DOI: 10.1111/tbed.13438

SUPPLEMENT ARTICLE

Transboundary and Emerging Diseases WILEY

In silico identification of immunotherapeutic and diagnostic targets in the glycosylphosphatidylinositol metabolism of the coccidian *Sarcocystis aucheniae*

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Funding information
Agencia Nacional de Promoción Científica y Tecnológica, (ANPCyT), Grant/Award Number: PICT start up 2014-3747; CONICET

Abstract

Meat of the South American camelids (SACs) llama and alpaca is an important source of animal protein and income for rural families in the Andes, and a product with significant growth potential for local and international markets. However, infestation with macroscopic cysts of the coccidian protozoon *Sarcocystis aucheniae*, a parasitosis known as SAC sarcocystosis, significantly hampers its commercialization. There are no validated methods to diagnose the presence of *S. aucheniae* cysts other than carcass examination. Moreover, there are no available drugs or vaccines to cure or prevent SAC sarcocystosis. Identification of relevant molecules that act at the host–pathogen interface can significantly contribute to the control of this disease. It has been shown for other pathogenic protozoa that glycosylphosphatidylinositol (GPI) is a critical molecule implicated in parasite survival and pathogenicity. This study focused on the identification of the enzymes that participate in the *S. aucheniae* GPI biosynthetic pathway and the repertoire of the parasite GPI-anchored proteins (GPI-APs). To this aim, RNA was extracted from parasite cysts and the transcriptome was sequenced and translated into amino acid sequences. The generated database was mined using sequences of well-characterized GPI biosynthetic enzymes of *Saccharomyces cerevisiae* and *Toxoplasma gondii*. Eleven enzymes predicted to participate in the *S. aucheniae* GPI biosynthetic pathway were identified. On the other hand, the database was searched for proteins carrying an N-terminal signal peptide and a single C-terminal transmembrane region containing a GPI anchor signal. Twenty-four GPI-anchored peptides were identified, of which nine are likely *S. aucheniae*-specific, and 15 are homologous to membrane proteins of other coccidians. Among the latter, 13 belong to the SRS domain superfamily, an extensive group of coccidian GPI-anchored proteins that mediate parasite interaction with their host. Phylogenetic analysis showed a great degree of intra- and inter-specific divergence among SRS family proteins. In vitro and in vivo experiments are needed to validate *S. aucheniae* GPI biosynthetic enzymes and GPI-APs as drug targets and/or as vaccine or diagnostic antigens.

Cecilia Decker Franco and Sarah N. Wieser contributed equally to this manuscript.

KEYWORDS
glycosylphosphatidylinositol, GPI, GPI metabolism, GPI-anchored proteins, *Sarcocystis aucheniae*, transcriptome

1 | INTRODUCTION

The domestic South American camelids (SACs) llama and alpaca are raised in the flatland regions close to the Andes mountain range of Argentina, Bolivia, Peru, Chile, and Ecuador (Wheeler, 1991). They are an important part of the life strategy of the rural communities that take care of their breeding, contributing transportation, manure for fuel, fibre for clothing and meat as a major source of protein (Leguía, 1991; Vilca, 1991). SACs are especially adapted to extreme environmental conditions, such as high altitude, lack of water, steep slopes and frost. However, they can thrive under varied climatic and topographic conditions and have been introduced as livestock to other regions of the world such as Australia (Saeed, Rashid, Vaughan, & Jabbar, 2018). A higher protein/cholesterol ratio in SAC meat as compared to beef and a lower environmental impact of SAC breeding as compared to European cattle fulfil the profile of environment-conscious, health-oriented consumers (Avilés Esquivel, Montero, & Barros-Rodríguez, 2018; Saeed et al., 2018). Thus, SAC meat is increasingly regarded as an attractive product for the local and international gourmet cuisine (Mamani-Linares & Gallo, 2014; Vilca, 1991).

One of the limitations for the production and commercialization of SAC meat is its frequent infestation with macroscopic cysts resembling rice grains, produced by the apicomplexan protozoon *Sarcocystis aucheniae* (Figure 1).These cysts correspond to the final stage of the parasite life cycle in its intermediate host and essentially consist of wall-encapsulated vesicles, each containing millions of live bradyzoites. Cysts give SAC meat an unappetizing aspect for the consumer and may lead to the confiscation of carcasses by sanitary authorities or the devaluation of their commercial value. In addition, ingestion by humans of insufficiently cooked cyst-infested meat provokes gastroenteritis (Decker Franco, Schnittger, & Florin-Christensen, 2018; Moré et al., 2016; Saeed et al., 2018).

