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Original article

Identification of the proteins of *Borrelia garinii* interacting with human brain microvascular endothelial cells

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ABSTRACT

Lyme borreliosis is one of the major tick-borne diseases in Europe. Events of the translocation of *Borrelia* across the blood-brain barrier (BBB) involve multiple interactions between borrelial surface proteins and receptors on the brain microvascular endothelial cells (hBMECs). In this study, we aimed to identify proteins of *Borrelia* that plausibly interact with hBMECs. The surface proteome of live *Borrelia* (a neuroinvasive strain of *B. garinii*) was crosslinked with biotin prior to its incubation with hBMECs. The interacting proteins were recovered by affinity purification, followed by SWATH-MS. Twenty-four interacting candidates were grouped into outer membrane proteins (n = 12) and inner membrane proteins (n = 12) based on the subcellular location as per the predictions of LocateP. Other algorithms like TMHMM 2.0 and LipoP, ontology search and literature review were subsequently applied to each of the identified protein candidates to shortlist the most probable interactors. Six proteins namely, LysM domain protein, BESBP-5, Antigen S1, CRASP-1 (Bg071), Erp23 protein and Mlp family Lipoprotein were selected to produce their recombinant forms and experimentally validate their interaction with hBMECs. All the recombinant proteins interacted with hBMECs, in ELISA and immunocytochemistry. We present here a high-throughput approach of generating a dataset of plausible borrelial ligands followed by a systematic bioinformatic pipeline to categorize the proteins for experimental validation.

1. Introduction

Lyme borreliosis is the most common tick-borne disease in the northern hemisphere caused by various genospecies of *Borrelia,* namely, *B. afzelii, B. garinii, B. burgdorferi* sensu stricto and *B. bavariensis* (Steere et al., 2017). *Borrelia* spreads systemically to various tissues, such as skin, joints, heart, and the central nervous system (CNS). In the case of neuroborreliosis, symptoms include meningoradiculitis, cranial nerve abnormalities, and altered mental status. Whereas in early neuroborreliosis headache, sleep disturbance, and symptoms associated with increased intracranial pressure can be observed. Neuroinvasive *Borrelia* (e.g. *B. bavariensis* or *B. garinii*) were suggested to traverse the blood-brain barrier (BBB), either via a paracellular route, i.e. crossing through the tight junctions of brain microvascular endothelial cells (BMECs) or via the transcellular passage involving receptor-mediated endocytosis. Interaction of *Borrelia* with endothelial cells and its paracellular crossing through an *In vitro* BBB model has been clearly documented (Szczepanski et al., 1990; Grab et al., 2005). On the other hand, transcellular passage was proposed in *in vitro* infected endothelial cells using electron microscopy (Comstock and Thomas, 1989). Of note, a recent study has shown the colonization of *Borrelia* in the meninges and dura mater (Divan et al., 2018). Additionally, the spirochetes were not only located in vascular regions, but also in perivascular and extravascular regions (Divan et al., 2018).

Traversal of *Borrelia* across the endothelial layer of BBB is a complex process that involves transient adhesion, short-term dragging interactions, a stationary adhesion and crawling through intercellular space (Hu et al., 1995; Coleman and Benach, 2003; Zhao et al., 2007; Moriarty et al., 2008). Surface proteins of *Borrelia* (borrelial adhesins) are essential for transient tethering-type and stationary adhesion on host cells (Comstock and Thomas, 1991). *Borrelia* expresses an array of adhesive molecules on its surface, and it seems that to interact with endothelial cells, it relies on multiple protein-protein interactions. Adhesive proteins, like outer surface proteins (Osp), P66 and protein-

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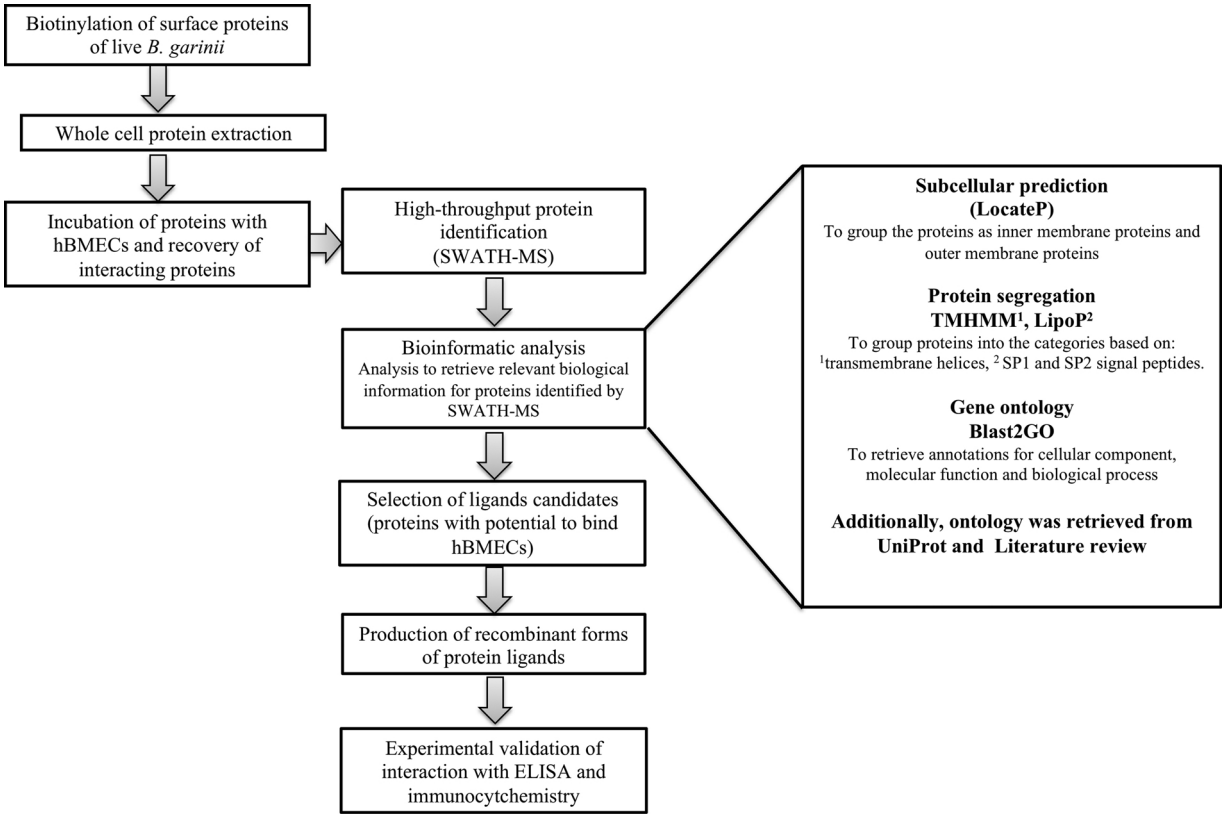

Fig. 1. An Overview of the experimental and bioinformatic pipeline applied in this study. The surface proteins of live *B. garinii* were biotinylated, whole-cell proteins were extracted and incubated with human brain microvascular endothelial cells (hBMECs). Interacting proteins were captured on NeutrAvidin beads and identified by SWATH-MS. In this study, we have used several bioinformatics tools. The first step of the bioinformatic analysis was to identify the subcellular location (LocateP). Prediction of transmembrane domains was performed with TMHMM 2.0 and the presence of signal peptides SP-I and SP-II were predicted with LipoP. The second step of the bioinformatic analysis was to retrieve gene ontology with Blast2GO, UniProt, pfam and literature review. Protein candidates shortlisted based on bioinformatic analysis were overexpressed in *E. coli.* Interaction of the selected protein candidates was validated experimentally with ELISA and immunocytochemistry using recombinant proteins.

ligand for b3-chain integrins were shown to bind the endothelial cell receptors (Comstock et al., 1993; Antonara et al., 2007). Likewise, Bgp and BBK32 were shown to bind glycosaminoglycan, while DbpA and DbpB (BBA25) have shown affinity to both glycosaminoglycan (GAG, dermatan sulfate) and proteoglycan decorin (Fischer et al., 2003; Parveen et al., 2003; Fischer et al., 2006). Previously we have reported that besides borrelial OspA and host CD40 interaction, other protein-protein interactions might be important for the adhesion of *Borrelia* to the endothelial cells (Pulzova et al., 2011). It was also demonstrated that *Borrelia* can interact with microvasculature even in the absence of BBK32 (Seshu et al., 2006). This implies that *Borrelia* employ multiple surface proteins for adhesion on endothelial cells and during translocation across the vasculature.

