Expression and phylogeny of multidrug resistance protein 2 and 4 in African white backed vulture *(Gyps africanus)*

Bono Nethathe [Corresp., 1, 2] , **Aron Abera** [3] , **Vinny Naidoo** [2]

[1] Department of Food Science and Technology, University of Venda, Thohoyandou, Limpopo, South Africa

[2] Department of Paraclinical Science, University of Pretoria, Onderstepoort, Pretoria, South Africa

[3] Inqaba Biotechnology, Sunnyside, Pretoria, South Africa

Corresponding Author: Bono Nethathe
Email address: bono.nethathe@univen.ac.za

Diclofenac toxicity in old world vultures is well described in the literature by both the severity of the toxicity induced and the speed of death. While the mechanism of toxicity remains unknown at present, the necropsy signs of gout suggests primary renal involvement at the level of the uric acid excretory pathways. From information in the chicken and man, uric acid excretion is known to be a complex process that involves a combination of glomerular filtration and active tubular excretion. For the proximal convoluted tubules excretion occurs as a two-step process with the basolateral cell membrane using the organic anion transporters and the apical membrane using the multidrug resistant protein to transport uric acid from the blood into the tubular fluid. With uric acid excretion seemingly inhibited by diclofenac, it becomes important to characterize these transporter mechanism at the species level. With no information being available on the molecular characterization/expression of MRPs of *Gyps africanus*, for this study we used next generation sequencing, and Sanger sequencing on the renal tissue of African white backed vulture (AWB), as the first step to establish if the MRPs gene are expressed in AWB. In silico analysis was conducted using different software to ascertain the function of the latter genes. The sequencing results revealed that the MRP2 and 4 are expressed in AWB vultures. Phylogeny of avian MRPs genes confirms that vultures and eagles are closely related, which could be attributed to having the same ancestral genes and foraging behavior. In silico analysis confirmed the transcribed proteins would transports anionic compounds and glucose.

1   **Expression and phylogeny of multidrug resistance protein 2 and 4 in African White Backed**
2   **vulture** *(Gyps africanus).*

3   Bono Nethathe[1, 3], Aron Abera[2], Vinny Naidoo[1]

4   [1]Department of Paraclinical Science, University of Pretoria, Onderstepoort, Pretoria, South
5   Africa.

6   [2]Inqaba Biotechnology, Pretoria, South Africa.

7   [3]Department of Food Science and Technology, University of Venda, Thohoyandou, Limpopo,
8   South Africa.

9   Corresponding Author:
10   Bono Nethathe[1]
11   University road, Thohoyandou, Limpopo, 0950, South Africa
12   Email address: bono.nethathe@univen.ac.za

14   **Abstract**

15   Diclofenac toxicity in old world vultures is well described in the literature by both the severity of
16   the toxicity induced and the speed of death. While the mechanism of toxicity remains unknown
17   at present, the necropsy signs of gout suggests primary renal involvement at the level of the uric
18   acid excretory pathways. From information in the chicken and man, uric acid excretion is known
19   to be a complex process that involves a combination of glomerular filtration and active tubular
20   excretion. For the proximal convoluted tubules excretion occurs as a two-step process with the
21   basolateral cell membrane using the organic anion transporters and the apical membrane using
22   the multidrug resistant protein to transport uric acid from the blood into the tubular fluid. With
23   uric acid excretion seemingly inhibited by diclofenac, it becomes important to characterize these
24   transporter mechanism at the species level. With no information being available on the molecular
25   characterization/expression of MRPs of *Gyps africanus*, for this study we used next generation
26   sequencing, and Sanger sequencing on the renal tissue of African white backed vulture (AWB),
27   as the first step to establish if the MRPs gene are expressed in AWB. In silico analysis was
28   conducted using different software to ascertain the function of the latter genes. The sequencing
29   results revealed that the MRP2 and 4 are expressed in AWB vultures. Phylogeny of avian MRPs
30   genes confirms that vultures and eagles are closely related, which could be attributed to having
31   the same ancestral genes and foraging behavior. In silico analysis confirmed the transcribed
32   proteins would transports anionic compounds and glucose.

33

34

35

1. Introduction.

In India, birds belonging to the *Gyps* genus (Oriental white-backed vulture, *Gyps bengalensis*; long-billed vulture, *G. indicus* and slender-billed vulture, *G.tenuirostris*) were in grave danger of extinction in the 1990s following their inadvertent exposure to veterinary diclofenac that found its way into their food chain (Oaks et al., 2004). Following their single exposure to quantities of drug that equated to a dose in the region of 0.8 mg/kg, birds were found dead within 48 hours of said exposure with signs of severe visceral gout and associated renal and hepatic damage (Oaks et al., 2004, Green et al., 2004; Shultz et al., 2004; Swan et al., 2006). Despite the diclofenac induced deaths being largely recognized as one of the worst environment intoxications in recent times, the mechanism behind the evident gout is still incompletely understood. One of the theories put forward, suggests that toxicity is due to the inhibition of the uric acid transporter channels in the renal tubular epithelial cells (Naidoo et al., 2007), which would be consistent with diclofenac inhibition of uric acid transport in mammals (Khamdang et al., 2002; Nozaki et al., 2007; Burckhardt, 2012).

The Multidrug resistance proteins (MRPs) are part of the uric acid transporters that play an important role in the excretion of uric acid from the intracellular environment, following their movement therein by the organic anionic transporters in mammals (Sweet, 2005, 2010; Van wert et al., 2010). The MRPs family also known as ATP-binding cassette subfamily C (ABCC) was first described as a drug resistance protein by Cole et al. (1992) when they managed to clone the MRP1 gene in human (Cole et al., 1992). They first associated it with transporting anti-cancer drugs (Haimeur et al., 2004). MRP1 is generally regarded as the godfather of the family and further research has since described five homologs named MRP2-6 (Kool et al., 1997, 1998, 1999).

In general, the MRP protein functions as an extrusion pump system responsible for moving substances from within the cell into the extracellular environment e.g. in biliary transport it moves substances into the biliary tract and bile (Taniguchi et al., 1996; Kool et al., 1997), while MRP2 expressed in the proximal renal tubule endothelial cells found in apical membrane plays a role in the excretion of small intracellular organic anions (Sekine et al., 2006). Examples of substance transported by renal MRP2 are lipophilic substances conjugated to glutathione, glucuronate, or sulfate (König et al., 1999). The MRP transport proteins are distributed throughout the body with MRP3 and MRP4 found in the pancreas, bladder, gut, lung, prostate, ovary, muscle, testis, kidney and gallbladder respectively while MRP5 being ubiquitous (Kool et al., 1997, 1999; Lee et al., 1998; Kiuchi et al., 1998; KÖnig et al., 1999). Nonetheless, the main MRPs transporters are located in the liver and kidney.

With diclofenac having the ability to induce severe hyperuricemia and gout in the vultures (Naidoo and Swan, 2009), this study focuses on characterizing the last step in uric acid transport. More specifically, we focused on the molecular characterizing of the MRP2 and MRP4

74   transporter in the kidney of *Gyps africanus* (African white backed) vulture. In addition to being
75   known to transport organic anions including uric acid, it is believed that these channels function
76   in the same manner as in mammals. The rationale for this approach, it that once the channel has
77   been characterized, more specific expression and cloning studies can be undertaken on these
78   channels. While no such studies have been undertaken in an avian species, studies on cloned
79   MRP transporter proteins from humans have been successfully used to evaluate the effect of
80   specific non-steroidal anti-inflammatory drugs as inhibitors of these transporters (El-Sheikh et
81   al., 2007). Moreover we compare the results to the recent study by Barik et al. (2019) which
82   characterized MRP4 gene on *Gyps Himalyanesis* for comparison to results with obtained in this
83   study (Barik et al., 2019).