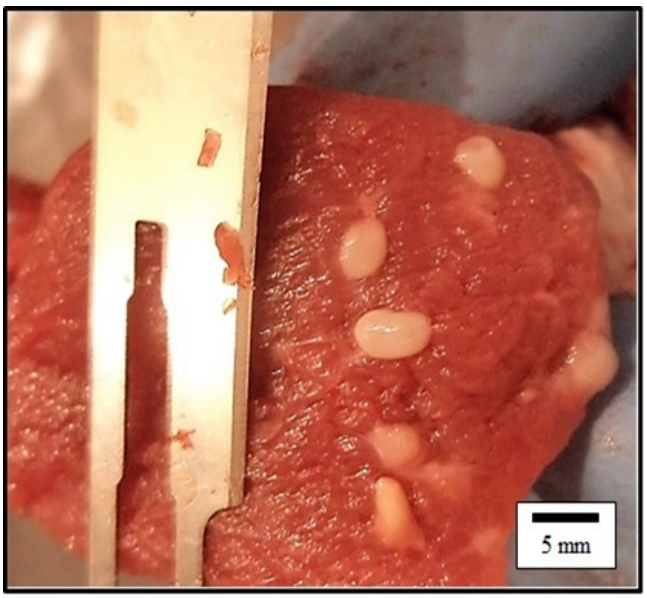

FIGURE 1 *Sarcocystis aucheniae* macroscopic cysts in SAC meat [Colour figure can be viewed at wileyonlinelibrary.com]

Currently, diagnosis of infestation with *S. aucheniae* cysts can only be carried out by visual examination of carcasses upon slaughter. Recently, we have reported a semi-nested PCR that allows detecting the parasite in SAC blood with high sensitivity (Decker Franco, Romero, Ferrari, Schnittger, & Florin-Christensen, 2018; Martín et al., 2016). However, it seems that this method exclusively detects the parasite immediately after infection when it circulates in the bloodstream, before its encystment in the muscles (Decker Franco, Romero, et al., 2018). It may be assumed that, in the current scenario of absence of any effective treatment, detection of anti-*S. aucheniae* antibodies is more representative of cyst infestation than parasite DNA detection in blood. This is so because cyst production is a slow process and antibodies produced upon first contact with the parasite are likely to remain in the serum after they have disappeared from the bloodstream. Although no *S. aucheniae*-specific serodiagnostic methods have been developed so far, high prevalence values, up to 96% of anti-*Sarcocystis* sp. antibodies found in some llama herds, suggest that the humoral response elicited upon infection is long-lasting (Moré et al., 2008; Romero et al., 2017). Additionally, vaccines or therapeutics to prevent or treat *S. aucheniae* infections are not available. Thus, the identification of diagnostic, vaccine and therapeutic targets is needed for this understudied parasite.

The glycolipid glycosylphosphatidylinositol (GPI) is highly abundant in pathogenic protozoa and is present either as surface protein anchor or as independent surface molecule being the latter form exclusive for lower eukaryotes (Rodríguez et al., 2014). Glycoinositol phospholipids (GIPLs) and lipophosphoglycans (LPGs) of the genus *Leishmania* are representatives of this protein-free form of GPI (Proudfoot, Schneider, Ferguson, & McConville, 1995). Inhibition of the GPI biosynthetic pathway or treatment of cells with phosphatidylinositol (PI)-specific phospholipase C hampers invasion of host cells by other Apicomplexa, highlighting the vital role of these molecules for intracellular protozoan parasites (Delorenzi et al., 2002; Rodríguez, Couto, Echaide, Schnittger, & Florin-Christensen, 2010; Rodríguez et al., 2014). In addition, GPIs have been shown to exert strong immunomodulatory effects during the infection of *Plasmodium falciparum*, which are detrimental for the host (Deroost,

Pham, Opdenakker, & Van den Steen, 2016). Synthetic GPIs and recombinant GPI-anchored proteins (GPI-APs) have been proposed as vaccine candidates, and some have been used in vaccine formulations against apicomplexan protozoa that elicited significant protection upon challenge (Hegazy-Hassan et al., 2018; Kapoor et al., 2018; Moubri et al., 2018). Additionally, GPI-APs of pathogenic protozoa are frequently immunodominant and have proven useful for diagnostic purposes, such as in the case of *Babesia bovis* MSA-2c, *Sarcocystis neurona* SnSAG2 and *Neospora caninum* NhSAG1 (Dangoudoubiyam et al., 2011; Dominguez et al., 2012).

We have hypothesized that the study of GPI biosynthesis and GPI-APs in *S. aucheniae* can lead to a pool of attractive vaccine, diagnostic and therapeutic targets. Thus, as a first approach, this work aimed to identify the enzymes involved in the biosynthesis of GPI and the array of GPI-APs of this parasite through in silico exploration of its transcriptome.

2 | MATERIALS AND METHODS

2.1 | Isolation of macrocysts and RNA extraction

Macroscopic cysts were isolated by visual examination of cervical muscles of a slaughtered llama from Turco, Bolivia. Twenty cysts measuring ~ 3–5 mm long were separated from llama tissues with sterilized tweezers, washed with 1xPBS, cut into small pieces and preserved in RNA*later* (Thermo Fisher Scientific) at −20°C until use. Total RNA was extracted with TRIzol™ Reagent (Invitrogen) as described by Chomczynski (1993). The RNA sample was examined by electrophoresis under denaturing conditions in 1.5% agarose gels containing 37% formaldehyde in MOPS buffer (100 mM MOPS/ 40 mM sodium acetate/ 5 mM EDTA, pH 7) and stained with ethidium bromide, revealing two main bands corresponding to 18S and 28S rRNA as well as an array of different sized mRNA bands. A total amount of 16.8 μg of RNA was obtained, with a 260/280 ratio of 2.16 and a 260/230 ratio of 1.98, as evaluated using a Nanodrop spectrophotometer (UV Nanodrop NS-1000).

