Veterinary Microbiology 232 (2019) 42–49

Contents lists available at ScienceDirect

Veterinary Microbiology

journal homepage: www.elsevier.com/locate/vetmic

Labeled quantitative mass spectrometry to study the host response during aspergillosis in the common bottlenose dolphin (*Tursiops truncatus*)

Guillaume Desoubeaux[a,b,c], Maria del Carmen Piqueras[d], Carolina Le-Bert[e], Vanessa Fravel[f], Tonya Clauss[g], Alexa J. Delaune[g], Risa Daniels[e], Eric D. Jensen[h], Jennifer E. Flower[i,1], Gregory D. Bossart[a,g], Sanjoy K. Bhattacharya[d], Carolyn Cray[a,*]

[a] University of Miami, Division of Comparative Pathology, Department of Pathology & Laboratory Medicine, Miller School of Medicine, Miami, FL, 33136, USA
[b] CHU de Tours, Service de Parasitologie, Mycologie, Médecine tropicale, 37044, Tours, France
[c] Université de Tours, CEPR - INSERM U1100 / Équipe 3, Faculté de Médecine, 37032, Tours, France
[d] University of Miami, Bascom Palmer Eye Institute, Mass Spectrometry Core Facility, Miller School of Medicine, Miami, FL, 33136, USA
[e] National Marine Mammal Foundation, San Diego, CA, 92106, USA
[f] Six Flags Discovery Kingdom, Vallejo, CA, 94589, USA
[g] Georgia Aquarium, Atlanta, GA, 30313, USA
[h] U.S. Navy Marine Mammal Program, San Diego, CA, 92152, USA
[i] Chicago Zoological Society's Brookfield Zoo, Brookfield, IL, 60513, USA

ARTICLE INFO

Keywords:
Aspergillosis
Dolphin
Interleukin
Orbitrap
Prepronociceptin
Protamine P1
Tursiops truncates

ABSTRACT

Aspergillosis is a fungal infection caused by *Aspergillus* molds that can affect both humans and animals. Despite advances in diagnostics and therapy, medical management of this disease remains difficult. Expansion of the basic knowledge regarding its pathophysiology in animals is critical to aid in the identification of new biomarkers of infection for diagnosis and therapeutic targets. For such a purpose, proteomics can be used by addressing protein changes during various disease processes. In the present study, a mass spectrometry analysis based on isobaric tagging for relative and absolute quantitation (iTRAQ®) was applied for direct identification and relative quantitation of proteins in blood collected from 32 *Aspergillus*-diseased common bottlenose dolphins (*Tursiops truncatus*, 32 samples) in comparison with blood from 55 other dolphins (55 samples from 41 clinically-normal controls and from 14 cetaceans with miscellaneous non-*Aspergillus* inflammation diseases) and ten convalescent dolphins (28 samples). Sixty-six and 40 proteins were found to be ≥2.0-fold over- and under-represented *versus* miscellaneous non-*Aspergillus* inflammatory dolphins, respectively, and most were confirmed *vs.* clinically-normal controls and convalescents. Many proteins which play a role in the adaptive immune response were identified, including MHC proteins and others involved in catalytic activity like the NADPH-ubiquinone oxido-reductases. Overall, iTRAQ® appears to be a convenient proteomic tool greatly suited for exploratory *ex vivo* studies focusing on pathophysiology. This technique should be considered as a preliminary step before validation of new diagnostic markers.

1. Introduction

Aspergillosis is an airborne fungal infection caused by saprophytic ubiquitous molds that belong to the *Aspergillus* genus (Latgé, 1999). It is responsible for several distinct respiratory diseases in both humans and animals. In marine mammals, *Aspergillus*-related disease is assumed to be rare, but it has been reported with increasing incidence: 66.7% of the 18 reported cases have been published after the year 2000 (Dagleish et al., 2008; Abdo et al., 2012). Aspergillosis can involve the lungs and brain in cetaceans (Latgé, 1999; Seyedmousavi et al., 2015). The major species responsible for infection belong to the *Fumigati* section (Lamoth, 2016), *Nigri* section and *Terrei* section in which *A. fumigatus stricto sensu (ss), A. niger ss* and *A. terreus ss* are the most frequently isolated (Balajee, 2009), respectively.