Historically, protein-protein interactions between the host and pathogen were studied using small-scale proteomic or genetic experiments. Although each piece of information in protein-protein interactions is valuable, like in a jigsaw puzzle the assembly of pieces is difficult and a complete picture of interactions between borrelial ligands and receptors on hBMECs is necessary. With the small-scale gel-based approach, the interaction between borrelial OspA and endothelial receptor has been shown earlier, wherein a monoclonal anti-OspA antibody was used to show adhesion of OspA on the HUVEC (Comstock et al., 1993). Classical gel-based proteomic methods pose several disadvantages (e.g. poor resolution of hydrophobic proteins, delayed output, low-throughput, and requirement of large input material (Goonetilleke et al., 2010; Pulzova et al., 2011; Bencurova et al., 2015; Mlynarcik et al., 2015). Due to these limitations, gel-based techniques are poorly suited for the study of neurovascular diseases, in which the material of the study is scanty (e.g. primary cells of the neurovascular unit). Further on, mutant or gene knockout *Borrelia* were developed to study the binding of surface proteins to the endothelium (Sadziene et al., 1995; Ristow et al., 2015). Obviously, it is extremely laborious to identify each borrelial interactor using such small-scale approaches. Hitherto, a high-throughput *in vivo* phage display was used to identify a set of borrelial adhesins (Antonara et al., 2007). Another approach to identify surface-exposed proteins is to use biotin cross-linking agents (Cullen et al., 2004), which typically label primary amines located on the side chain of lysine residues and at the amino terminus. Thence, biotin cross-linking agents can identify a broader range of surface-exposed proteins when compared to relatively non-specific proteases (such as proteinase K), which rely on the exposure of a protease cleavage site (Cullen et al., 2004). Since biotin cross-linking agents are relatively small molecules, these agents are more sensitive than antibodies or proteases for the detection of outer membrane proteins with limited surface-exposure. Moreover, we have recently demonstrated the use of biotin labeling to map the interactome of *Neisseria meningitidis* (Kánová et al., 2018) and *Streptococcus pneumoniae* (Jimenez-Munguia et al., 2018).

In the present study, the surface proteome of live *Borrelia* was biotinylated, proteins were extracted and incubated with hBMECs. The proteins interacting with hBMECs were recovered and identified with mass spectrometry (SWATH-MS). Thereafter, potential interactors (outer membrane proteins) were shortlisted by the systematic bioinformatics workflow and literature review. Lastly, ELISA and immunocytochemistry were employed to validate the binding ability of six outer membrane proteins using their recombinant forms. Results

presented in this study together with earlier published reports (Jimenez-Munguia et al., 2018; Kánová et al., 2018) validate the experimental and bioinformatic approach designed to uncover potential borrelial ligands interacting with the host cells. The present study shows that *Borrelia* is equipped with multiple surface proteins that may interact with the receptors on hBMECs.

2. Materials and methods

The overall experimental approach, bioinformatic analysis, and validation are illustrated in Fig. 1.

2.1. B. garinii culture

B. garinii (strain SKT-7.1, Serotype 4, GenBank accession number - GU906888.1) was grown in complete BSK-II medium (Sigma Aldrich, USA) enriched with 6% rabbit serum at 33 °C. After two weeks, cultures were examined under the dark field microscopy (40x magnification) to assess the shape and motility of *Borrelia*. Please note that this strain was renamed as *B. bavariensis* as per the revised nomenclature (Margos et al., 2009).

2.2. Biotin labeling of the surface proteome of live Borrelia

Before biotinylation, the quality of borrelial culture was assessed under the dark-field microscopy (Fig. 2A-I). *Borrelia* were motile without aggregation. To achieve biotinylation of the maximum number of surface protein candidates, it is necessary to use culture in the log phase when surface proteins are expressed abundantly (Sapi et al., 2012). Culture was centrifuged at 6000 × g for 10 min at room temperature and the pellet was washed two times with sterile 1x phosphate-buffered saline (PBS). The density of bacteria was measured with flow-cytometry (Accuri, Becton Dickinson, USA). 0.25 mg of Sulfo-NHS-SS-Biotin reagent (Thermo-Scientific, Slovakia) was added to one milliliter of *Borrelia* suspension (1×10^6 spirochetes resuspended in PBS) and incubation was carried out for 1 hour in the dark. Biotin labeled bacterial cells were centrifuged and the pellet was washed with PBS.

To assess the biotinylation of surface proteins, a small aliquot of biotinylated borrelial cells was incubated with streptavidin-FITC conjugate (1:10 000 in PBS, Promega, USA) and after six washings with PBS, borrelial cells were observed under the fluorescent microscope (480 nm, 100x magnification), in which uniform biotinylation of the borrelial surface was observed (Fig. 2A-II). Simultaneously, a negative control was also included in which non-biotinylated *Borrelia* were incubated with streptavidin-FITC conjugate. None of the *Borrelia* were fluorescent under the microscope (Fig. 2A-III). Biotinylated borrelial cells were stored at −80 °C until cell lysis.

2.3. Preparation of protein extract of Borrelia

Biotinylated borrelial cells were resuspended in a non-denaturating lysis solution containing 20 mM CHAPS, 300 mM NaCl, 0.1% sodium azide and 1x proteases inhibitors (Sigma-Aldrich). Subsequently, the cells were sonicated on ice (15 cycles, 100% amplitude, 30 s) and centrifuged at 26 000 × g for 10 min. A small aliquot of the resulting protein extract was checked for the presence of biotinylation through NeutrAvidin (Thermo-Scientific) capture. The capture of biotinylated proteins on NeutrAvidin agarose beads was performed according to the manufacturer´s instructions. Captured proteins were eluted in 200 µl of 50 mM dithiothreitol (DTT) in PBS (pH 7.2) and resolved on SDS-PAGE. Biotinylated proteins were enriched after NeutrAvidin capture (Fig. 2B-II), leaving non-biotinylated proteins in the flow-through (Fig. 2B-III). Negative control was also included in which lysate of the non-biotinylated *Borrelia* was incubated with NeutrAvidin beads. Bound proteins on the beads were eluted and resolved on SDS-PAGE. Absence of any protein in the eluate (Fig. 2C-II), indicates that borrelial proteins do not bind NeutrAvidin beads non-specifically.

The rest of the protein extract from biotinylated *Borrelia* was dialyzed against PBS and stored at −80 °C until further use. Protein concentration was measured with the Bradford assay.

2.4. Human brain microvascular endothelial cell (hBMECs) culture

Human BMECs were cultured as previously described (Jimenez-Munguia et al., 2018). In short, hBMEC/D3 cell line was obtained from Merck/Millipore (Prague, Czech Republic) and cultured in 25-mL cell culture flask coated with collagen type I (Sigma, USA) in EBM-2 medium (Lonza, UK) containing 10% FBS, gentamycin, 1.4 µM hydrocortisone (Sigma), 5 µg/mL ascorbic acid, 10 mM HEPES and 1 ng/mL bFGF (Sigma). Cells were incubated at 37 °C in a humid atmosphere of 5% CO_2 until confluence. Cells from the confluent monolayer (6^{th} passage) were either incubated with proteins of *Borrelia* or scrapped for protein isolation.

2.5. The interaction between biotinylated proteins of Borrelia and hBMECs

The interaction was performed by following earlier standardized protocols (Jimenez-Munguia et al., 2018; Kánová et al., 2018). In brief, 200 µg of biotinylated borrelial proteins were incubated on the confluent monolayer of hBMECs in a 25-ml cell culture flask for 1 hour at 37 °C in the presence of 5% CO_2. The experiment was performed in four replicates. After incubation, cells were subjected to four washes with Dulbecco's PBS (Sigma) to remove unbound biotinylated proteins. Thereafter, the cell monolayer was scraped in 2 ml of PBS and the suspension was centrifuged at 3000 × g for 10 min. The resulting supernatant (S1) was kept on ice until further use (it should be noted that S1 may contain biotinylated proteins). The cell pellet was resuspended in 200 µl of lysis solution (Cell Surface Protein Isolation Kit, Thermo-Scientific) followed by 30 min incubation on ice (after every 5 minutes, cells were vortexed for 5 s). Next, the supernatant S1 was added to the cell lysate prior to 5 cycles of sonication (100% amplitude, 30 s on ice). Subsequent centrifugation (10 000 × g, 5 min, 4 °C) yielded the supernatant (S2), which was kept on ice until the capture of biotinylated proteins.

To confirm the presence of biotinylated proteins, 50 µL of NeutrAvidin agarose beads were incubated with 200 µl of S2 for 1 hour. After washing the beads with PBS (five times), biotinylated proteins were eluted with 20 µl of 100 mM glycine buffer (pH 2.5). Subsequently, 2 µl of the eluate was spotted on the nitrocellulose membrane and blocked with 1% BSA in Tris-buffer saline containing 0.05% Tween 20 (TTBS) for 1 hour at room temperature. The membrane was washed and incubated with streptavidin-HRP conjugate (Amersham, 1:10 000 in TTBS containing 1% BSA) for 1 hour at room temperature. After six washings with TTBS, the membrane was incubated for 5 min with SuperSignal™ West Dura substrate (Thermo-Scientific) and signals were captured on C-DiGit Scanner (Li-cor, USA). Biotinylated proteins were present in S2 (Fig. 2D). As an input control, protein extract of biotinylated *Borrelia* immobilized on the nitrocellulose membrane was also detected with the streptavidin-HRP conjugate. Protein extract of hBMECs without any incubation with borrelial proteins served as the negative control (Fig. 2D).

