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1 Invited Review

2 **Vaccination against babesiosis using recombinant GPI-anchored proteins**

3

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15

16 Abstract

17 The increase in human babesiosis is of major concern to health authorities. In the USA,
18 most of these cases are due to infections with *Babesia microti*, whereas in Europe *B.*
19 *divergens* is the major cause of clinical disease in humans. Here we review the
20 immunological and biological literature of glycosylphosphatidylinositol (GPI)-anchored
21 merozoite proteins of human *Babesia* parasites with emphasis on their role in immunity,
22 and provide some new bioinformatical information on *B. microti* GPI-Anchored Proteins
23 (GPI-AP). Cattle can be vaccinated with soluble parasite antigens (SPA) of *Babesia*
24 *divergens* that are released by the parasite during proliferation. The major component in
25 SPA preparations appeared to be a 37 kDa merozoite surface protein that is anchored in the
26 merozoite membrane by a GPI anchor. Animals could be protected by vaccination with the
27 recombinant 37 kDa protein expressed in *Escherichia coli*, provided the protein had a
28 hydrophobic terminal sequence. Based on this knowledge, a recombinant vaccine was
29 developed against *Babesia canis* infection in dogs, successfully. In order to identify similar
30 GPI-anchored proteins in *B. microti*, the genome was analysed. Here it is shown that *B.*
31 *microti* encodes all proteins necessary for GPI assembly and its subsequent protein transfer.
32 In addition, in total 21 genes encoding for GPI-anchored proteins were detected, some of
33 which reacted particularly strongly with sera from *B. microti*-infected human patients.
34 Reactivity of antibodies with GPI-anchored merozoite proteins appears to be dependent on
35 the structural conformation of the molecule. It is suggested that the three-dimensional
36 structure of the protein that is anchored in the membrane is different from that of the
37 protein that has been shed from the merozoite surface. The significance of this protein's
38 dynamics in parasite biology and immune evasion is discussed. Finally, we discuss

39 developments in tick and *Babesia* vaccine research, and the role such vaccines could play
40 in the control of human babesiosis.

41

42 *Keywords: Babesia microti; Babesia divergens; Recombinant vaccine; GPI anchors;*

43 *Apicomplexa; Human babesiosis; Merozoite surface protein*

44

45

46 **1. Introduction**

47 The incidence of clinical babesiosis in humans is increasing, which is of concern to
48 health authorities (Vannier et al., 2015). A growing number of *Babesia* spp. has been
49 associated with human infections, the majority of which are due to *Babesia microti* in the
50 United States (USA) and *Babesia divergens* in Europe. Importantly, it has been shown that
51 animals can be immunised against *B. divergens* infection using a vaccine based on the
52 recombinant *B. divergens* antigen Bd37 (Delbecq et al., 2006). This antigen is a merozoite
53 surface protein that is anchored to the membrane by a specific structure called
54 glycosylphosphatidylinositol (GPI) anchor. It was later shown that a homologous GPI-
55 anchored protein (GPI-AP) of *Babesia canis* successfully protected dogs against virulent
56 challenge infection (Moubri et al., 2018). This holds promise for the development of
57 vaccines against human babesiosis. Here we review the immunological and biological
58 literature of GPI-anchored merozoite proteins of human *Babesia* parasites with emphasis on
59 their role in immunity, and provide some new bioinformatical information on *B. microti*
60 GPI-AP. In addition, we discuss developments in tick and *Babesia* vaccine research, and
61 the role such vaccines could play in the control of human babesiosis.

62

63 **2. Parasite antigens released during infection**

64 Natural infection with *Babesia* parasites follows transmission by a tick bite. In order
65 to feed successfully, ticks have developed a range of responses to counteract coagulation,
66 inflammation and the onset of immunity (reviewed by Schetters, 2018). The environment of
67 the biting site is anti-inflammatory, and due to the anti-coagulants that are injected with the
68 tick saliva, there is free movement and a high density of red blood cells. Hence, at the

69 moment *Babesia* parasites are injected with tick saliva into a naive host, the environment at
70 the injection site is favourable to them. The parasite rapidly invades an erythrocyte that
71 subsequently enters the blood circulation (some parasites, however, can be taken up with
72 the blood meal of the tick). This could be enough time to activate components of the innate
73 humoral immune systems, but is less likely to be enough time for interactions with white
74 blood cells. During the invasion process, however, the merozoite surface coat is shed and
75 left outside the red blood cell where it interacts with the host defence mechanisms (Igarashi
76 et al., 1988). The merozoite surface coat is a major constituent of supernatants of in vitro
77 cultures of the parasite (soluble parasite antigens; SPA), which have been shown to induce
78 protective immunity in a number of host-parasite models (Schetters and Montenegro-
79 James, 1995). The molecules in SPA preparations are proteinaceous and have sugar
80 moieties (sensitive to amylase), suggesting they are glycoproteins. Importantly, the
81 antigenicity of the SPA preparations was lost after 2-mercaptoethanol treatment, which
82 indicates that intact disulphide bonds implied in the structural organization of the molecule
83 are critical (reviewed in Ristic and Kakoma, 1988).