In marine mammals, and especially in dolphins, diagnostic tools are less developed than those for humans (Dagleish et al., 2008; Cassle et al., 2016). For instance, quantitative polymerase chain reaction (qPCR) has not been widely implemented in veterinary laboratories,

* Corresponding author.
E-mail address: ccray@med.miami.edu (C. Cray).
[1] Current address: Mystic Aquarium, Mystic, CT, 06355, USA.

https://doi.org/10.1016/j.vetmic.2019.03.030
Received 10 December 2018; Received in revised form 10 January 2019; Accepted 29 March 2019
0378-1135/

and advanced medical imaging (computed tomography (CT), magnetic resonance imaging (MRI)) may not be accessible. Moreover, a positive culture from respiratory specimens does not definitively diagnose a true infection as it may reflect a simple colonization of the upper airways or represent an environmental contaminant (Desoubeaux et al., 2014a). Also, as reported in humans, the sensitivity of blood cultures is very low for *Aspergillus* spp (Desoubeaux et al., 2014b). Furthermore, detection of galactomannan antigen (GM) was demonstrated to be unreliable (Desoubeaux et al., 2017a). Conversely, serologic testing by western blot has been demonstrated to accurately detect anti-*Aspergillus* antibody in dolphin blood (Desoubeaux et al., 2017a). Thus, additional and novel biomarkers for aspergillosis should be investigated to improve the basic understanding of the pathophysiology of this disease. Evaluation of biomarkers in the host response may be able to provide such information.

Within the last decade, proteomics has been largely used for addressing significant protein changes within diseased organisms or pathologic fluids (Fekkar et al., 2012; Desoubeaux et al., 2014b). Innovative mass spectrometry (MS) tools were initially developed for identification and qualitative characterization; however, some are now able to directly quantitate the relative amount of proteins identified. For instance, iTRAQ® (isobaric tags for relative and absolute quantitation) is an isobaric labeling method to identify and to determine the amount of proteins from different multiplexed sources within a single-run experiment (Ross et al., 2004; Desoubeaux et al., 2017b). This technique uses stable isotope-tagged molecules that can be covalently bonded to the N-terminus and side chain amines of trypsin-digested peptides (Bourassa et al., 2015; Desoubeaux et al., 2018). In the present study, through an original MS approach based on iTRAQ®, we attempted to bring new insight to the host protein response against *Aspergillus* in diseased common bottlenose dolphins (*Tursiops truncatus*).

2. Material and methods

2.1. Animals

2.1.1. Study population: inclusion and samples

Over 15 years, 115 blood samples were opportunistically collected during routine or clinical health assessments from 87 common bottlenose dolphins (*Tursiops truncatus*) under human care (from 2002 to 2016 (except for four samples obtained in 1991, 1993, 1993, and 1995), but 62.8% obtained over the last three years). The cetaceans were hosted in several different facilities throughout the United States of America (U.S.A.). All blood samples were centrifuged and plasma specimens were stored at -20 °C or − 80 °C prior to being utilized for MS.

No dolphins were anesthetized or euthanized for the purposes of this study. This study was conducted using archived samples collected from Six Flags Aquarium, Georgia Aquarium, Chicago Zoological Society (CZS), and US Navy marine mammals as part of their routine care. The US Navy Marine Mammal Program (MMP) houses and cares for a population of dolphins in San Diego Bay (CA, USA). The MMP is Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-accredited and adheres to the national standards of the United States Public Health Service on the Human Care and Use of Laboratory Animals and the Animal Welfare Act. Georgia Aquarium and CZS are USDA/APHIS approved and inspected facilities and members of the Association of Zoos and Aquariums and the Alliance of Marine Mammal Parks and Aquariums. Both have been awarded the Humane Conservation certification by American Humane. Biological samples for this study were taken as a part of a preventative medicine program or for medical monitoring purposes.

2.1.2. Case definitions

Aspergillosis classification in dolphins was derived from the definition utilized in human medicine (De Pauw et al., 2008; Arendrup et al., 2012; Marchetti et al., 2012). For example, the definitive diagnosis of “proven” aspergillosis was based upon histopathologic evidence (Desoubeaux et al., 2014a). The diagnosis of “probable” aspergillosis was based upon positive mycological culture in conjunction with clinical examination, imaging investigations, and any clinical signs which could corroborate the diagnosis. Since GM detection was shown to be unreliable in cetaceans, it was not considered to be a pivotal parameter for the diagnosis of “probable” infection (Desoubeaux et al., 2017a). Notably, samples obtained from dolphins with clinical signs consistent with possible aspergillosis, but with no positive mycological cultures, were excluded from the subsequent analysis, unless they had significant anti-*Aspergillus* antibody titers, *i.e.* equal or superior to 1: 512 ELISA titers (Desoubeaux et al., 2017a). Altogether, 32 dolphins (n_1) were included in the *Aspergillus*-diseased group. An additional category, referred to as convalescent, was defined for that recovered from aspergillosis, *i.e.* sampling date greater than three months after the diagnosis, if clinical improvement was noticed and/or long-term antifungal therapy was given. Thus, twenty-eight samples were obtained from ten of the n_1 aforementioned cetaceans. In average, convalescent dolphins were tested 2.8 ± 1.6 times, 47.7 ± 40.8 months after the initial diagnosis of aspergillosis.

