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Therefore do not worry about tomorrow,
for tomorrow will worry about itself.
Matthew 6:34

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ABSTRACT

Lyme borreliosis (LB) is a common tick-borne infectious disease in the Northern Hemisphere. The LB causing *Borrelia burgdorferi* sensu lato (*Borrelia*) spirochetes enter the human host via the tick bite. From the initial entry site *Borrelia* may disseminate in the blood vasculature to distant organs. In the disseminated stage of LB, the infection is manifested as inflammation in the joints, peripheral and central nervous system, skin or heart. LB patients are successfully treated with antibiotics. Without antibiotic treatment *Borrelia* adheres to the host molecules and is able to persist in the host tissues for a long time.

The aims of this study were to investigate how *Borrelia* survives despite its limited metabolic capacity, how the host molecule biglycan affects the infectivity and dissemination of *Borrelia in vivo*, and, what was the LB seroprevalence in Finland fifty years ago, when LB was still an unrecognized disease.

The study results showed that *Borrelia* expresses a periplasmic Basic membrane protein D involved in the salvage of purine nucleosides which are essential molecules for RNA and DNA synthesis. Further, the results revealed that biglycan affects the infectivity and dissemination of *Borrelia* in the mammalian host, and the host immune response. Finally, the results strongly suggested that LB was a common infectious disease in Finland half a century ago.

In summary, the study results broaden the knowledge on the metabolism and pathogenesis of *Borrelia*, which is important for the discovery of novel vaccines or drug treatments. Further, knowledge on the LB seroprevalence fifty years ago helps to understand and proportionate the LB seroprevalence today.

KEYWORDS: Lyme borreliosis, *Borrelia burgdorferi*, purine nucleoside, host molecule, biglycan, infectivity, immune response, seroprevalence

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TIIVISTELMÄ

Lymen borrelioosi on pohjoisella pallonpuoliskolla yleisin puutiaisvälitteinen infektiotauti. *Borrelia burgdorferi* sensu lato (borrelia) -spirokeettabakteerit pääsevät puutiaisen pureman kautta ihmisen ihoon ja leviävät verenkierrossa eri kudoksiin. Taudin levinneessä vaiheessa potilailla on usein tulehdusoireita nivelissä, ääreis- ja keskushermostossa, ihossa tai sydämessä. Borrelioosia voidaan hoitaa tehokkaasti antibiooteilla. Ilman antibioottihoitoa borreliabakteerit tarttuvat ihmisen omiin kudosmolekyyleihin sekä säilyvät kohdekudoksissa elinkykyisinä pitkään.

Tämän tutkimuksen tavoitteina oli selvittää, miten borreliabakteerit selviävät pitkään elinkykyisenä suppeasta aineenvaihduntakyvystä huolimatta, miten biglykaani-kudosmolekyyli vaikuttaa bakteerin infektiokykyyn sekä selvittää, mikä oli borrelioosin yleisyys Suomessa vuosina 1968–72, jolloin borrelioosin taudinaiheuttajabakteeri oli vielä tuntematon.

Tutkimustulokset osoittivat, että borreliabakteerit ottavat talteen elintärkeitä nukleosidejä RNA- ja DNA-synteesiä varten Basic membrane protein D (BmpD) -proteiinin avulla. Bakteerien sisäkalvolla sijaitseva BmpD-proteiini kuljettaa ainakin kahta eri puriininukleosidiä ihmisen kudosympäristöstä bakteerisolun sisälle. Toiseksi tutkimustulokset selvittivät, että biglykaani-kudosmolekyyli vaikuttaa borreliabakteerien infektiokykyyn ja leviämiseen sekä isäntäeläimen tulehdusvasteeseen. Lopuksi paljastettiin, että borrelioosi oli yleinen infektiotauti Suomessa 50 vuotta sitten.

Yhteenvetona voidaan todeta, että tutkimustulokset tuovat uutta tietoa borreliabakteerin metaboliasta ja taudinaiheuttamiskyvystä, mitä voidaan hyödyntää tulevaisuudessa mm. rokote- ja lääkekehityksessä. Lisäksi tieto borrelioosin yleisyydestä vuosina 1968–72 auttaa suhteuttamaan borrelioosin yleisyyttä tänä päivänä.

AVAINSANAT: Lymen borrelioosi, *Borrelia burgdorferi*, puriininukleosidi, kudosmolekyyli, biglykaani, infektiokyky, tulehdusvaste, seroprevalenssi

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Abbreviations

ABC	ATP-binding cassette
ACA	acrodermatis chronica atrophicans
AcGal	6-O-acylated cholesteryl β-d-galactopyranosides
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Amp	ampicillin
ATP	adenosine triphosphate
bgn	biglycan
Bmp	Basic membrane protein
bp	base pair
BSK-II	Barbour-Stoenner-Kelly II
C	carboxyl group
CCL	C-C motif chemokine ligand
cDNA	complementary deoxyribonucleic acid
CFSE	carboxyfluorescein diacetate succinimidyl ester
CI	confidence intervals
Cm	chloramphenicol
cp	circular plasmid
Cq	quantification cycle
CS	chondroitin sulfate
CSF	cerebrospinal fluid
CTP	cytidine triphosphate
CV	column volume
CXCL	C-X-C motif chemokine ligand
d	deoxy
DAPI	4′, 6-diamidino-2-phenylindole
DAMP	danger-associated molecular pattern
Dbp	decorin-binding protein
DNA	deoxyribonucleic acid
DS	dermatan sulfate
ECM	extracellular matrix

EDTA	ethylenediaminetetraacetic acid
EIU	enzyme immunoassay unit
ELISA	enzyme-linked immunosorbent assay
EM	erythema migrans
FMC	Finnish Mobile Clinic
GAG	glycosaminoglycan
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GMP	guanosine monophosphate
GTP	guanosine triphosphate
HRP	horseradish peroxidase
H_2SO_4	sulfuric acid
HtrA	serine protease high temperature requirement A
Ig	immunoglobulin
IL	interleukin
IM	inner membrane
IMP	inosine monophosphate
kDa	kilodalton
Km	kanamycin
KO	knock-out
LA	Lyme arthritis
LB	Lyme borreliosis
LC-MS	liquid chromatography-mass spectrometry
LED	light-emitting diode
lp	linear plasmid
LPS	lipopolysaccharide
M	molarity
Mbp	mega base pair
MCP	monocyte chemoattractant protein
MIP	macrophage inflammatory protein
mRNA	messenger RNA
MST	microscale thermophoresis
MyD88	myeloid differentiation primary response 88
n	nano
N	amino group
N	nucleoside
NaOH	sodium hydroxide
NF-κB	nuclear factor kappa B
Ni-NTA	nickel-nitrilotriacetic acid
NMP	nucleoside monophosphate
NSS	normal sheep serum

OD	optical density
OM	outer membrane
OPD	ortho-phenylenediamine
Opp	oligopeptide
OR	odds ratio
Osp	outer surface protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PG	proteoglycan
r	recombinant protein
RANTES	regulated upon activation, normal T cell expressed and secreted
rpm	rounds per minute
qPCR	quantitative polymerase chain reaction
RNA	ribonucleic acid
RT	room temperature
RT-qPCR	reverse-transcriptase quantitative polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SEC	size exclusion chromatography
ss	sensu stricto
T	Tween 20
TCDM	Turku Center for Disease Modeling
TLR	Toll-like receptor
TNF	tumor necrosis factor
TTP	thymidine triphosphate
U	enzyme unit
USA	United States of America
UTP	uridine triphosphate
VlsE	Vmp-like sequence
VZV	varicella zoster virus
WCS	whole-cell sonicate
WT	wildtype
XMP	xanthosine monophosphate

List of Original Publications

This dissertation is based on the following original publications, which are referred to in the text by their Roman numerals:

I Julia Cuellar, Mia Åstrand, Heli Elovaara, Annukka Pietikäinen, Saija Sirén, Arto Liljeblad, Gabriela Guédez, Tiina A. Salminen & Jukka Hytönen. Structural and biomolecular analyses of *Borrelia burgdorferi* BmpD reveal a substrate-binding protein of an ABC-type nucleoside transporter family. Accepted manuscript to *Infection and Immunity.*

II Julia Cuellar, Annukka Pietikäinen, Otto Glader, Heidi Liljenbäck, Mirva Söderström, Saija Hurme, Jemiina Salo & Jukka Hytönen. *Borrelia burgdorferi* Infection in Biglycan Knockout Mice. *The Journal of Infectious Diseases*, 2019; 220, (1): 116–126. https://doi.org/10.1093/infdis/jiz050

III Julia Cuellar, Timothée Dub, Jussi Sane & Jukka Hytönen. Seroprevalence of Lyme Borreliosis in Finland 50 years ago. *Clinical Microbiology and Infection*. In press. https://doi.org/10.1016/j.cmi.2019.10.003

1 Introduction

Lyme borreliosis (LB) is a tick-borne infectious disease common in Europe, Asia, and in North America. The LB causing spirochetes belong to the *Borrelia burgdorferi* sensu lato -complex (*Borrelia*) including about twenty different genospecies, where *Borrelia burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* are the most common human pathogens. *Borrelia*-infected *Ixodes* ticks transmit the spirochetes during feeding on a vertebrate host.

Borrelia spirochetes are exceptional bacteria as they survive and maintain their infectivity in different host environments, such as in ticks, rodents, birds, and in humans. *Borrelia* is an auxotroph bacterium with a limited metabolic capacity. For example, *Borrelia* lacks the enzymes required for *de novo* synthesis of amino acids, fatty acids, cofactors, and nucleosides. Hence, the spirochetes express multiple transporter proteins for the uptake of nutrients from the host environment. Further, the outer surface of *Borrelia* is covered with proteins mediating adhesion to the host molecules enabling the dissemination and persistence of *Borrelia* in the host vasculature and extravascular tissues, such as in joints, nervous tissue, skin, and heart. The comprehensive characterization of *Borrelia* proteins, their function and interaction with host molecules (Studies I and II) is important for the development of vaccines and novel drugs supplementing the traditional antibiotics currently in use.

LB was discovered forty years ago when first reports of tick bites, isolated *Borrelia* spirochetes, and subsequent arthritis reactions were published. Since the beginning of the 1990s, the LB seroprevalence among the healthy general population in various countries, regions, and in target groups with outdoor activities has been frequently studied. However, ticks and *Borrelia* have probably been circulating for thousands of years, and in order to elucidate LB occurrence in a longer retrospect, the seroprevalence of LB in Finland half a century ago was investigated (Study III).

2 Review of the Literature

2.1 Lyme borreliosis

2.1.1 History

The bacterial agent, *Borrelia burgdorferi* sensu lato (later referred to "*Borrelia*") and the resulting disease, Lyme borreliosis (LB), have been existing in the world probably for thousands of years as DNA of *Borrelia* was detected in the 5,300-year-old Tyrolean iceman (Keller et al., 2012). Already in 1883, the German physician Alfred Buchwald described a chronic skin condition, acrodermatitis chronica atrophicans (ACA), which is today a well-known clinical manifestation of disseminated LB (Buchwald, 1883). In the beginning of the 20th century, the Swedish physician Arvid Afzelius described the occurrence of a red skin lesion after a tick bite (Afzelius, 1910). Also, two French neurologists, Charles Garin and Antoine Bujadoux, reported of patients with neurological symptoms after a tick bite (Garin & Bujadoux, 1922). In 1977, a new and previously unrecognized disease entity, "Lyme arthritis" (LA), was described for the first time. The rheumatologist Allen Steere and colleagues discovered an unusual high number of children suffering from arthritis in the communities of Lyme, Old Lyme, and East Haddam in Connecticut in the northeastern parts of the USA (Steere et al., 1977b). The investigators suggested that the infectious agent of LA was transmitted by an arthropod vector, possibly by a sheep tick (Steere et al., 1977b).

Indeed, the ticks were found to be carriers of long and helical formed bacteria, the LA causing spirochetes. The spirochetes were successfully isolated and cultivated from ticks, *Ixodes scapularis* (formerly known as *I. damminii*) (Burgdorfer et al., 1982) and *I. pacificus* ticks in the USA (Burgdorfer et al., 1985), and *I. ricinus* ticks in Europe (Barbour, 1984; Burgdorfer, 1984). Also, antibodies in serum samples of LA patients bound the intact spirochetes isolated from the *I. scapularis* ticks (Burgdorfer et al., 1982). Later, the *Borrelia* spirochetes were isolated from blood, skin, and cerebrospinal fluid of Lyme disease patients (Benach et al., 1983; Steere et al., 1983). The name of the LA disease entity was broadened to Lyme disease in North America and to Lyme borreliosis in Europe as the multisystemic form of the infection was established (Steere et al., 1977a; Stanek et

al., 1988; Stanek & Strle, 2018). Today, LB is known as the most common tick-borne disease in the Northern Hemisphere (Stanek et al., 2012).