84

85   **2. Material and methods.**

86   **2.1. Sample collection**

87   Before the commencement of experiments, ethical clearance for collection of samples was
88   conducted according to the guidelines approved by University of Pretoria Animal Ethics
89   committee (V108-16). Two African white-backed (AWB) (*Gyps africanus*) vultures of unknown
90   age with a badly fractured wing and had not been treated with any analgesic previously were
91   euthanized at Faculty of Veterinary Science, University of Pretoria. The cranial lobe part of the
92   kidneys was harvested immediately after euthanasia and stored in cryogenic vials containing
93   RNAlater (Whitehead Scientific, Cape Town, South Africa) and stored at -80°C freezer until
94   analysed.

95   **2.2. Next generation sequencing (NGS)**

96   Total RNA was extracted from AWB1`s kidney using the RNeasy plus mini kit (Qiagen)
97   according to the manufacturer's instructions and transported to Agricultural Research Council
98   (ARC, Onderstepoort, Pretoria, South Africa) on dry ice for sequencing. DNA contamination
99   was removed from total RNA using DNase. The RNA fragments were reverse transcribed into
100   cDNA and sequencing adaptors were ligated. Finally, the ends of the cDNAs were sequenced
101   using Illuminia Truseq mRNA stranded Ran preparation kit on Hiseq 2500 v4
102   2x125bp chemistry model. The result obtained from sequencing was 50 million short reads
103   which were 125 nucleotides long each. Prior assembly of the raw reads, several quality control
104   steps were conducted on the raw reads using the online Galaxy platform (Blankenberg et al.,
105   2010) and fast quality check (FASTQC) (Andrews, 2014) to check overall quality of the
106   sequences, duplication and adapters. After checking the quality of the sequences, the adapters
107   and PCR duplicates were removed using the programme Trimmomatic (Bolger et al., 2014). The
108   pre-processed reads were assembled into transcripts using TRINITY (Grabherr et al., 2011). The
109   assembled transcriptome was converted to a local blast database NCBI (Altschul et al., 1990) and
110   sequences were identified based on the predicted MRP2 (XM_030031380.1) and MRP4
111   (XM_030036470.1) sequences from the Golden eagle genome. The Golden Eagle was selected

112   as another study completed in our laboratory has shown them to be closely related species as
113   well as having both channels present. Transcript quantification was undertaken using Sailfish
114   with reference transcripts from the de novo analysis. Alignment of AWB MRP4 and *Gyps*
115   *Himalyanesis* was also conducted using Clusta omega to reveal similarity.

116

117   **2.3. Confirmation of transcriptome MRP2 and MRP4 using Sanger sequencing.**

118   Fresh total RNA was extracted from AWB2`s kidney using Quick RNA Miniprep kit following
119   instructions from the manufacturer (Zymo Research, USA). cDNA synthesis was performed
120   using Lunascript RT supermix kit from New England Biolabs (NEB), (USA) according to the
121   manufacturer`s instructions. Primers of MRP2 and MRP4 were designed based on the
122   transcriptome sequences generated from illuminia next generation sequencing. The special
123   primers were synthesized at Inqaba Biotechnology, Pretoria, South Africa. Using the latter set of
124   primers, PCR was performed using One taq polymerase kit from NEB and AWB`s kidney cDNA
125   as a template. The amplification protocol for all the targets was as follows: Initial denature 94^0C
126   30 sec, 40 cycles of (denature 94^0C for 30sec; annealing 55^0C for 30sec and elongation 68^0C for
127   2min) and final elongation 68^0C 5min. However for MRP2 segment 1, the former did not work
128   so the following cycling conditions where used, Initial denature 98^0C 30 sec, 40 cycles of
129   (denature 98^0C for 30sec; annealing 65^0C for 30sec and elongation 72^0C for 2min) and final
130   elongation 72^0C 2min. The PCR products was run on 1% agarose gel. The gel was viewed for
131   desired bands using gel documentation system and PCR products of the desired bands was
132   further cleaned up using Exopsup from NEB and sequences were generated using ABI 3500X1
133   genetic analyser.

134

135   **2.4. Phylogenetic analyses**

136   After confirming the NGS data with Sanger sequencing, searches for homologous sequences
137   were performed using BLASTn (Altschul et al., 1990). Thirty-five (MRP2) and 36 (MRP4)
138   genes that were 85% and above identical to NGS sequences were downloaded from GenBank
139   (NCBI). A multiple sequence alignment was performed along with the downloaded sequences,
140   using muscle algorithm (Molecular Evolutionary Genetics Analysis version X (MEGA X))
141   software package (Edgar, 2004; Kumar et al., 2018). The evolutionary history was inferred by
142   using the Maximum Likelihood method and Tamura-Nei model for MRP2 (Tamura and Nei,
143   1993) while MRP4 was Maximum Likelihood method and General Time Reversible model (Nei
144   and Kumar, 2000). The bootstrap consensus tree inferred from 1000 replicates was taken to
145   represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches
146   corresponding to partitions reproduced in less than 50% bootstrap replicates were collapsed. The
147   percentage of replicate trees in which the associated taxa clustered together in the bootstrap test

148 (1000 replicates) are shown next to the branches (Felsenstein, 1985). Evolutionary analyses were
149 performed using MEGA X (Kumar et al., 2018).

150

151 **2.5. In silico analysis**

152 The obtained MRP2 and MRP4 NGS sequences were converted in Expasy (Gasteiger et al.,
153 2003) to protein sequences for their open reading frame. The deduced amino acids sequences
154 were analysed by following software; Transmembrane helix prediction using a Markov model
155 (Sonnhammer et al., 1998), Swiss model Protparam for functional prediction of the protein (De
156 Castro et al., 2006) and PHYRE2 (Kelly et al., 2015) for prediction of 2-D and 3-D structures.
157 PROTTER sequence database was used to predict N-glycosylation sites (Omasits et al., 2013)
158 and Expasy database was also used to examine other possible post-glycosylation and
159 phosphorylation. The Walker A, ABC signature motif and Walker B segments which are
160 hallmarks of human MRP 2 and 4 were evaluated for its presence (Hipfner et al., 1999;
161 Choudhuri and Klaassen 2006), to conclude that the proteins were functional MRP. This was
162 compared for both nucleotide binding domain 1&2 (Ren et al., 2004).

163

164 **3. Results.**

165 **3.1. Next generation sequencing.**

166 The assembled transcriptomes was compared to the Golden Eagle MRP2 (XM_030031380.1)
167 and MRP4 (XM_030036470.1) sequences. The predicted AWB MRP2 (MN691108) and MRP4
168 (MN691109) genes were submitted to NCBI and consisted of 5212 and 4061bp respectively. The
169 MRP2 and 4 alignment of the AWB vulture and the golden eagle were 98.20% and 99.16%
170 similarity respectively. Oddly the AWB and *Gyps Himalyanesis* (KX168697.1) alignment of
171 MRP4 gene revealed 94.06% similarity.