Animals with no apparent clinical signs were considered as healthy controls and classified into the clinically-normal group ($n2' = 41$), while those undergoing miscellaneous non-*Aspergillus* inflammatory processes were classified as non-*Aspergillus* inflammatory dolphins ($n2'' = 14$).

2.2. Proteomic analysis

2.2.1. Pre-processing step with iTRAQ®-tags labeling

This protocol was the same as described previously (Desoubeaux et al., 2018). Briefly, protein enrichment was completed using the Pierce Albumin / IgG removal® kit (ThermoFisher Scientific, Waltham, MA, U.S.A.) that allows for depletion of overrepresented proteins like albumin or immunoglobulins (Kumar et al., 2012). Total protein content was quantified by the Pierce BCA Protein assay® kit (ThermoFisher Scientific, Waltham, MA, U.S.A.), according to the manufacturer’s instructions (Kumar et al., 2012; Desoubeaux et al., 2014a). All depleted blood samples were thereafter pooled, on the basis of 1000 µg protein for each, in four distinct aliquots, according to the clinical group the dolphins were affiliated with: clinically-normal controls, non-*Aspergillus* inflammatory dolphins, *Aspergillus*-diseased animals, and convalescent subjects. Then, 100 µg of every pool was incubated with 30 µL dissolution buffer of 0.5 M triethylammonium bicarbonate (Sigma-Aldrich, Saint-Louis, MO, U.S.A.) at pH 8.5, and thereafter treated with 2.0% SDS-denaturant solution. One microliter of tris-(2-carboxyethyl)phosphine-reducing reagent was added, followed by vortex-shaking for 1 min, centrifugation at 18,000 x *g* for 5 min and incubation at 60 °C for 1 h. After another step of shaking and centrifugation, 84 mM iodoacetamide was added for a 30 min-long incubation in the dark at room temperature. Freshly prepared 0.1 µg/µL sequencing grade modified trypsin was then mixed with each sample, and digestion was carried out at 37 °C during 30 min. Thereafter, 1 µL trypsin was added to continue digestion overnight. The samples were dried in an Eppendorf Vacufuge® concentrator, and then reconstituted with 30 µL dissolution buffer. Every tube was mixed with one unique iTRAQ® reagent (Sigma-Aldrich, Saint-Louis, MO, U.S.A.) and reconstituted in isopropanol, according to the manufacturer’s recommendations. The tube containing all the blood samples pooled from clinically-normal controls was mixed with the 117-reagent, those from miscellaneous non-*Aspergillus* inflammatory dolphins with the 118-reagent, those from *Aspergillus*-diseased dolphins with the 119-reagent, and those from *Aspergillus*-convalescent dolphins with the 121-reagent. Thereafter, the contents of all these iTRAQ® reagents-labelled tubes were combined into one single tube, vortexed for 1 min, and centrifuged at 18,000 *g* for 5 min. The multiplexed specimen was thoroughly dried by centrifugal vacuum concentration as above.

Table 1
Baseline characteristics of the dolphins included in the study. The assignment was made according to the case definition for diagnosis of aspergillosis (for details, see Material & Methods section).

	Mean (± SD) or Number (%), [$_{95\%}$confidence interval]		
	Aspergillus-diseased cases (n_1 = 32)	Clinically-normal controls ($n_{2"}$ = 41)	Miscellaneous non-*Aspergillus* inflammatory animals[a] ($n_{2"}$ =14)
Age (years)	**22.5** (± 10.8), [18.4 – 26.5]	**15.8** (± 10.0), [11.8 – 19.8]	**17.5** (± 15.3), [10.5 – 24.5]
Sex (male)	**16** (50.0%), [32.7 – 67.3%]	**14** (34.1%), [23.4 – 45.4%]	**6** (42.9%), [28.9 – 56.9%]
Clinical signs			
- respiratory[e]	**20** (62.5%), [45.7 – 79.3%]	/	**1** (7.1%), [3.5 – 10.7%]
- disseminated[f]	**8** (25.0%), [10.0 – 40.0%]	/	**11** (78.6%), [70.1 – 87.1%]
Finding(s) for diagnosis of aspergillosis[b]			
- positive *in vitro* culture	**24** (75%) [60.0 – 90.0%]	/	/
- anti-*Aspergillus* antibody ELISA titer[c]	1024 (1536) [128 – 16384]	/	**256** (384) [0 – 8192]
- positive histopathology observation	**19** (59.4%) [42.4 – 76.4%][d]	/	/