2.1.2 Clinical manifestations

LB is a multisystemic infection that is traditionally divided into three stages 1) early localized, 2) early disseminated, and 3) late disseminated stage (Steere, 2001; Stanek & Strle, 2018).

In the early localized stage of LB, within days or weeks after the tick bite, a migrating skin lesion called erythema migrans (EM) occurs around the initial tick bite site in approximately 50–70% of European (Berglund et al., 1995; Santino & Longobardi, 2011) and in 70–80% of North American LB cases (Steere & Sikand, 2003). Additionally, the patient can experience flu-like symptoms, such as lymphadenopathy, fever, fatigue, joint, muscle pain, and malaise (Steere et al., 2016). Also, lymphocytosis, a lymphocytic infiltration, may occur in the nipples of adults or in the ear lobes of children. After a few weeks, the EM can disappear even without antibiotic treatment (Steere et al., 1980).

In the early disseminated stage, within weeks or months, the spirochetes have disseminated from the original entry site to other skin sites and distant organ sites, such as to the peripheral and/or central nervous system, or to the heart (Steere et al., 2016). The clinical symptoms can be presented as multiple EMs in the skin, as objective signs of neuroborreliosis, or as acute cardiac symptoms due to disrupted electrophysiological conductance, or, in rare cases, as inflammation of the heart tissue. Neuroborreliosis is a subacute inflammatory condition often presenting with painful meningoradiculitis (Garin-Bujadoux-Bannwarth syndrome), lymphocytic meningitis, and cranial neuritis presented as uni- or bilateral facial palsy (Stanek et al., 2011; Borchers et al., 2015).

In the late disseminated stage, usually months or years after the initial entry of the spirochetes, arthritis, and ACA might occur (Steere et al., 2016). Arthritis is presented as short, but recurrent attacks, or as long-lasting joint swelling and pain development affecting the large joints, such as knees (Stanek et al., 2011; Borchers et al., 2015). ACA is a persisting inflammatory skin condition starting with a bluish-red discolouration of the extensor surfaces of lower or upper extremities (Stanek et al., 2011; Borchers et al., 2015). In time, the skin condition slowly progresses to skin atrophy (Stanek et al., 2011).

The clinical symptoms of LB patients vary in different parts of the world as there are multiple *Borrelia* genospecies circulating (Stanek et al., 2012). For example, *B. afzelii* and *B. garinii* are mainly circulating in Europe. Thus, the persisting skin inflammation ACA and neuroborreliosis are often manifested in European patients (Ruzić-Sabljić et al., 2000; Strle et al., 2006). Whereas, *B. burgdorferi* sensu stricto

(ss) is the prevalent genospecies in North America. Hence, arthritis is a common symptom in North American patients (Steere & Sikand, 2003).

2.1.3 Diagnosis and treatment of LB

The diagnosis of EM is based on the clinical picture (Stanek et al., 2011). A red skin lesion qualifies as EM if the diameter is ≥5 cm (Borchers et al., 2015). Further, serological testing is not advised as only 50% of LB patients with EM have antibodies against *Borrelia* (Dessau et al., 2018).

The diagnosis of early and late disseminated LB is performed by serology. The serological tests are feasible as the LB patients develop antibodies against *Borrelia* in approximately 4–6 weeks after a tick bite (Borchers et al., 2015). The *Borrelia* antibodies in serum or cerebrospinal fluid (CSF) are detected by enzyme-linked immunosorbent assays (ELISA) and by immunoblotting (Stanek et al., 2011; Dessau et al., 2018). Commonly, a two-step approach is used. At first, immunoglobulin (Ig) M and G are detected by a sensitive screening assay with whole-cell sonicate of *Borrelia* or recombinant antigens, such as VlsE or C6 peptide, a conserved region of the VlsE antigen (Mygland et al., 2010). In the second confirmatory test, an immunoblot with specific antigens of *Borrelia* is performed.

Nowadays, *Borrelia* serological tests from different manufacturers vary in their antigenic composition and combination of recombinant proteins. For example, the first screening tests can contain whole-cell extracts of *B. burgdorferi* ss, *B. afzelii*, and/or *B. garinii* (Kodym et al., 2018). Also, the immunoblots are composed of a plethora of recombinant proteins, such as VlsE, flagellin, OspA, and OspC, derived from one or various *Borrelia* genospecies (Kodym et al., 2018). Further, numerous other proteins of *Borrelia* have been investigated for their performance as antigens in serologic tests, such as BmpA, P66, and BBK32 (Aguero-Rosenfeld et al., 2005). Although laboratories use several commercial *Borrelia* antibody tests, especially the IgG test results are reliable. Recently, the *Borrelia* antibody assays used in twelve Scandinavian laboratories were compared (Lager et al., 2019). The study showed that the sensitivity of the diagnostic assays used in different laboratories was high (average 88%) as IgG results were similar in the investigated serum samples of LB patients and healthy control persons.

The serologic tests are limited by the high background seroprevalence of the general population ranging from a few percent to over 20% in high risk groups (Mygland et al., 2010). As IgM and IgG towards *Borrelia* might persist for decades (Hammers-Berggren et al., 1993; Kalish et al., 2001), one cannot distinguish between acute and recurrent *Borrelia* infection by serologic tests (Steere et al., 2016). Hence, in cases of an atypical cutaneous manifestations or suspected LA, complementary laboratory tests for *Borrelia* detection can be performed (Stanek et

al., 2011; Dessau et al., 2018). For example, a skin biopsy sample can be cultured in Barbour-Stoenner-Kelly II (BSK-II) medium. Further, *Borrelia* nucleic acid amplification test with polymerase chain reaction (PCR) can be performed on skin biopsy samples from the EM, synovial fluid, or CSF samples.

The *Borrelia* infection is successfully treated with antibiotics of the class of β-lactams (e.g. penicillin, amoxicillin, and ceftriaxone), tetracyclines (e.g. doxycycline), and macrolides (e.g. azithromycin) with treatment time up to four weeks (Borchers et al., 2015). According to Finnish Current Care Guidelines, doxycycline and amoxicillin are the first line of treatment in cases of EM and lymphocytoma (https://www.kaypahoito.fi/hoi13020#s14; accessed 9.12.2019). Further, patients with LA, neuroborreliosis, and ACA are intravenously treated with ceftriaxone (Oksi et al., 2008). Azithromycin is recommended as an alternative to doxycycline and penicillin if the LB patients are children, pregnant, breast-feeding, or have a drug allergy (Borchers et al., 2015).

2.1.4 Vaccine development

In 1998, the first LB vaccine, Lymerix by SmithKline Beecham (now GSK), was approved by the Federal Drug Administration in the USA (Schuijt et al., 2011). The immunogenic antigen was Outer surface protein A (OspA) of *B. burgdorferi* ss, a protein expressed only during the *Borrelia* colonization in the tick midgut. The bacterial transmission was blocked in the tick as anti-OspA antibodies transported via the blood meal contributed to the elimination of the spirochetes inside the midgut of the tick. Even though the mechanism-of-action was a brilliant idea, the immune response was short-lasting in humans, and several booster vaccine doses were necessary to maintain sufficient antibody response. Further, concerns of OspA vaccine-induced arthritis, due to suspected molecular mimicry, were raised (Embers & Narasimhan, 2013). Only after four years, the human OspA vaccine was withdrawn from the market. In 2014, a vaccination study conducted by the Baxter BioScience company showed that a multivalent vaccine containing OspA epitopes of four prevalent *Borrelia* genospecies, *B. burgdorferi* ss, *B. afzelii*, *B. garinii*, and *B. bavariensis*, was safe and immunogenic in healthy adults (Wressnigg et al., 2013; Wressnigg et al., 2014). However, the vaccination study was discontinued. Currently, the Austrian biotechnology company Valneva is conducting phase I clinical vaccination studies using an OspA-based fusion protein VLA15. In the preclinical studies, the mice immunized with VLA15 were protected from *B. burgdorferi* ss, *B. afzelii*, *B. garinii*, and *B. bavariensis* infection (Comstedt et al., 2017). There are several commercial OspA vaccine on the market which are used in the veterinary field for vaccinating dogs (Schuijt et al., 2011) and as an “off-label” vaccine for horses (Divers et al., 2018).

The research for the development of a safe and effective LB vaccine is ongoing. In addition to the OspA protein, other *Borrelia* proteins and tick saliva proteins have been investigated as potential vaccine candidates (Table 1) either as single antigens or in cocktails of multiple antigens. For example, an immunization protocol with OspC in combination with OspA was studied (Grosenbaugh et al., 2018). However, the OspC/OspA fusion vaccine displayed lower immunogenicity compared to the monovalent OspA (Grosenbaugh et al., 2018). Also, an OspC cDNA tattoo vaccine, a novel vaccine technology, has been shown to prevent *B. afzelii* infection (Wagemakers et al., 2014). The plasmid expressing OspC was applied on the skin of mice, and was tattooed on the skin with a tattoo needle machine prior to the *B. afzelii* challenge (Wagemakers et al., 2014). The tattoo needle gun perforates the skin in thousands of spots, where the cDNA vaccine is deposited (Kim, 2017). The local skin trauma elicits a pro-inflammatory reaction, and thus, the DNA tattoo vaccination leads to a stronger and faster humoral and cellular immune response than traditional intramuscular vaccinations (Kim, 2017). Further, wildlife reservoir animals, mainly mice, have been vaccinated by oral bait vaccines containing vaccinia virus expressing OspA (Kern et al., 2016). Additionally, vaccinations of mice with live attenuated *B. burgdorferi* ss (Hahn et al., 2016), and different adjuvants boosting the OspA vaccine have been examined (Effenberg et al., 2017).

Table 1. List of potential vaccine antigens, their function, and mechanism of action. The table is modified from (Schuijt et al., 2011) and (Singh et al., 2017).

Vaccine antigen	Function	Mechanism of action	Reference
OspA	Colonization of *Borrelia* in the tick midgut.	*Borrelia* killed inside tick.	(de Silva et al., 1996) (Pal et al., 2004)
OspB	Colonization of *Borrelia* in the tick midgut.	*Borrelia* killed inside tick.	(Neelakanta et al., 2007) (Fikrig et al., 2004)
OspC	Transmission of *Borrelia* from tick to mammalian host.	*Borrelia* killed in mice.	(Gilmore et al., 1996)
DbpA	Binding of *Borrelia* to host decorin.	Protective after needle inoculation, not after tick infestation in mice.	(Hanson et al., 1998) (Hagman et al., 2000)
DbpB	Binding of *Borrelia* to host decorin.	Protective after needle inoculation in mice.	(Hagman et al., 1998)
BBK32	Binding of *Borrelia* to host fibronectin and mediating extravasation of *Borrelia*.	*Borrelia* killed inside and outside tick.	(Fikrig et al., 2000) (Moriarty et al., 2012)
RevA	Binding of *Borrelia* to host fibronectin.	*Borrelia* killed after passive immunization in mice.	(Floden et al., 2013)

Vaccine antigen	Function	Mechanism of action	Reference
AcGal	Major glycolipid in cell membrane of *Borrelia.*	Not determined.	(Stübs et al., 2009) (Stübs et al., 2011)
BBA65, BBA66, BBA69, BBA70, BBA73	*Borrelia* proteins with unknown function.	Not protective after tick infestation.	(Brandt & Gilmore, 2017)
BBI39	*Borrelia* protein expressed in ticks with unknown function.	*Borrelia* killed inside tick.	(Singh et al., 2017)
BmpA, BmpB	*Borrelia* proteins binding to purine nucleosides.	Not protective after passive immunization in mice.	(Pal et al., 2008) (Åstrand et al., 2019)
BB0405	*Borrelia* protein with unknown function.	*Borrelia* killed inside tick.	(Kung et al., 2016)
HtrA	*Borrelia* serine proteinase cleaving host proteins.	Not protective after tick infestation.	(Ullmann et al., 2015)
Salp15	Tick protein.	Enhanced phagocytosis of Salp15 coated *Borrelia* in mice.	(Dai et al., 2009)

2.1.5 LB prevention and future treatment forms

Currently, the prevention strategy of the tick-borne spirochete transmission is based on protective clothing, daily tick checks, and removal of the attached tick as early as possible. At the same time, it is important to bear in mind that removal of ticks within 24–48 hours leads to no or very low risk of *Borrelia* infection in humans (Eisen, 2018). In a recent study, the risk of developing LB after a tick bite in Scandinavia was estimated to be 2% (Wilhelmsson et al., 2016).