2.2 | Transcriptome sequencing

The transcriptome of *S. aucheniae* was sequenced by Macrogen (South Korea) using an Illumina sequencer. The sequencing raw data were converted into two *fastq* files, each one representing one end of the fragment. A random sample composed of 500,000 sequences from each *fastq* file was extracted and analysed with the FastQC program (www.bioinformatics.babraham.ac.uk/projects/fastqc/). The sequence quality score was on average above 34 in all positions, which is considered as "very good" by this program (above the threshold of 28). Sequences were analysed by Scythe (https://github.com/vs.buffalo/scythe) using default parameters (pairing of 8 bases or more) for adaptor detection and removal. A small percentage of approximately 0.04% of the sequences were contaminated with TrueSeq adapters. Then, sequences with low quality at the ends were eliminated with Seqtk (quality < 20, as assessed by the program; github.com/lh3/seqtk), and finally, shorter sequences than 75 bases were eliminated with Cutadapt (journal.embnet.org/index.php/embnetjournal/article/view/200).

2.3 | Assembly and preliminary annotation of the transcriptome

De novo assembly of the sequences was carried out with the program "Trinity" (github.com/trinityrnaseq/trinityrnaseq/), obtaining a database of 98,481 contigs or transcripts. The minimum and maximum lengths of contigs were 201 and 24,513, respectively. The median length of the contigs was 363, and the N50, 1802. The average GC content was 52%. For the preliminary annotation of the transcriptome, the software "Trinotate" (trinotate.github.io/) was used. Then, a peptide database with predicted amino acid sequences was created using "GeneiousPrime" (www.geneious.com/geneious). Six-frame translation of nucleotide sequences was carried out, and the most likely open reading frames as predicted by Trinity were chosen for subsequent analysis.

2.4 | In silico prediction of the GPI biosynthetic pathway

Enzymes involved in the GPI biosynthesis pathway of *Saccharomyces cerevisiae* and *Toxoplasma gondii* were searched within KEGG PATHWAY (www.genome.jp/kegg/pathway.html) and KEGG GENES (www.genome.jp/kegg/genes.html) (Kanehisa & Goto, 2000; Kanehisa et al., 2007). The peptide database of *S. aucheniae* was screened using *S. cerevisiae* and *T. gondii* GPI-biosynthesis enzyme sequences as queries. *S. aucheniae* sequences with an E_{value} lower than 0.05, and containing the corresponding conserved domains, as verified by Pfam (pfam.xfam.org/search#tabview = tab0) (Bateman et al., 2004) and/or InterPro (https://www.ebi.ac.uk/InterPro/, Mitchell et al., 2018) were selected. For identity and similarity percentage calculations, *S. cerevisiae*, *T. gondii* and *S. aucheniae* sequence segments corresponding to the conserved domains as predicted by InterPro were aligned by Clustal omega (www.ebi.ac.uk/Tools/msa/clustalo/) and compared in the Sequence Manipulation Suite website (www.bioinformatics.org/sms2/ident_sim.html).

2.5 | In silico prediction and characterization of GPI-anchored proteins from *S. aucheniae*

Predicted proteins containing a signal peptide (www.cbs.dtu.dk/services/SignalP) (Petersen, Brunak, Heijne, & Nielsen, 2011) were shortlisted, creating a database of 2,371 sequences. GPI-anchored proteins (GPI-AP) were identified from this database using the following GPI

anchor predictors: GPI-SOM (gpi.unibe.ch, Fankhauser & Maser, 2005); GPI Big-PI (mendel.imp.ac.at/home/Birgit.Eisenhaber, Eisenhaber, Bork, & Eisenhaber, 1999); and PredGPI (gpcr.biocomp.unibo.it/predgpi/pred.htm, Pierleoni, Martelli, & Casadio, 2008). Additionally, transmembrane regions were considered present when predicted by any of the following programs: DAS-TOPO (www.sbc.su.se/~miklos/DAS/maindas.html, Cserzö, Eisenhaber, Eisenhaber, & Simon, 2002), TOPCONS (topcons.cbr.su.se/, Tsirigos, Peters, Shu, Käll, & Elofsson, 2015), TMHMM (www.cbs.dtu.dk/services/TMHMM/, Krogh, Larsson, Heijne, & Sonnhammer, 2001) and Consensus TOPology (cctop.enzim.ttk.mta.hu/, Dobson, Reményi, & Tusnády, 2015). Proteins selected as GPI-anchored were those positively identified by 3 out of 5 of the GPI anchor predictor programs and carrying a transmembrane region in the C-terminus coinciding with the GPI anchor. Conserved domains were searched for with Pfam and InterPro and homology searches in the GenBank were carried out by BLASTp (blast.ncbi.nlm.nih.gov). A statistical E_{value} of 0.05 or lower was considered to identify all potential homologs. In the case of homologous proteins, percentages of identity and similarity were calculated in the Sequence Manipulation Suite website after Clustal omega alignment (see section 2.4). N-glycosylation sites were predicted with NGlycPred (bioinformatics.niaid.nih.gov/nglycpred). Sequences of the identified GPI-AP1 to 24 were deposited in the GenBank with the following accession numbers: MK825580, MK825581, MK825582, MK825583, MK825584, MK825585, MK825586, MK825587, MK825588, MK825589, MK825590, MK825591, MK825759, MK825760, MK825761, MK825889, MK825890, MK825891, MK831010, MK831011, MK831012, MK831013 MK831014 and MK831015.