172

173 **3.2. Sanger sequencing of data.**

174 To confirm the above NGS results, Sanger sequencing was conducted. The cDNA from the
175 AWB kidney was used as template. MRP2 was amplified into two segments using primers
176 design based on NGS data. The PCR products were viewed on a gel under UV documentation
177 and it revealed product size of 2754bp and 2550bp for segment 1 and 2 respectively
178 (Supplementary Figure 1). Moreover amplification of MRP4 was also divided into two segments
179 and PCR products were 2241bp and 2136bp for segment 1 and 2 in that order. The PCR products
180 were sequenced and the sequences were analysed for consensus which revealed 4537bp for
181 MRP2 and 3897bp for MRP4. While full coverage was not possible, based on the degree of
182 coverage and similarity between NGS and Sanger of 99.76% and 99.43% for MRP2 and MRP4

183 respectively was observed. The similarity gave us confidence that the NGS sequences are a true
184 representative of the MRPs sequences.These sequences could not deposited in the NCBI as a
185 result of stop codons within the sequences, which we believe were sequencing errors.

186

187 **3.3. Phylogenetic analysis.**

188 The AWB predicted genes were further analysed to confirm its homogeneity compared to the
189 MRP gene of other avian species. The phylogenetic tree topology revealed 2 distinct clades for
190 the vulture and chicken with the golden eagle, bald eagle and AWB vulture belonging to the
191 same clade. The percentage similarity between vulture, eagles and chicken is stated on the
192 internal nodes numbers representing the percentage of 1000 replicates for which the same
193 branching patterns were attained. (Fig 1 and 2).

194

195

196

197 **3.4. In silico predictions.**

198 The predicted AWB MRP2 sequence generated open reading frame (ORF) containing 1552
199 amino acids while MRP4 ORF contained 1287 amino acids with a predicted molecular weight
200 173610.3 and 145113.90 Daltons respectively. Scan prosite results revealed that the above
201 proteins are likely ABC transporter integral membrane type 1 fused domain (ABC-TMF1) and
202 ABC transporter 2. Secondary structure prediction with PHYRE2 revealed that the predicted
203 MRP2 amino acids contain 62% of alpha-helix, 7% of beta- strand and 30% transmembrane
204 helix. While MRP4 PHYRE2 results showed the former to contain 61% alpha-helix, 9% of beta-
205 helix and 26 to be coiled in nature. TMHMM software analysis for MRP2 revealed the presence
206 of two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) consisting
207 of 6 and 5 transmembrane helices (TMH) respectively with an extra transmembrane domain
208 (TMD) with 5 TMH and for MRP4 it revealed their presence of two NBDs and two TMDs
209 containing 5 and 6 TMH each. PROTTER sequence database was used for prediction of N-
210 glycosylation sites for MRP2 and were found at position 15, 106, 284, 551, 620, 725, 773, 886,
211 1019, 1193, 1254, 1383, 1443 and MRP4 sites were at position 428, 706, 716, 1009, 1138 for
212 both AWB vulture and golden eagle moreover confirming the above results (Figure 3). The
213 quaternary structures predicted by PHYRE2 from MRPs amino acids revealed the presence of
214 two NBDs for both MRPs protein (Figure 3). The Walker sequence and ABC signature motif
215 are presented in Figure 4. For both MRP 2 and 4, these sequences were present with one amino
216 acid difference, when a difference was present, in comparison to human MRP2&4.

4. Discussion.

217

218 Previous studies revealed that the *Gyps* species found dead in India was due to the presence of
219 residues of diclofenac in the carcass they fed upon (Oaks et al., 2004), which caused signs of
220 abnormally high level of plasma uric acid prior to death (Naidoo and Swan 2009). With uric acid
221 excretion primarily occurring in the kidneys, it has been speculated that diclofenac inhibited uric
222 acid excretion within the renal tubules. The one shortcoming of this hypothesis is that the
223 mechanisms of uric acid excretion is not yet completely understood in vultures. Thus to
224 understand the mechanism of toxicity of diclofenac, the mechanism of uric acid excretion needs
225 to be elucidated.

226 From studies in the chicken, it is known that uric acid in the plasma is excreted by the renal
227 tubules by the organic anion transporters which transports uric acid from the plasma into the cell;
228 while subsequent transport from within cell into the renal tubule being mediated by the MRP
229 transporters (Sweet, 2005, 2010; Van wert et al., 2010). For this study we focused on the MRP2
230 and 4 transporters, since Haritova et al. (2010) and Bataille et al. (2008), were respectively able
231 to demonstrate the presence of MRP2 and MRP4 in the chicken kidney. More specifically, we
232 used the AWBV as our test species, which as a vulture species was the second species
233 demonstrated to be highly sensitive to the toxic effects of diclofenac, and more so under
234 controlled laboratory conditions by Swan et al (2006). In a later rebuttal, Cuthbert et al. (2007)
235 indicated that a sample size of two birds was sufficient to demonstrate toxicity due to the
236 reported LD_{50} in other vulture species. Also in a subsequent study, when the plasma diclofenac
237 plasma concentrations was subjected to non-compartmental analysis by Naidoo et al (2009), they
238 were able to demonstrate that toxicity in the study vulture species was associated with a very
239 long half-life of 11 and 22h in the two birds.

240 For this study both the study methods (NGS and Sanger sequencing) were able to demonstrate
241 that the MRP2 and MRP4 transporters were expressed in the AWB vulture`s kidney. With no
242 primers being described in the study species, that we were aware off, led to our first step of using
243 NGS. NGS as a tool is known to sequence hundreds to thousands of genes at one time without
244 the need for specific primers. The latter method provides the power to discover uncommon or
245 novel variants through deep sequencing (Alekseyev et al., 2018). In order to verify the results
246 obtained from NGS, Sanger sequencing was subsequently carried out since it is the golden
247 standard for sequencing (Sanger, 1975). As for Swan et al. (2006) we also relied on the use of
248 two AWBV. While the sample size is low, the high sensitivity of the species to toxicity suggests
249 a highly conserved mechanism of uric acid excretion. Also, due to the endangered nature of the
250 species under study, it is extremely difficult to obtain fresh tissue for analysis i.e. fresh tissue is
251 only obtainable when birds are euthanised for ethical reasons. This study also relied on *in silico*
252 analysis to predict the structure and functions of the proteins sequenced as a first step for cloning
253 and functional studies.