In addition to novel LB vaccines, new treatment forms to combat LB infection have also been under investigation. Currently, *Borrelia* is susceptible to antimicrobial treatment as described above. However, worldwide, the antimicrobial resistance is an emerging health threat (López-Jácome et al., 2019). Hence, anti-adhesion based drugs have been envisioned to supplement the antibiotics currently in use (Ofek et al., 2003). For example, sugar side chains on host molecules are important binding targets for *Borrelia* (Lin et al., 2017). These interactions could be interfered by small molecules that either mimic or block directly the host binding components necessary for *Borrelia* adhesion (Lin et al., 2017). Further, the determination of the structure of *Borrelia* proteins can lead to the design of small molecules that interfere or inhibit protein function, and thus, the colonization and persistence of *Borrelia* in the host. For example, after solving the structure of a manganese transporter of *Borrelia*, small molecule drugs were discovered from existing drug libraries that inhibited the transporter function in *Borrelia*, and led to the rupture of *Borrelia* cells *in vitro* (Wagh et al., 2015).

2.1.6 Epidemiology

2.1.6.1 Historical perspective

The long-lasting *Borrelia* antibodies in human serum samples can be used in epidemiological studies, for example to investigate the seroprevalence of LB in a certain region or population group. The first LB seroprevalence studies using human serum samples were conducted in the late 1980s.

Smith and colleagues were one of the first investigators, who studied the occupational risk of LB in outdoor workers compared to indoor workers by investigating *Borrelia* antibodies in sera of national park and office workers collected in 1986 (Smith et al., 1988). In all study participants, the total LB seroprevalence was 6.5%. However, the investigators could only suggest that outdoor workers had higher risk of *Borrelia* infection as the number of indoor workers was too low for statistical analyses. During the same time period, the LB seroprevalence in Swiss orienteers was investigated in 1986 to 1987 (Zhioua et al., 1998). The orienteers presented high seroprevalence rates ranging from 28.1 to 30.3% compared to the control groups consisting of non-orienteers and healthy blood donors with seroprevalences of 3.9 to 6.0%. The study results confirmed that outdoor activities in areas with high tick density led to high LB seroprevalence. Meanwhile in Mexico, the LB seroprevalence was studied using serum samples collected in 1987 to 1988 (Gordillo et al., 1999). The seroprevalence in Mexican population was determined as 0.3% indicating that LB is non-endemic in Mexico.

The oldest serum samples investigated for *Borrelia* antibodies are collected from gray wolves in Minnesota and Wisconsin, USA, during the years 1972 to 1989 (Thieking et al., 1992). The first *Borrelia* positive serum samples in the gray wolves were detected in the years 1975 to 1977 (Thieking et al., 1992), which is same time period when the first human LB patients were described (Steere et al., 1977b). In total, 3% of the collected historical wolf serum samples contained *Borrelia* antibodies indicating that the gray wolves had been exposed to *Borrelia* infected ticks, and that wolves, similar to humans, are susceptible to LB (Thieking et al., 1992).

Since the 1990s until today, the LB seroprevalences among healthy population in different regions (Table 2) and in various target groups have been well-characterized. However, no LB seroprevalence studies have been conducted in the decades pre-dating the early 1980s, when the LB disease entity was described for the first time.

2.1.6.2 Present-day data

At the present times, the LB epidemiological data in different geographical regions are obtained with diverse methodologies and from multiple sources (Sykes &

Makiello, 2017). In some countries, health authorities collect and release up-to-date surveillance data on LB cases in disease registries. However, there are differences whether only cases with disseminated LB or also cases with early localized LB are reported (Sajanti et al., 2017). Further, in some countries, the reporting is implemented only in certain regions within the country. For example in Germany, the reporting of LB cases is mandatory in 9 out of 16 federal states (Enkelmann et al., 2018). Commonly, the LB surveillance data is based on epidemiological studies. The LB epidemiological data are reported either as case numbers, as LB incidence rates (number of cases per 100,000 population) or as LB seroprevalence (proportion of population with serum antibodies towards *Borrelia* at a certain time point).

The LB incidence rates and, thus, the LB seroprevalence in the general population, vary largely among European countries (Sykes & Makiello, 2017). In Western Europe, the unweighted mean incidence is estimated as 56 LB cases per 100,000 persons. This would equal to over 200,000 LB cases annually in Western European countries alone, where the total population is approximately 412 million. For comparison, the U.S. national health authorities estimated the LB incidence rate as 107 cases per 100,000 persons. Hence, in the USA, there are approximately 329,000 LB cases annually with a total population of 312 million (https://www.census.gov/quickfacts/fact/table/US/PST045218; accessed 21.5.2019).

In summary, the LB seroprevalences in Europe are lowest in the Southern countries, intermediate in the Northern countries, and highest in Central and Eastern Europe (Table 2) (Sykes & Makiello, 2017). The lowest LB incidence rate with 0.001 cases per 100,000 persons has been described in Italy, and the lowest LB seroprevalence among healthy population with 0.3% in Greece. In Central European countries (Belgium, France, Germany, The Netherlands, and Switzerland) the incidence rates range from 9 to 134 cases per 100,000 persons and the seroprevalences are approximately 1–10%. High seroprevalences of over 10% have been described in Eastern European countries (Poland and Slovakia). In Northern European countries (Denmark, Finland, Iceland, and Norway) the incidence rates fall to less than 31 cases per 100,000 persons, and the seroprevalences decrease to below 5%. Interestingly, Sweden is a country of high LB seroprevalence as in different regions the seroprevalence rates range from below 10% to over 20%. Also, one of the highest incidence rates of 464 LB cases per 100,000 persons in Europe has been described in an endemic region of Blekinge in Southeastern Sweden (Bennet et al., 2006).

Another important fact in LB epidemiology is the considerably high LB seroprevalence in certain target groups. The LB seroprevalence is approximately 20% in residents of LB endemic regions, such as in the Åland Islands (Carlsson et al., 1998), and in persons with occupational exposure to outdoors or with outdoor activities, such as forestry workers, hunters, farmers, and orienteers (Rath et al.,

1996; Zhioua et al., 1998; Cinco et al., 2004; Johansson et al., 2017; Zajac et al., 2017; De Keukeleire et al., 2018).

Table 2. List of LB seroprevalence in healthy population in selected European countries.

	Country	LB seroprevalence (%)	Reference
Low	Greece	0.3	(Stamouli et al., 2000)
	Belgium	1.1–2.9	(De Keukeleire et al., 2017; Lernout et al., 2019)
	Italy	1.2–1.5	(Santino et al., 2004; Sonnleitner et al., 2015)
Intermediate	Finland (mainland)	3.9	(van Beek et al., 2018)
	Norway	2.5–4.4	(Vestrheim et al., 2016; Hvidsten et al., 2017)
	The Netherlands	5.0	(Kuiper et al., 1993)
High	Germany	4.0–9.4	(Dehnert et al., 2012; Wilking et al., 2015)
	Austria	7.2	(Sonnleitner et al., 2015)
	Sweden	8.0–22.0	(Tjernberg et al., 2007; Johansson et al., 2017)
	Serbia	8.6	(Jovanovic et al., 2015)
	Poland	12.5	(Chmielewska-Badora et al., 2012)
	Slovakia	12.8	(Bazovska et al., 2005)
	Spain	13.3	(Lledó et al., 2014)
	Åland Islands	19.5	(Carlsson et al., 1998)

In Finland, there are approximately 2,000 cases with disseminated LB (Figure 1) and over 3,500 LB patients with EM based on clinical diagnosis each year (Sajanti et al., 2017). Hence, the LB incidence rate was 120 cases per 100,000 population. The Åland Islands were excluded from the Finnish incidence rate calculation due to the fact of being an LB hyperendemic region with over 1,500 LB cases per 100,000 population. Importantly, the annual number of Finnish LB cases has been increasing since 1995 when the notification of microbiologically confirmed LB cases by the Finnish National Institute of Health and Welfare authorities started (Figure 1). In 1995, there were only 340 LB cases. In 2018, the number of LB cases had increased six-fold to more than 2,100 notified cases. The highest number of over 2,300 cases was reported in the year 2017.

In mainland Finland, the LB seroprevalence among healthy population was 3.9% in 2011(van Beek et al., 2018). The main risk factors associated with LB seroprevalence were male gender and age of 60 years and older (van Beek et al., 2018). In contrast to mainland Finland, the LB seroprevalence in the Ålands Islands was 19.5% (Carlsson et al., 1998).

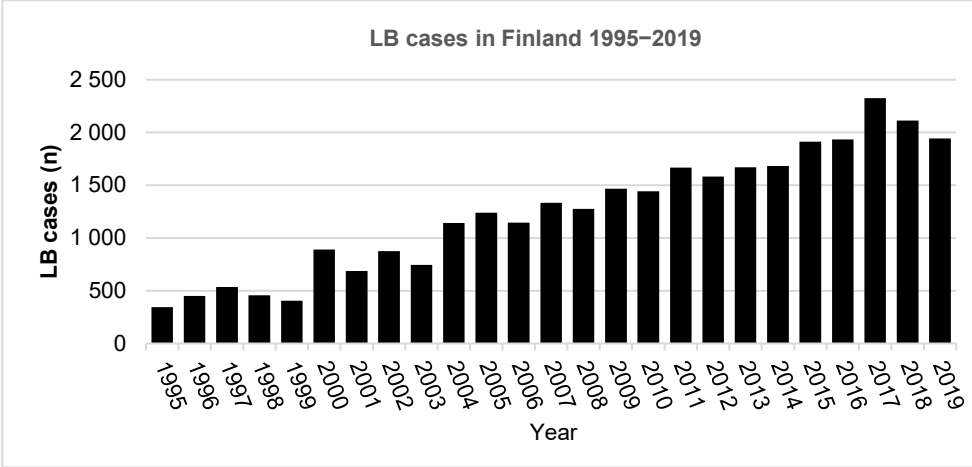

Figure 1. The total number of disseminated LB cases in Finland including the Åland Islands during the years 1995 to 2019 as reported by the Finnish National Institute of Health and Welfare. The last update of the table was 25th November, 2019. (https://sampo.thl.fi/pivot/prod/fi/ttr/shp/fact_shp?row=area-12260&column=time-12059&filter=reportgroup-12465; accessed 4th December, 2019).

2.2 *Borrelia burgdorferi* sensu lato

2.2.1 Taxonomy

The LB causing *Borrelia* spirochetes are classified in the kingdom of bacteria, in the phylum Spirochaetes, and in the order of Spirochetales (Norris et al., 2006). The order of Spirochetales includes three families, Brachyspiraceae, Leptospiraceae, and Spirochaetaceae. The family of Brachyspiraceae contains the bacteria of the genus *Brachyspira*. The Leptospiraceae family includes bacteria of the genera *Leptonema* and *Leptospira*. The Spirochaetaceae family consists of six genera, *Borrelia*, *Brevinema*, *Cristispira*, *Spirochaeta*, *Spironema*, and *Treponema*. The genus *Borrelia* includes the relapsing fever *Borrelia* and the LB *Borrelia*. Recently, the LB *Borrelia* has been proposed to be renamed as *Borreliella* (Adeolu & Gupta, 2014). The novel nomenclature is based on distinct phylogenetic and molecular markers between the relapsing fever and LB *Borrelia* species (Adeolu & Gupta, 2014). However, the proposed renaming has not been widely accepted in the *Borrelia* research community due to various reasons, such as confusion of the public and potential misdiagnosis of patients (Bergström & Normark, 2018; Stevenson et al., 2019).

The LB causing *Borrelia* (or *Borreliella*), also known as the *Borrelia burgdorferi* sensu lato -complex, includes about twenty genospecies (Table 3) (Cutler et al., 2017). The most important human LB pathogens are *B. burgdorferi* ss, *B. afzelii*, and *B. garinii* (Stanek & Reiter, 2011; Mead, 2015). *B. valaisiana*, *B. spielmanii*, *B.*

lusitaniae, *B. bissettii*, *B. bavariensis*, *B. kurtenbachii*, *Candidatus* B. andersonii, *B. americana*, and *Ca*. B. mayonii have been occasionally isolated from LB patients (Diza et al., 2004; Maraspin et al., 2006; de Carvalho et al., 2008; Rudenko et al., 2008; Clark et al., 2014; Margos et al., 2014; Markowicz et al., 2015; Pritt et al., 2016). Yet, the potential and importance of all these genospecies as human pathogens is not clearly established (Stanek et al., 2012).