84

85 **3. Development of recombinant vaccines**

86 The fact that animals could be protected by vaccination with *Babesia* antigens from
87 supernatants of in vitro cultures of the parasite stimulated the search for immunoprotective
88 moieties from such preparations (Timms et al., 1983). Fractionation of the supernatants of
89 in vitro cultures of the human *B. divergens* parasite (strain Rouen) led to the discovery of a
90 37 kDa protein that was recognized by sera from humans, bovines and gerbils that were
91 infected with *B. divergens* (Bd37; Précigout et al., 1991). The protein appeared to be a

92 merozoite surface protein, and metabolic labelling studies indicated that it was glycosylated
93 and palmitoylated, indicative of a GPI-anchored surface protein (Carcy et al., 1995). After
94 incubation with GPI-specific phospholipase D, a soluble, hydrophilic form of Bd37 was
95 released from the merozoite surface in the supernatants of in vitro cultures of *B. divergens*
96 (Delbecq et al., 2002). A monoclonal antibody that was generated in mice that were
97 vaccinated with partially purified Bd37 protein, recognized the Bd37 antigen and was
98 shown to confer protection in vivo (Précigout et al., 2004). Subsequently, the Bd37 gene
99 could be cloned from an expressed sequence tag (EST) library and expressed in
100 *Escherichia coli*. The recombinant glutathione S-transferase (GST) fusion protein was used
101 to vaccinate gerbils. Results showed that gerbils were fully protected from a virulent *B.*
102 *divergens* challenge infection (Hadj-Kaddour et al., 2007). Importantly, where vaccination
103 with the native Bd37 protein from culture supernatants induced immunity that was largely
104 strain-specific (Précigout et al., 1991), vaccination with the recombinant protein induced a
105 broad spectrum of protection (Hadj-Kaddour et al., 2007). Additional studies suggested that
106 induction of broad spectrum immunity depended on the structural conformation of the
107 protein, which might be influenced by the addition of hydrophobic terminal peptide
108 sequences to the core Bd37 protein (Delbecq et al., 2006, 2008). Using the knowledge
109 acquired from the Bd37 vaccine project, a recombinant vaccine against *B. canis* for dogs
110 was developed. The recombinant protein used was a 40 kDa merozoite surface protein of *B.*
111 *canis* that has a predicted GPI-anchoring site. Vaccination of dogs with the recombinant
112 protein induced immunity against virulent challenge infection (Moubri et al., 2018). These
113 two examples suggest the feasibility of a more general strategy involving GPI-anchored

114 merozoite surface antigens to develop protective vaccines against other *Babesia* spp.
115 including *B. microti*.

116 Information on the degree of polymorphism of *B. microti* GPI-anchored proteins is
117 still missing. In the case of bovine *Babesia* spp., sequencing studies showed a variety of
118 scenarios from high level of strain conservation, as in the case of *B. bovis* merozoite surface
119 antigen 2c (MSA-2c) to a high level of polymorphism as is the case of *B. bigemina* gp45/55
120 (McElwain et al., 1991; Wilkowsky et al., 2003; Carcy et al., 2006). Interestingly, even in
121 the case of moderate polymorphism, conservation among strains at the level of
122 neutralization-sensitive B-cell epitopes has been shown (Suarez et al., 2000; Dominguez et
123 al., 2010).

124 Despite the presence of conserved neutralization-sensitive B-cell epitopes in *B.*
125 *bovis* GPI-anchored proteins, protection upon challenge using recombinant forms of some
126 of these antigens has so far been unsuccessful. It could be that, similar to the situation with
127 Bd37 of *B. divergens*, addition of hydrophobic terminal peptide sequences to the core
128 protein induces conformational changes in the molecule, thus exposing the neutralization-
129 sensitive epitopes. Clearly, similar studies and studies on delivery systems and
130 immunomodulators that could elicit protective responses in the bovine host are much
131 needed in *Babesia* vaccine development (reviewed in Florin-Christensen et al., 2014).

