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The *Aotus nancymaae* erythrocyte proteome and its importance for biomedical research

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ABSTRACT

The *Aotus nancymaae* species has been of great importance in researching the biology and pathogenesis of malaria, particularly for studying *Plasmodium* molecules for including them in effective vaccines against such microorganism. In spite of the forgoing, there has been no report to date describing the biology of parasite target cells in primates or their biomedical importance. This study was thus designed to analyse *A. nancymaae* erythrocyte protein composition using MS data collected during a previous study aimed at characterising the *Plasmodium vivax* proteome and published in the pertinent literature. Most peptides identified were similar to those belonging to 1189 *Homo sapiens* molecules; >95% of them had orthologues in New World primates. GO terms revealed a correlation between categories having the greatest amount of proteins and vital cell function. Integral membrane molecules were also identified which could be possible receptors facilitating interaction with *Plasmodium* species. The *A. nancymaae* erythrocyte proteome is described here for the first time, as a starting point for more in-depth/extensive studies. The data reported represents a source of invaluable information for laboratories interested in carrying out basic and applied biomedical investigation studies which involve using this primate.
Significance: An understanding of the proteomics characteristics of *A. nancymaae* erythrocytes represents a fascinating area for research regarding the study of the pathogenesis of malaria since these are the main target for *Plasmodium* invasion. However, and even though *Aotus* is one of the non-human primate models considered most appropriate for biomedical research, knowledge of its proteome, particularly its erythrocytes, remains unknown. According to the above and bearing in mind the lack of information about the *A. nancymaae* species genome and transcriptome, this study involved a search for primate proteins for comparing their MS/MS spectra with the available information for *Homo sapiens*. The great similarity found between the primate's molecules and those for humans supported the use of the monkeys or their cells for continuing assays involved in studying malaria. Integral membrane receptors used by *Plasmodium* for invading cells were also found; this required timely characterisation for evaluating their therapeutic role. The list of erythrocyte protein composition reported here represents a useful source of basic knowledge for advancing biomedical investigation in this field.

1. Introduction

Animal research has been essential for understanding and studying some human diseases, particularly those having the greatest impact around the world, such as malaria. For example, using rodents (BALB/c, C57BL/6, NOD/SCID or humanised strains) has led to obtaining valuable information about this parasite pathogenesis [1,2]. Rodent parasite species (*Plasmodium chabaudi, Plasmodium vinckei, Plasmodium berghei and Plasmodium yoelii*) are different to those infecting humans (*Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale* and *Plasmodium knowlesi*), therefore having differences regarding their biology and immune response [3]; this means that extrapolating such studies in humans is not always reliable.

Non-human primates represent another model; they have been shown to be the most suitable for studying pathogenesis, immunology and anti-malarial vaccine development, given that they are genetically and immunologically more similar to humans [1]. It is worth noting that some of these primates (mainly *Saimiri* sp. [4,5] and *Aotus* sp. [6,7]) have been widely used in basic and applied biomedical research.

Aotus spp. has been used since its susceptibility to experimental infection by parasites from the genus *Plasmodium* was shown in the

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1960s [8]; several parasite strains have been adapted since then in this model for studying malaria and developing possible pharmacological treatments or vaccines [9]. Within the genus, the *Aotus nancymaae* species has been infected with different *Plasmodium* strains (*P. falciparum*: Santa Lucia, Indochina I/CDC and Uganda Palo Alto strains; *P. vivax*: Chesson, ONG, Vietnam Palo Alto, Salvador I and Honduran I/CDC; *P. malariae*: Uganda I/CDC), as reported by Collins and his group several years ago [10].

The *Aotus* species has led to an enormous advance regarding preclinical studies highlighting the immunological and protective role of various molecules or parts of them from the *P. falciparum* FVO strain; taking into account that the complex machinery involved in erythrocyte invasion used by this parasite for infecting cells is partly known today, the *Aotus* model has been essential for describing the fundamental basis when identifying vaccine components against this parasite species [11,12]. On the other hand, these primates develop very reproducible infection following experimental infection with the *P. vivax* VCG-1 (Vivax Colombia Guaviare-1) strain, having high levels of parasitaemia (>5%) after 22 passages [13]; this has been of great importance for advancing molecular (MSP-7, Pv38, RAP-1 and RBP-1 between others) [14–17] and immunological (MSP-1_{20} and MSP-1_{14} from MSP-1_{33} fragment) [18,19] characterisation studies of some molecules from the *P. vivax* species and evaluating their usefulness in developing an effective vaccine. These findings highlight the fact that using *A. nancymaae* in combination with the *P. falciparum* FVO or *P. vivax* VCG-1 strains is valuable for screening suitable vaccine candidates for later testing in humans.