Table 3. List of genospecies belonging to the *Borrelia burgdorferi* sensu lato -complex. Table is modified from (Yano et al., 1997; Masuzawa et al., 2001; Chu et al., 2008; Casjens et al., 2011; Hamer et al., 2012; Skuballa et al., 2012; Norte et al., 2013; Foley et al., 2014; Margos et al., 2014; Mead, 2015; Margos et al., 2016; Cutler et al., 2017; Johnson et al., 2017; Mysterud et al., 2019).

Genospecies	Human pathogen	Competent host	Geographical location
B. afzelii	Yes	Mice, shrews, voles, squirrels, hares, badgers, hedgehogs	Europe, Asia
B. americana	-	Birds	North America
Ca. B. andersonii	-	Birds, rabbits	North America
B. bavariensis	Yes	Rodents, birds, hedgehogs	Europe, Asia
B. bissettii	Possible	Rats	Europe, North America, Asia
B. burgdorferi sensu stricto	Yes	Mice, birds, squirrels, foxes, hares	Europe, North America
B. californiensis	-	Rats	North America
B. carolinensis	-	Voles, mice	North America
B. chilensis	-	Unknown	South America
Ca. B. finlandensis	-	Unknown	Europe
B. garinii	Yes	Birds, squirrels, hares	Europe, Asia
B. japonica	-	Voles, mice	Asia
B. kurtenbachii	-	Rodents	North America
B. lusitaniae	Possible	Lizards, birds, mice	Europe
Ca. B. mayonii	-	Mice, squirrels	North America
B. sinica	-	Rodents	Asia
B. spielmanii	Yes	Hedgehogs	Europe
B. tanukii	-	Rodents, raccoon dogs	Asia
B. turdi	-	Birds	Asia
B. valaisiana	Possible	Birds, squirrels, badgers	Europe
B. yangtzensis	-	Rodents	Asia

Yes ~ Established human pathogens. Possible ~ Genospecies isolated from few patients. - ~ Not determined.

2.2.2 Cell biology

Borrelia is a spirochete with a long thin spring-like morphology of 10−30 µm length and approximately 0.2 µm in diameter (Burgdorfer et al., 1982). The length of *Borrelia* varies among the genospecies. For example, the wildtype *B. garinii* SBK40 and *B. burgdorferi* ss B31 are longer than laboratory strains, such as *B. burgdorferi* ss B313 and JF105 (personal observation). In Figure 2D, *B. garinii* SBK40 are adhering to the human brain microvascular endothelial cells. The length of *B. garinii* is estimated as 20 µm as the diameter of the cell nuclei is about 10 µm (Jiménez-Munguía et al., 2018).

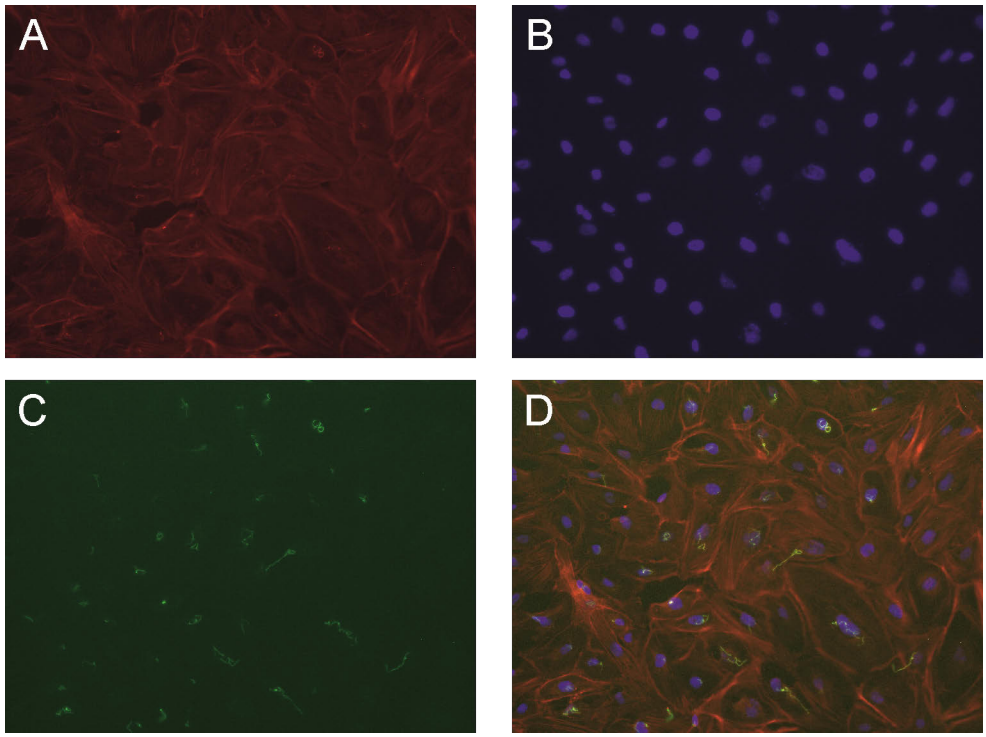

Figure 2. *Borrelia* adhering to endothelial cells. (A) The red actins and (B) blue nuclei of human brain microvascular endothelial cells stained with fluorescent anti-phalloidin and DAPI, respectively. (C) The green *Borrelia* stained with CFSE. (D) Merged image. All images magnified 200x. Photographs by Julia Cuellar.

The didermic cell body of *Borrelia* includes an outer and inner membrane (Figure 3). Using Gram staining, the resulting pink *Borrelia* is characterized as Gram-negative bacteria. In contrast to the typical Gram-negative bacteria, such as *Escherichia coli*, the outer membrane of *Borrelia* is covered with lipoproteins instead of lipopolysaccharides (LPS) (Radolf et al., 2012). The outer surface

lipoproteins have diverse functions, such as adhesion to host tissues, mediating colonization, and evading from the host immune system (Caine & Coburn, 2016). Further, transporter proteins on the outer and inner membrane allow the uptake of nutrients from the host environment (Fraser et al., 1997). The *Borrelia* membranes consist mainly of lipids, phosphatidylcholine, phosphatidylglycerol, and cholesterol derivatives, such as 6-O-acylated cholesteryl β-d-galactopyranoside (AcGal) (LaRocca et al., 2010). Between the outer and inner membrane is the periplasmic space where the intracellular flagella are located (Figure 3) (Radolf et al., 2012). The flagella are important molecules for maintaining the coiled morphology and motility of *Borrelia*. A flagellum consists of multiple linear filaments attached to the both ends of *Borrelia* where intricate motor proteins fueled by chemiosmotic energy allow the rapid movement of *Borrelia*.

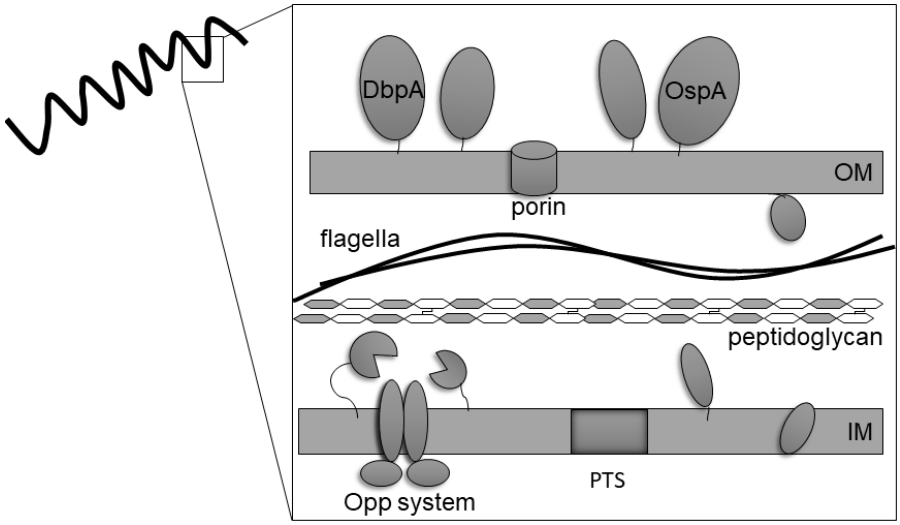

Figure 3. The schematic cell envelope of *Borrelia* shows the outer membrane (OM), inner membrane (IM), and the periplasmic space.The OM is covered with outer surface lipoproteins. The IM is composed of multiple membrane-spanning transporter proteins. Figure modified from (Radolf et al., 2012). DbpA ~ decorin-binding protein A, Opp ~ oligopeptide, OspA ~ outer surface protein A, PTS ~ phosphotransferase systems.

The small, but fragmented and complex genome of *Borrelia* is unique among bacteria. The genome of *B. burgdorferi* ss B31 was the first *Borrelia* isolate to be completely sequenced (Fraser et al., 1997). Later, the complete genome of several isolates of *B. burgdorferi* ss, *B. bissettii*, *B. spielmanii*, *B. valaisiana*, *B. garinii*, and *B. afzelii* have been sequenced (Schutzer et al., 2011; Schutzer et al., 2012; Schüler et al., 2015; Jabbari et al., 2018). The complete or partial genomes of more than 30 LB or relapsing fever *Borrelia* isolates have been deposited in the online database

BorreliaBase (Di et al., 2014). The *Borrelia* genome consists of one linear chromosome (approximately 1 Mbp) and of multiple linear and circular plasmids (in total approximately 0.6 Mbp) (Fraser et al., 1997). The linear chromosome contains housekeeping genes encoding for proteins involved in e.g. cellular processes, cell division and replication, and for multiple transporter proteins (Fraser et al., 1997; Casjens et al., 2012). The plasmids carry genes encoding virulence factors for infection and persistence in tick and mammalian hosts. Also, the plasmids can be easily lost during *in vitro* propagation of *Borrelia*.

Although the number of linear and circular plasmids varies between genospecies (Casjens et al., 2012; Casjens et al., 2017), the core genome of *Borrelia* comprises the linear chromosome, the linear plasmid 54 (lp54), and the circular plasmid 26 (cp26). The lp54 harbors genes encoding proteins essential for tick colonization (*ospA*), adhesion proteins (*dbpAB*), and pH- and temperature-regulated proteins (Maruskova et al., 2008). The cp26 carries genes essential for *Borrelia* survival, a telomere resolvase (*resT*), transporter proteins for peptides (*oppA1-A5*), glucose (*bbb29*), chitobiose (*chbA-C*), and purine bases (*bbb22* and *bbb23*), enzymes involved in the purine salvage pathway (*guaAB*), and an important protein for tick-to-host transmission (*ospC*) (Byram et al., 2004; Jewett et al., 2009; Jain et al., 2012).

The limited metabolism is one additional striking feature of *Borrelia* (Fraser et al., 1997). *Borrelia* lacks the ability of *de novo* synthesis of amino acids, carbohydrates, fatty acids, enzyme cofactors, and nucleic acids. Hence, *Borrelia* needs to scavenge the nutrients from its host environment or from the nutrient-rich BSK-II medium. *Borrelia* expresses multiple transporters for amino acids, carbohydrates, anions, and cations (Fraser et al., 1997). Short peptides consisting of 3 to 7 amino acids (proline and histidine in combination with leucine, valine or phenylalanine) are transported by the oligopeptide transporter system (OppABCDEF) (Lin et al., 2001; Wang et al., 2004; Groshong et al., 2017). The Opp transporter system is a typical bacterial ATP-binding transporter cassette (ABC) transporter (Figure 4) (Maqbool et al., 2015) as it comprises five substrate-binding proteins for oligopeptides (OppA1-A5), two heterodimeric permeases (OppB1C1), and a heterodimeric nucleotide-binding domain (OppEF) (Groshong et al., 2017). Also, carbohydrates (glucose, mannose, maltose, chitobiose, glycerol, N-acetylglucosamine) are transported via specific transporters (von Lackum et al., 2005; Rhodes et al., 2010; Corona & Schwartz, 2015). Accordingly, transporter proteins for anions and cofactors, such as phosphate (Brautigam et al., 2014), riboflavin (Deka et al., 2013), and manganese (Ouyang et al., 2009) have been described.