132

133 **4. In silico prediction of the GPI synthesis pathway of *B. microti***

134 GPI has been shown to be abundantly present in the membranes of apicomplexan
135 and kinetoplastid pathogenic protozoa such as *Plasmodium falciparum*, *Toxoplasma gondii*,
136 *B. bovis*, *Trypanosoma brucei* and *Leishmania donovani* (Smith et al., 1997; Ferguson,

137 1999; Rodriguez et al., 2010). The molecule is present in free and protein-bound forms, and
138 is comprised of a conserved core structure and variable moieties. The core structure is
139 characterized by glucosamine linked to myo-inositol in position 1, and to the first of a chain
140 of commonly three mannose residues in position 4 (Fig. 1). Myo-inositol can be linked to
141 diacylglycerol, 1-alkyl-2-acylglycerol or ceramide, forming different kinds of inositol
142 phospholipids. In the case of GPI anchors, the last mannose residue is conjugated via
143 phosphoethanolamine to the C terminus of a protein. In addition to the different lipid
144 moieties that can be bound to myo-inositol, the latter may or may not be acylated, and the
145 number of sugar residues varies among different organisms (Ferguson et al., 2009). In *B.*
146 *bovis*, the most abundant free GPI molecule has a more simple structure consisting of a
147 chain of two mannose residues, N-glucosamine and a non-acylated inositol linked to
148 diacylglycerol (Rodriguez et al., 2010). *Babesia bovis* protein-bound GPI molecules, as
149 well as free and protein-bound GPIs of other piroplasmids, are yet to be structurally
150 characterized.

151 All enzymes necessary for the assembly of man-man-GlcN-inositol-diacylglycerol,
152 the main free GPI of *B. bovis*, have been identified in its in silico proteome (Rodriguez et
153 al., 2010). To analyze if *B. microti* possesses the metabolic capacity to generate GPI, its
154 proteome was searched for GPI biosynthetic enzymes orthologous with those described for
155 *P. falciparum* and *B. bovis* (Delorenzi et al., 2002; Rodriguez et al., 2010). The results
156 show that this parasite, which has the smallest genome among apicomplexans (Cornillot et
157 al., 2012), encodes all proteins necessary for GPI assembly and its subsequent protein
158 transfer (Table 1). Interestingly, the genomes of both *B. bovis* and *B. microti* encode
159 phosphatidylinositol glycan anchor biosynthesis-W (PIG-W), the enzyme that catalyzes

160 acylation of myo-inositol, hence acylated inositol is likely present in the GPI molecules
161 synthesized by these parasites. The differential sensitivity of the Bd37 GPI anchor to
162 specific phospholipases C and D also indicates an acylated inositol in *B. divergens*
163 (Delbecq et al., 2002). The *P. falciparum* PIG-B orthologs, responsible for adding the third
164 mannose residue, could neither be found in the *B. bovis* nor in the *B. microti* genome. This
165 finding suggests that either all GPI molecules of these *Babesia* parasites include a chain of
166 two mannose residues instead of the commonly found chain of three mannoses, or that
167 another yet unknown enzyme adds a third mannose residue. It is concluded that all the
168 genes encoding for the factors necessary for GPI synthesis are predicted to be present in the
169 genome of *B. microti*.

170

171 **5. Identification and analysis of the GPI-anchored proteome of *B. microti***

172 Proproteins, to which GPI is attached post-translationally, share the following
173 features: (i) an N-terminal signal peptide sequence targeting them to the endoplasmic
174 reticulum (ER); (ii) a C-terminal transmembrane domain for transient anchoring to the ER
175 membrane, and (iii) the GPI-attachment or omega site, where cleavage of the proprotein
176 takes place and which is located a few amino acids before the C-terminal hydrophobic
177 domain. Upon cleavage of the transmembrane domain, the protein is transferred onto a GPI
178 anchor (Ferguson et al., 2009). Prediction of these three features with bioinformatic
179 algorithms allows the in silico identification of GPI-anchored proteomes (Fig. 2), thus
180 providing an attractive pool of antigens for vaccine and diagnostic test development.
181 Seventeen proteins predicted to be GPI-anchored were identified in the *B. microti* proteome
182 (Table 2). Most of these proteins coincide with the predictions of Cornillot et al. (2016),

183 who obtained a list of 19 predicted GPI-APs. Two of the proteins found in the present study
184 (GenBank Accession numbers XP_012647776.1 and XP_012650569.1) were not identified
185 earlier, which brings the total of predicted GPI-APs to 21.

186 Interestingly, the genes encoding two of the identified GPI-APs (BmGPI12 and
187 BmGPI13) are among the most expressed in both mice and hamsters (Silva et al., 2016).
188 Similarly, in *B. bovis*, the gene encoding GPI-anchored Merozoite Surface Antigen-1 is one
189 of the most actively transcribed in the merozoite stage (Pedroni et al., 2013), highlighting
190 the importance of GPI-APs for membrane structure and/or function.