254 In silico analysis with Expasy revealed that the ORFs of MRP2 and MRP4 proteins consisted of
255 1552 and 1287 amino acids respectively for both the AWB vulture and golden eagle. This
256 finding is similar to human MRP2 and MRP4 proteins which comprises of 1545 and 1325 amino
257 acids respectively (Borst et al., 2000). Moreover a recent study by Barik et al., 2019 also

258 revealed that *Gyps Himalyanesis* MRP4 consisted of 1349 amino acids while Expasy analysis we
259 undertook for the *Gallus gallus* MRP2 (accession number XM_015288821.1) and MRP4
260 (accession number NM_001030819) consisted of 1550 and 1330 amino acids respectively. While
261 the similarity in the above protein sequences of MRP2 and MRP4 in size and length maybe
262 revealing that their structures are conserved, the phylogenetic tree only showed a 100%,
263 similarity between vulture and eagle for both MRP2 and MRP4 channels, while the chicken
264 belongs to separate clade. This was in accordance with previous findings by Zhang et al. (2014)
265 who explored macroevolution. In their evaluation using the full genomes of 48 avian species
266 representing all main extant clades, their phylogenetic tree revealed that vulture and eagles share
267 the same clade distinct from the chickens. Jarvis (2014) and his team showed that vultures and
268 eagles also belonged to the same family called accipitrimorphae (Jarvis et al., 2014). The
269 similarity of vulture and eagle is likely attributed to their foraging behaviour as eagles and
270 vultures share a carnivorous diet while chicken is more an omnivore. With uric acid production
271 related to dietary protein concentration and catabolism, it not also surprising that the eagles and
272 vulture would share similar transport mechanism as they would be exposed to the same level of
273 uric acid as an end product of purine metabolism.

274 In the first step in determining if the evaluated protein was effective, the protein homology
275 results from PHYRE2 were evaluated. For the protein homology, the programme evaluated the
276 similarity of residue probability distributions for each position, secondary structure similarity and
277 the presence or absence of insertions and deletions. For both MRP 2 and 4, the proteins were
278 57% and 39% similar to bovine MRP 1, with near 100% confidence, supporting the assertion
279 that the channel sequenced where MRP channels. PROTTER and TMHHM results confirmed the
280 two TMDs and NBDs for both proteins. Research on few high-resolution structures of ABC
281 transporters revealed that the former has two TMDs and two NBDs (also known as ABCs) which
282 aligns with our results (Hollenstein et al., 2007; Oldham et al., 2008; Rees et al., 2009). The
283 TMDs and NBDs are important for protein functionality, since MRPs are ATP-binding cassettes
284 (ABC) transporters that require the use of ATP to facilitate the movement of an extensive range
285 of substance within the cells (Linton and Higgins, 1998). In general for the transport, the TMDs
286 binds the transported substrates and NBDs provide the transport energy by binding and
287 hydrolyzing up to two molecules of ATP (Newstead et al., 2009; Khare et al., 2009; Oldham
288 et al., 2007; Ward et al., 2007). Nonetheless some differences have been recorded, with some
289 ABCs transporter that are not specific transporters with energy also being needed to reorient the
290 TMDs, which is also provided by the NBDs (Jardetzky, 1966; Smith et al., 2002).

291 Also important in the functionality of the MRP is the presence of the Walker A and B segments
292 which are the ATP binding segments of the NBDs. Comparison of the MRP with their respective
293 human sequence showed a high degree of similarity for both NBDs. With the single amino acid
294 difference, there appears to be no difference in the ATP binding sequence as the difference was
295 at a variable point as reported by Walker et al. (1982). This leads to the conclusion that the ATP
296 binding sites on the protein was functional. Also of importance is the ABC signature motif. The
297 motif was minimally different to that of the human reported sequence as for the Walker
298 segments. This is an important feature as studies have shown that a mutation in this region
299 renders the transporter unable to function. TMHMM and PROTTER predicted the

300 transmembrane helices and the N-glycosylation sites of the latter proteins with MRP2 having 13
301 and MRP4 having 5 N-glycosylation sites. The importance of glycosylation for the MRP has
302 been evaluated in other studies. It has been demonstrated that glycosylation is important for the
303 translocation of the MRP from the endoplasmic reticulum to the cell membrane (Zhang et al.,
304 2005). In the absence of glycosylation, the transporter remains inactive. Based on this result, we
305 are confident that the evaluated sequence would result in the expression of a viable drug efflux
306 transporter.

307 The presence of MRP2 and 4 in the vulture supports the presence of the described uric acid
308 pathways as for other species which provides the first step for the further evaluation of the
309 mechanism of toxicity. The importance of the channels in toxicity is evident from the study by
310 Konig (1999, 2003) who showed that rat mutants and humans lacking MRP2 gene had
311 compromised excretion of organic anions from the liver leading to mild liver disease and
312 inherited jaundice (Konig et al., 1999; 2003). This finding was supported by an MRP4
313 functionality study, where Bakos et al., 1997 and later Ren et al., 2004 demonstrated that the
314 replacement of a highly conserved glycine in NBD1 with an aspartate in vector-transfected cells
315 resulted in intracellular accumulation of the evaluated drugs, confirming their role as efflux
316 transporters. The importance of MRP4 channel was further highlighted in Abcc4−/− mice study,
317 where the absence of the transporter played a role in acute 9-(2-phosphonylmethoxyethyl)
318 adenine (PMEA) toxicity, suggesting a protective role for MRP4 more in the bone marrow,
319 gastrointestinal tract, thymus and spleen (Abla et al., 2008). A similar study on humans
320 comparing two antiviral agents, adefovir (PMEA) and azidothymidine (AZT), by measuring the
321 intracellular accumulation, showed a significantly higher intracellular accumulation of PMEA
322 and AZT in cells without the functional allele in comparison to the controls since these cells
323 were unable to extrude the mentioned drugs (Alba et al., 2008). In addition to the absence of
324 proper expression playing a role in the intracellular accumulation of excretory substrates, the
325 functioning of the MRP2 and MRP4 may be altered by the NSAIDs such as diclofenac. This is
326 best seen by the excretion of prostaglandins by the kidney being inhibited by probenecid,
327 indomethacin, and PAH which all interact with MRP4 (Adachi et al., 2002; Reid et al., 2003).
328 Also in a study by El-Sheikh et al., (2007), the excretion of methotrexate by the MRP 2 and 4
329 was inhibited by numerous NSAIDs including diclofenac, ketoprofen and phenylbutazone all
330 known to be toxic in vultures.

331

332 **5.5. Conclusion**

333 For this study we show the presence of MRP2 and MRP4 in AWB vulture`s kidney, supporting
334 the presence of the expected cellular pathways in uric acid excretion. Phylogenetic analysis also
335 confirmed that vulture and eagle are on the same clade in contrast to chicken moreover inslico
336 revealed two nucleotide binding domains (NBDs) and two transmembrane domains (TMDs) for
337 the latter channels with an extra transmembrane domain (TMD) for MRP4. The former confirm
338 that the transporters function the same as other species. Based on this results, further studies can
339 focus on clonal expression of these transporters to ascertain if they are inhibited by the NSAIDs
340 and thereby explain the hyperuricemia associated with diclofenac toxicity in vultures.

341 **5.7. References**

342 Abla, N., Chinn, L.W., Nakamura, T., Liu, L., Huang, C.C., Johns, S.J., Kawamoto, M., Stryke,
343 D., Taylor, T.R., Ferrin, T.E. and Giacomini, K.M., 2008. The human multidrug resistance
344 protein 4 (MRP4, ABCC4): functional analysis of a highly polymorphic gene. *Journal of*
345 *Pharmacology and Experimental Therapeutics*, *325*(3), pp.859-868.

346 Adachi, M., Sampath, J., Lan, L.B., Sun, D., Hargrove, P., Flatley, R., Tatum, A., Edwards,
347 M.Z., Wezeman, M., Matherly, L. and Drake, R., 2002. Expression of MRP4 confers resistance
348 to ganciclovir and compromises bystander cell killing. *Journal of biological chemistry*, *277*(41),
349 pp.38998-39004.