In spite of *A. nancymaae* species having led to a promising advance in developing an anti-malarial vaccine, the biology of its erythrocytes still remains unknown (these being vital hosts for *Plasmodium*). Most studies have focused on establishing the similarity between primate and human genes encoding proteins related to the immune response [20–22]. The revolution in omic sciences represented by Baylor College of Medicine's Human Genome Sequencing Centre (BCM-HGSC) has led to the genome and transcriptome of species being studied through the Owl Monkey Genome Project. However, no study describing primate protein composition has been carried out to date. Taking the importance of studying *A. nancymaae* erythrocytes into account, our group was thus interested in obtaining the greatest amount of information possible about the proteome of these cells using data obtained from a previous study by our research group [23] and evaluating it in terms of protein composition and function.

2. Material and methods

2.1. Reanalysing proteome data

Tandem mass spectrometry (MS/MS) data came from a sample consisting of a mixture of mature erythrocytes and reticulocytes (*P. vivax* infected and non-infected ones); two samples had a 50:1 ratio (mature erythrocytes:reticulocytes) whilst the other had a 1.11:1 ratio as it had previously been subjected to a Percoll gradient to enrich infected reticulocytes (preferential invasion target for *P. vivax*) [23]. Data were used for searching for similar peptides, using the human proteome reported in the UniProt database [24]. Mascot [25] and SEQUEST algorithms [26] and Thermo Scientific Proteome Discoverer software were used with astringent search parameters. In brief, the most recent UniProt *H. sapiens* (AUP000005640), *P. vivax* (AUP000008333) and *P. falciparum* (AUP000001450) proteomes were used for compiling a FASTA file containing common non-human contaminants (trypsin, Lys-C and BSA). Thermo's Proteome Discoverer (version 1.4.0.288) was then used for analysing each file in batch and in MudPit [27] for replicates from the same sample; the latter led to identifying low quantity proteins. Results having a <0.01 (high confidence) q-value were filtered using a Mascot Score threshold above 20 and 1.5, 2.0, 2.25, 2.5, 2.75, 3, 3.2, 3.4 for SEQUEST HT (XCorr) for charge states from 1 to 7 and from 3.4 for values >7. An Excel file was generated for each filter showing protein identification details (accession code, description and coverage), including all scores and identified peptides. Redundant UniProt access codes were manually eliminated so that the total list of molecules identified here could be reported.

2.2. Searching orthologous genes in New World monkeys

The search strategy for *H. sapiens* orthologous molecules with New World primates involved using the biological DataBase network (bioDBnet) [28], an online web resource enabling the search for orthologue identifiers in different species. UniProt accession codes from *A. nancymaae-H. sapiens* analysis were used for searching for orthologous molecules in *Callithrix jacchus*, the only species from the primate family phylogenetically related to *Aotus* for which proteome data is available to date. Molecules identified as non-orthologous were analysed again using the OrthoDB database [29].

2.3. Identifying erythrocyte proteins

The proteins identified here were compared to the most extensive profiling of human erythrocyte RNAs published to date [30]. UniProt accession codes were converted into Ensembl gene ID codes with bioDBnet [28] and then compared to 8092 genes expressed as a >0.5 threshold according to an erythrocyte transcriptome study [30].

2.4. Protein annotation according to gene ontology terms

Gene ontology (GO) annotations available in the UniProt database were analysed using the Software Tool for Rapid Annotation of Proteins (STRAP, version 1.5) [31], developed by Boston University School of Medicine's Cardiovascular Proteomics Centre (Boston, MA). The National Institute of Allergy and Infectious Diseases (NIAID) Database for Annotation, Visualization, and Integrated Discovery (DAVID) [32,33] was also used for categorising molecules according to GO terms; stringent parameters were used to ensure statistical significance (thresholds: EASE value = 0.001 and Count = 2).