191 *Babesia microti* experimentally infected mice reacted with recombinant forms of
192 several of the GPI-APs shown in Table 2 in a microarray antibody assay. Among these
193 proteins, BmGPI12 mounted particularly strong IgM and IgG antibody responses.
194 Moreover, in a pilot study, sera from *B. microti*-infected human patients showed ~35-fold
195 higher and ~16-fold higher IgG and IgM reactivity, respectively, against recombinant
196 BmGPI12 than the control non-infected group. These results indicate that BmGPI12 can be
197 considered a biomarker for *B. microti*-infections (Cornillot et al., 2016). Recently, a fully-
198 automated prototype antibody assay was developed based on recombinant BmGPI12
199 which, when applied to human samples, showed a high level of positive concordance with
200 results of *B. microti* infection diagnosis by PCR or immunofluorescence. The estimated risk
201 of human babesiosis among blood donors of tick-endemic areas is relatively elevated and
202 higher than HIV. Thus, this type of sensitive diagnostic assay can help mitigate the spread
203 of *B. microti* in affected countries (Cheng et al., 2018).

204

205 **6. Structural analysis of GPI-APs in apicomplexans: domain repertoire**

206 One of the first classifications of GPI-APs in apicomplexans relies on the size of
207 these proteins. Medium-sized proteins (< 40 kDa) are generally constituted by a single
208 domain (or a duplicated small domain). In contrast, in large proteins as found in
209 *Plasmodium*, a multi-domain organisation is mainly found. As reviewed in Anantharaman
210 et al. (2007), for host cell invasion apicomplexan parasites use either specific protein
211 domains that are found only in apicomplexans, or conserved domains that are also found in
212 other organisms, in particular in the genome of their host. Some of these domains of
213 mammalian origin have been acquired through lateral gene transfer. Transmembrane
214 parasite proteins that are involved in host cell invasion contain a large number of domains
215 that are also found in mammals (e.g. Epidermal Growth Factor-like domain (EGF),
216 Thrombospondin type-1 Repeat domain (TSR) coagulation factors). In contrast, only a few
217 domains that are specific to apicomplexans, such as Duffy Binding Like domain (DBL) and
218 Microneme Adhesive Repeat domain (MAR; Blumenschein et al., 2007), have been
219 reported.

220 It is likely that the domain repertoires used at each parasite stage are shaped by
221 different evolutionary pressures; for instance, malaria sporozoites that invade liver cells,
222 which are structurally organized in an organ, are likely to have evolved adaptations that are
223 different from those of merozoites that invade free-moving erythrocytes. It is tempting to
224 speculate that mammalian domains are mainly involved in invasion of nucleated cells while
225 parasite-specific domains are involved in invasion of erythrocytes. In addition, the large
226 number of specific domains in parasite GPI-AP repertoires could reflect some adaptation to
227 the relatively long exposure of these parasite surface proteins to the immune system during
228 infection, in contrast to the “just-in-time” release of proteins from secretory organelles.

229 The three-dimensional (3D) structures of two GPI-anchored merozoite surface
230 molecules of *Babesia* parasites (Bd37.1 in *B. divergens* and Bc28.1 in *B. canis*) have been
231 resolved (Delbecq et al., 2008; Yang et al., 2012). Although both are involved in the
232 binding of merozoites to erythrocytes, these two proteins bear no structural relationship
233 with each other nor with other known GPI-AP structures in parasites. The immunological
234 epitopes at the surface of Bd37.1 are highly clustered at one side of the proteins and at the
235 unstructured parts, leaving one side of the proteins without detectable antibody-binding
236 regions (Delbecq et al., 2008). A 3D structural analysis of Bd37.1 suggests that the protein
237 can adopt different conformations which could be involved in immune escape (Delbecq et
238 al., 2008). In contrast, the Bc28.1 protein appears to be stably structured around a single
239 core, which is not likely to allow drastic conformational changes (Yang et al., 2012).
240 Nevertheless, an unusually long α -helix in the C-terminal position suggests a large degree
241 of freedom in protein dynamics at the merozoite surface. Both Bd37.1 and Bc28.1 contain
242 additional unstructured parts and positively charged patches, but the significance of these
243 features is not known.

244

245 **7. Soluble form of proteins: functions and the notion of membrane context**

246 One of the intriguing features of apicomplexan GPI-APs is the fact that they can be
247 released from the parasite cell surface. As a result, these proteins can be found in the
248 plasma of infected animals and in the supernatants of in vitro cultures of the parasite. This
249 shedding could result from cleavage by a phospholipase, but for most known GPI-APs the
250 exact mechanisms have not been determined. Since GPI-APs can be recognized by effector

251 molecules of the host immune system, shedding allows parasites to escape immune
252 destruction. The fate and role of shed GPI-APs themselves in the host-parasite relationship
253 is not known. It has been suggested that the glycan part of the GPI anchor interacts with
254 host defence mechanisms, which could play a role in pathogenesis (Debierre-Grockiego
255 and Schwartz, 2010). Indeed, in *B. canis*-infected dogs, fever was positively correlated with
256 the level of SPA in plasma (Schetters et al., 2009). The fact that vaccination with
257 supernatants of in vitro cultures of *B. canis* could protect dogs from clinical disease without
258 an effect on the parasitaemia but on the level of SPA in plasma supports this view
259 (Schetters et al., 1996).