350 Alekseyev, Y.O., Fazeli, R., Yang, S., Basran, R., Maher, T., Miller, N.S. and Remick, D., 2018.
351 A next-generation sequencing primer—how does it work and what can it do?. *Academic*
352 *pathology*, *5*, p.2374289518766521.

353 Altschul, S.F., Gish, W., Miller, W., Myers, E.W. and Lipman, D.J., 1990. Basic local alignment
354 search tool. Journal of molecular biology, 215(3), pp.403-410.

355 Andrews, S., 2014. FastQC: a quality control tool for high throughput sequence data. Version
356 0.11. 2. Babraham Institute, Cambridge, UK http://www. bioinformatics. babraham. ac.
357 uk/projects/fastqc.

358 Bakos, É., Klein, I., Welker, E., Szabó, K., Müller, M., Sarkadi, B. and Váradi, A., 1997.
359 Characterization of the human multidrug resistance protein containing mutations in the ATP-
360 binding cassette signature region. Biochemical Journal, 323(3), pp.777-783.

361 Barik, S., Saini, M., Rana, M. and Gupta, P.K., 2019. Multidrug resistance protein 4 (MRP4) is
362 expressed as transcript variants in both Gallus domesticus and Gyps himalyanesis. Gene, 689,
363 pp.172-182.

364 Bataille, A.M., Goldmeyer, J. and Renfro, J.L., 2008. Avian renal proximal tubule epithelium
365 urate secretion is mediated by Mrp4. American Journal of Physiology-Regulatory, Integrative
366 and Comparative Physiology, 295(6), pp.R2024-R2033.

367 Blankenberg, D., Gordon, A., Von Kuster, G., Coraor, N., Taylor, J., Nekrutenko, A. and Galaxy
368 Team, 2010. Manipulation of FASTQ data with Galaxy. Bioinformatics, 26(14), pp.1783-1785.

369 Bolger, A.M., Lohse, M. and Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina
370 sequence data. Bioinformatics, 30(15), pp.2114-2120.

371 Borst, P., Evers, R., Kool, M. and Wijnholds, J., 2000. A family of drug transporters: the
372 multidrug resistance-associated proteins. Journal of the National Cancer Institute, 92(16),
373 pp.1295-1302.

374 Burckhardt, G., 2012. Drug transport by organic anion transporters (OATs). Pharmacology &
375 therapeutics, 136(1), pp.106-130.

376   Choudhuri, S. and Klaassen, C.D., 2006. Structure, function, expression, genomic organization,
377   and single nucleotide polymorphisms of human ABCB1 (MDR1), ABCC (MRP), and ABCG2
378   (BCRP) efflux transporters. International journal of toxicology, 25(4), pp.231-259.

379   Cole, S.P.C., Bhardwaj, G., Gerlach, J.H., Mackie, J.E., Grant, C.E., Almquist, K.C., Stewart,
380   A.J., Kurz, E.U., Duncan, A. and Deeley, R.G., 1992. Overexpression of a transporter gene in a
381   multidrug-resistant human lung cancer cell line. Science, 258(5088), pp.1650-1654.

382   Cuthbert, R., Pain, D.J., Green, R.E., Swan, G. and Swarup, D., 2007. Comparative toxicity
383   studies of NSAIDs in birds: A criticism of Reddy et al. *Anim. Conserv, 9*, pp.349-354.

384   De Castro, E., Sigrist, C.J., Gattiker, A., Bulliard, V., Langendijk-Genevaux, P.S., Gasteiger, E.,
385   Bairoch, A. and Hulo, N., 2006. ScanProsite: detection of PROSITE signature matches and
386   ProRule-associated functional and structural residues in proteins. Nucleic acids research,
387   34(suppl_2), pp. W362-W365.

388   Edgar, R.C., 2004. MUSCLE: multiple sequence alignment with high accuracy and high
389   throughput. Nucleic acids research, 32(5), pp.1792-1797.

390   El-Sheikh, A.A., Van Den Heuvel, J.J., Koenderink, J.B. and Russel, F.G., 2007. Interaction of
391   nonsteroidal anti-inflammatory drugs with multidrug resistance protein (MRP) 2/ABCC2-and
392   MRP4/ABCC4-mediated methotrexate transport. Journal of Pharmacology and Experimental
393   Therapeutics, 320(1), pp.229-235.

394   Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap.
395   Evolution, 39(4), pp.783-791.

396   Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R.D. and Bairoch, A., 2003.
397   ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic acids
398   research, 31(13), pp.3784-3788.

399   Grabherr, M.G., Haas, B.J., Yassour, M., Levin, J.Z., Thompson, D.A., Amit, I., Adiconis, X.,
400   Fan, L., Raychowdhury, R., Zeng, Q. and Chen, Z., 2011. Full-length transcriptome assembly
401   from RNA-Seq data without a reference genome. Nature biotechnology, 29(7), p.644.

402   Green, R.E., Newton, I.A.N., Shultz, S., Cunningham, A.A., Gilbert, M., Pain, D.J. and Prakash,
403   V., 2004. Diclofenac poisoning as a cause of vulture population declines across the Indian
404   subcontinent. Journal of Applied ecology, 41(5), pp.793-800.

405   Haimeur, A.G.R.G.S.P., Conseil, G., Deeley, R.G. and Cole, S.P., 2004. The MRP-related and
406   BCRP/ABCG2 multidrug resistance proteins: biology, substrate specificity and regulation.
407   Current drug metabolism, 5(1), pp.21-53.

408   Haritova, A.M., Schrickx, J. and Fink-Gremmels, J., 2010. Expression of drug efflux transporters
409   in poultry tissues. Research in veterinary science, 89(1), pp.104-107.

410   Hipfner, D.R., Deeley, R.G. and Cole, S.P., 1999. Structural, mechanistic and clinical aspects of
411   MRP1. *Biochimica et Biophysica Acta (BBA)-Biomembranes, 1461*(2), pp.359-376.

412 Hollenstein, K., Dawson, R.J. and Locher, K.P., 2007. Structure and mechanism of ABC
413 transporter proteins. Current opinion in structural biology, 17(4), pp.412-418.

414 Jardetzky, O., 1966. Simple allosteric model for membrane pumps. Nature, 211(5052), p.969.

415 Jarvis, E.D., Mirarab, S., Aberer, A.J., Li, B., Houde, P., Li, C., Ho, S.Y., Faircloth, B.C.,
416 Nabholz, B., Howard, J.T. and Suh, A., 2014. Whole-genome analyses resolve early branches in
417 the tree of life of modern birds. Science, 346(6215), pp.1320-1331.

418 Kelley, L.A., Mezulis, S., Yates, C.M., Wass, M.N. and Sternberg, M.J., 2015. The Phyre2 web
419 portal for protein modeling, prediction and analysis. Nature protocols, 10(6), p.845.

420 Khamdang, S., Takeda, M., Noshiro, R., Narikawa, S., Enomoto, A., Anzai, N., Piyachaturawat,
421 P. and Endou, H., 2002. Interactions of human organic anion transporters and human organic
422 cation transporters with nonsteroidal anti-inflammatory drugs. Journal of Pharmacology and
423 Experimental Therapeutics, 303(2), pp.534-539.