2.5. Predicting cell membrane molecules

The Red Blood Cell Collection (RBCC) database was used for predicting Surface molecules; RBCC integrates the proteome of human RBC proteins identified to date [34]. The search parameters involved being a highly confident match in both hRBCD and BSc_CH, a blood group or CD marker, experimentally tested in the Sarkadi-lab. The UniProt accession codes for each protein so identified were then manually downloaded for compiling the supplementary table.

3. Results

3.1. Aotus protein prediction

The flow chart in Fig. 1 shows how *A. nancymaae* proteins were identified and analysed. *A. nancymaae* mass spectra were initially imported in Proteome Discoverer software and compared to the information available for the *H. sapiens* proteome by data Mascot and SEQUEST search algorithms; 1084 fully-tryptic and 1052 semi-tryptic molecules were identified using digestion search parameters; 901 were recognised by both parameters, whilst 183 fully-tryptic and 151 semi-tryptic ones were only recognised by one parameter (Supplementary data 1). Primate peptides had great similarity with those from humans, representing 1189 molecules (SD 2). The bioDBnet and OrthoDB tools were used for confirming *A. nancymaae* proteins by comparing the orthology of molecules found in this study with the available information regarding New World monkey proteomes in the UniProt database (Fig. 1). It was found that 95.7% (1138 proteins) of the proteins

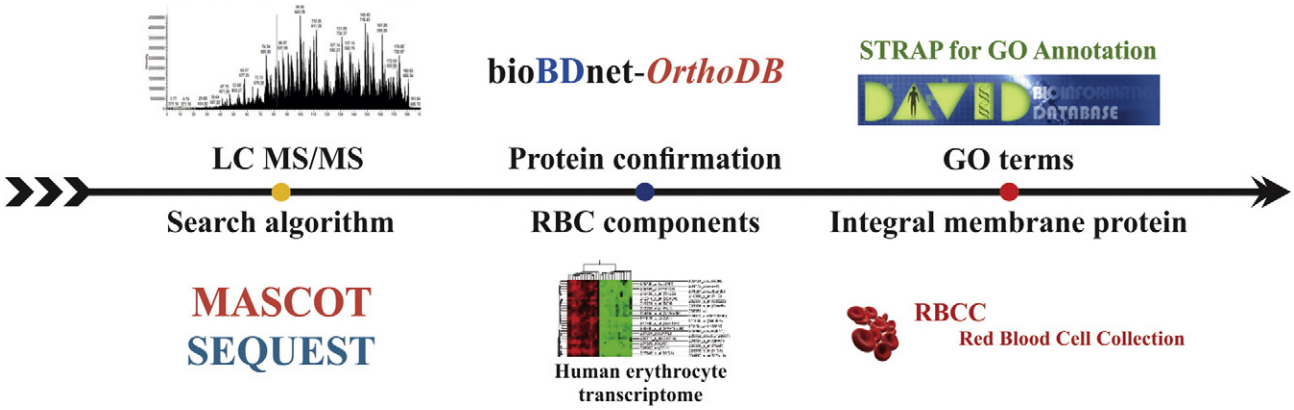

Fig. 1. A flow chart showing the analysis for identifying and confirming *A. nancymaae* erythrocyte proteins.

identified had orthologues in New World primates whilst 4.3% (51 molecules) of them did not (SD3); they were therefore excluded from further analysis.

3.2. Specific erythrocyte proteins

Aotus RBC proteins were discriminated by comparing the transcriptome data reported for human erythrocytes (Fig. 1) [30]. According to such analysis, 811 *A. nancymaae* proteins were typical of erythrocytes, given the evidence of transcription found (SD4). There was no evidence of transcription for 327 proteins which might have been due to most encoding genes (222) not complying with transcription inclusion criteria used in this study (RPKM >0.5). Furthermore, some proteins may have had reduced transcription, this being typical during erythrocyte maturation. Other proteins were associated with cell receptors (i.e. immune components (C3b (P01024)/C4b (P04003)) or proteins from serum (serum albumin (A0A087WWT3)) (SD4, no transcription evidence sheet) which are non-typical for RBC and hence do not contain an encoding gene. Transcriptome analysis of *A. nancymaae* erythrocytes is required to validate such hypothesis.