260 Results from *Babesia* Bd37.1 and *Theileria* PIM proteins suggest that the cleaved,
261 soluble forms of the GPI proteins have different binding characteristics compared with their
262 membrane-bound counterparts (Casanova et al., 2006). It is suggested here that the location
263 of the GPI protein in the context of the parasite plasma membrane (referred to as
264 ‘membrane context’) affects the conformation, orientation and association with other
265 molecules, as has been described for PrP, a GPI-AP prion protein found in mammals
266 (Mahal et al., 2012). Coexistence of multiple conformations of a protein has been
267 associated with enzyme allostery and was suggested to occur in antibodies (reviewed in
268 James and Tawfik, 2003). Such structural dynamics could be crucial for parasite survival.
269 First, if the soluble form of the protein were structurally similar to the membrane-bound
270 form that is used to enter the host cell, it would compete for the host cell receptor (Fig. 3).
271 Hence, switching to the alternative conformation would prevent the soluble protein from
272 binding to the host cell receptor, thus allowing the parasites to bind to and enter the host
273 cell. A second advantage of structural dynamics could be related to evasion of the immune

274 response. For instance, if the cleaved protein had a conformation similar to the protein in
275 the membrane context, then antibodies against the soluble protein would also recognize the
276 protein at the parasite surface. This could then lead to, e.g., blocking of binding to the host
277 cell receptor, or destruction of the parasite by immune phagocytosis and/or lysis through
278 complement activation. Hence, it would be advantageous if the soluble protein would
279 change its structural conformation and prevent the generation of antibodies that reacted
280 with the membrane-bound form of the protein. This is supported by results from the *B.*
281 *canis*-dog model; dogs that were vaccinated with SPA from in vitro cultures were protected
282 against disease after a virulent *B. canis* infection without affecting the parasite proliferation
283 (Schetters et al., 1996). Protection was associated with the presence of antibodies against a
284 39 kDa antigen, which appeared to be the soluble hydrophilic form of a 40 kDa merozoite
285 surface membrane protein (Moubri et al., 2018). Apparently, antibodies against the soluble
286 protein did not negatively affect parasite proliferation. In contrast, vaccination with the
287 recombinant hydrophobic 40 kDa protein induced antibodies that reacted with the 40 kDa
288 merozoite surface protein. When the dogs were challenged, protection was reflected in a
289 strong anti-parasite response (Moubri et al., 2018).

290 This model is further corroborated by the analysis of the recombinant Bd37.1-based
291 vaccine against *B. divergens*. Using this vaccine, an efficient immune response was
292 obtained only with hydrophobic recombinant antigens, but not with the hydrophilic
293 versions of the recombinant Bd37.1, although similar levels of antibody were induced
294 (Delbecq et al., 2006). Protection was reflected in a strong anti-parasite response. These
295 results suggest that the hydrophobic protein exhibits parasite epitopes that are also
296 expressed in GPI-AP in the membrane context. As a result the immune response is directed

297 against the merozoite. Similar epitopes appear to not be present on the hydrophilic version
298 of the Bd37.1 protein. Importantly, vaccination with the hydrophobic recombinant Bd37.1
299 protein induced protection against heterologous infection, which indicates that the
300 hydrophobic protein expresses epitopes are present in a wide range of genetically different
301 *B. divergens* strains. In contrast, vaccination with the native soluble (hydrophilic) form of
302 Bd37 from supernatants of in vitro cultures induced immunity that was highly strain-
303 dependent (Précigout et al., 2004).

304

305 **8. Outlook**

306 Control of babesiosis in animals comprises a number of measures, which depend on
307 the host species. In production animals, tick control by acaricide treatment and vaccination
308 of animals with live attenuated *Babesia* vaccines is practised in countries with high parasite
309 prevalence. With increasing acaricide resistance, tick control by vaccination appears to
310 become an important additional tool (Schetters, 2018). It has the added advantage that there
311 is no withdrawal period for meat or milk. With the advent of effective recombinant *Babesia*
312 vaccines, live-attenuated vaccines will eventually be replaced. In canine babesiosis,
313 vaccination with subunit vaccines has been practised (Schetters and Montenegro-James,
314 1995). With the discovery of a recombinant *Babesia* antigen that induces protection against
315 experimental infection in dogs, it is expected that vaccines with recombinant antigens will
316 become commercially available (Moubri et al., 2018).