After confirming the presence of biotinylated proteins in S2 through dot blot, the rest of S2 (2 ml) was incubated with NeutrAvidin agarose beads (200 µl) and eluted in 200 µl of 50 mM dithiothreitol (DTT) in PBS (pH 7.2). It should be noted that EZ-Link NHS-SS-Biotin contains a disulfide bond in its spacer arm, enabling labeled proteins to be cleaved from the biotin group by treatment with reducing agents like DTT. The eluate was vacuum dried in a concentrator (Speed-Vac, Thermo-Scientific). Moreover, removal of the biotin group from the proteins increases the accuracy of the protein identification with mass spectrometry. Proteins in the eluate were identified by mass spectrometry (SWATH-MS, AB Sciex, USA) as described previously (Jimenez-

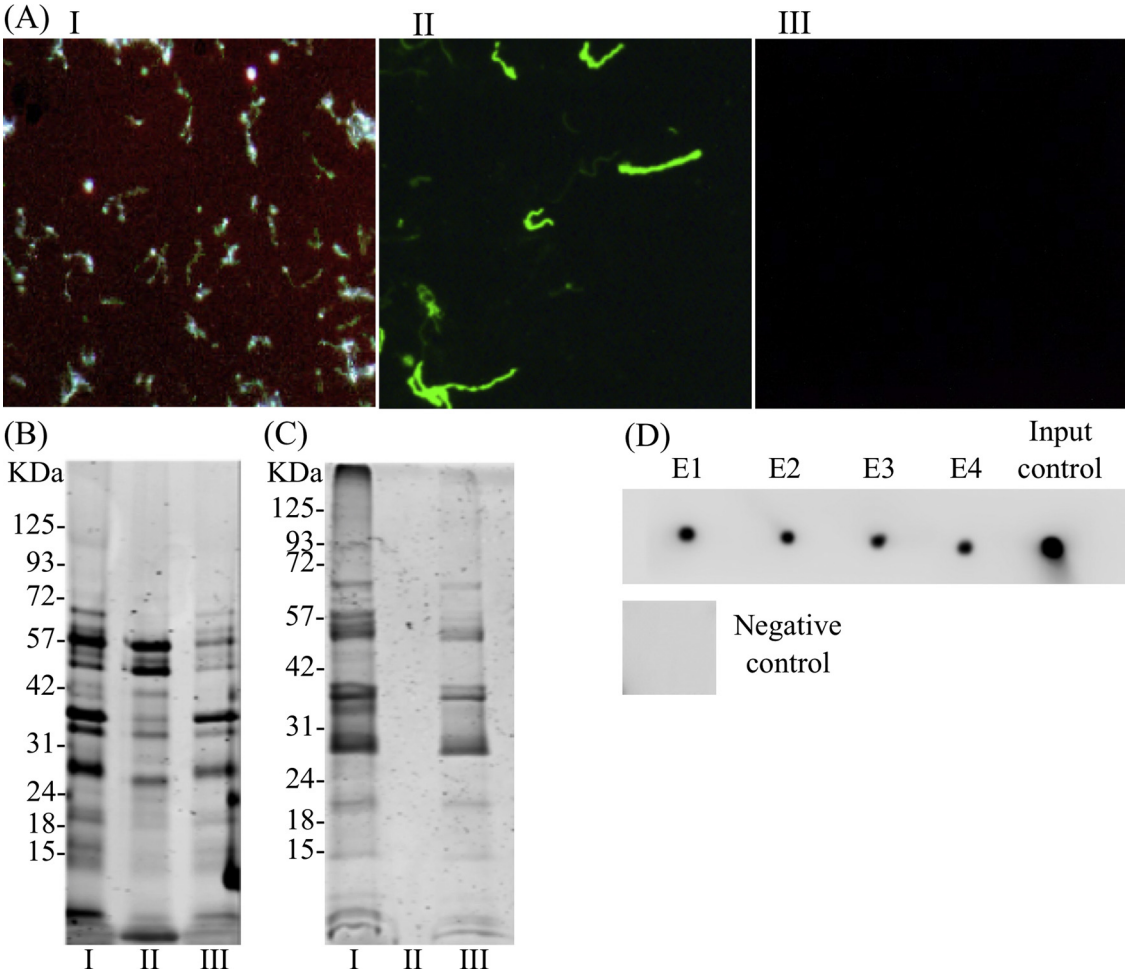

Fig. 2. Biotinylation of live *Borrelia* and confirming the presence of biotinylated proteins bound to hBMECs. **(A)** *Borrelia* under dark field microscopy (40x magnification), which were used for biotinylation (I), biotinylated *Borrelia* (II) or non-biotinylated *Borrelia* (III) incubated with the streptavidin-FITC conjugate, washed and visualized under the fluorescent microscope (480 nm, 100 x magnification). **(B)** Whole-cell extract of biotinylated *Borrelia* (lane I), biotinylated proteins were captured with NeutrAvidin beads, eluted and separated on SDS-PAGE (lane II), proteins remaining in flow-through after capture (lane III). **(C)** Whole-cell extract of non-biotinylated *Borrelia* (lane I), proteins captured with NeutrAvidin beads, eluted and resolved on SDS-PAGE (lane II), proteins remaining in flow-through after capture (lane III). **(D)** Biotinylated proteins of *Borrelia* adhering on the hBMECs were captured from the cell lysate of hBMECs with NeutrAvidin beads and eluted. 2 µl of each eluate (E1 to E4, four replicates) spotted on the nitrocellulose membrane and the presence of biotinylated proteins was detected with the streptavidin-HRP conjugate. Input control - biotinylated proteins of *Borrelia* spotted on the membrane and detected with the streptavidin-HRP conjugate. Negative control - protein extract of hBMECs (without incubation with borrelial proteins) spotted on the membrane and incubated with the streptavidin-HRP conjugate.

Munguia et al., 2018). In brief, the vacuum dried eluate was re-suspended in 50 µL of 8 M urea in 50 mM Tris-HCl (pH 8.0). Proteins were reduced in 5 mM DTT for 30 min at 37 °C and alkylated with 15 mM iodoacetamide for 30 min at 25 °C. Thereafter, the trypsin/LysC mix was added to the proteins (1:20) and incubated for 4 hours at 37 °C. Next, 50 mM Tris-HCl (pH 8.0) was added to bring the urea concentration to 1 mol/L and incubated for the next 8 h at 37 °C. Trifluoroacetic acid (TFA) was added to stop the proteolytic reaction. Digested peptides were separated on a nano-LC (Ultimate 3000 Thermo-Scientific). Further desalting (mobile phase 2% acetonitrile, 0.05% TFA) was performed by Pre-column (Acclaim PepMap µ, Dionex) and the peptides were separated on a C18 column (25 cm Acclaim PepMap, the flow rate of 250 nL/min, gradient starting at 4% to 50% of elution solution (80% ACN with 0.1% formic acid, elution time 180 min). Lastly, peptides were analyzed with TripleTOF 5600+ (Sciex, USA). Information dependent data acquisition (IDA) was performed with survey scans ranging between 400–1600 m/z. The most intense precursors with a higher than two charge state with a minimum of 90 counts per second were selected for fragmentation. Ion scans were collected for MS2 in the range of 90–1700 m/z for 120 ms. Data were processed with ProteinPilot Software version 5.0 (Sciex, USA). For protein search, the UniProt database was restricted to *Borrelia (B. garinii)*. Mass tolerance in MS mode was set between 0.001 Da in MS and 0.01 Da in MS/MS mode for the search. The sample parameters were: trypsin digestion, cysteine alkylation - iodoacetamide, search effort - rapid ID. False discovery rate analysis (FDR) was performed using the integrated tools in ProteinPilot. The global false discovery rate was set to < 1%. IDA identification results were used to create the SWATH ion library with the MS/MS with SWATH Acquisition MicroApp 2.0 in PeakView 2.2 (both Sciex, USA).

2.6. Bioinformatics

Subcellular localization of the proteins identified in SWATH-MS was performed with the freely available algorithm LocateP (http://www.cmbi.ru.nl/locatep-db/cgi-bin/locatepdb.py) (Zhou et al., 2008). The presence of transmembrane domains was predicted by TMHMM 2.0 (http://www.cbs.dtu.dk/services/TMHMM/) (Krogh et al., 2001). Type-I and type-II signal peptides were predicted with LipoP (http://www.cbs.dtu.dk/services/LipoP) (Juncker et al., 2003). Gene ontology was retrieved using Blast2GO program (https://www.blast2go.com), which provided annotations from the characterized proteins. Whereas

for non-annotated proteins, annotations were retrieved from their orthologs (> 95% identity). For proteins lacking annotations in Blast2GO, a search was performed in UniProt (http://www.uniprot.org/) and in earlier published studies (literature survey) to obtain biologically relevant data.

2.7. Synthesis of the recombinant form of the shortlisted proteins

The recombinant form of borrelial proteins - LysM domain protein (BGAPBR_0326), Antigen S1 (BGAPBR_A0008), CRASP-1 (BGAPBR_A0071), Erp23 protein (BGAPBR_Q0067), BESBP-5 (BGAPBR_0334) and Lipoprotein of Mlp family (BGAPBR_V0029) were used to validate their interaction with the proteins of hBMECs. In brief, gene fragments encoding the surface-exposed region of aforestated proteins were amplified by PCR from the genomic DNA of *B. garinii*. Detailed information on primers, amplicon length, and names of restriction enzymes used for cloning are presented in Table S1 of Supplementary material 1. Digestion of amplified PCR products, ligation into a pQE-30-mCherry-GFP plasmid (Fig. S1 in Supplementary material 1), transformation into *E. coli* expression system, and selection of clones were performed as described earlier (Jimenez-Munguia et al., 2018). The presence of the inserted gene was confirmed by sequencing with vector-specific primers UA Insertom F and R, (Table S2 of Supplementary material 1). Protein expression and purification with metal affinity chromatography were carried out as described previously (Jimenez-Munguia et al., 2018). Proteins were desalted with gel filtration (Sephadex G25) using FPLC (Äkta, GE healthcare) and the purity of recombinant proteins was assessed by SDS-PAGE, while molecular mass was measured by MALDI-TOF MS.

