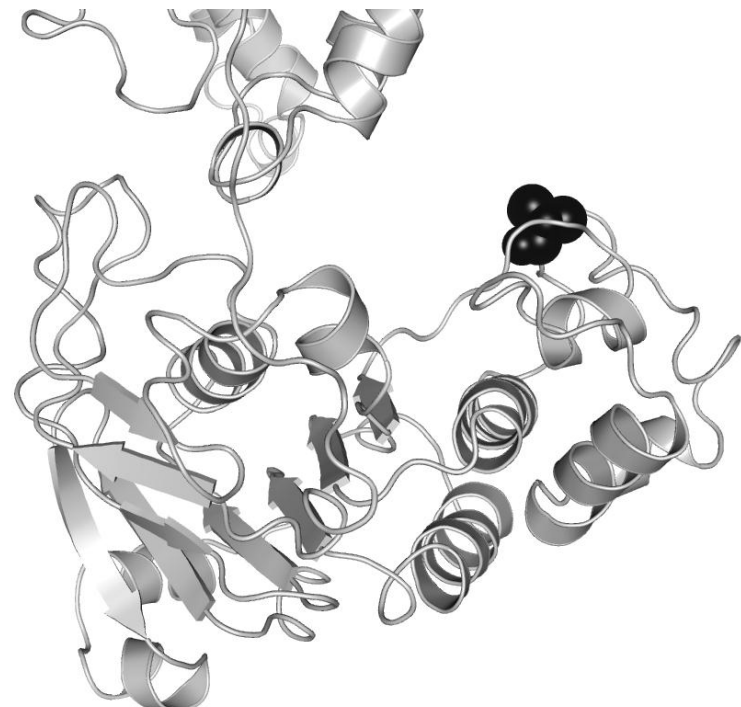


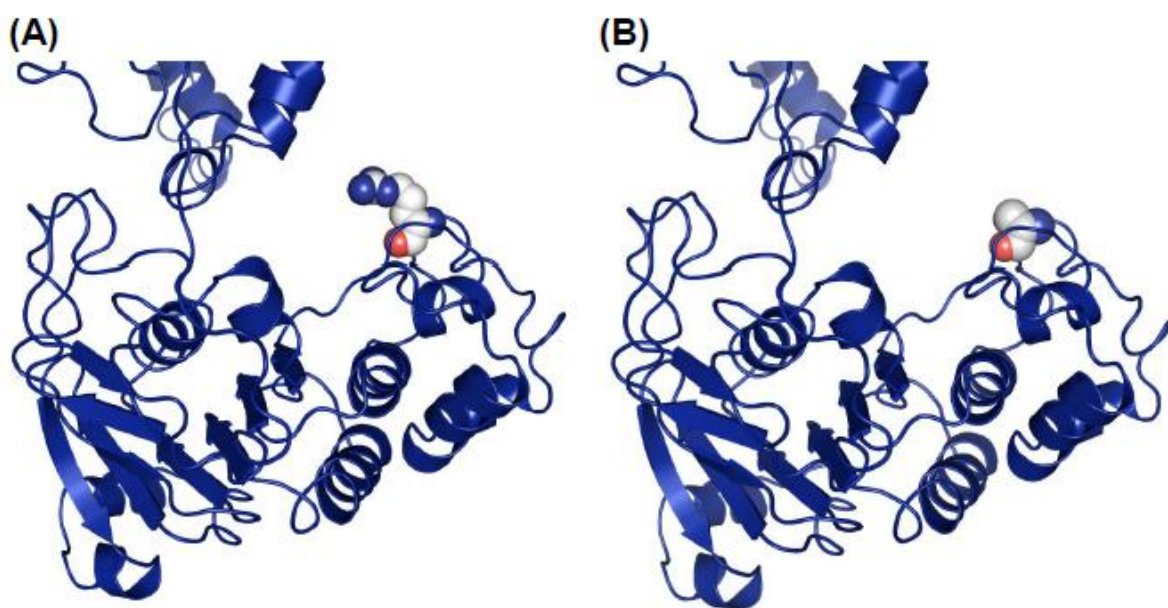
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(A)



(B)





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