317 The GPI-anchored *Babesia* proteins that are located at the merozoite surface appear
318 attractive vaccine candidate antigens, despite the fact that many of them are polymorphic in
319 nature. The results obtained with recombinant forms of GPI-APs suggest that the 3D

320 structure of the protein influences the expression and/or immunodominance of epitopes that
321 are common on geographically different *Babesia* strains. Results further indicated that
322 increased relative hydrophobicity is crucial for the induction of protective immunity that
323 transcends strain variation. Although this seems to apply to *B. divergens* and *B. canis*, it
324 remains to be determined whether this also applies to other *Babesia* spp. including *B.*
325 *microti*.

326 Although the incidence of *Babesia* infection in humans is increasing, it is not likely
327 that a vaccine against human *Babesia* parasites, or against the ticks that transmit the
328 parasites, will be developed and introduced at a large scale in the human population. It
329 could be envisaged, however, that a vaccine is used to protect humans who are particularly
330 vulnerable to infection such as elderly or immunocompromised subjects. In addition,
331 healthy subjects who have a high chance of becoming infected, such as foresters who work
332 in highly endemic areas, could benefit from vaccination. Until that time, hygienic measures
333 e.g. wearing protective clothing that may be impregnated with an acaricide, visual
334 inspection and removal of ticks after visiting a potentially tick-infested area, and
335 chemotherapeutic treatment of *Babesia* infection remain the methods of control.

336

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341

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478

479 **Figure legends**

480

481 Fig. 1. Biosynthetic pathway of *Babesia microti* glycosylphosphatidylinositol (GPI). The
482 biosynthesis starts at the cytoplasmic side of the endoplasmic reticulum (ER) and continues
483 in the ER lumen. The first Uridine diphosphate-N-acetylglucosamine (UDP-GlcNac) is
484 linked to a phosphatidylinositol (PI) molecule by a complex formed by the enzymes PIG
485 (PI glycan anchor biosynthesis)-A and GPI-1 (step 1). Then, the N-acetyl group is removed
486 by PIG-L (step 2) and the GlcPI is translocated to the ER lumen by an uncharacterized
487 flippase (step 3). A fatty acid is added to the PI inositol ring by PIG-W, forming GlcN-acyl-
488 PI (step 4). Two mannose (Man) molecules are sequentially added to GlcN-acyl-PI
489 catalyzed by PIG-M and PIG-V (steps 6 and 7). The mannose donor substrate is dolichol-
490 phosphate-mannose, synthesized from dolichol-phosphate (Dol-P) and GDP-mannose by
491 DPM1 (Dolichol-phosphate mannosyltransferase 1) at the cytoplasmic side of the ER (step
492 5), and transported across the ER membrane. When the GPI is destined to function as an
493 anchor, ethanolamine-phosphate is added by PIG-O, and finally, a protein is translocated to
494 the GPI molecule by the GPI anchor attachment 1 protein (GAA1) and GPI-8 complex (not
495 shown).

496

497 Fig. 2. Strategy for the identification of glycosylphosphatidylinositol (GPI)-anchored
498 proteins in *Babesia microti*. The proteome of *B. microti* was scanned with SignalP to
499 identify the secretome, i.e. signal peptide-containing proteins. Then, the presence of a

500 transmembrane helix in the C-terminus was detected by five programs: TOPCONS,
501 DASTMfilter, TMHMM, HMMTOP and Constrained Consensus TOPology server. The
502 resulting protein list was scanned with four GPI predictor programs: GPI-SOM, big-PI
503 (Protozoa), big-PI (Plants) and PredGPI. Proteins detected by at least two of these programs
504 are shortlisted in Table 2.

505

506

507 Fig. 3. Immunobiological interactions of glycosylphosphatidylinositol (GPI)-anchored
508 proteins (GPI-AP) from *Babesia* merozoites. GPI-AP are relatively abundant due to
509 differential expression and differential shedding of the anchor. The potential heterogeneity
510 in the osidic anchor could affect shedding, packing and orientation (1). GPI-AP interact
511 with the host cell surface for penetration. The membrane vicinity induces electrostatic
512 perturbation and stimulates conformational changes, which affect packing and orientation
513 of GPI-AP that could increase avidity for the host cell (2). Soluble GPI-AP could
514 potentially stick to the host cell surface, with low affinity (3) and bind to antibodies (4).
515 Membrane-bound GPI-AP could be recognized by specific antibodies (5). It is thought that
516 immune protection/escape relies on the equilibrium between these two interactions (4 and
517 5). A continuous release of GPI-AP during the active penetration of the cell (shedding) or
518 by enzymatic cleavage of the anchor (by lipase or protease) from plasma membrane could
519 overwhelm the antibody production (6).