2.6 | Phylogenetic analysis of SRS proteins

One of the *S. aucheniae* SAGs (SaGPI-AP12) was used as query to search for paralogs in the genomes of coccidians available in the GenBank and EuPathDB (eupathdb.org/eupathdb) using a threshold of E < 0.05. Identified sequences were verified for the presence of SRS domains in Pfam and cut out of the sequences for the phylogenetic analysis. The following sequences were identified by this search procedure: *T. gondii*: XP_018636471.1, XP_018637047.1, XP_002365842.2, XP_002368595.2 and XP_002368205.2; *Neospora caninum*: XP_003882969.1, XP_003884871.1, XP_003884866.1, XP_003884898.1, XP_003884868.1, XP_003884865.1, XP_003881285.1 and XP_003883768.1; *Eimeria tenella:* XP_013232589.1; *Sarcocystis neurona*: SN3_00700580, SN3_00300451, SRCN_6668, SRCN_2786, SRCN_3434, SRCN_2490, SRCN_1229; *S. aucheniae*: SaGPI-AP12 to 18. ADP domain-containing proteins of *E. tenella* ETH, *N. caninum* Liverpool and *T gondii* ME49 were included as outgroup. Sequences were aligned by MUSCLE (https://www.ebi.ac.uk/Tools/msa/muscle), and a neighbour joining tree was assembled with MEGA6 (https://www.megasoftware.net/, Tamura, Stecher, Peterson, Filipski, & Kumar, 2013). The Dayhoff model was used with G = 6.7, and there was a total of 83 positions in the final dataset. The bootstrap was tested by 1,000 replicates. The evolutionary distance was calculated as the number of exchanged amino acids per site.

3 | RESULTS

3.1 | In silico identification and characterization of the *S. aucheniae* GPI biosynthetic pathway

In silico searches in the *S. aucheniae* transcriptome allowed the identification of 11 proteins homologous to *S. cerevisiae* and *T. gondii* enzymes involved in the synthesis of the glycan part of a GPI molecule and the attachment of a nascent protein to GPI (Table 1). It is possible to infer a pathway constituted by phosphatidylinositol N-acetylglucosaminyltransferase (PIG)-subunit A (PIG-A), PIG-subunit C (PIG-C), PIG-subunit L (PIG-L), PIG-subunit W (PIG-W), dolichol phosphate-mannose biosynthesis regulatory protein (DPM-1), PIG-subunit *M* (PIG-M), PIG-subunit V (PIG-V) and PIG-subunit B (PIG-B). This pathway leads to the synthesis of a glycan composed of N-glucosamine and three mannose residues bound to phosphatidylinositol (Figure 2). Eventually, a complex formed by three transamidases, PIG-subunit K (PIG-K), GPI transamidase component GAA1 (GAA-1) and PIG-subunit U (PIG-U), which were identified in this study, catalyzes the transfer of a nascent protein to the GPI molecule. Attachment takes place via a phosphoethanolamine residue bound to the last mannose of the chain by the action of PIG-subunit O (PIG-O). The latter enzyme, however, could not be found in the *S. aucheniae* transcriptome database.

3.2 | In silico prediction and characterization of *S. aucheniae* GPI-anchored proteins

GPI-anchored proteins can be bioinformatically predicted due to their distinctive features, including an N-terminal signal peptide and a GPI anchor signal in the hydrophobic C-terminal end of the protein (Figure 3). Altogether 24 *S. aucheniae* proteins were in silico identified to be GPI-anchored. Protein lengths varied from 100 to 448 aa, and 15 had 1 to 5 N-glycosylation sites in the region corresponding to the mature protein (Figure 4).

Nine of these proteins (SaGPI-AP1 to 9) might be *S. aucheniae*-specific since no significant similarities were found with any so far deposited sequence and they exhibit no conserved domains. Notably, 7 were found to be very short (100 to 170 aa) but corresponded to complete open reading frames with start and stop codons.

SaGPI-AP10 and SaGPI-AP11 have homologues in other coccidians such as *Hammondia hammondi* (Hh), *Neospora caninum* (Nc) and *Toxoplasma gondii* (Tg). SaGPI-AP10 is homologous to Hh XP_0088868 with an identity percentage (%id) of 18.7 and a similarity percentage (%s) of 29.4, Nc XP_00387984 (%id: 21.1; %s: 30.7) and Tg XP_018638496 (%id: 18.2; %s: 29.6); while SaGPI-AP11 is homologous to HhXP_0088868 (%id: 27.2; %s: 46.2), Nc XP_00387971 (%id: 26.9; %s: 44.7) and Tg XP_002370248 (%id: 27.0; %s: 44.3). Interestingly, the latter proteins belong to the conserved SNARE family of fusogens

TABLE 1 GPI biosynthetic pathway enzymes found in *Sarcocystis aucheniae* and their homologs in *S. cerevisiae* and *Toxoplasma gondii*. InterPro conserved domains nomenclature are shown for each enzyme, as well as percentages of identity (%id) and similarity (%s) between *S. aucheniae* conserved domain sequences and those of *S. cerevisiae* and *T. gondii*