Abbreviations: /, 0.0% (0.0%) [0.0 – 0.0%]; ELISA, enzyme-linked immunosorbent assay; %, percentage; SD, standard deviation.
[e] *i.e.*, coughing, abnormal vocalizations, hard chuffing, radiological changes and all signs related to tracheitis, bronchitis, pneumonia, pleurisy ….
[f] *i.e.,* at least to distinct organs were shown to be involved.
[a] Included samples from dolphins undergoing miscellaneous non-*Aspergillus* inflammation like four cases of candidiasis (two were due to *Candida glabrata* and two caused by *C. tropicalis*), two cases of cryptococcosis, two cases of histoplasmosis, four cases of mucormysis, and two cases of coccidioidomycosis.
[b] Medical imaging was not included as pivotal criterion of aspergillosis diagnosis, as it is difficult to perform and rarely achieved in cetaceans.
[c] Median (interquartile range), [min. – max. value], estimated in comparison with a positive control at 1: 1024 titer for optical density at 0.2, according to Desoubeaux et al. (Desoubeaux et al., 2017a).
[d] For one case, necropsy revealed concomitant aspergillosis and mucormycosis involving the trachea.

2.2.2. Processing steps by mass spectrometry

The multiplexed iTRAQ® specimen was re-suspended in 2.0% acetonitrile and loaded onto a high performance liquid chromatographic (HPLC) instrument. HPLC analysis was conducted in an Easy Nano LC 1000® model (ThermoFisher Scientific, Waltham, MA, U.S.A.) where a 75 µm i.d. x 15 cm column, packed with Acclaim PepMap® RSLC C18-2 µm 100 Å column (ThermoFisher Scientific, Waltham, MA, U.S.A.), was in-line connected to a Acclaim PepMap® 100 75 um x 2 cm, nanoviper C18-3 µm 100 Å pre-column (ThermoFisher Scientific, Waltham, MA, U.S.A.). Peptides were eluted following a 75 min gradient from 2.0% to 98.0% acetonitrile (ThermoFisher Scientific, Waltham, MA, U.S.A.) with a water flow of 350 nL/min into a QExactive® Orbitrap mass spectrometer (ThermoFisher Scientific, Pittsburg, PA, U.S.A.) in data-dependent mode, with an automatic gain control (AFC) target of 1.0×10^6 for full MS at 70,000 resolution, and of 2.0×10^5 for dd-MS^2 at 17,500 resolution in positive mode. The isolation window was fixed to 1.5 *m/z* with normalized collision energy of 28 eV, underfill ratio of 1.0%, and dynamic exclusion of 3.0. First mass was fixed to 103 *m/z*. QExactive® Orbitrap typical technical specifications are evaluated as follows: high resolving power of 140,000 full width at half maximum, defined at *m/z* 200, and elevated resolution at $\Delta_{m/z} = 0.001$, with a 50-4,000 *m/z* range (Holčapek et al., 2012).

2.2.3. Mass spectrometry data analysis and identification/quantitation of host proteins

The bioinformatics analysis was performed using Proteome Discoverer® software (ThermoFisher Scientific, Pittsburg, PA, U.S.A.). Specific SwissProt® reviewed non-redundant databases (http://www.uniprot.org/uniprot/) were used for the analysis of dolphin or dolphin-like (according to the following genera: *Tursiops, Cephalorhynchus, Grampus, Lagenorhynchus, Platanista, Sotalia, Stenella, Inia, Lipotes*, and *Sousa*) proteome using Sequest® HT search engine (Washington DC, U.S.A.). The parameters of the study set trypsin as the enzyme used for digestion, with a maximum number of missed cleavage sites of two, 10 ppm as precursor mass tolerance, and 0.02 Da for fragment mass tolerance. Alkylation, as well as N-terminal / lysine modifications and dynamic iTRAQ® modifications were selected as fixed. Maximum value for delta correlation was set to 0.05. False discovery rate target value was 0.01 for strict rate and 0.05 for relaxed one, considering the *q*-value as a validation reference. At the quantification level, the analysis included K-means clustering, *i.e.* supervised and heuristic algorithm, for both protein and peptide quantitation ratios. The software actually worked by grouping proteins, based on the peptide spectral match (PSMs). Then, a customized ratio was calculated for every protein group as the median of all PSMs included in the protein group.

2.3. Statistical analysis

Statistical analyses were performed using XLStat® v.2016.6.04 software (Addinsoft, Paris, France). Missing data, *e.g.* when the total volume of sample was insufficient to complete all the analyses, were managed by the method of mean imputation. The α-risk was adjusted at 0.05.

Regarding specifically the iTRAQ® data, we based inference on one-way analysis of variance (ANOVA) models, with the data analysis conducted one protein at a time (Unwin et al., 2005; Keshamouni et al., 2006), combining both normalization, *i.e.* bias removal, and assessment of differential protein expression in a single model fit to the collection of reporter ion peak areas, *i.e.* corrected for isotopic overlap, from all observed tandem mass spectra. Significant cutoff for determination of over- or underrepresentation was set at ≥ 2-fold in comparison of the *Aspergillus*-diseased cases with the other three groups (Desoubeaux et al., 2018). Principal component analysis (PCA) was used to facilitate interpretation of the multivariate proteome dynamics dataset (Rao and Li, 2009). Data were z-scored prior to model building.