424 Khare, D., Oldham, M.L., Orelle, C., Davidson, A.L. and Chen, J., 2009. Alternating access in
425 maltose transporter mediated by rigid-body rotations. Molecular cell, 33(4), pp.528-536.

426 Kiuchi, Y., Suzuki, H., Hirohashi, T., Tyson, C.A. and Sugiyama, Y., 1998. cDNA cloning and
427 inducible expression of human multidrug resistance associated protein 3 (MRP3) 1. FEBS letters,
428 433(1-2), pp.149-152.

429 König, J., Nies, A.T., Cui, Y., Leier, I. and Keppler, D., 1999. Conjugate export pumps of the
430 multidrug resistance protein (MRP) family: localization, substrate specificity, and MRP2-
431 mediated drug resistance. Biochimica et Biophysica Acta (BBA)-Biomembranes, 1461(2),
432 pp.377-394.

433 König, J.Ö.R.G., Nies, A.T., Cui, Y.U.N.H.A.I. and Keppler, D.I.E.T.R.I.C.H., 2003. MRP2, the
434 apical export pump for anionic conjugates (pp. 423-443). Academic Press, London.

435 Kool, M., de Haas, M., Scheffer, G.L., Scheper, R.J., van Eijk, M.J., Juijn, J.A., Baas, F. and
436 Borst, P., 1997. Analysis of expression of cMOAT (MRP2), MRP3, MRP4, and MRP5,
437 homologues of the multidrug resistance-associated protein gene (MRP1), in human cancer cell
438 lines. Cancer research, 57(16), pp.3537-3547.

439 Kool, M., Van Der Linden, M., De Haas, M., Baas, F. and Borst, P., 1999. Expression of human
440 MRP6, a homologue of the multidrug resistance protein gene MRP1, in tissues and cancer cells.
441 Cancer Research, 59(1), pp.175-182.

442 Kool, M., Wijnholds, J., Evers, R., Holló, Z., Scheffer, G.L., de Haas, M., van der Linden, M.,
443 Mol, C.A.A.M., van Deemter, L., Scheper, R.J. ., Baas, F., Borst, P., 1998. Proc. AACR 39 167
444 (Abstract).

445 Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K., 2018. MEGA X: molecular
446 evolutionary genetics analysis across computing platforms. *Molecular biology and*
447 *evolution*, *35*(6), pp.1547-1549.

448   Lee, K., Belinsky, M.G., Bell, D.W., Testa, J.R. and Kruh, G.D., 1998. Isolation of MOAT-B, a
449   widely expressed multidrug resistance-associated protein/canalicular multispecific organic anion
450   transporter-related transporter. Cancer research, 58(13), pp.2741-2747.

451   Linton, K.J. and Higgins, C.F., 1998. The Escherichia coli ATP-binding cassette (ABC) proteins.
452   Molecular microbiology, 28(1), pp.5-13.

453   Naidoo, V. and Swan, G.E., 2009. Diclofenac toxicity in Gyps vulture is associated with
454   decreased uric acid excretion and not renal portal vasoconstriction. Comparative Biochemistry
455   and Physiology Part C: Toxicology & Pharmacology, 149(3), pp.269-274.

456   Naidoo, V., Duncan, N., Bekker, L. and Swan, G., 2007. Validating the domestic fowl as a
457   model to investigate the pathophysiology of diclofenac in Gyps vultures. Environmental
458   toxicology and pharmacology, 24(3), pp.260-266.

459   Naidoo, V., Wolter, K., Cuthbert, R. and Duncan, N., 2009. Veterinary diclofenac threatens
460   Africa's endangered vulture species. Regulatory toxicology and pharmacology, *53*(3), pp.205-
461   208

462   Nei, M. and Kumar, S., 2000. Molecular evolution and phylogenetics. Oxford university press.

463   Newstead, S., Fowler, P.W., Bilton, P., Carpenter, E.P., Sadler, P.J., Campopiano, D.J., Sansom,
464   M.S. and Iwata, S., 2009. Insights into how nucleotide-binding domains power ABC transport.
465   Structure, 17(9), pp.1213-1222.

466   Nozaki, Y., Kusuhara, H., Kondo, T., Iwaki, M., Shiroyanagi, Y., Nakayama, H., Horita, S.,
467   Nakazawa, H., Okano, T. and Sugiyama, Y., 2007. Species difference in the inhibitory effect of
468   nonsteroidal anti-inflammatory drugs on the uptake of methotrexate by human kidney slices.
469   Journal of Pharmacology and Experimental Therapeutics, 322(3), pp.1162-1170.

470   Oaks, J.L., Gilbert, M., Virani, M.Z., Watson, R.T., Meteyer, C.U., Rideout, B.A., Shivaprasad,
471   H.L., Ahmed, S., Chaudhry, M.J.I., Arshad, M. and Mahmood, S., 2004. Diclofenac residues as
472   the cause of vulture population decline in Pakistan. Nature, 427(6975), p.630.

473   Oldham, M.L., Davidson, A.L. and Chen, J., 2008. Structural insights into ABC transporter
474   mechanism. Current opinion in structural biology, 18(6), pp.726-733.

475   Oldham, M.L., Khare, D., Quiocho, F.A., Davidson, A.L. and Chen, J., 2007. Crystal structure of
476   a catalytic intermediate of the maltose transporter. Nature, 450(7169), p.515.

477   Omasits, U., Ahrens, C.H., Müller, S. and Wollscheid, B., 2013. Protter: interactive protein
478   feature visualization and integration with experimental proteomic data. Bioinformatics, 30(6),
479   pp.884-886.

480   Rees, D.C., Johnson, E. and Lewinson, O., 2009. ABC transporters: the power to change. Nature
481   reviews Molecular cell biology, 10(3), p.218.

482   Reid, G., Wielinga, P., Zelcer, N., van der Heijden, I., Kuil, A., de Haas, M., Wijnholds, J. and
483   Borst, P., 2003. The human multidrug resistance protein MRP4 functions as a prostaglandin

484   efflux transporter and is inhibited by nonsteroidal antiinflammatory drugs. *Proceedings of the*
485   *National Academy of Sciences*, *100*(16), pp.9244-9249.

486   Ren, X.Q., Furukawa, T., Haraguchi, M., Sumizawa, T., Aoki, S., Kobayashi, M. and Akiyama,
487   S.I., 2004. Function of the ABC signature sequences in the human multidrug resistance protein 1.
488   Molecular pharmacology, 65(6), pp.1536-1542.

489   Sanger, F. and Coulson, A.R., 1975. A rapid method for determining sequences in DNA by
490   primed synthesis with DNA polymerase. *Journal of molecular biology*, *94*(3), pp.441-448.

491   Sekine, T., Miyazaki, H. and Endou, H., 2006. Molecular physiology of renal organic anion
492   transporters. American Journal of Physiology-Renal Physiology, 290(2), pp.F251-F261.

493   Shultz, S., Baral, H.S., Charman, S., Cunningham, A.A., Das, D., Ghalsasi, G.R., Goudar, M.S.,
494   Green, R.E., Jones, A., Nighot, P. and Pain, D.J., 2004. Diclofenac poisoning is widespread in
495   declining vulture populations across the Indian subcontinent. Proceedings of the Royal Society
496   of London. Series B: Biological Sciences, 271(suppl_6), pp.S458-S460.