Table 1. Identification of *Babesia microti* proteins predicted to participate in the synthesis of glycosylphosphatidylinositol (GPI) anchors and the attachment of GPI anchors to nascent proteins.

Enzymatic activity	Protein name	GenBank Accession number	GenBank annotation
Glycosyltransferase	PIG-A	XP_021337772.1	Phosphatidylinositol glycan, class A
	GPI-1	or XP_021338686.1	Phosphatidylinositol glycan, class Q
	PIGQ		
de-N-acetylase	PIG-L	XP_021337725.1	N-acetylglucosaminyl phosphatidyl inositol deacetylase
Flippase	Nd	Nd	Nd
Acyltransferase	PIG-W	XP_021338156.1	Multiple TM. Not a GPI protein
Dol-P-Man synthase	DPM-1	XP_021337246.1	Dolichol-phosphate mannosyltransferase; DPM1
Mannosyltransferase MT-I	PIG-M	XP_021338330.1	phosphatidylinositol glycan, class M

Mannosyltransferase MT-II	PIG-V	XP_012650107.1	Conserved protein, unknown function
Etanolamine-P transferase	PIG-O	XP_021338017.1	phosphatidylinositol glycan, class O
Transamidase	GAA1	XP_021337605	glycosylphosphatidylinositol anchor attachment 1 protein
	GPI-8	XP_012650207.2	GPI-anchor transamidase

Nd, not determined

Table 2. Identification and description of glycosylphosphatidylinositol (GPI)-anchored proteins of *Babesia microti*.

No	GenBank ID	GenBank Annotation	SignalP	GPIsom	PredGPI	BigPI	C-term	Length (aa)	Exons	Domains	<i>B. microti</i> - specific
1	XP_021337223	BmGPI15, Sexual stage antigen, Pfam s48/45	+	+	HP	+	+	1024	1	None	Yes
2	XP_012647442 ^a	BmGPI5, Sexual stage antigen, Pfam s48/45	+	+	HP	+	+	881	1	6-Cys PS51701	No
3	XP_012648325 ^a	BmGPI8, Sexual stage antigen, Pfam s48/45	+	+	HP	+	+	924	1	6-Cys PS51701	No
4	XP_012647158	BmGPI1, <i>B. microti</i> -specific	+	+	HP	+	+	798	1	None	Yes
5	XP_012647167 ^b	BmGPI4, <i>B. microti</i> -specific, 24 tandem	+	+	HP	+	+	648	1	GmlU TIGR01173	No