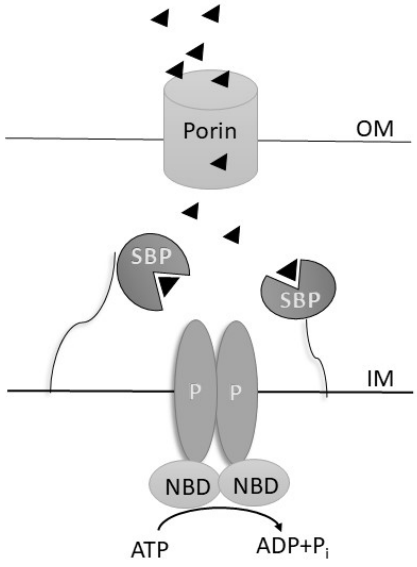

Figure 4. Schematic presentation of a bacterial ATP-binding cassette (ABC) transporter system for nutrient uptake. Nutrients (black triangles) enter the periplasmic space via porins located in the outer membrane (OM). Substrate-binding protein (SBP) anchored to the inner membrane (IM) transport the nutrient to permeases (P) which import the nutrients into the bacterial cell. The nucleotide-binding domains (NBD) drive the nutrient transport by hydrolyzing ATP to ADP. Modified from (Groshong et al., 2017).

In contrast, no genes encoding for fatty acid or nucleic acid transporters were identified by genomic sequencing in 1997 (Fraser et al., 1997). The uptake of fatty acids by passive diffusion might circumvent the need for a specific transporter as the outer membrane is relatively permeable due to lack of LPS (Cox & Radolf, 2001). On the other hand, in a recent study, members of the Basic membrane protein (Bmp) family, BmpA, BmpB, BmpC and BmpD, were predicted as components of the ABC transporter for purine nucleosides in *Borrelia* (Åstrand et al., 2019). The BmpA-D are encoded in the chromosome (*bb0383*, *bb0382*, *bb0384*, *bb0385*, respectively) suggesting that Bmps are important housekeeping proteins for *Borrelia* survival (Ramamoorthy et al., 1996; Bryksin et al., 2005). The first known bacterial purine nucleoside transporter was discovered in the *Treponema pallidum* spirochete and named as purine nucleoside receptor A (PnrA) (Deka et al., 2006). At the amino acid sequence level, the Bmps of *Borrelia* are related to *T. pallidum* PnrA with amino acid sequence identities of 27.5 to 31.3% (Åstrand et al., 2019). Strikingly, the 3D modeling structure of Bmps *in silico* suggest their function to be substrate-binding proteins possibly preferring purine nucleoside as ligands. However, the *in silico* structure predictions need to be confirmed with protein crystallization.

2.2.3 Purine and pyrimidine salvage

As described above, *Borrelia* lacks the ability to synthesize nucleic acids and its derivatives *de novo*. Hence, *Borrelia* depends on purine and pyrimidine salvage from extracellular sources. The nucleotides, nucleosides, purine and pyrimidine bases (Figure 5) are available in the tick midgut and saliva after a blood meal, and in the

host tissues at the infection sites due to cell lysis and nucleic acid degradation by the host innate immunity (Lawrence et al., 2009).

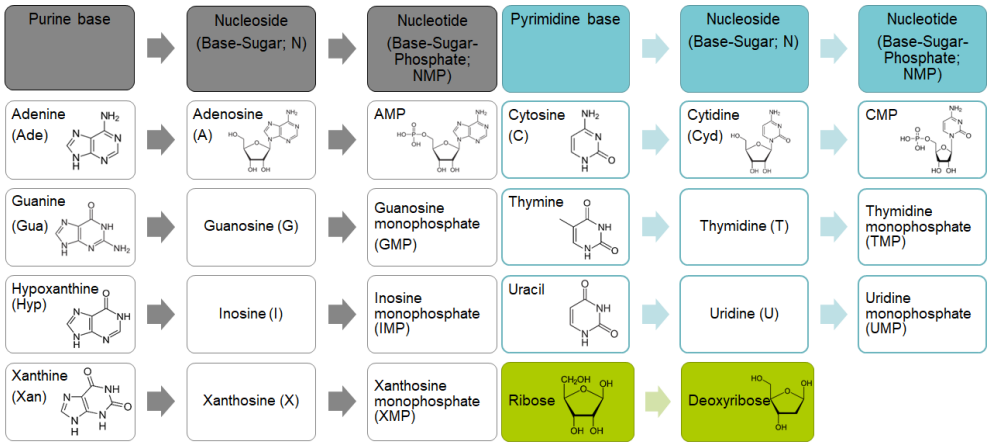

Figure 5. The purine (grey boxes) and pyrimidine bases (blue boxes), nucleosides and nucleotides essential for *Borrelia*. The nucleosides are either ribosylated (left green box) or deoxyribosylated (right green box). The abbreviations marked in the parentheses are used in Figures 6 and 7.

The complex *Borrelia* salvage pathway for purine and pyrimidine bases and nucleosides is depicted in Figure 6 and 7, respectively. First, the nucleotides, nucleosides, and their deoxygenated forms, deoxynucleotides and deoxynucleosides, and the free purine and pyrimidine bases traverse the outer membrane of *Borrelia*, possibly through porins allowing the diffusion of small hydrophilic molecules (Bárcena-Uribarri et al., 2010). In the periplasmic space, the nucleotides are dephosphorylated by nucleotidases to nucleosides. For example, the AMP nucleotide, is converted by a putative nucleotidase BB0504 to adenosine, its nucleoside form (Lawrence et al., 2009). Then, adenosine and other nucleosides are imported by a putative nucleoside ABC transporter (BB0677–79) into the cytoplasm (Lawrence et al., 2009; Gherardini et al., 2010; Corona & Schwartz, 2015).

In contrast to the purine nucleotides, the purine bases can freely enter the bacterial cytoplasm via specific transporters for purine bases (Figure 6). Adenine, hypoxanthine, and guanine are imported inside the bacterial cytoplasm by two characterized *Borrelia* purine transporters encoded by the *bb222* and *bb223* in the cp26 (Byram et al., 2004; Jain et al., 2012). Although individual BB222 and BB223 have different affinities towards hypoxanthine, adenine, and guanine, both transporters are required for maintaining the infectivity of *Borrelia* in the mammalian host, but not for *in vitro* growth as BSK-II medium is rich of nutrients containing nucleotides, nucleosides, and free bases (Jain et al., 2012; Jain et al., 2015).

The purine base hypoxanthine plays a central role in the purine salvage pathway (Figure 6). Hypoxanthine is either directly imported from the host environment (Jain et al., 2012) or adenine is deaminated by adenine deaminase to hypoxanthine (Jewett et al., 2007; Lawrence et al., 2009; Jain et al., 2015). For DNA synthesis, hypoxanthine is first converted to deoxyinosine by deoxyribosyl transferase, and then, is phosphorylated to deoxyinosine monophosphate (dIMP) by a deoxynucleotide kinase (Lawrence et al., 2009; Gherardini et al., 2010). Alternatively, for RNA synthesis, hypoxanthine is directly converted to IMP by xanthine-guanine phosphoribosyl transferase. The final enzymatic steps before DNA and RNA synthesis are similar. The (d)IMP is converted by IMP dehydrogenase to (d)XMP, and by GMP synthase to (d)GMP (Jewett et al., 2009). Two phosphate groups are added to the (d)GMP by a nucleoside di-phosphate kinase (BB0463) to gain (d)GTP before incorporating dGTP for DNA and GTP for RNA synthesis (Lawrence et al., 2009; Gherardini et al., 2010).

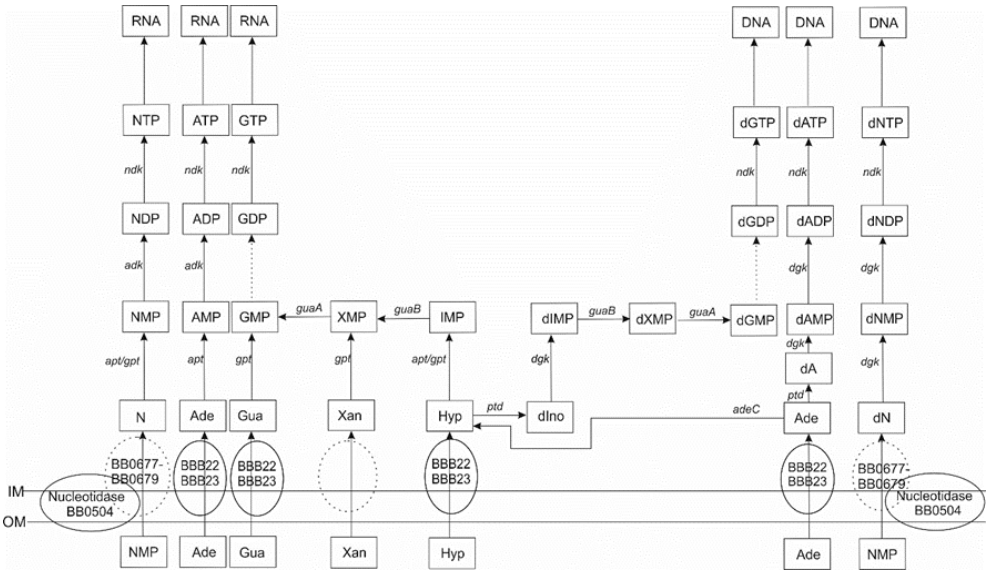

Figure 6. Putative purine salvage pathway of *Borrelia*. The free purine bases, adenine (Ade), guanine (Gua), xanthine (Xan), hypoxantine (Hyp), or any nucleoside monophosphate (NMP) are traversing the outer membrane (OM) of the bacterial cell possibly through porins. The NMPs are dephosphorylated to nucleosides by a nucleotidase, such as BB0504. The free bases are imported by known transporters expressed on the inner membrane (IM), such as BBB22 and BBB23, and the nucleosides by presumed transporters (dashed ellipses), such as BB677−79, or by unknown transporters (empty dashed ellipses). The bases or nucleosides are enzymatically converted to ribonucleotides or deoxyribonucleotides for RNA and DNA synthesis. The enzymes are listed in Table 4. The dashed arrows indicate unknown enzymes. Modified from (Gherardini et al., 2010).

Only two pyrimidine nucleosides, cytidine and deoxycytidine are rescued (Figure 7) (Zhong et al., 2006; Lawrence et al., 2009; Gherardini et al., 2010). Cytidine is the precursor for two RNA pyrimidine bases, cytidine triphosphate (CTP), and uridine triphosphate (UTP). Deoxycytidine is the precursor for deoxycytidine triphosphate (dCTP) and deoxythymidine triphosphate (dTTP) for DNA incorporation.

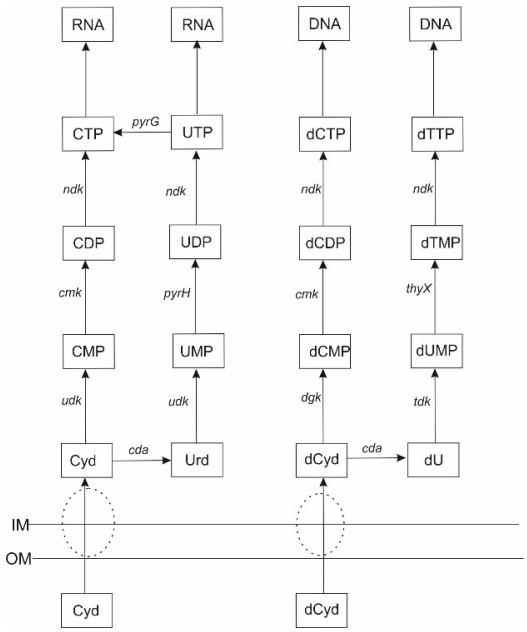

Figure 7. Putative pyrimidine salvage pathway of *Borrelia*. The free pyrimidine bases, cytidine (Cyd) and deoxycytidine (dCyd), are traversing the outer membrane (OM) of the bacterial cell, possibly by porins. The free bases are imported by unknown transporters (empty dashed ellipses). The bases are enzymatically converted to ribonucleotides or deoxyribonucleotides for RNA and DNA synthesis. The enzymes are listed in Table 4. Modified from (Gherardini et al., 2010).

The enzymes involved in the *Borrelia* purine and pyrimidine salvage pathway are listed in Table 4. *Borrelia* lacks three enzymes of the classical purine salvage pathway that are encoded by the relapsing fever strains, *Borrelia hermsii* and *Borrelia turicatae* (Pettersson et al., 2007). First, the ribonucleotide reductase catalyzing the conversion of ribosylated nucleotides to their deoxyribosylated form is not present in *Borrelia*. Also, the adenylosuccinate synthase and lyase catalyzing the formation of IMP to AMP are lacking. Hence, *Borrelia* is not able to convert ribosylated purines (ATP and GTP) to deoxyribosylated forms (dATP and dGTP). Further, the scavenging of adenine is essential as the conversion of IMP to AMP is not feasible.