497   Smith, P.C., Karpowich, N., Millen, L., Moody, J.E., Rosen, J., Thomas, P.J. and Hunt, J.F.,
498   2002. ATP binding to the motor domain from an ABC transporter drives formation of a
499   nucleotide sandwich dimer. Molecular cell, 10(1), pp.139-149.

500   Sonnhammer, E.L., Von Heijne, G. and Krogh, A., 1998, July. A hidden Markov model for
501   predicting transmembrane helices in protein sequences. In Ismb (Vol. 6, pp. 175-182).

502   Swan, G.E., Cuthbert, R., Quevedo, M., Green, R.E., Pain, D.J., Bartels, P., Cunningham, A.A.,
503   Duncan, N., Meharg, A.A., Lindsay Oaks, J. and Parry-Jones, J., 2006. Toxicity of diclofenac to
504   Gyps vultures. Biology letters, 2(2), pp.279-282.

505   Sweet, D.H., 2005. Organic anion transporter (Slc22a) family members as mediators of toxicity.
506   Toxicology and applied pharmacology, 204(3), pp.198-215.

507   Sweet, D.H., 2010. Renal organic cation and anion transport: From physiology to genes.

508   Tamura, K. and Nei, M., 1993. Estimation of the number of nucleotide substitutions in the
509   control region of mitochondrial DNA in humans and chimpanzees. Molecular biology and
510   evolution, 10(3), pp.512-526.

511   Taniguchi, K., Wada, M., Kohno, K., Nakamura, T., Kawabe, T., Kawakami, M., Kagotani, K.,
512   Okumura, K., Akiyama, S.I. and Kuwano, M., 1996. A human canalicular multispecific organic
513   anion transporter (cMOAT) gene is overexpressed in cisplatin-resistant human cancer cell lines
514   with decreased drug accumulation. Cancer Research, 56(18), pp.4124-4129.

515   Van Wert, A.L., Gionfriddo, M.R. and Sweet, D.H., 2010. Organic anion transporters: discovery,
516   pharmacology, regulation and roles in pathophysiology. Biopharmaceutics & drug disposition,
517   31(1), pp.1-71.

518 Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J., 1982. Distantly related sequences in the
519 alpha-and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and
520 a common nucleotide binding fold. *The EMBO journal*, *1*(8), pp.945-951.

521 Ward, A., Reyes, C.L., Yu, J., Roth, C.B. and Chang, G., 2007. Flexibility in the ABC
522 transporter MsbA: Alternating access with a twist. Proceedings of the National Academy of
523 Sciences, 104(48), pp.19005-19010.

524 Zhang, G., Li, C., Li, Q., Li, B., Larkin, D.M., Lee, C., Storz, J.F., Antunes, A., Greenwold,
525 M.J., Meredith, R.W. and Ödeen, A., 2014. Comparative genomics reveals insights into avian
526 genome evolution and adaptation. Science, 346(6215), pp.1311-1320.

527 Zhang, P., Tian, X., Chandra, P. and Brouwer, K.L., 2005. Role of glycosylation in trafficking of
528 Mrp2 in sandwich-cultured rat hepatocytes. *Molecular pharmacology*, *67*(4), pp.1334-1341.

529

530

Figure 1

MRP2 phylogenetic tree

Reconstruction of phylogenetic relationship between MRP2 genes of different avian species by maximum likelihood best model: bootstrap.

Figure 2

MRP4 phylogenetic tree

Reconstruction of phylogenetic relationship between MRP4 genes of different avian species by maximum likelihood best model: bootstrap.

White-throated tinamou

Southern ostrich

North island brown kiwi

Mallard

Domestic geese

Helmeted guinea fowl

Turkey

Chicken

Himalayan vulture

Barn owl

Speckled mousebird

Chimney swift

Peregrine falcon

Saker falcon

Red-legged seriema

Great tit

Common starling

American crow

Golden-collared manakin

Rock pigeon

Common cuckoo

Ruff

Killdeer

Bar-tailed trogon

Sun bittern

Little egret

Kea

Budgerigar

Chuck-wills-widow

Stinkbird

Adlie penguin

Dalmatian pelican

Asian crested ibis

Bald eagle

XM 011586Golden Eagle

Predicted Vulture MRP4

0.020

100

78

100

100

100

100

84

74

100

26

100

99

99

100

99

75

74

100

28

48

30

28

83

100

Figure 3

Presentation of transmembrane helices with N-gyclan motif sites and 3-D structures.

Pictorial presentation of transmembrane helices and N-gyclan motif sites from A) MRP2 containing 13 N-glycan motif sites with 16 TMH and B) MRP4 comprising of 5 N-glycan motif sites with 11 TMH. Also shown are the corresponding 3-D structures of MRP2 and MRP4, illustrated by the PHYRE2 software, showing the presence of two NDBs.

Pane A

MRP2

NDB I

NDB II

Pane B

MRP4

NDB II

NDB I
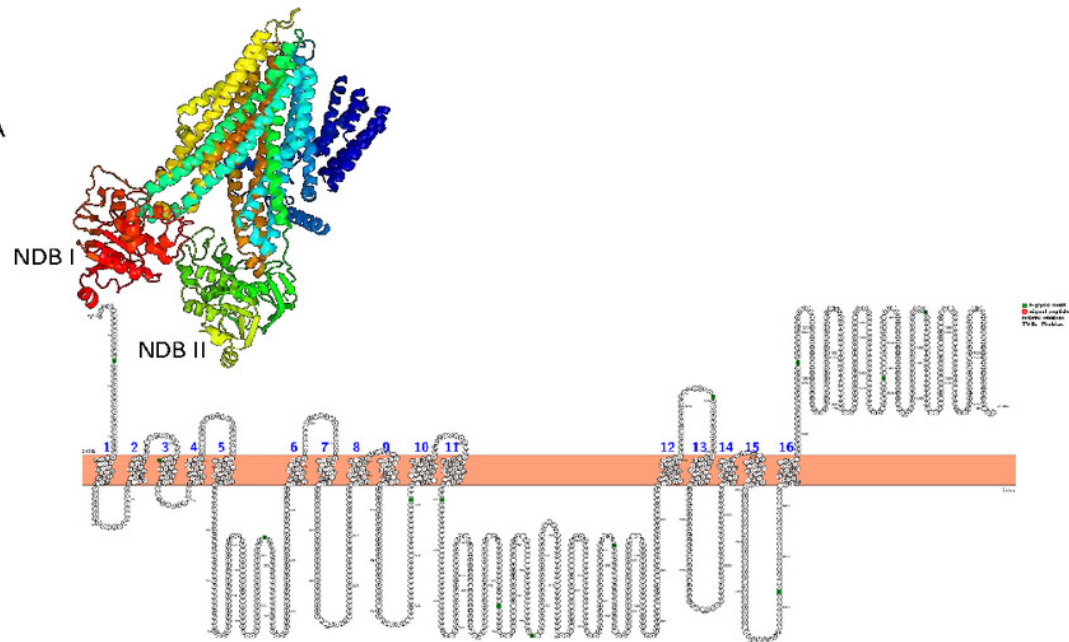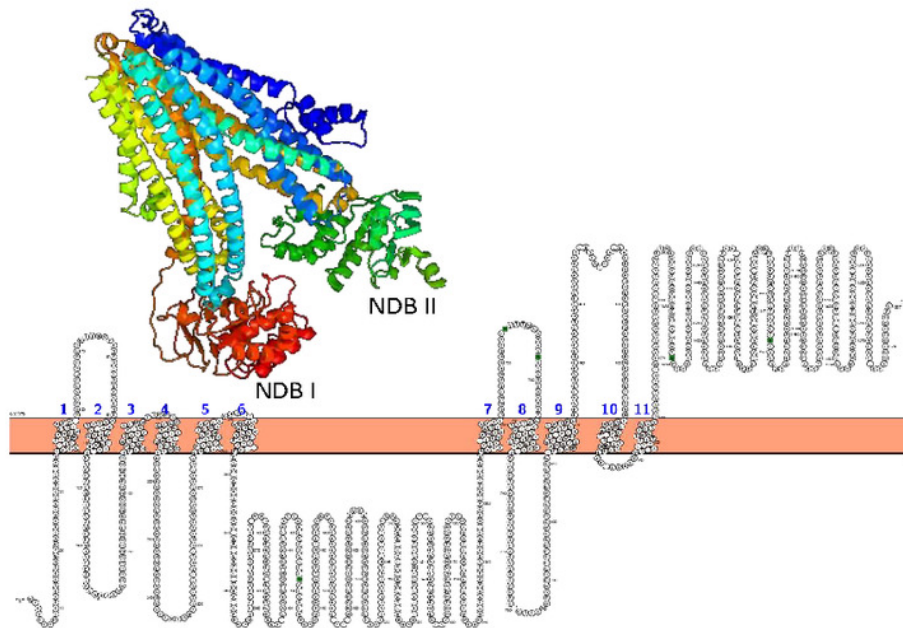
Figure 4