S. aucheniae enzyme	Conserved domain		*S. cerevisiae* (acc. number)	%id	%s	*T. gondii* (acc. number)	%id	%s
	Id	Family description						
PIG-A	IPR013234	PIGA, GPI anchor biosynthesis	NP_015150.2	51.1	63.3	XP_002366736.1	74.2	77.5
PIG-C	IPR009450	*N*-acetylglucosaminyl-phosphatidylinositol transferase subunit C	NP_015249.1	6.7	13.9	XP_002371772.1	29.8	34.0
PIG-L	IPR039516	*N*-acetylglucosaminyl-phosphatidylinositol de-*N*-acetylase	NP_014008.1	14.5	20.9	XP_018637001.1	24.7	31.5
PIG-W	IPR009447	Phosphatidylinositol anchor biosynthesis protein PIGW/GWT1	NP_012444.2	11.9	20.9	XP_018635716.1	18.5	29.7
DPM–1	IPR039528	DPM1-like	NP_015509.1	29.0	45.4	XP_002370501.2	73.3	86.4
PIG-M	IPR007704	GPI mannosyltransferase 1	NP_012547.2	18.0	26.8	XP_002364256.1	28.9	33.4
PIG-V	IPR007315	GPI mannosyltransferase 2	NP_009558.2	8.1	14.7	XP_018636607.1	24.2	36.6
PIG-B	IPR039521	GPI mannosyltransferase 3	NP_011373.1	9.7	17.1	XP_018638410.1	20.8	29.4
PIG-K	IPR028361	GPI-anchor transamidase	NP_010618.1	25.6	33.9	XP_002369352.1	29.8	34.0
GAA1	IPR007246	GPI transamidase component Gaa1	NP_013189.1	4.7	8.6	XP_018638017.1	8.5	11.2
PIG-U	IPR009600	GPI transamidase subunit PIG-U	NP_013564.1	13.5	30.6	XP_018635164.1	33.7	42.8

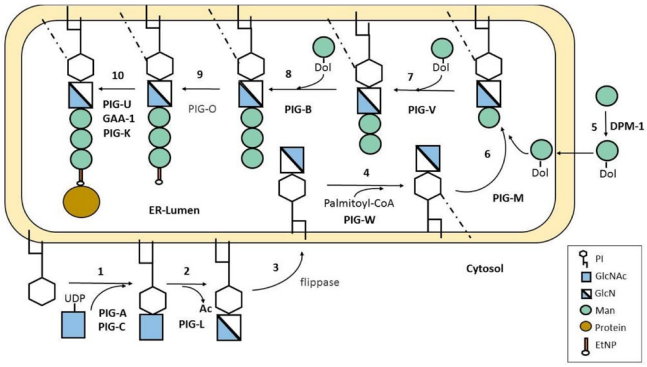

FIGURE 2 Predicted GPI biosynthetic pathway of *Sarcocystis aucheniae*. All enzymes shown in bold characters were found in the transcriptome database. First, N-acetylglucosamine (NAcGlc) is transferred to phosphatidylinositol (PI) in the cytoplasmic side of the rough endoplasmic reticulum (RER) by PIG-A and PIG-C, forming NAcGlc-PI. Then, de-N-acetylation of NAcGlc-PI, catalysed by PIG-L, generates GlcN-PI, which is translocated to the RER luminal side by an uncharacterized flippase, not identified in this study (shown in grey). There, the inositol moiety is acylated by PIG-W generating acylated GlcN-PI. Next, three mannose residues (Man) are added to the acylated GlcN-PI, catalysed by the sequential action of PIG-M, PIG-V and PIG-B. These enzymes use Man-dolichol-P as Man donor, synthesized by DPM1. An ethanolamine-phosphate (EtNP) residue is then transferred to the third Man, and this process is catalysed by PIG-O in other organisms (not found in this study, shown in grey). Finally, a complex formed by GAA1, PIG-K and PIG-U catalyzes the transfer of a nascent protein to the GPI molecule [Colour figure can be viewed at wileyonlinelibrary.com]

(cl22856) which, in higher eukaryotes, have been described to intervene in cell fusion events (Hernandez & Podbilewicz, 2017).

The remaining 13 *S. aucheniae* GPI-APs were classified as SRS proteins since they carry one or two SRS domains as identified by Pfam (PF04092) and InterPro (IPR007226) (SaGPI-AP12 to 18), or exclusively by InterPro (SaGPI-AP19 to 24).

Neighbour-joining analysis of the Pfam SRS domain of SaGPI-AP12 to SaGPI-AP18, together with those of SRS proteins of other coccidians, is shown in Figure 5. It can be observed that *S. aucheniae* sequences SaGPI-AP12, 13, 15, 16 and 17 segregate into a single clade supported with a moderate bootstrap of 58, forming a 5-member family (cluster 1). Of these, two pairs of related proteins, SaGPI-AP16 and 17, and SaGPI-13 and 15, grouped with a very high bootstrap. SaGPI-AP14 segregates with the SRS protein SRCN_3434 from *S. neurona*, isolate SOSN1, in a single clade with a low bootstrap (cluster 2). On the other hand, *S. aucheniae* SaGPI-AP18 showed no relationship with any other sequences of the SRS proteins included in the tree.