Table 4. Existing and absent enzymes involved in the classical purine and pyrimidine salvage pathway of *Borrelia*. Modified from (Pettersson et al., 2007; Gherardini et al., 2010).

Enzyme name	Gene designation	Open reading frame in *Borrelia*
Adenine deaminase	*adeC*	*bbk17*
Adenine phosphoribosyltransferase	*apt*	*bb0777*
Adenylate kinase	*adk*	*bb0417*
Deoxynucleoside kinase	*dgk*	*bb0239*
Deoxyribosyl transferase	*ptd*	*bb0426*
GMP synthase	*guaA*	*bbb18*
IMP dehydrogenase	*guaB*	*bbb17*
Guanine-hypoxanthine permease	*pbuG*	*bbb22*, *bbb23*
Nucleoside diphosphate kinase	*ndk*	*bb0463*
Nucleotidase	-	*bb0504*
Xanthine-guanine phosphoribosyltransferase	*gpt*	*bb0103*
Cytidine deaminase	*cda*	*bb0618*
Thymidine kinase	*tdk*	*bb0793*
CTP synthase	*pyrG*	*bb0575*
Uridylate kinase	*pyrH*	*bb0571*
Uridine kinase	*udk*	*bb0015*
Cytidylate kinase	*cmk*	*bb0819*, *bb0128*
Thymidylate synthase	*thyX*	*bba76*
Hypoxanthine-guanine phosphoribosyltransferase	*hpt*	-
Adenylosuccinate synthase	*purA*	-
Adenylosuccinate lyase	*purB*	-
Auxiliary (NrdI) protein	*nrdI*	-
Ribonucleotide-diphosphate reductase alpha subunit	*nrdE*	-
Ribonucleotide-diphosphate reductase beta subunit	*nrdF*	-

2.3 Pathogen-host interaction

2.3.1 The tick vector

Borrelia is a zoonotic bacterium that naturally circulates between its arthropod vector, the *Ixodes* ticks (Figure 8), and the competent reservoir animals (rodents, squirrels, birds) and the incompetent large animals (deer) (Mysterud et al., 2019). The host animals are classified as (in)competent depending on their (un)ability to become infected with *Borrelia* and to be a source of infection for their vector (Mysterud et al., 2019).

In general, ticks are ectoparasites temporarily living on vertebrate hosts for blood meal acquisition, and can transmit multiple infectious agents, such as bacteria, viruses, and parasites (Estrada-Peña & de la Fuente, 2014; Boulanger et al., 2019). There are 900 identified tick species which are mainly divided into two families: hard ticks (Ixodidae) and soft ticks (Argasidae) (Boulanger et al., 2019). For example, the relapsing fever *Borrelia* are transmitted by soft ticks, *Ornithodoros* and *Argas* ticks, except for *B. recurrentis* and *B. miyamotoi* which are transmitted by body lice and *I. ricinus* ticks, respectively. Examples of the hard ticks, *I. ricinus* and *I. persulcatus*, transmit, in addition to *Borrelia*, the tick-borne encephalitis (TBE) virus. Another hard tick, *Hyalomma marginatum* prevalent in warm climate countries, carries the Crimean-Congo hemorrhagic fever virus.

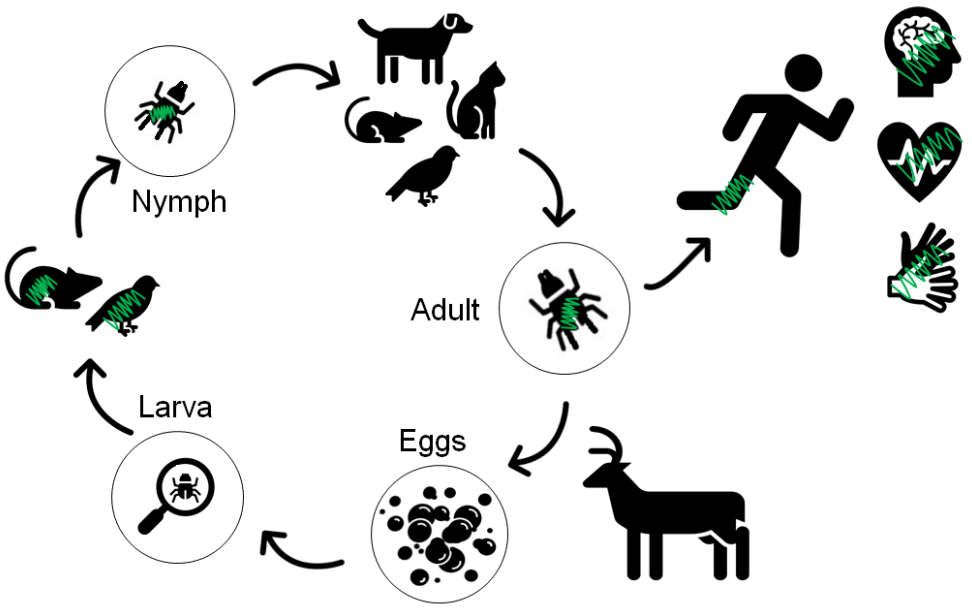

Figure 8. The life cycle of an *Ixodes* tick starts with the larva hatching from the eggs. The larva molts to a nymph and later to an adult. Between each developmental stage, the tick acquires a blood meal from host animals. The tick becomes infected if it feeds on an infected host. The tick can transmit the infectious agent to the following host animal. Humans are accidental hosts. From the intial tick bite site, *Borrelia* (green) can disseminate and cause inflammation in the joints, nervous system, heart, or skin. Figure modified from (Radolf et al., 2012).

There are four species of *Ixodes* ticks transmitting *Borrelia* infection. *I. ricinus* and *I. persulcatus* are the prevalent ticks in Europe and *I. persulcatus* in Asia (Radolf et al., 2012; Estrada-Peña et al., 2018). In Finland, both *I. ricinus* and *I. persulcatus* exist due to its proximity to the Eurasian border (Laaksonen et al., 2017). 16.9% and

1.6% of the ticks in Finland are infected with *Borrelia* and TBE virus, respectively. *I. scapularis* and *I. pacificus* are the main ticks in eastern and western North America, respectively (Radolf et al., 2012; Dumic & Severnini, 2018). Factors such as climate change, milder winters, faster developmental rates of ticks, and increasing host density lead to the spread of ticks towards northern latitudes and higher altitudes (Laaksonen et al., 2017; Estrada-Peña et al., 2018).

The length of a tick life cycle varies from one to four years depending on the climate (Estrada-Peña & de la Fuente, 2014). In cold climate regions, the life cycle of a tick is longer. In countries with continental climate, ticks are active from March to October, whereas in oceanic countries, ticks can be active all year round (Boulanger et al., 2019). In Finland, *I. ricinus* is active from April to November and *I. persulcatus* from April to July with high seasonal activity peak in the early summer months (Laaksonen et al., 2017).

The life cycle of ticks involves four stages: egg, larva, nymph, and female or male adult (Figure 8) (Estrada-Peña & de la Fuente, 2014; Boulanger et al., 2019). The larval tick hatches from the eggs laid by a female tick. The newly hatched larva acquires its first blood meal from a small vertebrate animal, such as a rodent, squirrel or a bird. After feeding, the larva drops to the ground and molts to a nymph. During the nymphal stage, the tick acquires its second blood meal before molting to an adult. The resulting adult tick feeds on a third host, such as a deer, then mates, and the female tick lays thousands of eggs on the ground. If the tick feeds on an infected host animal, the tick will become infected with *Borrelia*. In Europe, mainly rodents and migratory birds are infected with *B. afzelii* and *B. garinii*, respectively, and mice are infected with *B. burgdorferi* ss in North America (Table 3) (Radolf et al., 2012).

Notably, the transmission of *Borrelia* in ticks is transstadial as the female adult cannot pass on the infection to its offspring (Estrada-Peña & de la Fuente, 2014). In contrast, the transmission of the relapsing fever *B. miyamotoi* is transovarial (Richter et al., 2012; van Duijvendijk et al., 2016). Hence, larvae may be infected with *B. miyamotoi* and can transmit the spirochetes to their first host animal. In rare cases *Borrelia* infected larvae are also detected (Richter et al., 2012; van Duijvendijk et al., 2016). Most likely, the acquisition of the *Borrelia* occurred during feeding on the host, but the feeding was interrupted, and larvae could not molt to the nymphal stage.

2.3.2 Infection of the host

Humans and companion animals, such as dogs and cats, are accidental hosts of the ticks (Radolf et al., 2012; Estrada-Peña & de la Fuente, 2014; Boulanger et al., 2019). During outdoor activities humans and companion animals visit forests and fields, the typical habitats of ticks, and may encounter a questing tick. A questing tick sitting on the tip of grasses or leaves detects a passing animal or human with its complex

sensory system (sensory bristles, pedipalps, and Haller's organ on front legs) and attaches to the skin of the host. Once on the skin, the tick locates a warm and humid spot, perforates the skin with its feeding mouth parts, and releases a cement-like substance securing the attachment of the tick to the skin. After a few hours, the tick starts to insert saliva into the feeding cavity. The tick saliva is abundant of pharmacologically active compounds inducing cells lysis around the feeding cavity, enabling blood flow into the feeding cavity, and evading the host inflammatory response (Šimo et al., 2017). Often the tick bite is not noticed as pain is inhibited by bradykininases cleaving bradykinin, the pain and inflammation signaling molecule in humans (Šimo et al., 2017).

The warm blood meal entering the tick midgut is the escape signal for *Borrelia*. The *Borrelia* residing in the unfed tick midgut are firmly attached as OspA expressed on the *Borrelia* binds to the tick receptor for OspA (TROSPA) expressed on the surface of the tick midgut (Pal et al., 2004). After the blood arrives in the tick midgut, the OspA expression on *Borrelia* is downregulated and the OspC is upregulated which is an important protein for establishing *Borrelia* infection in the vertebrate host (Schwan & Piesman, 2000). The arriving blood contains serum proteins of the complement system of the vertebrate host innate immune system. Thus, *Borrelia* also expresses complement regulator-binding proteins to survive the complement-mediated killing (Hart et al., 2018). *Borrelia* released from the tick midgut, spreads first to the tick salivary glands via the haemocoel, and then enters the host skin via the tick saliva (Schwan & Piesman, 2000). As shown by experimental transmission, after 12 hours of tick attachment, *Borrelia* is transmitted from the nymphal ticks and after 24 hours from adult ticks to mice (Sertour et al., 2018).

2.3.3 Host immune response

During the early phase of infection, the activated host complement system continues to fight the entering *Borrelia* (Kraiczy, 2016). Further, *Borrelia* is recognized by the innate immune cells residing in the skin of the host (Radolf et al., 2012). The first arriving innate immune cells are the neutrophils, which recognize and kill effectively the invading *Borrelia* (Steere et al., 2016). Within 24 hours, the neutrophils disappear, but other innate immune cells, such as dendritic cells, monocytes and macrophages, and adaptive immune cells, such as T cells, remain (Steere et al., 2016). *Borrelia* is detected by pattern-recognition receptors which are expressed on the host innate immune cells and non-immune cells (epithelial cells, endothelial cells, and fibroblasts) for recognition and phagocytosis of microbes or pathogenic host cell particles (Takeuchi & Akira, 2010). The family of Toll-like receptors (TLRs) represents the major pattern-recognition receptors in *Borrelia* infection (Steere et al., 2016). In general, the TLRs detect bacteria, viruses, and protozoa by pathogen-associated

molecular patterns, such as flagella, di- or triacylated lipoproteins and single- or double stranded DNA and RNA (Takeuchi & Akira, 2010).