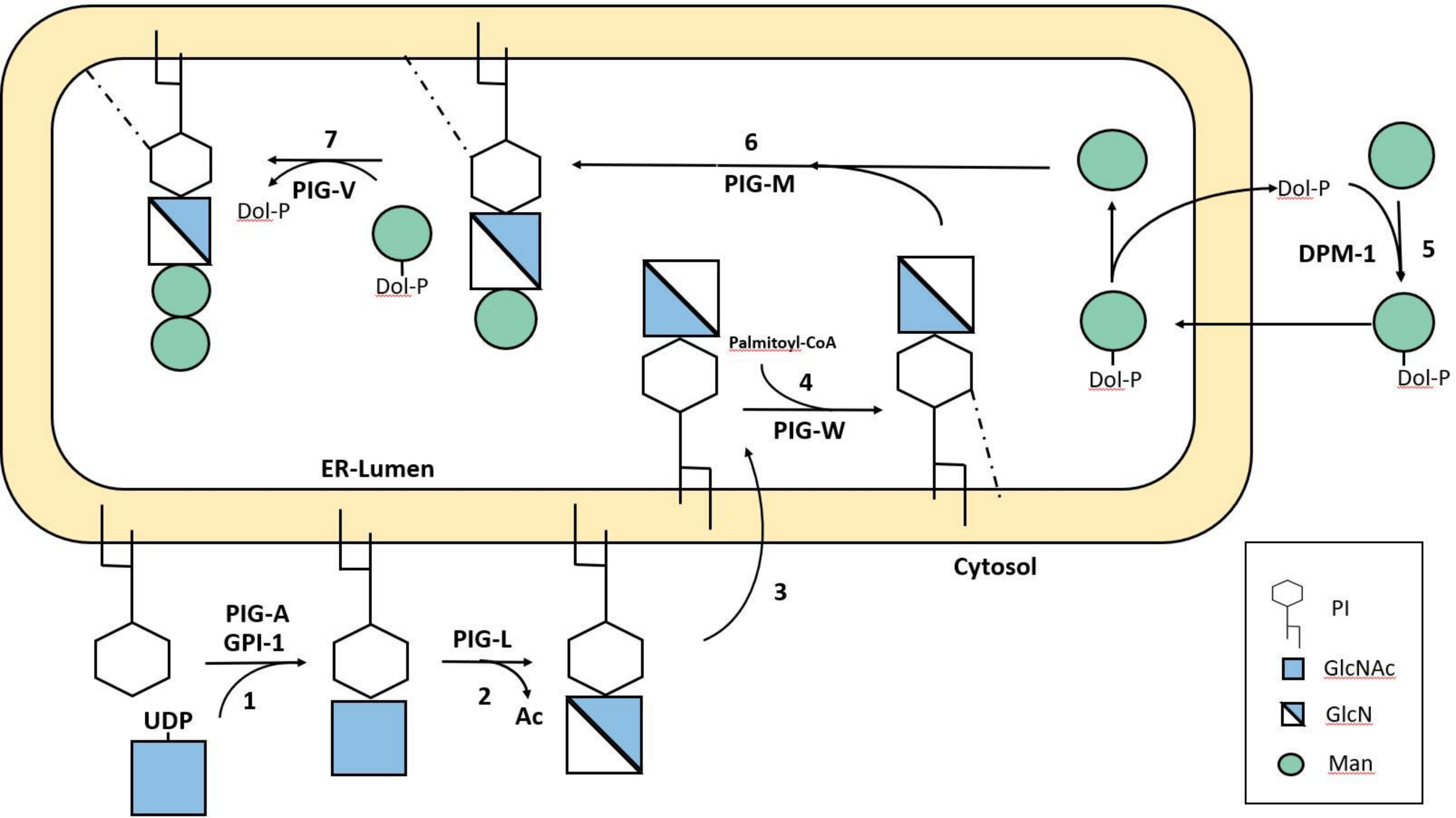
		repeat, IPR011004										
6	XP_012648768	BmGPI13,	B.	+	+	HP	+	+	319	1	IG_MHC	Yes
		<i>microti</i> -specific									PS00290	
7	XP_012649764	BmGPI18,	acid	+	+	WP	+	+	380	4	GAP	No
		phosphatase									PTZ00422	
8	XP_021338453 ^c	BmGPI11,		weak	+	HP	0	+	298	8	MOLO-1	No
		Conserved protein,									Pfam 17175	
		unknown function										
9	XP_021338712	BmGPI17,		+	+	HP	0	+	456	2	None	No
		Conserved protein,										
		unknown function										
10	XP_012648767 ^d	BmGPI12,	BMN1	+	+	HP	0	+	328	1	None	Yes
		family, BMN1-9,										
		BmSA1 orthologue										
11	XP_012649179	BmGPI16,		+	+	HP	0	+	437	1	None	Yes
		Chemotaxis domain										
12	XP_012648610 ^d	BmGPI10,	BMN1	+	+	HP	0	+	304	1	None	Yes

		family, N1-21a orthologue									
13	XP_012647166 ^b	BmGPI3, <i>B. microti</i> -specific	+	+	HP	0	+	530	1	None	Yes
14	XP_021338061 ^c	BmGPI6, Conserved protein, unknown function	+	+	P	0	+	252	8	MOLO-1 Pfam17175	No
15	XP_012647776^d	BMN1 family	+	+	P	0	+	194	2	None	Yes
16	XP_012650569^d	BMN1 family	+	+	P	0	+	280	2	None	Yes
17	XP_012648607 ^d	BmGPI9, BMN1 family	+	+	WP	0	+	281	1	None	Yes

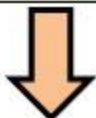
Proteins positively predicted with at least two GPI-anchor prediction programs, with a signal peptide and a C-terminal transmembrane domain, and a mature protein predicted as hydrophilic are included in decreasing order, according to the strength of their GPI-anchor prediction. A plus sign (+) and 0 show positive and no prediction, respectively, using the default parameters of each program. HP, P and WP

correspond to prediction with high, medium or low probability, respectively. In the case of XP_021338453, prediction of a signal peptide is considered weak, since a signal peptide was not predicted with the default parameters of the program, but could be detected with a lower cut-off value. Proteins with the same superindex are paralogs. Proteins not predicted as GPI-anchored in the analysis of Cornillot et al. (2016) are in bold. Domains: 6-Cys: 6-cysteine domain; IG-MHC: Immunoglobulins and major histocompatibility complex proteins signature; GAP: glideosome-associated protein 50; MOLO-1: Modulator of levamisole receptor-1; GmlU: UDP-N-acetylglucosamine diphosphorylase/glucosamine-1-phosphate N-acetyltransferase.

aa, amino acid

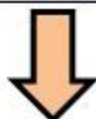


Proteome
3567 proteins



Signal peptide

Secretome
302 proteins



Transmembrane helix in
C-terminus

**Membrane-associated in
C-terminus**
98 proteins



GPI anchor signal

GPI-anchored proteome
17 proteins