The recombinant form of non-related proteins (truncated form of OspB - Q09090.1 and mCherry - AST15061.1) was also produced as described above to use them as negative controls. Detailed information on the primers used to amplify the coding regions, length of amplicon and names of restriction enzymes employed in cloning are presented in Table S1 of Supplementary material 1. Ligation, overexpression and protein purification were performed as described above. Recombinant OspA and domain III of protein E of the West Nile virus used as positive control were from our previous work (Mlynarcik et al., 2015; Kánová et al., 2018).

Bradford assay was used to measure the concentration of recombinant proteins and their aliquots were stored at −20 °C in 20% glycerol until further use.

2.8. Confirmation of the interaction between recombinant ligands of B. garinii and proteins of hBMECs

ELISA was performed as described before with minor modifications (Jimenez-Munguia et al., 2018). Briefly, the wells were coated with protein extract of hBMECs diluted in coating buffer (8 µg of protein in 10 mM Na_2CO_3, 40 mM $NaHCO_3$, pH 7.2). Thereafter, various concentrations of each recombinant ligand (125 pMol, 250 pMol or 500 pMol, Table S3 in Supplementary material 1) were incubated for 1 hour at room temperature. Unbound proteins were washed away with PBS containing 0.05% Tween-20. The binding of recombinant ligands to hBMECs proteins was detected by HisProbe-HRP (1 µg/ml dissolved in PBS containing 0.05% Tween-20) during 30 min of incubation at room temperature and the reaction was developed with 1-step Ultra TMB (Thermo-Scientific). Absorbance was measured at 450 nm. As a negative control, 8 µg of the hBMECs extract was coated in the wells and incubated with HisProbe-HRP. Non-related proteins (His-tagged truncated OspB and His-tagged mCherry, Table S3 in Supplementary material 1) were used as another negative control. As a positive control, 8 µg of the hBMECs extract was incubated with recombinant domain III of protein E of West Nile Virus (A previously known protein that interacts with hBMECs, Table S3 in Supplementary material 1) and its detection was carried out using HisProbe-HRP. Absorbance obtained from the negative control wells (without recombinant proteins) was used for background subtraction. The assay was performed in triplicate.

2.9. Confirmation of binding of ligands to the cultured hBMECs

Immunocytochemistry was performed as described earlier with minor modifications (Jimenez-Munguia et al., 2018). Briefly, hBMECs were cultured on the coverslips coated with collagen type I (Sigma, USA) until 70% confluency. Cells were then incubated with purified recombinant ligands (500 pMol resuspended in 1 mL EBM-2 medium, Table S3 of Supplementary material 1) for 1 hour at 37 °C in 5% CO_2. Washing of the cells with PBS containing 0.05% Tween 20 (PBST) was performed prior to their fixing with ethanol/acetone (2:8 v/v) for 10 min. After conducting 3 washes with PBST, the presence of bound recombinant proteins was detected with anti-His antibody conjugated with FITC (Abcam). In the case of negative control assay, recombinant ligands were excluded. Moreover, non-related proteins (His-tagged truncated OspB and His-tagged mCherry, 500 pMol Table S3 of Supplementary material 1) were also used in the immunocytochemistry as additional negative controls. hBMECs incubated with domain III of protein E of West Nile Virus or OspA served as a positive control (500 pMol Table S3 in Supplementary material 1). Photo-documentation was performed on the LSM-710 microscope (Zeiss, Germany). Fluoroshield with DAPI (Sigma) was used to mount the cells. The experiment was performed in biological triplicate.

3. Results

3.1. Multiple proteins of B. garinii may interact with hBMECs

The protein extract of the biotinylated *Borrelia* was incubated with hBMECs, the proteins that bound to the endothelial cells were selectively captured on NeutrAvidin beads and identified with SWATH-MS. The list of proteins identified in four replicates is in Supplementary material 2. Among them, 12 protein candidates were present in all the replicates (Table 1). The present study has identified several proteins that are uncharacterized to date (viz., BGAPBR_0055, BGAPBR_0443, BGAPBR_0546, BGAPBR_0560 and BGAPBR_0614). These uncharacterized proteins were present in the 1st and 4th replicates (Table 1). We also document 14 proteins with known functions (e.g. OspC, BmpA, CRASP-1, DbpA, etc.), whereas two candidates are designated as putative lipoprotein and putative membrane protein (BGAPBR_A0064 and BGAPBR_0072). Two lipoproteins (BGAPBR_V0029 and BGAPBR_H0004) identified in the present study lack functional annotations.

All the identified proteins were subjected to the series of *in silico* analyses to predict subcellular localization, presence of transmembrane domain or lipid moiety. An additional search was also performed to retrieve information on the ontology and function of each identified protein candidate.

3.2. In silico analysis

Realizing the importance of bacterial surface proteins for the adhesion of pathogens to host cells, we applied bioinformatics tools to shortlist outer surface proteins (Fig. 1). The identified proteins were grouped as either outer (12 candidates) or inner membrane proteins (12 candidates) to designate their subcellular location with the help of the LocateP server (Table 1). The presence of transmembrane domains was predicted by TMHMM 2.0, wherein 13 proteins were found to possess an outer membrane domain. Apart from the LysM domain protein (BGAPBR_0326), which has only one transmembrane domain (Table 1), four inner membrane proteins (rseP, ftsH, BGAPBR_0756 and BGAPBR_0603) possess more than one transmembrane domain. Multiple transmembrane domains anchor the protein in the membrane at multiple sites therefore, their exposure from the outer part of the

Table 1
Potential surface ligands of *B. garinii* identified in our study by SWATH-MS

No.	Protein hit from SWATH-MS (Mascot search)	Present in replicates#	Protein name in serotype 4 *B. garinii* / paralog	LocateP	TMHMM	LipoP
Outer membrane proteins						
1	*Borrelia* lipoprotein OspA [B8F1J0]*, (BGAPBR_A0017)†	1,2,3,4	Outer surface protein A	Outer membrane lipoprotein	Outside	SP-II
2	Outer surface protein C [B8F168], (BGAPBR_B0020)	1,2,3,4	Outer surface protein C	Outer membrane lipoprotein	Outside	SP-II
3	Erp23 protein (ErpL) [B8F1E4], (BGAPBR_Q0067)	1,2,3,4	ErpL	Outer membrane lipoprotein	Outside	SP-II
4	Basic membrane protein A/Immunodominant antigen P39 [B7XT52], (BGAPBR_0384)	1,2,3,4	Basic membrane protein A	Outer membrane lipoprotein	Outside	SP-II
5	Antigen S1 [B8F1I1], (BGAPBR_A0008)	1,2,3,4	Antigen, S1	Outer membrane lipoprotein	Outside	SP-II
6	BESBP-5 [B7XT01], (BGAPBR_0334)	1,2,3,4	Bacterial extracellular solute-binding protein, family 5 (BGAPBR_0334)	Outer membrane lipoprotein	Outside	SP-II
7	Lipoprotein of Mlp family [B8F0W5], (BGAPBR_V0029)	2,3,4	Mlp family contains several paralogs however their functions are still unknown.	Outer membrane lipoprotein	Outside	SP-II
8	Putative lipoprotein [B8F1N7], (BGAPBR_A0064)	1,2,3,4	No information available in the literature	Outer membrane lipoprotein	Outside	SP-II
9	CRASP-1 [B8F1P4], (BGAPBR_A0071)	1,2,3,4	Bga71 paralog	Outer membrane lipoprotein	Outside	Cytoplasmic
10	LysM domain protein [B7XSZ3] (BGAPBR_0326)	1,2,3,4	Bg0324 paralog	Outer membrane lipoprotein	1 TMD	SP-II
11	*Decorin binding protein A [B8F1J8], (BGAPBR_A0025)*	*2,3,4*	*Decorin binding protein A*	*Intracellular protein*	*Outside*	*Cytoplasmic*
12	*Enolase (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase) [B7XT07], (eno)*	*3,4*	*enolase*	*Intracellular protein*	*Outside*	*Cytoplasmic*
Inner membrane proteins						
13	Zinc metalloprotease [B7XTK5], (rseP)	3	Zinc metalloprotease	Integral inner membrane	5 TMD	Cytoplasmic
14	ATP-dependent zinc metalloprotease FtsH [B7XTU2], (ftsH)	1,2,3,4	FtsH	Integral inner membrane protein	2 TMD	TMD
15	Transporter, dicarboxylate/amino acid:cation (Na + or H+) symporter (Daacs) family [B7XSB7], (BGAPBR_0756)	1,2,3,4	Glutamate transporter (gltP)	Integral inner membrane	9 TMD	TMD
16	Conserved hypothetical integral membrane protein [B7 × R × 6], (BGAPBR_0603)	1,3,4	Conserved hypothetical integral membrane protein	Integral inner membrane	12 TMD	TMD
17	Uncharacterized protein [B7XTE2], (BGAPBR_0055)	1,4	Protein-export membrane protein SecG (BG0053)	Integral inner membrane	TMD	TMD
18	Putative membrane protein [B7XTF9] (BGAPBR_0072)	2,3,4	No information available in the literature	Integral inner membrane	SP-I	SP-I
19	Uncharacterized protein [B7XRH5], (BGAPBR_0443)	1,4	No information available in the literature	Integral inner membrane protein	TMD	TMD
20	Uncharacterized protein [B7XRY7], (BGAPBR_0614)	1,4	No information available in the literature	Integral inner membrane	Cytoplasmic	Cytoplasmic
21	Uncharacterized protein [B7XRS3], (BGAPBR_0546)	1,4	No information available in the literature	Integral inner membrane protein	TMD	TMD
22	Uncharacterized protein [B7XRT4], (BGAPBR_0560)	1,4	No information available in the literature	Periplasmic/Integral inner membrane protein	SP-I	SP-I
23	*Borrelia lipoprotein [B8F1F6], (BGAPBR_H0004)*	*1,2,3,4*	No information available in the literature	*Intracellular protein*	*Outside*	*Cytoplasmic*
24	*Spermidine/putrescine import ATP-binding protein PotA [B7XS33], (potA)*	*1,3*	*spermidine*	*Intracellular protein*	*Outside*	*Cytoplasmic*