The host immune response against *Borrelia* infection via TLR2 is the best characterized signaling pathway, although TLR5, TLR7, TLR8, and TLR9 and other signaling pathways are also involved (Wooten et al., 2002; Shin et al., 2008; Petnicki-Ocwieja & Kern, 2014; Petzke & Schwartz, 2015). The TLR2 forms a heterodimer with TLR1 on the surface of innate immune cells, and recognizes the tripalmitoyl-S-glycerylcysteine lipid modified *Borrelia* outer surface proteins (Hirschfeld et al., 1999; Wooten et al., 2002). The binding of TLR2 to *Borrelia* leads to the cellular downstream effect of translocation of the nuclear factor κB (NF-κB) via the myeloid differentiation primary response gene 88 (MyD88) -dependent signaling pathway (Petnicki-Ocwieja & Kern, 2014; Petzke & Schwartz, 2015). The NF-κB activates the expression of proinflammatory cytokines, such as interleukin (IL) 1β, IL-6, IL-8, IL-12, tumor necrosis factor (TNF) α, type 1 interferon, and adhesion factors, such as E-selectin, vascular cell adhesion molecule 1, and intracellular adhesion molecule 1 (Shin et al., 2008; Cervantes et al., 2011; Petnicki-Ocwieja & Kern, 2014; Petzke & Schwartz, 2015). Further, TLR2 signaling induces the internalization and phagocytosis of *Borrelia* in the phagosomes of monocytes (Cervantes et al., 2011). The pivotal roles of TLR2 and MyD88 in the phagocytosis of *Borrelia* are evident from the infection studies using TLR2- or MyD88-deficient mice (Wooten et al., 2002; Bolz et al., 2004). Mice lacking TLR2 or MyD88 were unable to control spirochetemia as the amount of *Borrelia* genomes in the investigated tissues were significantly higher in TLR2- and MyD88-deficient mice than in the tissues of wildtype mice. Notably, TLR2- or MyD88-deficiency did not reduce production of antibodies.

During the disseminated phase of *Borrelia* infection, the adaptive immunity is crucial for clearing of bacteria and for controlling the inflammatory response (Radolf et al., 2012). Despite the short blood-borne phase during dissemination, *Borrelia* releases antigens which are eventually passing through the spleen (Belperron et al., 2007). In the spleen, the marginal zone B cells (MZB) detect the *Borrelia*-specific antigens and recognize those as foreign antigens, and subsequently, the MZBs start producing IgM antibodies targeting *Borrelia*. Later, the B cells with the help of the T cells start producing *Borrelia*-specific IgG antibodies which are detected earlierst after two weeks of infection by serologic tests (Tunev et al., 2011; Steere et al., 2016; Tracy & Baumgarth, 2017). IgG antibodies towards *Borrelia* are produced in 99% of patients (Dessau et al., 2018), and the IgGs remain elevated for more than ten to twenty years and probably longer (Kalish et al., 2001).

Interestingly, the combined effect of the innate and adaptive immunity is able to clear *Borrelia* infection within several months or years (Steere et al., 2016). Even

the clinical symptoms resolve eventually without antibiotic treatment (Steere et al., 1987), except for the persisting ACA skin inflammation (Steere et al., 2016).

2.3.4 Dissemination and persistence of *Borrelia* in the human host tissues

Despite the host innate immune response during the early phase of infection, *Borrelia* establishes infection using multiple immune evasion strategies. First, the expression of OspC on the outer surface of *Borrelia* is required for mammalian infection (Grimm et al., 2004). OspC binds to tick protein Salp15 leading to the evasion from the complement-mediated killing (Schuijt et al., 2008). Further, OspC protects *Borrelia* from phagocytosis by the host macrophages (Carrasco et al., 2015). At the same time, the highly expressed OspC has its disadvantage as it is easily recognized by host immune cells leading to the production of antibodies (Magnarelli et al., 1996). Hence, within two to three weeks, the OspC expression in *Borrelia* is downregulated (Liang et al., 2002; Liang et al., 2004), and the significance of multiple other virulence factors, such as the antigenic variation protein VlsE (Vmp-like sequence E), becomes more prominent (Liang et al., 2004; Radolf et al., 2012).

Borrelia starts to disseminate from the initial tick bite site within two days (Shih et al., 1992). As described above, *Borrelia* is motile and can spread between the cells in the extracellular matrix (ECM) consisting of macromolecules surrounding all types of organs and tissues (Radolf et al., 2012). To facilitate its movement in the ECM and to enter the blood vasculature by piercing the blood vessels walls, *Borrelia* activates the host matrix metalloproteases, and the host fibrinolytic system including plasminogen, and its activator, urokinase, to degrade the ECM macromolecules (Grab et al., 2005; Behera et al., 2006).

Borrelia disseminates to distant organ sites via the blood vessels and probably via the lymphatic vessels (Radolf et al., 2012; Tracy & Baumgarth, 2017). In the bloodstream, *Borrelia* evades the complement-mediated killing by binding to Factor H and Factor H -like protein 1 with its surface-exposed complement regulator-acquiring surface proteins (CRASPs) (Mühleip et al., 2018). The extravasation of *Borrelia* to the surrounding target tissue, is mediated by the vascular-adhesion protein BBK32 (Moriarty et al., 2012). BBK32 binds to glycosaminoglycans (GAGs) and fibronectin on the vascular surface resulting in slowing down of the movement of *Borrelia* and in tethering and dragging interactions of *Borrelia* with the vascular endothelium before entering the extravascular tissue (Moriarty et al., 2012; Ebady et al., 2016).

In the extravascular tissues, such as in the joints, peripheral and central nervous system, skin or in the heart, *Borrelia* is able to persist for a long time as it adheres to multiple ECM macromolecules, such as to fibronectin, laminin, proteoglycans and to GAGs (Caine & Coburn, 2016). For example, the *Borrelia* outer surface protein

RevA binds to fibronectin (Brissette et al., 2009a), Erpx and BmpA bind to laminin (Brissette et al., 2009b; Verma et al., 2009) and DbpA and B are known to bind to two proteoglycans, biglycan and decorin (Guo et al., 1995; Guo et al., 1998; Salo et al., 2011; Salo et al., 2016). Importantly, the adhesion proteins of *Borrelia* display redundancy and multifunctionality (Caine & Coburn, 2016).

2.3.5 Biglycan and decorin

The ECM consisting of multiple macromolecules (collagens, elastins, fibronectin, laminins, glycoproteins, proteoglycans and gylcosaminoglycans) provides a physical scaffold for the cells in organs and tissues, but also regulates multiple cellular functions (Theocharis et al., 2016). The proteoglycans (PGs) are the largest family of ECM macromolecules with diverse structures and functions. The PGs are composed of a core protein and of one or several attached GAG chains. The GAGs are linear polysaccharides consisting of repeating di-saccharides of an amino sugar and uronic acid or galactose. The GAGs can be classified as chondroitin sulfate (CS), dermatan sulfate (DS) and to heparan sulfate, heparin, keratan sulfate and hyaluronan glucosaminoglycans.

Biglycan and decorin are PGs belonging to the class I of the small leucine-rich proteoglycans that are expressed overall in the ECM. Biglycan has a core protein of 42 kDa with two attached GAG chains (Figure 9) (Fisher et al., 1989; Bianco et al., 1990). The core protein of decorin is approximately 45 kDa with one attached GAG chain (Fisher et al., 1989; Theocharis et al., 2016). The GAG chains are either DS or CS depending on the tissue site. Biglycan has two and decorin has three attachment sites for N-linked oligosaccharides possibly preventing the self-aggregation of the PG (Iozzo, 1998).

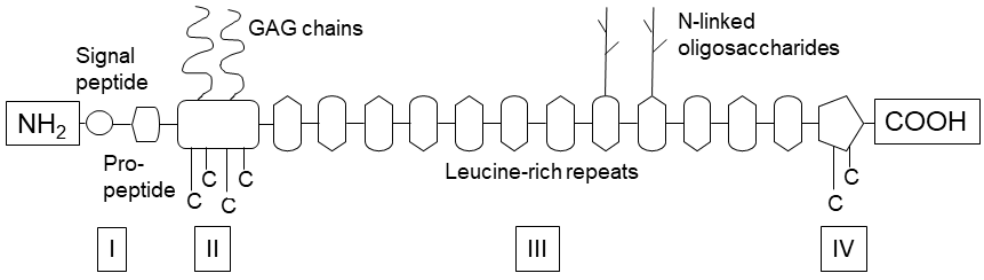

Figure 9. Schematic structure of biglycan. The biglycan core protein contains four domains (I–IV). Domain I consists of a signal peptide and a pro-peptide that are cleaved before secretion into the extracellular matrix. Domain II consists of four cysteines and the attachment sites for two glycosaminoglycan (GAG) side chains. Domain III consists of the leucine-rich tandem repeats and the attachment sites for two N-linked oligosaccharides. Domain IV consists of two cysteines. Modified from (Iozzo, 1998; Sainio & Järveläinen, 2019).

The name decorin derives from the function of the core protein as it "decorates" collagen bundles (Theocharis et al., 2016). Hence, decorin is found in the ECM of collagen-rich connective tissues, such as in skin, joints, tendons, in the connective sheaths of skeletal muscles, in peripheral nerves and in the eye cornea (Bianco et al., 1990). Notably, decorin deficiency leads to a fragile skin phenotype (Corsi et al., 2002). Biglycan is also expressed in the ECM of connective tissues, but in specialized cell types. For example, both decorin and biglycan are expressed in the skin (Bianco et al., 1990). Decorin is found in the dermal matrix of the skin, whereas, biglycan is expressed in the keratinocytes of the epidermis and in the endothelial cells of dermal capillaries. Although both PGs are expressed in the cartilage and bones, only biglycan deficiency leads to decreased skeletal growth and to a severe osteoporosis phenotype (Xu et al., 1998). Importantly, only biglycan is expressed in endothelium of myofibers, peripheral nerves, and kidneys as well as in aorta (Bianco et al., 1990) and in blood vessels in the skin and brain (Salo et al., 2016), in kidneys (Schaefer et al., 2000), and in the liver (Högemann et al., 1997).

In addition to their structural function, biglycan and decorin display various roles in cell signaling, in tumorigenesis and in promoting inflammation (Schaefer & Schaefer, 2010; Theocharis et al., 2016). Following sterile inflammation (tissue stress, injury, and cell death), both PGs are released from the ECM (Schaefer, 2014). Soluble biglycan and decorin are identified as danger-associated molecular patterns (DAMP) by innate immune cells, mainly macrophages, via the TLR2 and TLR4 receptors (Schaefer, 2014; Frevert et al., 2018). Subsequently, the macrophages release proinflammatory cytokines and chemokines to attract more innate and adaptive immune cells (macrophages, neutrophils, B and T cells) to the site of infection. Examples of biglycan induced cytokines are TNF-α, MIP (macrophage inflammatory protein) -1α and -2, MCP (monocyte chemoattractant protein) -1, RANTES (regulated upon activation, normal T cell expressed and secreted), IL-1β, CCL (C-C motif chemokine ligand) 2 and 5, and CXCL (C-X-C motif chemokine ligand) 13 (Schaefer et al., 2005; Babelova et al., 2009; Moreth et al., 2010; Zeng-Brouwers et al., 2014). The newly attracted immune cells amplify the local inflammatory response by synthesizing additional biglycan *de novo* which, in turn, leads to the release of more proinflammatory cytokines (Nastase et al., 2012). Interestingly, after an LPS injection mimicking a bacterial infection, soluble biglycan possibly increased the severity of the lung sepsis as seen by shorter survival time of the biglycan wildtype mice compared to the longer survival of the biglycan deficient mice (Schaefer et al., 2005).

The host molecules, biglycan and decorin, are important for *Borrelia* pathogenesis. The adhesion of decorin-binding proteins (Dbp) A and B of *Borrelia* to decorin have been extensively studied *in vitro* and *in vivo* using *Borrelia* lacking either DbpA, DbpB, or DbpA and B (Guo et al., 1998; Brown et al., 1999; Shi et al.,

2006; Shi et al., 2008a; Shi et al., 2008b; Fortune et al., 2014; Lin et al., 2014; Salo et al., 2015). If one or both adhesins were lacking, the overall virulence and infectivity of *Borrelia* were reduced (Shi et al., 2008a; Salo et al., 2015). Further, mice deficient of decorin were similarly infected with *Borrelia* than wildtype mice, except that the bacterial joint colonization and joint inflammation were decreased (Brown et al., 2001). Brown and colleagues suggested that *Borrelia* overcomes the decorin deficiency by adhering to another ligand, such as biglycan. Recently, the DbpA and B adhesins were shown to bind to biglycan *in vitro* (Salo et al., 2016). Interestingly, the interaction of biglycan and DbpA and B varied depending on the *Borrelia* genospecies and depending on the study conditions. The DbpA and B of *B. garinii* bound effectively biglycan under flow conditions mimicking the conditions when *Borrelia* is circulating in the human blood vessels. In contrast, under stationary conditions only DbpB of *B. garinii* and of *B. burgdorferi* ss bound strongly to biglycan. The DbpA and B of *B. afzelii* did not bind to biglycan neither under flow nor under stationary conditions. Despite the well-characterized interaction of biglycan and DbpA and B *in vitro*, the *in vivo* studies are important to establish the role of biglycan in the pathogenesis of *Borrelia*.