As expected, key proteins in gas exchange were identified (SD4), such as carbonic anhydrase 1 (E5RII2) and 2 (P00918), aquaporin-1 (P29972) and β-globin (P68871) as well as other subunits from the major cytoplasmic components (haemoglobin alpha (P69905), gamma (E9PBW4), delta (P02042), zeta (P02008), theta (P09105) and mu (Q6B0K9) chains). Proteins comprising the ankyrin (ankyrin (P16157), band3 (P02730), Rh (P18577) and 4.2 (P16452) proteins) or 4.1R (band 4.1R (P11171), glycophorin C (P04921), the Kx protein (P51811), dematin (Q08495), adducin (alpha (P35611) beta (P35612) and gamma (Q9UEY8) chains), tropomodulin-1 (P28289) and several tropomyosin proteins) complexes, together with other molecules which are essential for maintaining erythrocyte structure and function (such as spectrin alpha (P02549) and beta (P11277) chains) were also detected (SD4, marked by asterisks).

3.3. Analysis of functional distribution

STRAP was used for scanning the subcellular localisation and molecular and biological functions for the 811 proteins for which there was evidence of transcription (Fig. 1) [31]. Exploring the known functions reported in UniProt revealed that the proteins identified in this study participated in the following biological processes: cellular, developmental, immune system, interaction with cells and organisms, localisation, metabolic, regulation and response to biological stimuli (Table 1). Regarding molecular function terms, most were implicated in binding function (439 molecules) or were associated with catalytic activity (326 molecules) whilst fewer were known for having a role in antioxidant activity (10 molecules), molecular transduction (9 molecules) and molecular structure (36 molecules) (Fig. 2A). Regarding cell localisation, some were extracellular, cytoplasm, nucleus and/or membrane components (Fig. 2B).

DAVID software was used for analysing GO term enrichment [32,33] in the search for the most relevant groups having statistical significance (Fig. 1). Regarding the 477 molecules identified by the software, the functional annotation chart showed that the commonest biological process (BP) terms determined were as follows: establishing localisation (175 molecules, $p = 1.8E^{-25}$), transport (173 molecules, $p = 4.9E^{-25}$), localisation (180 molecules, $p = 1.1E^{-21}$), generating precursor metabolites and energy (48 molecules, $p = 1E^{-19}$) and ATP metabolic processes (27 molecules, $p = 7.9E^{-17}$) (SD5). Regarding molecular function (MF), 77 were related to nucleoside-triphosphatase, pyrophosphatase or hydrolase activity ($p = 1.3E^{-22}$ to $1.8E^{-21}$), 38 to GTPase activity ($p = 1.1E^{-18}$), 325 to protein binding ($p = 2.1E^{-16}$) and 237 to catalytic activity ($p = 2, 9E^{-16}$) (SD5). Most proteins in the cell component (CC) category were intracellular, the most common ones being cytoplasm, mitochondria or organelle envelop (SD5). In spite of similarity between some terms stated by STRAP and DAVID software (e.g. BP: localisation, transport and metabolic processes; MF: binding and catalytic activity; CC: cytoplasm and mitochondria), there was discrepancy regarding the number of molecules comprising them, probably due to the different training sets and statistical analysis used by each tool. Hence, and because of the databases' dynamic nature, the analysis reported here cannot be considered as absolute.

3.4. Predicting integral membrane or surface-associated proteins

The RBCC database was used for predicting cell surface protein localisation, aimed at determining cellular receptors which could

Table 1
Proteins involved in biological processes.

Biological function	Number of proteins
Cellular	429
Developmental	104
Immune system	51
Interaction with cells and organisms	91
Localisation	190
Metabolic	77
Regulation	354
Response to stimulus	110
Other function	234