This table summarizes the results obtained from SWATH-MS and identification of proteins with Proteinpiloa t using Mascot search engine. #Protein hits in all replicates are presented in Supplementary material 2. * number in square brackets presents the uniport number. † round bracket presents the ORF name rederived from uniport for the given protein entry. Categories for the subcellular location were established according to LocateP. LipoP. The presence of transmembrane domains was predicted by TMHMM 2.0 that showed anchoring of proteins in the membrane. LipoP discriminate proteins containing an SP-I type and SP-II type (lipoproteins) signal peptides. Four proteins (in italics) according to LocateP and LipoP were predicted as cytoplasmic proteins, however, Uniprot and Pfam showed these proteins as membrane proteins.

Table 2
Ontology and literature review-based categorization of the surface interactome

No	Entry	Protein name	Gene	CC	MF				VD	SD	Host-pathogen interaction			Colonization of host tissue	Other	Involved in crossing of BBB	References
				PM	Ion binding	Stress	Peptidase	Transport			Adhesion	Binding host factor H	Binding host plasminogen				
Outer membrane proteins																	
1	B8F1J0	*Borrelia* lipoprotein OspA	BGAPBR_A0017	X							X	X	X				(Sadziene et al., 1993; Fuchs et al., 1994; Sigal et al., 1998; Steere et al., 1998; Kovacs-Simon et al., 2011; Pulzova et al., 2011)
2	B8F168	Outer surface protein C (OspC)	BGAPBR_B0020	X		X			X	X						X	(Coleman et al., 1995; Schuijt et al., 2011; Caine and Coburn, 2016)
3	B7XT52	Basic membrane protein A (Immunodominant antigen P39)	BGAPBR_0384	X						X	X						(Simpson et al., 1990; Verma et al., 2009)
4	B7XT01	Bacterial extracellular solute-binding protein, family 5	BGAPBR_0334	X				X				X					(Bhide et al., 2009)
5	B8F1E4	Erp23 protein (ErpL)	BGAPBR_Q0067						X		X				X		(Coleman et al., 1995; Brissette et al., 2008; Schuijt et al., 2011; Lin et al., 2015)
6	B8F1P4	CRASP-1 (Bga71)	BGAPBR_A0071								X	X	X			X	(Hellwage et al., 2001; Kraiczy et al., 2004; Hallström et al., 2010)
7	B8F1I1	Antigen S1	BGAPBR_A0008												X		(Xu et al., 2010)
8	B7XSZ3	LysM domain protein (Bg0324)	BGAPBR_0326								X				X		(Buist et al., 2008)
9	B8F0W5	Mlp Lipoprotein	BGAPBR_V0029						X		X				X		(Juncker et al., 2003; Kovacs-Simon et al., 2011){Kovacs-Simon, 2011, Lipoproteins of bacterial pathogens}
10	B8F1N7	Putative lipoprotein	BGAPBR_A0064						X		X				X		(Kovacs-Simon et al., 2011)
11	B8F1J8	Decorin binding protein A	BGAPBR_A0025								X		X	X		X	(Fischer et al., 2003)
12	B7XT07	Enolase (EC 4.2.1.11) (2-phospho-D-glycerate hydro-lyase) (2-phosphoglycerate dehydratase)	eno	X	X								X		X		(Floden et al., 2011)
Inner membrane proteins																	
13	B7XTK5	Zinc metalloprotease	rseP	X	X		X								X		(Hu et al., 2001)
14	B7XTU2	ATP-dependent zinc metalloprotease FtsH	ftsH	X	X									X	X		(Chu et al., 2016)
15	B7XSB7	Transporter, dicarboxylate/ amino acid:cation (Na + or H+) symporter (Daacs) family	BGAPBR_0756	X				X							X		(Rahman et al., 2017)
16	B7 × R × 6	Conserved hypothetical integral membrane protein	BGAPBR_0603	X				X									
17	B7XTE2	Uncharacterized protein	BGAPBR_0055	X				X									
18	B7XTF9	Putative membrane protein	BGAPBR_0072	X													

(*continued on next page*)

Table 2 (*continued*)

No	Entry	Protein name	Gene	CC	MF				VD	SD	Host-pathogen interaction			Colonization of host tissue	Other	Involved in crossing of BBB	References
				PM	Ion binding	Stress	Peptidase	Transport			Adhesion	Binding host factor H	Binding host plasminogen				
19	B7XRH5	Uncharacterized protein	BGAPBR_0443	X													
20	B7XRY7	Uncharacterized protein	BGAPBR_0614	X													
21	B7XRS3	Uncharacterized protein	BGAPBR_0546					X									
22	B7XRT4	Uncharacterized protein	BGAPBR_0560														
23	B8F1F6	*Borrelia* lipoprotein	BGAPBR_H0004	X											X		(Kovacs-Simon et al., 2011)
24	B7XS33	Spermidine/putrescine import ATP-binding protein PotA (EC 3.6.3.31)	potA					X							X		(Lin et al., 2017)

[a]Categories of the functional analysis were established according to BLAST2GO predictions; CC-cellular component, MF-molecular function, PM-plasma membrane.

[b]Categories assigned based on literature review; VD: vaccine design; SD: serodiagnostic; RT: includes processes such as the colonization of the respiratory tract or pneumonia.

Empty cells- not assigned function or not described in the literature.

Other - included functions, such as a role in infections, binding albumin, and elastin, heparan sulfate binding, hydrolase and glycosylase activity, modulation of inflammatory processes, degradation of collagen, digestion of proteoglycans, regulation of the cellular response to environmental stress or transport of proteins.

membrane gets hidden. Among the groups of inner membrane proteins, two proteins BGAPBR_0072 (putative membrane protein) and BGAPBR_0560 (uncharacterized protein) had transmembrane segments in the N-terminal region and it often turns out to be a signal peptide. One uncharacterized protein (BGAPBR_0614) from the same group was predicted as cytoplasmic protein and two proteins (*Borrelia* lipoprotein - BGAPBR_H0004 and potA) were predicted to be localized outside by TMHMM 2.0 (Table 1).

The presence of type-I and type-II signal peptides were predicted with LipoP. For almost all outer membrane proteins, LipoP predicted type-II signal peptide that is characteristic for lipoproteins. Interestingly, complement regulated acquiring surface protein 1 (CRASP-1; BGAPBR_A0071) and DbpA (BGAPBR_A0025) were predicted as cytoplasmic proteins by LipoP, however, they are well-characterized surface-exposed proteins binding to complement factor H and decorin, respectively (summarized in Table 2).

3.3. Ontology search and literature review

Annotations for the protein candidates were also retrieved from Blast2GO software. As a result, additional annotations were found for five out of twelve outer membrane proteins. Outer surface protein A (BGAPBR_A0017), Outer surface protein C (BGAPBR_B0020) and Basic membrane protein A (BGAPBR_0384) are having an association with the plasma membrane. On the other hand, bacterial extracellular solute-binding protein family 5 (BESBP-5; BGAPBR_0334) is involved in transport and Enolase (eno) is associated with magnesium ion binding and plasminogen binding. No annotations were available for the remaining 7 proteins (Table 2).

Except for three proteins (BGAPBR_0546, BGAPBR_0560 and Spermidine), all other inner membrane proteins were annotated as “cellular components of the plasma membrane proteins”. Five proteins were annotated to participate in transport (BGAPBR_0756, BGAPBR_0603, BGAPBR_0055, BGAPBR_0546 and potA), whereas, ATP-dependent zinc metalloprotease (FtsH) and zinc metalloproteinase (rseP) were annotated to participate in ion binding. The zinc metalloproteinases also possess peptidase activity. However, no relevant information was available for BGAPBR_0560 (uncharacterized protein) even in repositories that can be retrieved with the help of Blast2GO software (Table 2).

For a better understanding of the identified proteins, a literature review was also performed to obtain information on all non-annotated outer membrane proteins. Table 2 summarizes earlier reported functions of the non-annotated protein candidates. CRASP-1 (Bga071 paralog, BGAPBR_A0071) and Erp23 protein (BGAPBR_Q0067, a homolog of the ErpL protein) were reported to participate in the adhesion process (Hellwage et al., 2001; Kraiczy et al., 2004; Brissette et al., 2008; Hallström et al., 2010). ErpL was also reported to bind heparan sulfate glycosaminoglycans of the host cells (Lin et al., 2015). BESBP-5 was documented to bind host factor H (Bhide et al., 2009). Additionally, OspA (BGAPBR_A0017), CRASP-1 and DbpA (BGAPBR_A0025) binding to host plasminogen was proclaimed (Fuchs et al., 1994; Coleman et al., 1995; Fischer et al., 2003; Brissette et al., 2008; Hallström et al., 2010). Participation of LysM domain protein (BGAPBR_0326), Mlp Lipoprotein (BGAPBR_V0029) and putative lipoprotein (BGAPBR_A0064) in adhesion has also been described (Buist et al., 2008; Kovacs-Simon et al., 2011).