Walker sequence and ABC signature motif

comparison of the Walker A, ABC Signature motif and Walker B sequence of the first
nucleotide binding domain of the vulture MRP2 and MRP4 in comparison on human MRP (The
protein sequences used with their GenBank accession numbers were MRP2-NP_000383.2,
MRP3-AAD02845.1,MRP4NP_005836.2, MRP5-NP_005679.2

Nucleotide Binding Domain 1 (NBD1)

```
             1                                                          60
hMRP2    AGQLVAVI GPVGSGKSS LISAMLGEMENVHGHITIKGTTAYVPQQSWIQNGTIKDNILFG    722
hMRP3    KGALVAVV GPVGCGKSS LVSALLGEMEKLEGKVHMKGSVAYVPQQAWIQNCTLQENVLFG    712
hMRP4    PGELLAVV GPVGAGKSS LLSAVLGELAPSHGLVSVHGRIAYVSQQPWVFSGTLRSNILFG    494
hMRP5    EGKLVGIG GSVGSGKTS LISAILGQMTLLEGSIAISGTFAYVAQQAWILNATLRDNILFG    646
vMRP2    PGSLVAVV GAVGSGKSS LVSAMLGEMENIKGHINIQGSLAYVPQQAWIQNATLKDNILFG    735
vMRP4    RGELLAVI GPVGAGKSS LLSAVLGELPKDKGLINVTGRIAYVSQQPWVFSGTVRSNILFD    454
                     Walker A
```

```
hMRP2    TEFNEKRYQQVLEACALLPDLEMLPGGDLAEIGEKGIN LSGGQKQRISLARA TYQNLDIY    782
hMRP3    KALNPKRYQQTLEACALLADLEMLPGGDQTEIGEKGIN LSGGQRQRVSLARA VYSDADIF    772
hMRP4    KKYEKERYEKVIKACALKKDLQLLEDGDLTVIGDRGTT LSGGQKARVNLARA VYQDADIY    554
hMRP5    KEYDEERYNSVLNSCCLRPDLAILPSSDLTEIGERGAN LSGGQRQRISLARA LYSDRSIY    706
vMRP2    SELDEARYQQVIKACALLPDLELLPAGDQTEIGEKGIN LSGGQKQRVSLARA VYSNADIY    795
vMRP4    KEYEKEKYEKVLKVCALKKDLELLANGDLTVIGDRGAT LSGGQKARVNLARA VYQDADIY    514
                                                 Signature Motif
```

```
hMRP2    LLDDPLSAVD AHVGKHIFNKVLGPNGLLKGTRLLVTHSMHFLPQVDEIVVLGNGTIVEK    842
hMRP3    LLDDPLSAVD SHVAKHIFDHVIGPEGVLAGKTRVLVTHGISFLPQTDFIIVLADGQVSEM    832
hMRP4    LLDDPLSAVD AEVSRHLFELCIC--QILHEKITILVTHQLQYLKAASQILILKDGKMVQK    614
hMRP5    ILDDPLSALD AHVGNHIFNSAIR--KHLKSKTVLFVTHQLQYLVDCDEVIFMKEGCITER    764
vMRP2    ILDDPLSAVD AHVGKYLFEHVLGPKGLLQKKTRILVTHSISFLPQVDNIVVLVAGTVSEH    855
vMRP4    LLDDPLSAVD AEVGRHLFEKCIC--QALHQKISVLVTHQLQYLRAANQILILKDGKMVGK    572
          Walker B
```

Nucleotide Binding Domain 2 (NBD2)

```
hMRP2    FNNYQVRYRPELDLVLRGITCDIGSMEKIGVVGRT GAGKSSLTI CLFRILEAAGGQIIID    1361
hMRP3    FRNYSVRYRPGLDLVLRDLSLHVHGGEKVGIVGRT GAGKSSMTL CLFRILEAAKGEIRID    1350
hMRP4    FDNVNFMYSPGGPLVLKHLTALIKSQEKVGIVGRT GAGKSSLIS ALFRLSEP-EGKIWID    1101
hMRP5    FENAEMRYRENLPLVLKKVSFTIKPKEKIGIVGRT GSGKSSLGI ALFRLVELSGGCIKID    1254
vMRP2    FVDYKVRYRPELELVLQGITCNIGSTEKVGVVGRT GAGKSSLTI CLFRVLEAAGGTIIID    1368
vMRP4    FENVNFTYSLDGPLVLRHLSVLIKPKEKVGIVGRT GAGKSSLIA ALFRLAEP-EGRIWID    1063
                                          Walker A
```

```
hMRP2    LQLGLSHEVTEAGGN LSIGQRQLLCLGR ALLRKSKII VLDEATA AVDLETDNLIQTTIQN    1481
hMRP3    QPAGLDFQCSEGGEN LSVGQRQLVCLAR ALLRKSRII VLDEATA AIDLETDNLIQATIRT    1470
hMRP4    LPGKMDTELAESGSN FSVGQRQLVCLAR AILRKNQIL IIDEATA NVDPRTDELIQKKIRE    1221
hMRP5    LPLKLESEVMENGDN FSVGERQLLCIAR ALLRHCKIL ILDEATA AMDTETDLLIQETIRE    1374
vMRP2    LPEGLLHLVSEAGEN LSVGQRQLVCLAR ALLRKAKII ILDEATA AVDLETDHLIQTTIRS    1488
vMRP4    LPNKMEMQLAESGSN FSVGQRQLVCLAR AVLKKNRIL IIDEATA NVDPRTDEFIQKTIRE    1183
                     Signature Motif           Walker B
```