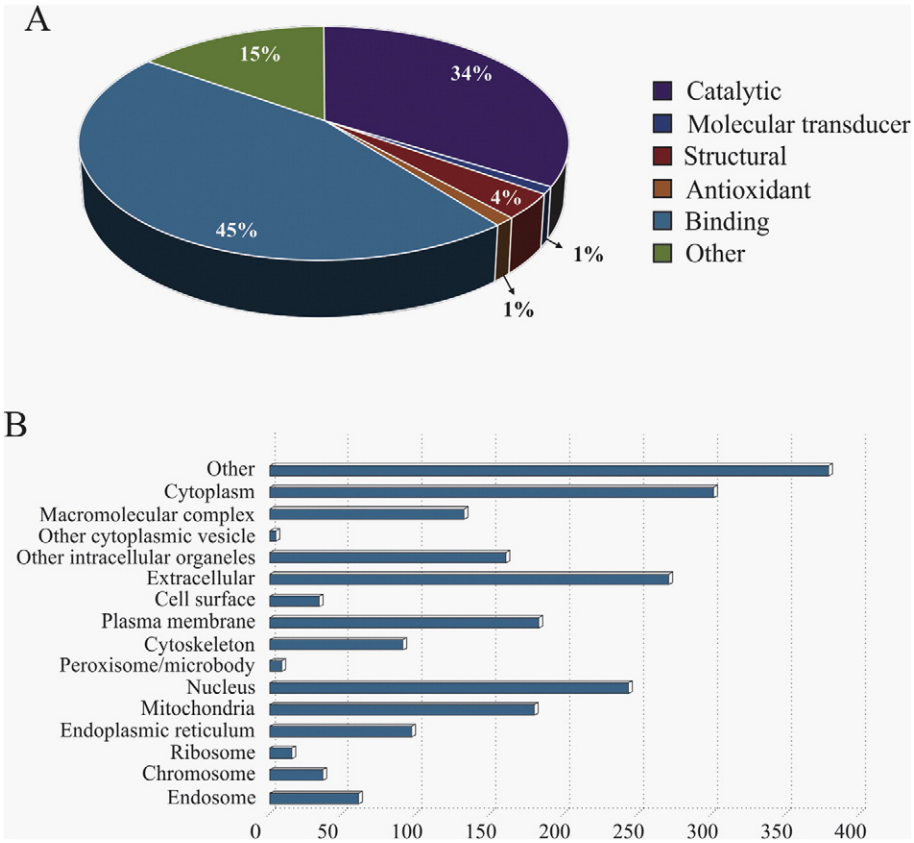

Fig. 2. In silico prediction of biological processes for *Aotus* proteins identified here. A) Distribution of proteins involved in molecular function. B) A bar chart showing protein distribution according to cell location.

determine infection by *Plasmodium* species (Fig. 1) [34]; 155 molecules were identified, 51 of which were annotated as integral membrane proteins and 54 as being membrane-associated (SD6). Receptors having a known *P. falciparum* and *P. vivax* binding pattern were found, such as glycophorin C (*Pf*EBA140 receptor [35]), band 3 (*Pf*MSP-1 [36], *Pf*MSP-9 [37] and *Pv*TRAg38 receptor [38]) and Kx (*Pf*AMA1 receptor [39]), and one having an unknown binding pattern (as its absence reduced parasite invasion of cells) such as that for ICAM-4 [40] (Fig. 3). Other receptors used by Plasmodia, such as the CR1 (*Pf*Rh4 receptor [41]), basigin (*Pf*Rh5 receptor [36]) and CD44 [42], were found manually, as