Based on earlier studies the identified proteins such as OspC, and DbpA could be associated with adhesion to the endothelial cells (Table 2) (Coleman et al., 1995; Fischer et al., 2003; Pulzova et al., 2011). Similarly, the plasminogen-binding property of enolase and CRASP-1 indicates that they may also involve in borrelial translocation across the endothelial barrier (Table 2) (Coleman et al., 1995; Hallström et al., 2010; Floden et al., 2011). The enolase is a plasminogen receptor released in outer membrane vesicles (Nogueira et al., 2012; Toledo et al., 2012).

3.3.1. Confirmation of binding of ligands to hBMECs

Outer surface proteins are the interface between bacterial cells and the external milieu and are also the key effectors during bacterial translocation across the BBB. Thus, candidates predicted as outer membrane proteins or those possessing a lipid moiety were selected for confirmation of their binding to hBMECs. Four outer membrane proteins were selected (LysM domain protein, BGAPBR_0326; Antigen S1, BGAPBR_A0008; CRASP-1, BGAPBR_A0071; and Erp23 protein, BGAPBR_Q0067). The participation of these 4 candidates in the host-pathogen interactions is presented in the literature, however, in the ontology search, no annotations were available (Table 2). One protein possessing a lipid moiety (Mlp Lipoprotein, BGAPBR_V0029) was also selected, whose function is still unknown (Table 2). The 6th selected protein was bacterial extracellular solute-binding protein family 5 (BESBP-5, BGAPBR_0334). Ontology search and literature review indicated that BESBP-5 binds host factor H and is involves in transport. Naturally, proteins predicted as inner membrane proteins were not included in further assays to confirm their binding to hBMECs. Other outer membrane proteins identified in the SWATH-MS, viz. OspA, OspC, DbpA, and BmpA were exempted from further validation, as their function and their level of expression are well studied. Moreover, the interaction of OspA with the CD40 is well documented in our earlier studies (Pulzova et al., 2011; Mlynarcik et al., 2015). OspC is shown to interact with endothelial cells (Antonara et al., 2007), however, this protein is upregulated mainly during the initial stage of the infection and is dramatically downregulated in the later stage (Cadavid et al., 2000). Expression of DbpA is upregulated in the host environment and its interaction with decorin expressing cells is well studied (Salo et al., 2011). The BmpA is an important surface protein of *Borrelia* that interacts with laminin, however, expressed mainly in joints in comparison to other host organs (Pal et al., 2008).

Recombinant forms of the selected ligands were produced to validate the interaction with hBMECs (Table 3). Correct insertion of the gene of interest in the transformants was checked with PCR (plasmid-specific primers, Fig. 3A). The purity of the recombinant proteins was evaluated by SDS-PAGE and their molecular masses were assessed by MALDI-TOF as shown in Fig. 3B and 3C. SDS-PAGE confirmed that these proteins were devoid of non-specific impurities and proteolysis that may occur during the purification process.

Validation of the interaction was performed by ELISA (Fig. 4). Different concentrations of the recombinant proteins were used in ELISA to check dose-dependent interaction with hBMECs. Among the set of recombinant ligands, Antigen S1 showed the strongest binding affinity. Of note, dose-dependent affinity was observed only in the case of Antigen S1 (Fig. 4). On the other hand, increasing concentration of LysM, CRASP-1, and Erp23 proteins decreased their binding ability (expressed in terms of OD value) (Fig. 4).

Immunocytochemistry was also performed to corroborate the interaction between recombinant ligands and hBMECs. The assay revealed that all ligands possess an affinity to the hBMECs *in situ* (Fig. 5). OspA protein of *Borrelia* and domain III of protein E of West Nile virus used as positive control showed significant binding affinity to the endothelial cells, whereas, mCherry used as non-related protein (negative control) showed no interaction with the cells (Fig. 5). Another protein, a truncated form of OspB (a protein, which was not identified as a plausible interacting protein in SWATH-MS), used as negative control also showed no interaction with endothelial cells (Fig. 5). The absence of binding of non-related proteins in the assay confirms that the interaction between recombinant ligands and endothelial cells is specific.

4. Discussion

Borrelial surface proteins play an important role in the evasion of the mammalian immune system, cell communication and activation of the cell processes required for invasion. Several experimental approaches have been developed to unfold the interactions between pathogen ligands and the cell receptors (Cullen et al., 2004; Rodríguez-Ortega et al., 2006; Dreisbach et al., 2010; Pulzova et al., 2011; Gesslbauer et al., 2012; Olaya-Abril et al., 2012). Among them, shaving of surface proteins from the intact cells helps in identifying the bacterial surface interactome (Rodríguez-Ortega et al., 2006; Dreisbach et al., 2010; Olaya-Abril et al., 2012), however, trypsin used to cleave the proteins could disrupt the interacting site that might negatively influence the protein-protein interactions. The ligand-capture assay is another alternative to identify bacterial proteins interacting with endothelial cells (Pulzova et al., 2011), however, it is laborious and less sensitive. The detergent-based approach is also suitable to solubilize outer membrane proteins for downstream protein-protein interactions assays, however, ionic detergent like SDS can denature the proteins. On the other hand, nonionic detergents may trigger the proteolysis of membrane proteins (Cullen et al., 2004). In the previous study, biotinylation of live *Borrelia* was found as the most efficient approach to isolate and identify outer membrane proteins than the detergent-based isolation or trypsin digestion of borrelial surface (Gesslbauer et al., 2012). The femtomolar dissociation constant between biotin and streptavidin enables the recovery of extremely low abundant biotinylated proteins present in the biological material. It is important to note that, the biotin used in our study was water-soluble and its spacer arm includes a cleavable disulfide bond for reversible labeling of proteins, which is advantageous for downstream MS applications. Although several biotinylation reagents are available (Elia, 2008), it is important to check their compatibility with the biological material (e.g. with live cells). Biotin labeling was used successfully by other researchers to study protein-protein interactions (Horvatić et al., 2016; Chang et al., 2017) or to label bacterial surface proteins (Cullen et al., 2004; Gesslbauer et al., 2012; Voss and Cover, 2015). It is noteworthy that, because of the thick capsule, not all live bacteria (e.g. *N. meningitidis* or *S. pneumoniae*) can be biotinylated (Jimenez-Munguia et al., 2018).

In silico predictions of *Borrelia* proteome comprised of 825 proteins (33 lipoproteins, 158 proteins with transmembrane domains and 38 designated as outer membrane proteins with β-barrel (Kenedy et al., 2016)). By using the experimental approach, 44 proteins have been identified associated with membrane (Gesslbauer et al., 2012). Membrane proteins of Gram-negative bacteria predominantly consist of large hydrophobic antiparallel β-barrels (Jeanteur et al., 1991; von Heijne, 1992), which makes it difficult to resolve on 2D-PAGE. This

Table 3
Potential ligands of *B. garinii* selected to produce their recombinant form

No.	Entry Uniprot	Protein name	Gene	Amino acid positions in recombinant form	Mw (kDa)	
					Theoretical	Observed in SDS-PAGE
1	B7XSZ3	LysM domain protein	BGAPBR_0326	E27-P374	69.548	≈ 69
2	B7XT01	BESBP-5	BGAPBR_0334	V41-N520	84.183	≈ 84
3	B8F1I1	Antigen, S1	BGAPBR_A0008	D26-N360	67.517	≈ 67
4	B8F1P4	CRASP-1	BGAPBR_A0071	D12-A169	47.186	≈ 47
5	B8F1E4	Erp23 protein (ErpL)	BGAPBR_Q0067	S31-A226	50.099	≈ 50
6	B8F0W5	Lipoprotein of Mlp family	BGAPBR_V0029	L14-C137	42.672	≈ 42