3. Results

3.1. Study population and samples

Characteristics of included dolphins are detailed in Table 1. Measurement of GM antigen in blood was not diagnostic, displaying the following mean values: 0.2 ± 0.1 *versus* 0.2 ± 0.2 ng/mL for *Aspergillus*-diseased cases and controls. There was a significant difference between anti-*Aspergillus* antibody titers in diseased and control dolphins ($p < 0.0001$).

3.2. Proteomic analysis

Overall, MS analysis achieved identification of 1385 proteins in dolphin blood. Sixty-six proteins were statistically overexpressed in

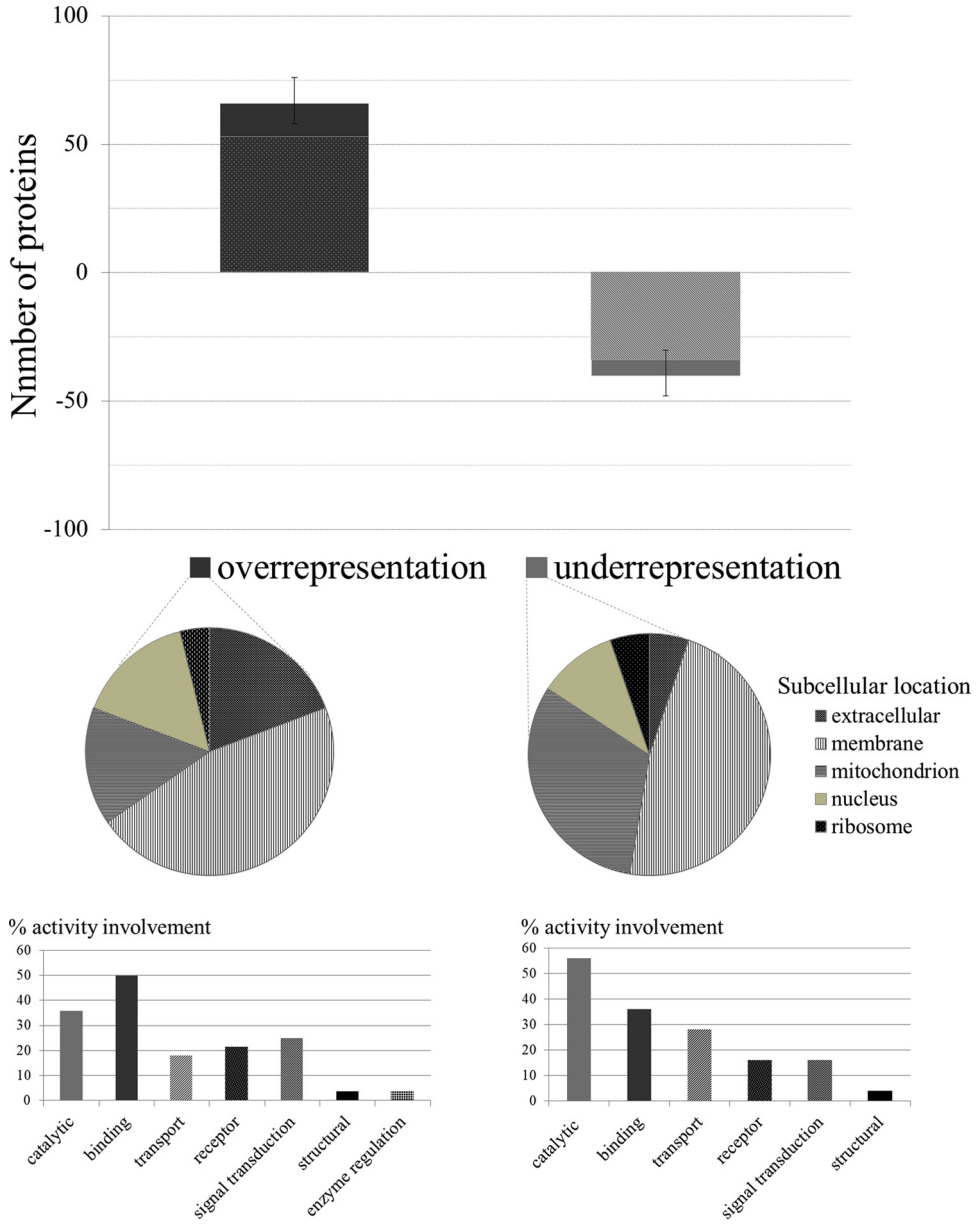

Fig. 1. Global protein changes in *Aspergillus*-diseased common bottlenose dolphins (*Tursiops truncatus*) *versus* non-*Aspergillus* inflammatory animals. In the upper panel, the bar graph indicates the absolute number of proteins that were found positively or negatively ≥ 2.0-fold differentially-expressed. The extreme upper and lower parts of the bars (no hatching) represent the protein moiety ≥ 4.0-fold differentially-expressed. In the middle panel, the pie charts display the distribution of the subcellular locations for each differentially-expressed protein. In the lower panel, the bar graph shows the molecular functions of these proteins.