4 | DISCUSSION

Sarcocystosis is a neglected disease that affects SAC meat consumption and commercialization, causing significant economic loss and hampering an incipient regional economy. In Bolivia, sarcocystosis has been estimated to cause in average an up to 20% reduction of the yearly income of owners when llama meat is sold through an abattoir (Rooney, Limon, Vides, Cortez, & Guitian, 2014).

Most aspects of the life cycle of *S. aucheniae*, the causative agent of sarcocystosis, are still unknown. Control tools are unavailable. A sensitive and specific molecular diagnostic method applicable to live animals was recently developed, but results of this test are not predictive for the presence or absence of cysts in muscles (Decker Franco, Romero, et al., 2018). The aim of the present work was to identify relevant *S. aucheniae* proteins that act at the host–pathogen interface through transcriptome sequencing and in silico analyses. In particular, we focused on proteins

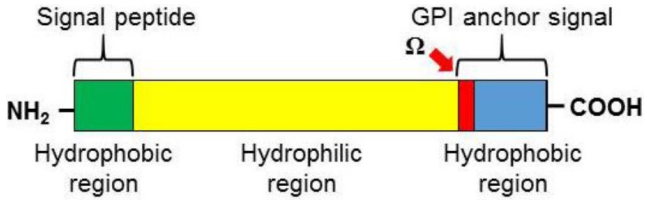

FIGURE 3 Topology of a generic GPI-anchored protein. Particular features include hydrophobic N and C termini, a signal peptide in the N-terminal, a GPI anchor signal with a C-terminal omega site where cleavage takes place, and a hydrophilic core with no additional transmembrane domains [Colour figure can be viewed at wileyonlinelibrary.com]

related to GPI, either participating in its biosynthesis or using it as membrane anchor, because of their potential relevance as targets for therapies, vaccines and/or diagnosis (Kinoshita & Fujita, 2016).

Since protocols for the in vitro cultivation of different parasite stages have not yet been developed, the bradyzoite stage contained within macroscopic cysts was chosen as a source of abundant and essentially host-free parasite RNA.

In silico analysis of the sequenced transcriptome allowed to obtain a database of *S. aucheniae* hypothetical proteins. This database does not directly correspond to the genome or predicted proteome of the parasite, due to repetition of sequences, alternative splicing and/or stage-related differential gene expression. The sequencing of the *S. aucheniae* genome, which is currently underway in our laboratory, will complement and enhance these studies. Nevertheless, searches in the collection of transcripts identified several enzymes implicated in the biosynthesis of GPI, as well as an array of predicted GPI-APs.

GPIs are synthesized in the rough endoplasmic reticulum (RER) with the participation of several housekeeping enzymes that sequentially add the different molecule components. GPIs have a common basic structure, characterized by the presence of glucosamine, bound in position 1 to myo-inositol and in position 4 to the first of a chain of usually three mannose residues. Furthermore, a phospholipid is linked to the C-1 hydroxyl group of myo-inositol. In protein anchors, the third mannose is conjugated, through a phosphoethanolamine residue, to the C-terminal end of a nascent protein that is inserted in the RER membrane via a signal peptide. This basic GPI structure presents some variations among different species. Changes are found in the number of mannose and phosphoethanolamine residues, the presence/absence of other sugars than mannose, the presence/absence of an acyl chain in the inositol moiety and the nature of the phospholipid attached to the glycan which can have a diacylglycerol or a ceramide backbone (Kinoshita & Fujita, 2016). This variety is directly connected to the repertoire of GPI biosynthetic pathway enzymes encoded in each genome, but in spite of structure polymorphisms, GPIs have common functions, including the formation of protective coats, host cell adhesion, recognition and invasion of host cells, transport and signalling (Mehlert & Ferguson, 2009).

In this study, we analysed whether transcripts of genes encoding GPI biosynthetic enzymes could be identified in *S. aucheniae* bradyzoites. Searches in the transcriptome using *S. cerevisiae* and *T. gondii* genes as queries allowed the identification of the *S. aucheniae* enzymes implicated in the generation of a GPI molecule containing 3 mannose residues, although this structure needs experimental validation. In addition, transcripts for three members of the transamidase complex, GAA1, PIG-K and PIG-U, could be identified. This complex catalyzes the transfer of a nascent protein to the GPI, attaching it to phosphoethanolamine, but the latter moiety first needs to be added to the last mannose in the chain by PIG-O. Although transcripts of *S. aucheniae* PIG-O could not be found in this study, the finding of GAA1, PIG-K and PIG-U transcripts strongly suggests that post-translational attachment of proteins to GPI anchors is operational in *S. aucheniae* bradyzoites. The GPI biosynthetic pathway can provide interesting targets for drug development, given the essential role of these glycolipids for parasite survival. Indeed, several molecules have been shown to block the GPI pathways of pathogenic protozoa at