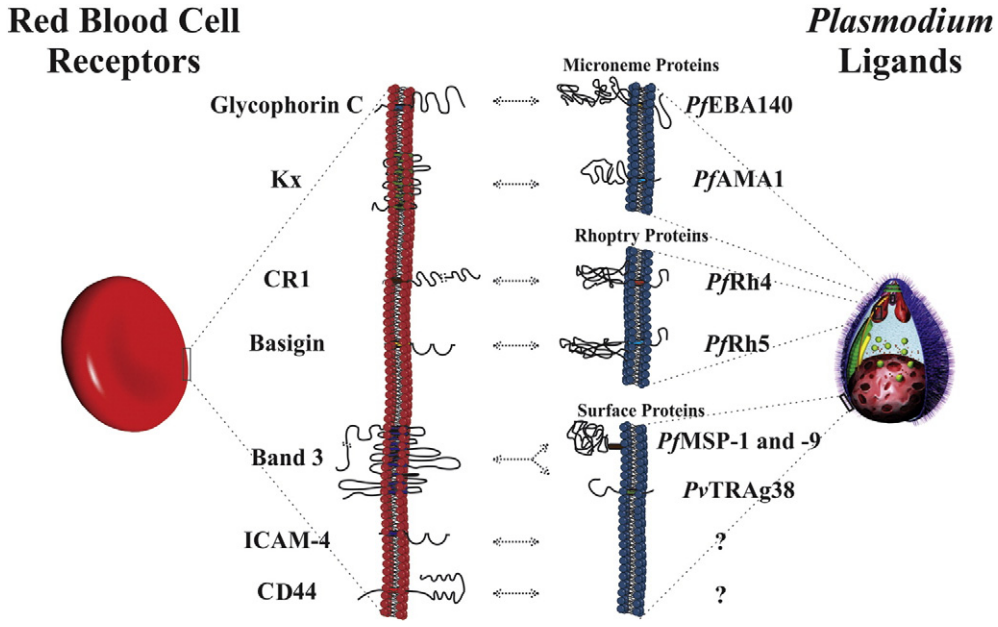

Fig. 3. A schematic representation showing integral membrane proteins identified here which have been experimentally validated (in humans) as erythrocyte receptors (glycophorin C, band3, Kx, CR1, basigin, ICAM-4 and CD44) and their respective ligands in *Plasmodium* (*Pf*EBA140, *Pf*MSP-1, *Pf*MSP-9, *Pv*TRAg38, *Pf*AMA1-II, *Pf*RH4 and -5).

their prediction by the RBCC database was not accurate due to the codes used by such databases (e.g. CR1 was P17927, basigin was P35613, CD44 was P16070).

4. Discussion

Given *A. nancymaae* species' experimental importance, this study was focused on an analysis based on the similarity of MS/MS spectra with those for *H. sapiens*; the aim was a comparative identification of new proteins, taking advantage of the additional data available, collected during a previous study [23]. Such comparison revealed similarity between both species for various peptides; this was reasonable due to phylogenetic closeness between primates and humans (SD1 and SD2). Such finding supported the fact that the primates' proteome being acquired from the search in human databases is an acceptable search strategy, as shown recently [43]. On the other hand, the relatively low amount of identified *Aotus* proteins (1189 molecules) could have resulted from using *P. vivax* infected samples as a source for the analysis. We must highlight that our original goal was to characterise the *P. vivax* proteome [23] and for such purpose, samples were processed to remove most biological "contaminants" from RBC; this resulted in a greater abundance of parasite proteins masking *Aotus* peptide detection.

The search for orthologues in New World primates using bioDBnet and OrthoDB databases led to confirming 1138 proteins (95%) (SD3); this approach was used as *A. nancymaae* genome or transcriptome data had not been released. Some of them were just identified by one database, possibly due to differences between their mapping algorithms. Unlike bioDBnet, which allowed comparison with *C. jacchus* (the only species from the family having its proteome available at the time), OrthoDB has recently been shown to have the advantage of making predictions using some *A. nancymaae* data. However, it is not practical to analyse a whole data set one-by-one because this requires a tremendous amount of time for processing it.

A filter based on transcriptome analysis was applied for determining the *Aotus* erythrocyte proteome, once the sample used for the initial MS/MS analysis consisted of erythrocytes and reticulocytes [23]. It was found that 811 molecules were mature erythrocyte components, as determined by transcript evidence for each of them (SD4). The attempt to identify genuine reticulocyte components proved unsuccessful as this cell, just like mature RBC, transcribed all genes encoding the proteins identified here (data not shown). The forgoing has been supported by a recent study showing that mature erythrocytes and reticulocytes consist of the same molecules but differ in abundance [44]. Considering the 811 molecules as erythrocyte components, these were compared to a list of the 4135 *H. sapiens* RBC molecules identified to date by MS/MS [44–56]; evidence was found regarding 403 of them (SD4, accession codes shown in bold).

Various molecules were identified here (SD4) which are abundantly expressed in reticulocytes and then become reduced (integrin β1 (P05556), some ribosome subunits, sodium/potassium-dependent ATPase and tubulin subunits) or completely lost as cells matured (such as transferrin receptor 1 (P02786)). This was to be expected given that the sample was heterogeneous and such cell types were not eliminated for initial analysis [23]. Likewise, even though the erythrocytes were devoid of organelles, some of their components were found, possibly because they are cell remains which should have become removed as they matured [57]. Complement receptor 1 (CR1) was found, possibly playing the same role in immune-adherence clearance as described in a previous study [58]. The forgoing was justified, since MS/MS spectra for immune components such as C3b and C4b were also found in the initial list of molecules (SD2).

Interestingly, several cytoplasm and cytoskeleton molecules which are very important for maintaining cell integrity [59] were also found (SD4, marked by asterisks). Given that Plasmodium infection affects cell morphology during invasion and growth [60,61] and several of such molecules could be affected during parasite infection and host cell remodelling, it would be interesting to compare primate proteomes of uninfected versus infected red blood cells in future studies.