Aspergillus-diseased dolphins *vs.* miscellaneous non-*Aspergillus* inflammatory dolphins (Fig. 1), *i.e.* 2.0% of the whole dolphin (*Tursiops truncatus*) proteome, and 52 / 66 (78.8%) were also overrepresented as compared to clinically-normal dolphins. Out of the 66 proteins overrepresented, 48 / 66 (72.7%) were also in overabundance *vs*. clinically-normal controls, and 46.15% were located at the cell membrane and 19.2% in the extracellular compartment. Fifty percent represented binding activity, whereas 35.7% and 25.0% were involved in catalytic processes and signal transduction (Fig. 1). Thirteen (13 / 66; 19.7%) were dramatically increased (≥ 4.0-fold) when compared to miscellaneous non-*Aspergillus* inflammatory dolphins (Table 2), primarily potassium inwardly-rectifying channel J1-like protein. There was a major presence of proteins belonging to the major histocompatibility complex (MHC): major histocompatibility complex class II beta chain and MHC class I antigen, increased by > 100.00-fold and 4.48-fold. Similarly, some nicotinamide adenine dinucleotide phosphate-(NADPH-) ubiquinone oxido-reductases were also increased with *Aspergillus* infection. Except for NADH-ubiquinone oxidoreductase chain 5 and endothelin-1 receptor, all of these 13 markedly-increased proteins returned to normal levels during the convalescent period.

Forty proteins were underrepresented in *Aspergillus*-diseased dolphins *vs.* miscellaneous non-*Aspergillus* inflammatory dolphins (Fig. 1), which represents 1.1% of the dolphin proteome, and 31 / 40 (77.5%) were confirmed to be lower when compared to clinically-normal controls. Among these 40 proteins, 47.4% were located at the membrane cell, and 31.6% in the mitochondrion. Fifty-six percent, 36.0% and 28.0% expressed catalytic, binding and transport activities, respectively (Fig. 1). Six proteins / 40 (15.0%) were reduced by ≥ 4.0-fold in *Aspergillus*-diseased dolphins *vs.* miscellaneous non-*Aspergillus* inflammatory dolphins: retinoblastoma binding protein 8, prepronociceptin, extra-spindle poles-like protein 1, NADH dehydrogenase subunit 2, interleukin 4 (IL-4), and NADH-ubiquinone oxidoreductase

Table 2
List of the host proteins with significantly-high increased representation ≥ 4-fold or significantly-low decreased expression ≤ 0.25-fold during *Aspergillus*-disease in dolphins *versus* control dolphins. Fold changes in the *Aspergillus*-diseased dolphins (*i.e.* encompassing all the subjects for which blood samples were tagged with 119-tag) were calculated with respect to the protein expression in non-*Aspergillus* controls either undergoing inflammation due to any miscellaneous causes (118-tag reagent), clinically-normal cetaceans (117-tag reagent) or convalescent subjects (121-tag reagent).