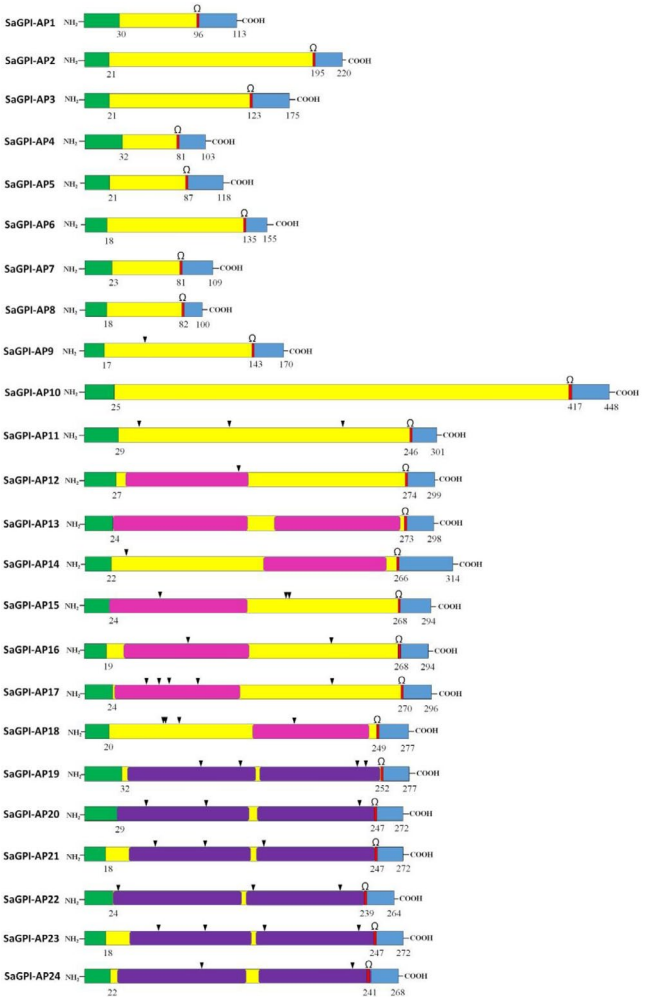

FIGURE 4 *Sarcocystis aucheniae* predicted GPI-anchored proteins. The diagram shows the sequences found by in silico searches in the *S. aucheniae* transcriptome drawn in scale according to their length in amino acids. Relevant amino acid positions are marked with numbers. The signal peptide is shown in yellow, the GPI anchor signal in blue, the omega site in red and the N-glycosylation sites with inverted triangles. Pfam SRS domains are marked in pink, and InterPro SRS signatures are marked in purple [Colour figure can be viewed at wileyonlinelibrary.com]

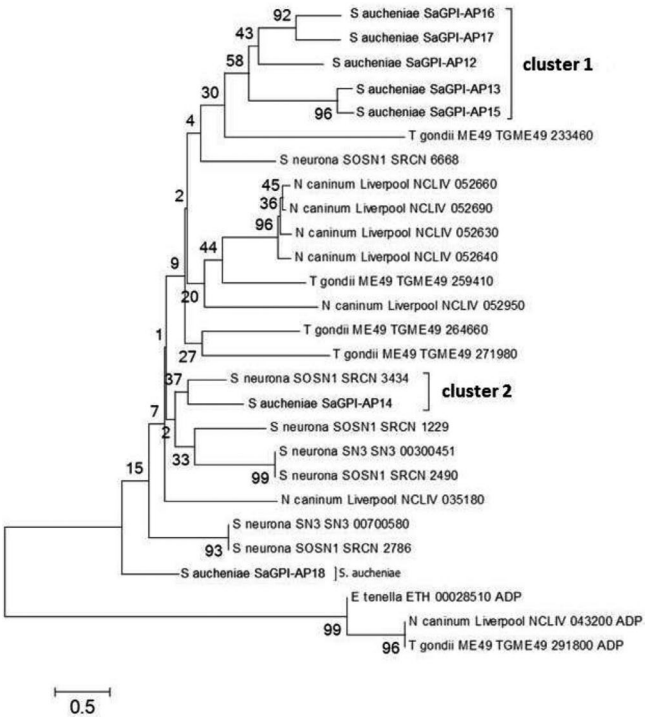

FIGURE 5 Phylogenetic analysis by neighbour joining of coccidian SRS proteins. Amino acid sequences corresponding to the Pfam SRS domain of 24 proteins of *Sarcocystis aucheniae*, *Toxoplasma gondii* strain ME49, *S. neurona* strains SN3 and SOSN1 and *N. caninum* strain Liverpool were included in the tree construction. ADP domain-containing proteins of *E. tenella* ETH, *Neospora caninum* Liverpool and *T gondii* ME49 were used as outgroup. The bootstrap was tested by 1,000 replicates. The evolutionary distance is given by the number of exchanged amino acids per site

different levels, in some cases in a species-specific way (de Macedo, Shams-Eldin, Smith, Schwarz, & Azzouz, 2003; Yadav & Khan, 2018). A most promising target is the GlcNAc-*de*-acetylase, which catalyzes the removal of an acyl group from PI-GlcNAc in an early step during GPI biosynthesis (Figure 2, Step 2). This enzyme varies significantly between *Trypanosoma brucei* and humans. Thus, synthetic GlcNAc-PI analogues, recognized by the parasite enzyme but not by its human counterpart, have been shown to be potent specific parasite suicide inhibitors (Smith et al., 2001). Also, salicylic hydroxamic acid (SHAM) was proven to be a non-substrate analog inhibitor specifically acting on the trypanosomal deacetylase (Urbaniak et al., 2014).