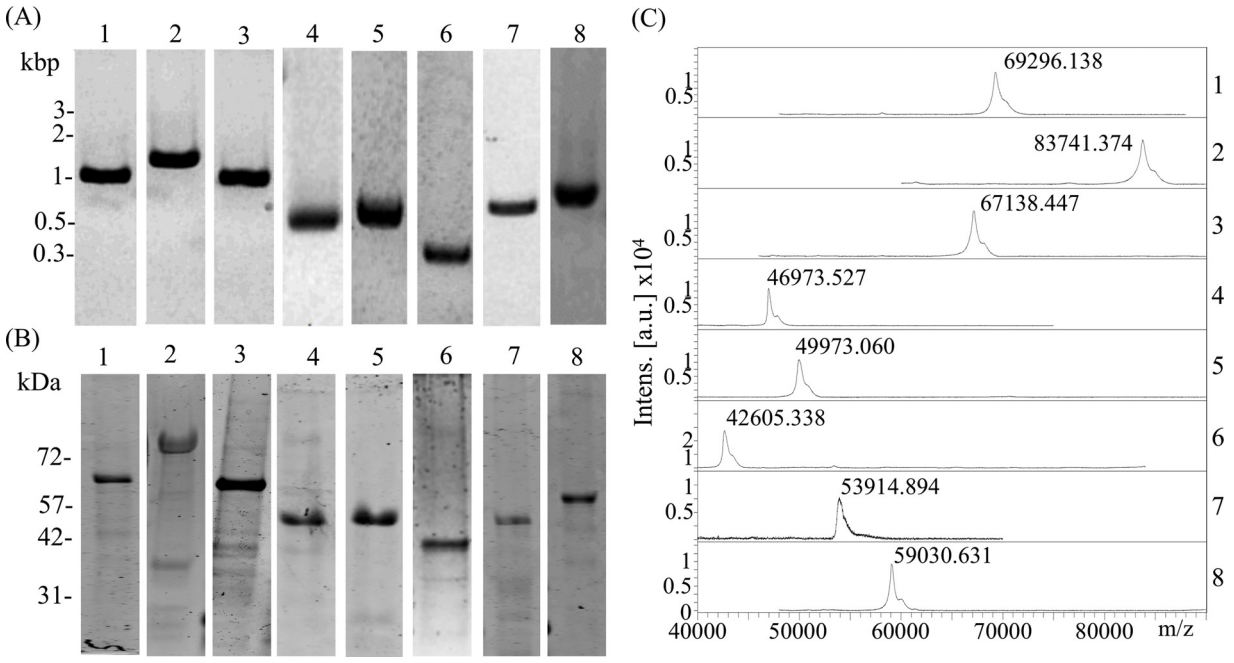

Fig. 3. Production of recombinant forms of the selected protein candidates. **(A)** amplicons of coding fragments resolved on the agarose gel. **(B)** Purified recombinant proteins separated with SDS-PAGE. **(C)** The molecular mass of recombinant proteins confirmed with MALDI-TOF/MS. 1 - LysM domain protein (BGAPBR_0326); 2 - BESBP-5 (BGAPBR_0334); 3 - Antigen, S1 (BGAPBR_A0008); 4 - CRASP-1 (BGAPBR_A0071); 5 - Erp23 protein (BGAPBR_Q0067); 6 - Lipoprotein (BGAPBR_V0029), 7 - truncated OspB (Q09090.1) and 8 - mCherry (AST15061.1). OspB and mCherry were produced to use as the negative control (non-related proteins) in immunocytochemistry.

limitation could be circumvented with the high-throughput data-independent acquisition by mass spectrometry -SWATH-MS (Ortea et al., 2016). Due to the high sensitivity of SWATH-MS, even the minor interactors (or low abundant proteins) were detected in this study. Recently, we have demonstrated biotin labeling of bacterial proteome and SWATH-MS to identify the interactome of *N. meningitidis* (Kánová et al., 2018) and *S. pneumoniae* (Jimenez-Munguia et al., 2018). Likewise, chemical cross-linking and MS were used to demonstrate interspecies protein-protein interactors in human lung epithelial cells infected with *Acinetobacter baumannii* (Schweppe et al., 2015).

In the present study, the LocateP algorithm applied to the set of identified candidates, predicted twelve proteins as outer membrane proteins and another twelve were categorized as inner membrane proteins. Although the presence of inner membrane proteins is beyond expectation, cytoplasmic or inner membrane proteins were identified in an earlier study after biotinylation of live *Borrelia* (Gesslbauer et al., 2012). As described above, biotin is a relatively small molecule and any minute variation in outer membrane integrity of *Borrelia* can lead to the labeling of subsurface proteins such as inner membrane proteins (Cullen et al., 2004), which can interact with hBMECs and appear in mass spectrometry.

It is predicted that several protein-protein interactions between *Borrelia* and the host cells participate in the process of pathogen translocation across the BBB (Pulzova et al., 2009; Bencurova et al., 2011; Pulzova et al., 2011; Pulzova and Bhide, 2014). In the case of borrelial translocation, transient tethering and stationary adhesion (both mediated through protein-protein interactions) on the endothelial cell surface followed by crawling of spirochete through the endothelial

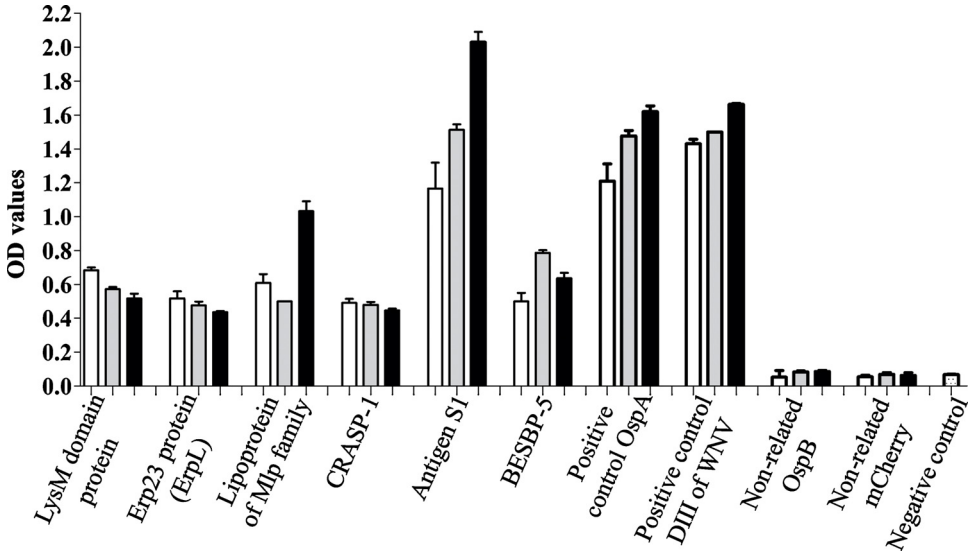

Fig. 4. Validation of the interaction between selected candidates of *B. garinii* and proteins of hBMECs. Semi-quantitative ELISA performed to confirm the interaction between proteins of hBMECs and bacterial ligands. The interaction was detected with HisProbe conjugated with HRP and TMB substrate. As positive control - domain III protein E of West Nile virus was used. Negative control (non-related protein) - protein not identified in SWATH-MS analysis (OspB). Another non-related protein, mCherry, was also used in the assay as the negative control. Negative control - no ligand was added. Different concentrations of the recombinant proteins were used in ELISA - white bars – 125 pMol, grey bars - 250 pMol and black bars – 500 pMol.

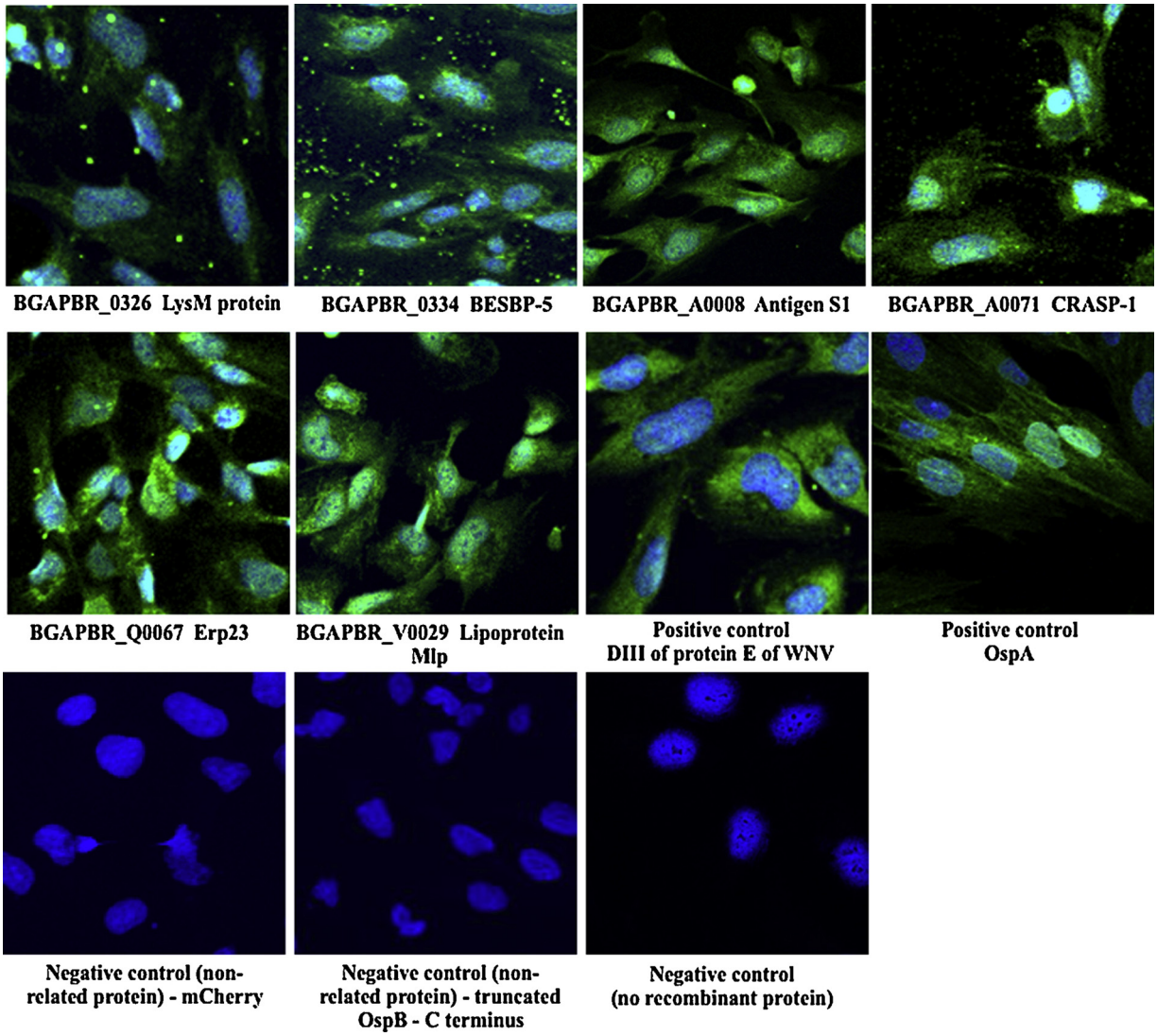

Fig. 5. Validation of the interaction between selected candidates of borrelial proteins and hBMECs by Immunocytochemistry. The interaction of selected proteins of *B. garinii* with cultured hBMECs. Nuclei were stained with DAPI. Recombinant proteins bound to hBMECs were detected with anti-His antibody conjugated with FITC. As positive control - domain III protein E of West Nile virus and OspA were used (proteins known to interact with hBMECs). Negative control – recombinant ligand was excluded from the assay. Negative control (non-related protein) - protein not identified in SWATH-MS analysis (OspB) was used in immunocytochemistry. Another non-related protein, mCherry, was also used in the assay as the negative control. The assay was performed in triplicate.