Protein identification	Access. number (UniProt®)	Fold change			% Seq. coverage	No. unique peptide (s)	No. aa	Theoretical MW (kDa)	Calc. pI	Gene name (GenBank®)	Known function	Subcellular location
		vs. inflammatory controls	*vs.* clinically-normal controls	*vs.* convalescents								
Potassium inwardly-rectifying channel J1-like protein (Fragment)	F2Y5W8	⇑ 100.000	100.00	100.000	23.316	6	193	21.5	8.12	KCNJ1	regulation of biological process (transporter activity); transport	membrane
Major histocompatibility complex class II beta chain (Fragment)	B2DC80	⇑ 100.000	2.282	100.000	61.644	2	73	8.9	5.54	Tutr-DQB	response to stimulus	membrane
Sperm protamine P1	C8C436	⇑ 15.424	21.428	33.774	42.857	4	49	6.3	11.96	PRM1	cell differentiation; cell organization and biogenesis (DNA binding); development; metabolic process	chromosome; nucleus
Cytochrome b (Fragment)	G5D6F5	⇑ 7.675	12.218	8.113	6.648	1	361	40.4	6.84	cytb	metabolic process (catalytic activity); transport (metal ion binding; transporter activity)	membrane; mitochondrion
NADH-ubiquinone oxidoreductase chain 2	A5H2W1	⇑ 6.785	8.246	4.786	26.512	1	347	39.2	9.88		metabolic process (catalytic activity); transport	membrane; mitochondrion
Mediator complex subunit 13-like protein (Fragment)	F2Y6H0	⇑ 6.548	10.462	4.585	29.629	1	135	14.8	4.67	MED13L		
NADH-ubiquinone oxidoreductase chain 5	Q2YG56	⇑ 5.642	3.844	1.633	27.722	11	606	68.5	8.95	nadh5	metabolic process (catalytic activity); transport	membrane; mitochondrion
Endothelin-1 receptor (Fragment)	A0A140GN19	⇑ 4.715	4.939	1.249	29.039	12	427	48.6	8.28	EDNRA	regulation of biological process; response to stimulus (receptor activity; signal transducer activity)	membrane
Cytochrome c oxidase subunit 1	Q70RT4	⇑ 4.612	2.412	5.55	5.232	2	516	57.2	6.67	Soch-I	metabolic process (catalytic activity); transport (metal ion binding)	membrane; mitochondrion
MHC class I antigen (Fragment)	B3SGM1	⇑ 4.48	2.834	3.898	63.265	1	49	5.9	5.01	COX1	response to stimulus	
Mediator complex subunit 13 (Fragment)	F4YEE3	⇑ 4.412	5.001	6.423	38.362	1	232	25.3	5.07	MED13	metabolic process; regulation of biological process (protein binding;); response to stimulus (receptor activity)	membrane; nucleus
Mitochondrial ribosomal protein L16 (Fragment)	F2Y6K8	⇑ 4.122	4.105	3.917	58.252	3	103	11.9	10.26	MRPl16	metabolic process (RNA binding; structural molecule activity)	ribosome
Testis expressed 11 (Fragment)	L0BW61	⇑ 4.095	10.266	3.701	51.851	1	27	3.1	9.29	ND5	cell organization and biogenesis; metabolic process; regulation of biological process	chromosome

(*continued on next page*)

Table 2 (*continued*)

Protein identification	Access. number (UniProt®)	Fold change			% Seq. coverage	No. unique peptide (s)	No. aa	Theoretical MW (kDa)	Calc. pI	Gene name (GenBank®)	Known function	Subcellular location
		vs. inflammatory controls	*vs.* clinically-normal controls	*vs.* convalescents								
Retinoblastoma binding protein 8 (Fragment)	F2Y791	⇓ 0.229	0.287	0.542	73.404	2	94	10.5	5.48	RBBP8	metabolic process (catalytic activity); response to stimulus	nucleus
Prepronociceptin (Fragment)	G5CYG5	⇓ 0.191	0.269	0.81	61.052	1	95	10.5	10.35	PNOC	cell communication; regulation of biological process; response to stimulus	
Extra-spindle poles-like protein 1 (Fragment)	F2Y871	⇓ 0.171	0.199	0.147	51.489	4	235	25	9.06	Espl1	metabolic process (catalytic activity; structural molecule activity)	nucleus; ribosome
NADH dehydrogenase subunit 2	A0A1L2M7D3	⇓ 0.001	0.001	0.001	17.867	1	347	39	9.79	ND2	metabolic process (catalytic activity); transport	membrane; mitochondrion
Interleukin 4 (Fragment)	B3V270	⇓ 0.001	0.001	0.171	52.830	3	53	6.1	9.45		response to stimulus (protein binding)	extracellular
NADH-ubiquinone oxidoreductase chain 2	Q2YG65	⇓ 0.001	0.001	0.051	17.003	2	347	39.1	9.83	ND2	metabolic process (catalytic activity); transport	membrane;mitochondrion

Abbreviations: aa, Amino acids; Access., Accession; calc., Calculated; kDa, Kilo dalton; MW, Molecular weight; No, Number; pI, Isoelectric point; Seq., Sequence.

chain 2. The last four were also decreased in convalescent dolphins.

4. Discussion

In marine mammals, development of aspergillosis varies fundamentally in comparison to the infection in humans. For instance, in dolphins, it appears to be associated with a chronic invasive process; generally suggestive of another disease and/or (su**b-**)acute physiologic stress (Desoubeaux et al., 2017a) rather than associated with severe immunosuppression and profound neutropenia as observed in humans (Seyedmousavi et al., 2015). Underlying pulmonary disease may affect host defense mechanisms, leading to long-term colonization in dolphin lungs and potential invasion of bronchial tissue by *Aspergillus* spp (Reidarson et al., 1998). Other organs, including the brain, may also be infected following systemic dissemination (Dagleish et al., 2008; Abdo et al., 2012). Thus, as observed in this study, the diagnosis of aspergillosis is particularly challenging in dolphins (Seyedmousavi et al., 2015; Desoubeaux et al., 2017a). For example, GM antigen is not contributive and true positive mycological cultures are infrequent; there is a common observation of colonization or chronic infection compared to humans. Therefore, it is critical to describe new biomarkers of aspergillosis to aid diagnosis and prognosis, to suggest alternative therapeutic targets, and to decrease the related morbidity and mortality in dolphins.