In the present study, transcripts for 24 GPI-APs were in silico identified in the *S. aucheniae* transcriptome. No homologues were found for nine of them, suggesting that they could be *S. aucheniae*-specific. Since GPI-anchored proteins are often immunodominant in pathogenic protozoa, these proteins are of particular interest for further exploration as diagnostic antigens (Dangoudoubiyam et al., 2011; Dominguez et al., 2012). Predicted B-cell epitopes were found in all 24 SaGPI-APs (data not shown). Thus, subsequent studies will concentrate on evaluating the usefulness of the above-mentioned GPI-anchored proteins and/or B-cell epitope-bearing peptides, in the development of serological tests.

Thirteen of the identified GPI-APs contain a conserved Pfam SRS domain and/or an InterPro SRS signature, which places them among the SRS superfamily. The SRS domain was first described for *T. gondii* SAG1, the prototypic SRS family member in this coccidian, and contains several cysteine residues that lead to disulphide bridge formation. SRS proteins can have one or two SRS domains, which can be located in the N or C-terminal region (He, Grigg, Boothroyd, & Garcia, 2002). *T. gondii* SRS proteins have been implicated in receptor–ligand interactions that promote host cell attachment and in the stimulation of immune responses during infection (Jones, Korcsmaros, & Cardinga, 2017). Indeed, monoclonal and polyclonal antibodies directed to SAG1 inhibited invasion of cells by tachyzoites, probably by interfering with parasite attachment (Jacquet et al., 2001). *T. gondii* tachyzoite mutants lacking SAG3 showed less virulence in vivo than wildtype parasites, indicating that this protein could be involved in mechanisms of pathogenicity (Jacquet et al., 2001; Rachinel et al., 2004; Tomavo, 1996). Importantly, native and recombinant *T. gondii* SAG1 preparations stimulated host humoral and cellular immunity and provided protection against challenge (Wang & Yin, 2014).

Genomic analysis showed that *T. gondii* and *N. caninum* contain 109 and 246 SRS protein-encoding genes, respectively, many times organized as tandemly-arranged sets of paralogs (Reid et al., 2012). In the case of *S. neurona*, a smaller set of 23 SRS genes was recently identified in its genome (Blazejewski et al., 2015). Expression of SRS genes of *S. neurona* and *T gondii* is differentially regulated according to the parasitic stage, suggesting that changes at the surface antigen level could be essential to complete the life cycles of these coccidians (Crowdus et al., 2008; Howe et al., 2005; Gautam, Dubey, Saville, & Howe, 2011). Since only the bradyzoite stage of *S. aucheniae* was analysed in our study, it can be speculated that additional SRS members are expressed in other parasite stages. It can also be hypothesized that these proteins fulfil vital roles in *S. aucheniae* as has been observed for other coccidia.

Phylogenetic analysis of SRS proteins of *S. aucheniae* and other coccidians supports the notion that this is an abundant family of intra- and inter-species divergent proteins, as indicated by Blazejewski et al. (2015) when analysing the SRS repertoire of *T. gondii*, *S. neurona* and *N. caninum*. Since SRS proteins are surface-exposed, the selection pressure of the hosts' immune systems could have driven the accumulation of mutations that provoked their divergence from a common ancestor.

Prior to the exploitation of the different GPI-APs found in this study as vaccine and/or diagnostic candidates, sequence polymorphism analysis among geographic *S. aucheniae* isolates needs to be carried out. In addition, expression and immunogenicity studies need to be performed by analysing the recognition of recombinant GPI-APs or peptides bearing species-specific B-cell epitopes by *S. aucheniae* immune sera from different regions.

GPI molecules appear as key factors in pathogenic protozoan infections, both because of their importance as abundant components of the cell membrane, as well as for the anchoring of proteins of relevance for infection and physiology, and their immunoregulatory

action on the host. The present results, which are the first that elucidate protein sequences functionally highly relevant for *S. aucheniae*, will facilitate the development of tools for the control of SAC sarcocystosis.

ACKNOWLEDGMENT

Financial support from Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT), Argentina (PICT start up 2014-3747) and doctoral fellowships from ANPCyT (SNW) and CONICET (CDF) are gratefully acknowledged. The authors also want to thank Med. Vet. Eleuterio Choque López, from the Municipality of Turco, Bolivia, for providing cyst samples.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

ETHICAL APPROVAL

The cysts of *Sarcocystis aucheniae* analysed in this study were obtained during slaughtering of a llama in an abbatoir of the locality of Turco, Oruro, Bolivia. Llama slaughtering at this abbatoir has been authorized by the National Service of Agricultural Health and Food Safety of Bolivia (SENASAG), and activities are carried out under criteria of good practices of livestock management, as recommended by SENASAG.

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How to cite this article: Decker Franco C, Wieser SN, Soria M, de Alba P, Florin-Christensen M, Schnittger L. In silico identification of immunotherapeutic and diagnostic targets in the glycosylphosphatidylinositol metabolism of the coccidian *Sarcocystis aucheniae*. *Transbound Emerg Dis*. 2020;67(Suppl. 2):165–174. https://doi.org/10.1111/tbed.13438