junctions are reported as major events (Bencurova et al., 2011; Moriarty et al., 2012). Each protein candidate identified in the study, their functions and their binding abilities to various cell receptors are discussed below in detail. The recombinant form of borrelial proteins, LysM domain protein (BGAPBR_0326), Antigen S1 (BGAPBR_A0008), CRASP-1 (BGAPBR_A0071), Erp23 protein (BGAPBR_Q0067), BESBP-5 (BGAPBR_0334) and Mlp Lipoprotein (BGAPBR_V0029), were selected in our study to validate their interaction with proteins of hBMECs. We understand that the recombinant version of these proteins may have a different topology from that produced on the spirochete surface and it would be ideal to generate mutants with a gain of function for precise assessment of adhesion in further studies.

Major adhesins like OspA and OspC are present among the 24 proteins detected in this study. With the gel-based approach, it was shown previously that the OspA interacts with the rat endothelial cells targeting the CD40 receptor (Pulzova et al., 2011). The crystal structure of OspA was revealed (21 antiparallel β-strands and an α-helix at the C terminus) in which the binding pocket formed by the charged residues (Arg139, Glu160, Lys189) was predicted (Li et al., 1997). Later it was proved that this pocket is essential for binding of OspA to CD40 (Mlynarcik et al., 2015). The OspC was also among the interactors identified in the present study. The affinity of OspC to vascular endothelium in mice was proposed earlier using *in vivo* phage display (Antonara et al., 2007), and its role in the establishment of infection was proved (Xu et al., 2007; Önder et al., 2012; Sen and Sigal, 2013). The CRASP-1 protein interacting with hBMECs (Fig. 4 and Fig. 5) is known to bind complement factor H, plasminogen (Kraiczy et al., 2001), fibronectin, laminin and several types of collagen (Hallström et al., 2010). Similarly, Erp23 (ErpL, the OspF-related Erp protein) is known to bind heparin or heparan sulfate (Lin et al., 2015). In the present study, we demonstrate that this protein can also bind the hBMECs cell line *in vitro* (Fig. 4 and Fig. 5). BESBP-5, another protein among the interactors, is a putative lipoprotein that can have signal peptide as predicted by LipoP (Table 1) and typical feature for lipoproteins of spirochetes (Setubal et al., 2006). Lipoproteins are usually considered structural components of the cell, however, surface-exposed lipoproteins of *Borrelia* can also be involved in the interactions with host tissues (Fraser et al., 1997; Casjens et al., 2000). Up to 4.9% of the chromosomal genes and 14.5% of the plasmid genes of *B. burgdorferi* B31 genome encode putative lipoproteins (Casjens et al., 2000). Many

borrelial lipoproteins mediate adhesion to integrins and host extracellular matrix molecules (Cabello et al., 2007). In the present study, most of the proteins found to interact with hBMECs were predicted as lipoprotein by LipoP prediction (Table 1). Among these lipoproteins, the Basic membrane protein A is known to bind laminin (Brissette et al., 2008; Verma et al., 2009), whereas DbpA binds decorin (Fischer et al., 2003). Decorin is expressed in the CNS and cerebral endothelial cells. A strong affinity of *Borrelia* to decorin suggests that this protein might have a central role in the bacterial colonization of the CNS. The high affinity of borrelial surface ligands to cell receptors and integrins is crucial for stationary adherence of *Borrelia* to the cerebral endothelium under constant blood flow, which is an essential step for the initiation of CNS colonization.

LysM domain protein, which showed the binding ability to the proteins of hBMECs in ELISA and live cells in immunocytochemistry in our study, contains a Lysin Motif. This motif is typically 44 to 65 amino acids long (Buist et al., 2008). LysM containing proteins have been identified in several bacterial species and are predicted to involve in protein-protein interactions (Pereira et al., 2019). This protein was reported as an essential factor for mammalian infection and *in vivo* survival of *Borrelia* (Kariu et al., 2013; Thakur et al., 2017). Albeit, the function of the LysM domain protein in *Borrelia* remains unexplored. Similarly, Antigen S1 (BBA05 or P55) was shown to elicit antibody responses in patients with early or late-stage Lyme disease (Xu et al., 2010), however, its function is not fully determined. Here we add a plausible adhesion function to this protein as validated by ELISA and immunocytochemistry (Fig. 4 and Fig. 5).

The proteomic approach presented here is a promising high-throughput tool to map plausible interacting proteins, nevertheless, the following limitations should be kept in mind when interpreting the findings of this study. *Borrelia* is known to alter the expression of surface proteins dramatically in response to the external environment. The relative abundance of each protein differs between *in vitro* culture and *in vivo* infection. Thus, some proteins identified in the present study might not be important in the pathogenesis because of their low or no expression *in vivo*. The expression levels of the proteins identified in this study are summarized in Table S4 in Supplementary material 1.

Some of the proteins that bind murine endothelial cells (e.g. BmpD, VlsE, and Lmp1) were not identified in the present study (Fischer et al., 2003). This might be because of the different origin of the endothelial cells (murine and human) or the low abundance of those proteins in *in vitro* conditions. Thus, the physiological importance of the protein candidates found in this study should be evaluated carefully based on their expression levels *in vivo*. Another caveat of this study is the use of the D3 hBMEC cell line. The primary endothelial cell line may lack some of the receptors, which might influence the identification of borrelial proteins. For example, We do not know if the D3 hBMEC cell line produces fibronectin in the sufficient amount, as the important fibronectin-binding adhesin BBK32 (Fischer et al., 2006) was not found in our study. Finally, it is important to note that in some cases *in silico* prediction algorithms may fail to categorize the protein candidate according to its subcellular locations. For example, DbpA, a known outer surface-exposed lipoprotein was designated as a cytoplasmic protein by LocateP and LipoP algorithms (Table 1). Thus, the results of *in silico* predictions should be used with caution and have to match with the data deposited in other repositories like UniProt and Pfam, to avoid exclusion of important protein candidates from downstream analysis.

In summary, *in vivo* biotinylation of *Borrelia*, high-throughput SWATH-MS followed by the bioinformatics tools used in the present study is an ideal pipeline to map large sets of proteins that are involved in interactions with the host cells. Several proteins with known adhesion function (like OspA, OspC, DbpA) were detected in this study using the above mentioned experimental pipeline. It is noteworthy that, this is the first study that demonstrates plausible adhesion of proteins like LysM domain protein, BESBP-5, Antigen S1, CRASP-1, Erp23 protein and Lipoprotein to the hBMECs. The systematic combination of proteomics and bioinformatic tools presented in this study can potentially be applied to other pathogens to decipher their interactome in varying environmental conditions.

5. Conclusion

The present study reveals that *Borrelia* possesses multiple surface proteins that may interact with hBMECs. From the biotinylated surface proteome of *Borrelia* 24 proteins were identified with SWATH-MS, which bind to the endothelial cells. Among them, 12 proteins were identified as outer surface proteins using bioinformatics tools. The binding ability of six proteins (LysM domain protein, BESBP-5, Antigen S1, CRASP-1, Erp23 protein, and Mlp Lipoprotein) was corroborated in this study using recombinant forms in ELISA and immunocytochemistry. Here, we present a high-throughput approach of generating a dataset of plausible borrelial ligands that may interact with hBMECs, followed by a systematic bioinformatic pipeline to categorize the proteins for experimental validation, and finally we validated the binding ability of six surface proteins of *Borrelia* to hBMECs. This study widens our understanding of the ligand-receptor interaction between *Borrelia* and hBMECs, which is the primary step in the crossing of the endothelial barrier.

Author contributions

MB conceived the project. MB, IJM, LBP, and EM designed experiments. Biotinylation was performed by ZT, IJM, EM, and LBP. KB performed sequencing. hBMEC cells were grown by KB and Andrej Kovac. Immunocytochemistry was performed by P. Mertinkova and photo-documentation was done by P. Majerova. Cloning was performed by IJM. Recombinant proteins were produced by ZT, LBP, and EM. Bioinformatics analyses were performed by ZT and LBP. AK performed ELISA. ZT, IJM, AK, and MB prepared the manuscript. All authors read and approved the final manuscript.

Declaration of Competing Interest

None of the authors has any conflicting interests to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.ttbdis.2020.101451.

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