Through its sensitivity and by comprehensively addressing the pathophysiology, proteomics may contribute to the discovery of new markers and their clinical validation in diseased animals (Desoubeaux et al., 2014b). In such a context, iTRAQ® appears to be an accurate and innovative technique based on an affordable ready-to-use kit (Kumar et al., 2012; Bourassa et al., 2015; Desoubeaux et al., 2017b, 2018), provided there is availability of a performant MS instrument. For this reason, we had to circumvent several technical limitations. First, we included relevant controls for reliable comparison: we rigorously enforced recruitment of samples from dolphins which experienced various inflammatory diseases, including other fungal infections like mucormycosis and histoplasmosis. Second, to reduce disease misclassification, we used a composite criterion based on clinical data, histopathology, and mycological culture as it is consensually approved for disease classification in human infections (Desoubeaux et al., 2014b). Lastly, given the probability of homologous proteins, we decided to consult broad SwissProt® databases. The latter voluntarily encompassed ten dolphin and dolphin-like genera in order to allow identification of more proteins when compared to browsing the database restricted to *Tursiops truncatus* species. Unfortunately, it was not possible to analyze protein enrichment according to the Gene Ontogeny (GO) (http://www.geneontology.org/) (Desoubeaux et al., 2018), because all the current classification system, including PANTHER classification systems version 13.1 (released 2018-02-03) (Mi et al., 2017), are lacking data related to cetaceans. This highlights the critical need to increase current databases that poorly represent marginal micro- or macro-organisms.

Our MS study described significant qualitative and quantitative changes in the dolphin blood proteome. This protein spectrum brought new insight into the host defense mechanisms and showed major involvement of proteins with binding and catalytic functions as previously observed with rats and penguins (Desoubeaux et al., 2018). A major moiety was expressed in the extracellular compartment or at the cell membrane which suggests that these proteins are massively discarded into circulation during *Aspergillus* infection. The major representation of proteins related to MHC underscores the role of adaptive immunity during aspergillosis in dolphins (Beineke et al., 2010). This also reemphasizes how anti-infectious responses vary based on the host: for instance in humans, adaptive immunity plays a minor role against *Aspergillus* infection, while polymorphonuclear neutrophils and macrophages are primarily recruited (Dagenais and Keller, 2009). We made similar observations in a neutropenic rat model (Chandenier et al., 2009; Desoubeaux et al., 2018).

Several NADPH-ubiquinone oxido-reductase proteins and cytochrome *b* were described as overrepresented in the current study. They are believed to be required for catalysis which functions in the transfer of electrons to the respiratory chain required for enzymatic activity to fight against aspergillosis. IL-4 was found largely decreased in our study. In other studies, IL-4-deficient mice developed attenuated airway inflammation compared with wild-type rodents (Choi and Reiser, 1998), and we also observed increased protein expression related to the interleukin signaling pathway in our acute rat model of aspergillosis (Desoubeaux et al., 2018). Similarly, prepronociceptin concentration was moderately lower in *Aspergillus*-diseased dolphins *vs.* non-*Aspergillus* inflammatory animals, which is consistent with previous findings showing significant increase in systemic chronic inflammation models (Liu et al., 2012). In the dolphin, decreased prepronociceptin concentration can be hypothesized as a physiological adaptation to chronic infection caused by *Aspergillus*. As up-regulation of the prepronociceptin system was previously demonstrated to alter airway hyper-responsiveness (Singh et al., 2013), down-regulation can be assumed to play an opposite role in modulating airway inflammation and airway tone. Interestingly, bacterial infections were demonstrated to have significant negative impact on protamine P1 concentration (Zeyad et al., 2018) whereas *Aspergillus*-infection appeared to induce positive effects in dolphins, by increasing it more than 15-fold. This finding may suggest a potential clue for differential diagnosis between bacterial and fungal infections. Other studies have shown significant antimicrobial activity of protamine (Kim et al., 2015).

Overall, the present study highlighted the potential of MS analysis for characterizing the pathophysiology of aspergillosis. Use of the iTRAQ® protocol provided new insight on the host inflammatory response against *Aspergillus* in naturally-infected mammals. This study may serve as a reliable preliminary trial towards the discovery of new biomarkers of infection and the suggestion of therapeutic targets. Further studies are warranted to validate these results and expand upon the construction of functional protein networks in aspergillosis. The example of iTRAQ® protocol application for aspergillosis in dolphins should be easily reproducible for other diseases and in various host species.

Conflicts of interest

The authors have no conflicts of interest to declare.

Funding

This work was supported by internal laboratory funding and partly by NIH center grant P30-EY14801. There is no disclosure of conflict of interest.

Acknowledgments

The authors thank all the veterinarians and practitioners that enrolled their animal patients. They are grateful to all the multi-institutional animal care and biological staff dedicated to the care and monitoring of the bottlenose dolphins in this study.

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