Analysis of GO term enrichment revealed a correlation between biological processes formed by most proteins and vital cell functions (SD5). For example, molecule localisation and transport are essential for correct cell structure organisation; various proteins participate in this as they are implicated in macromolecular complex formation, such as ankyrin and 4.1R, others forming the cytoskeleton and some responsible for phospholipid transport (such as flippases and scramblases) whose function is to maintain membrane stability and integrity [59]. Important proteins were also found regarding precursor metabolite and energy generation which is very essential for maintaining a number of vital cell functions, such as metabolism [62]. Likewise, there was clear correlation between molecular function and cell viability; proteins implicated in nucleoside-triphosphatase, hydrolase and GTPase activity could be participating in ubiquitin-dependent cell degradation which is essential for a cell's correct maturation, as already established [46]. The amount of molecules grouped in terms of cell localisation was quite significant, coinciding with proteins grouped in terms of major significance in biological processes and molecular function.

It is well-known that RBC membrane proteins have functional heterogeneity since they act as transport molecules and also as cell adhesion and interaction proteins [59]. Such proteins are of interest in the biomedical field, given that some pathogens such as those from the genus *Plasmodium* use them as binding receptors to interact with cells [63,64]. For example, proteins having sialic acid (SA), such as glycophorin A, B or C, are receptors for proteins from the *P. falciparum* erythrocyte binding ligand (EBL) family whilst those lacking SA, such as the CR1, basigin, semaphorin, band 3 and Kx, facilitate the adhesion of proteins from the reticulocyte-binding-like protein homologue (RH) family. This gives rise to the observation that *P. falciparum* can bind to RBC using SA-dependent or -independent pathways [65,66]. Regarding *P. vivax* infection, it has been described that the parasite only invades reticulocytes and one of the few receptors described so far is the Duffy antigen receptor for chemokines (DARC) [67].

Concerning the 51 integral membrane proteins identified by the RBCC database, 5 receptors previously reported for *P. falciparum* were found [63]: one receptor from the SA-dependent pathway (GPC) and four from the SA-independent route (CR1, basigin, band3 and Kx). Band3 has recently been identified as a receptor for a *P. vivax* molecule belonging to the tryptophan-rich protein family [38]. DARC was not identified in this study, possibly due to it being a protein whose peptides generated by digestion with trypsin are long and are thus not detected by MS [68]. The fact that these integral membrane components are Plasmodium receptors (as experimentally validated on humans) suggested that the remaining 46 identified here might also be essential for or be implicated in parasite entry to cells. Further research should thus involve assays aimed at confirming which of them are determinants regarding the pathogenesis of malaria. Identifying other receptors and a complete understanding of erythrocyte-parasite interactions represents a key area for research and, in the future, developing a rational therapeutic method allowing malarial infection to be controlled.

4.1. Concluding remarks and perspectives

The present study has reported the characterisation of the *A. nancymaae* erythrocyte proteome for the first time. The in silico approaches to the large datasets led to ascertaining this primate's erythrocyte proteomic profile and to interpreting its biological and molecular characteristics. Combining the data gathered here with the early release of *A. nancymaae* genomics and transcriptomics data provided by the Baylor College of Medicine's Human Genome Sequencing Centre (BCM-HGSC) will be enormously useful for strengthening bioinformatics analyses.

Integral membrane proteins were amongst the most important receptors described here, some of which have been shown to be involved

in *P. falciparum* merozoite invasion of human erythrocytes (e.g. glycophorin C, Kx, CR1, basigin, Band3, ICAM-4 and CD44). The *P. vivax* invasion pathway depends on Duffy antigen interaction with the DBP ligand [67]. Earlier studies have described the presence of this pathway in the *A. nancymaae* primate [69], suggesting that some integral membrane proteins identified here may also be involved in such host-parasite interactions; further receptor-ligand interaction studies are thus needed to validate and fully describe the complex invasion process led by *P. vivax* parasites in invading monkey erythrocytes. These findings will further highlight *Aotus* primates as an appropriate model for basic and applied biomedical investigation.

Supplementary data to this article can be found online at doi:10.1016/j.jprot.2016.10.018.

Transparency document

The Transparency document, associated with this article can be found, in online version.

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