3 Aims

The function of multiple *Borrelia* proteins and the interactions between *Borrelia* and host molecules are not completely understood in the pathogenesis of Lyme borreliosis. Further, the seroprevalence of Lyme borreliosis in the time prior to the characterization of the disease entity has not been studied. Hence, following aims were set to shed light on these questions.

1. To study the role of Basic membrane protein D in the pathogenesis of *Borrelia* infection.
2. To study the role of the host molecule biglycan in *Borrelia* infection *in vivo*.
3. To study the historical seroprevalence and the associated risk factors of Lyme borreliosis in Finland.

4 Materials and Methods

4.1 Bacterial experiments

4.1.1 Bacterial strains and culture conditions (Studies I and II)

The wildtype *Borrelia* strains, *B. burgdorferi* ss B31 and N40, *B. garinii* SBK40 and *B. afzelii* A91, were cultivated in BSK-II medium at 33°C under microaerophilic conditions (Table 5). *Borrelia* were counted after reaching the logarithmic growth phase with Neubauer chamber and phase-contrast microscope.

Table 5. Bacterial strains used in this study. The antibiotic selection is mentioned in the square brackets.

Bacterial strain	Special feature	Reference	Study
B. burgdorferi ss B31	Isolated from a tick	(Burgdorfer et al., 1982)	I and II
B. burgdorferi ss N40	Isolated from a tick	(Anderson et al., 1990)	II
B. garinii SBK40	Isolated from skin biopsy	(Heikkilä et al., 2002b)	II
B. afzelii A91	Isolated from skin biopsy	(Heikkilä et al., 2002b)	II
E. coli BL21(DE3)pLys + BmpD [Cm]	Transformed with pET-30 Ek/LIC + *bmpD*/ *B. burgdorferi* ss B31 [Km]	Study I	I
E. coli BL21(DE3)pLys + DbpA/ *B. burgdorferi* ss [Cm]	Transformed with pET-30 Ek/LIC + *dbpA*/ *B. burgdorferi* ss N40 [Km]	(Salo et al., 2011)	II
E. coli BL21(DE3)pLys + DbpB/ *B. burgdorferi* ss [Cm]	Transformed with pET-30 Ek/LIC + *dbpB*/ *B. burgdorferi* ss N40 [Km]	(Salo et al., 2011)	II
E. coli M15(pREP4) + DbpA/ *B. garinii* [Km]	Transformed with pQE30 + *dbpA*/ *B. garinii* SBK40 [Amp]	(Heikkilä et al., 2002b)	II
E. coli M15(pREP4) + DbpB/ *B. garinii* [Km]	Transformed with pQE30 + *dbpB*/ *B. garinii* SBK40 [Amp]	(Heikkilä et al., 2002a)	II
E. coli M15(pREP4) + DbpA/ *B. afzelii* [Km]	Transformed with pQE30 + *dbpA*/ *B. afzelii* A91 [Amp]	(Heikkilä et al., 2002b)	II
E. coli M15(pREP4) + DbpB/ *B. afzelii* [Km]	Transformed with pQE30 + *dbpB*/ *B. afzelii* A91 [Amp]	(Heikkilä et al., 2002a)	II

The *Escherichia coli* strains used for cloning were DH5α and for expression of recombinant proteins were BL21(DE3)pLys (Novagen, Madison, WI, USA) and M15 (Qiagen, Hilden, Germany) (Table 5). *E. coli* were propagated in Luria Bertani (LB) medium or on agar plates under antibiotic selection of 30 µg/ml kanamycin (Km; Sigma-Aldrich), 34 µg/ml chloramphenicol (Cm; USB Corporation, Cleveland, USA) or 100 µg/ml ampicillin (Amp; Sigma-Aldrich) at 37°C.

4.1.2 Proteinase K-assay (Study I)

The localizations of proteins on the outer membrane or in the periplasmic space of *Borrelia* were detected by enzymatic degradation assay using proteinase K (Sigma-Aldrich, Darmstadt, Germany). The *B. burgdorferi* ss B31 in the mid-logarithmic stage were washed twice with phosphate buffered saline (PBS) containing 5 mM $MgCl_2$, and diluted to $1x10^8$ bacteria/ml. Proteinase K was added to the bacterial cells at concentrations of 0, 50, 100 or 200 µg/ml at room temperature (RT) for one hour. The bacteria were washed twice with the aforementioned buffer before analyzing the bacterial lysate samples by Western blot as described below.

4.2 Mouse experiments

4.2.1 Mouse strains (Studies I and II)

All animal studies were approved by the National Animal Experiment Board in Finland (permissions ESAVI/5507/04.10.07/2014 and ESAVI/6265/04.10.07/2017), and performed according to the 3R-principle (Replacement, Reduction, and Refinement) as well as according to relevant guidelines and regulations by the Finnish Act on the Use of Animals for Experimental Purposes and directives of the European Union. All mouse strains used in this study are listed in Table 6.

Table 6. Characteristics of mouse strains used in this study.

Mouse strain	Gene modification	Sex	Provider	Study
C3H/HeNHSd	Wildtype (WT)	Female	Envigo, Horst, The Netherlands	I
C3H/HeNHSd	Wildtype (WT)	Female and male	Envigo	II
C3H/HeNHSd	Biglycan knock-out (KO)	Female and male	Turku Center of Disease Modeling (TCDM), Turku, Finland	II

In Study I, four-week-old wildtype (WT) female mice were used for the immunization and passive immunization study. In Study II, four-week-old WT or biglycan knock-out (KO) female and male mice were used for the infection studies. The KO mice in the C57BL/6 background (Xu et al., 1998) were kindly provided by Dr. Marian Young (National Institute of Dental and Craniofacial Research, Bethesda, MD, USA), and were backcrossed 7 generations into the C3H/HenHSd background (Envigo) by TCDM. The genotype of the WT and KO mice was verified by PCR as described in (Xu et al., 1998) by TCDM. The phenotypes of WT and KO were verified by RT-qPCR and immunohistochemistry as described below.

4.2.2 BmpD immunization study (Study I)

Mice (n=14) were subcutaneously immunized with 100 µl of solution containing 50 µg BmpD in PBS diluted 1:1 with TiterMax Gold adjuvant (Sigma-Aldrich). The control mice (n=15) received 100 µl PBS diluted 1:1 with the adjuvant. Mice received one booster dose at day 21. Blood samples were collected from tail vein at day 14 and by cardiac puncture at day 28. The blood samples were collected in heparinized tubes and centrifuged 1000 x g at RT for ten minutes. The antibodies towards rBmpD in serum samples collected at days 14 and 28 day after immunization were measured with ELISA as described below.

For passive immunization studies, mice were intravenously injected with (1) 5 ml/kg mouse serum containing anti-BmpD antibodies, (2) serum from PBS immunized mice, or (3) 50 µl saline as a control pretreatment. After 48 hours, the BmpD immunized mice (n=12) and mock immunized mice (n=12) were infected intradermally with 10^5 *B. burgdorferi* ss B31. The positive control mice (n=10) were infected similarly. The negative control mice (n=4) received 100 µl PBS intradermally. The course of infection was followed by collecting ear biopsy samples at days 7, 11, 14 and 21 post-infection, and measuring the joint swelling in a blindfolded manner once a week. After four weeks post-infection, mice were euthanized, and ear, tibiotarsal joint, heart, bladder, and whole-blood specimens were collected.

4.2.3 *Borrelia* infection of biglycan KO/WT mice (Study II)

The biglycan KO mice (n = 7–8/ experiment) and WT mice (n = 7–8/ experiment) were intradermally infected with 10^5 *B. burgdorferi* ss N40, *B. garinii* SBK40 or *B. afzelii* A91. The infected mice were followed up for 28 days (experiment I) or 56 days (experiments II–IV). The course of infection was followed by collecting ear biopsy samples at days 7, 11, 14, 21 and 42 post-infection depending on the experiment. The joint swelling was monitored in a blindfolded manner once a week.

After mice were euthanized, ear, tibiotarsal joint, heart, bladder, and whole blood specimens were collected.

4.3 Analyses of mouse tissue specimens

4.3.1 *Borrelia* culture (Studies I and II)

The tissue samples of *Borrelia* infected and mock treated mice were cultured in BSK-II medium supplemented with 50 µg/ml phosphomycin (Sigma-Aldrich) and 100 µg/ml rifampicin (Sigma-Aldrich) for six weeks. Every two weeks the growth of *Borrelia* in the tissue samples was monitored by dark-field microscopy.

4.3.2 Histology and immunohistochemistry (Study II)

For histologic examination, mouse tissue samples were fixed in 10% phosphate buffered formaldehyde, dehydrated in a graded ethanol series and embedded in paraffin and sectioned at 5 µm. Before paraffin embedding, tibiotarsal joint specimens were decalcified in EDTA. The tissue samples were stained with haematoxylin and eosin (H&E) for light microscopy using routine histology techniques. Further, heart specimens were stained with Weigert Van Gieson -method to highlight the collagen fibers in case of heart fibrosis.

The inflammation in joints (experiments I–IV) and in hearts (experiments I and II) were evaluated in sagittal tibiotarsal joint and heart sections by an experienced pathologist blinded to the experimental protocol. The joint inflammation was graded from no inflammation to severe inflammation by scoring the synovial proliferation and active and chronic inflammation (numerical scale 0 to 6). The myocardial inflammation and fibrosis were graded from no inflammation or fibrosis to severe inflammation with fibrosis (numerical scale 0 to 8).

For immunohistochemistry, tissues were processed until paraffin embedding as for histological analysis. For biglycan staining, the tissue samples were first digested with 0.0045 U/ml chondroitinase ABC (Sigma-Aldrich), and then stained with a rabbit IgG polyclonal antibody LF-159 (1:500; Kerafast, Boston, USA). Immunodetection was performed using the avidin-biotin complex method (Sigma-Aldrich), diaminobenzidine was used as the chromogen, and finally, the sections were counterstained with H&E.

4.3.3 DNA extraction and qPCR (Studies I and II)

The bacterial load in tissue specimens was analyzed by quantitative PCR (qPCR) as described in (Ivacic et al., 2007). The total DNA in mouse tissue samples were

extracted with the High Pure PCR Template Preparation Kit (Roche Diagnostics). Then, a 102 bp product of the *ospA* gene of *Borrelia* was amplified using the primers listed in Table 7, and with LightCycler 480 Probes master kit (Roche Diagnostics) and LightCycler 480 II equipment (Roche Diagnostics). The protocol contained the following steps: 1) initial denaturation at 95 °C for 5 minutes, 2) 45 cycles of denaturation at 95 °C for 10 seconds, primer annealing at 58 °C for 10 seconds and extension at 72 °C for 12 seconds, 3) melting curve, and 4) cooling at 40 °C. To calculate the actual *Borrelia* load in each sample, the bacterial load was compared to the standard curve of 100 to 1,000,000 bacteria. The samples were analyzed three times. The samples were accepted as positive, when the bacterial load could be analyzed at least two times. The data were expressed as the number of bacterial genomes per 100 ng of extracted total DNA.

4.3.4 RNA extraction and RT-qPCR analysis (Study II)

The biglycan mRNA expression in mouse tissues was detected by RNA extraction and by reverse transcriptase quantitative PCR (RT-qPCR). Lung tissue samples of uninfected biglycan KO and WT mice and multiple tissues from uninfected and *B. garinii* infected WT mice were collected and stored in RNAlater (Qiagen, Hilden, Germany) at -20 °C. The total RNA was extracted by RNeasy Mini kit (Qiagen), and reverse transcribed to cDNA by QuantiTect Rerverse Transcription Kit (Qiagen) according to manufacturer's protocols.

The LightCycler 480 SYBR Green I Master mix (Roche Diagnostics) and Light-Cycler 480 II equipment (Roche Diagnostics) were used to detect the gene expression of biglycan and the reference gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) with primers listed in Table 7. The protocol included the following steps: 1) pre-incubation at 50 °C for 2 minutes, 2) initial denaturation at 95 °C for 10 minutes, 3) 40 cycles of denaturation at 95 °C for 10 seconds, primer annealing at 60 °C for 10 seconds and extension at 72 °C for 10 seconds, 4) melting curve, and 5) cooling at 40 °C. The biglycan mRNA abundance was determined from the quantification cycle (Cq) for biglycan and normalized against the Cq for GAPDH using the $2^{-\Delta\Delta Cq}$ method (Rao et al., 2013). Data were expressed as fold changes

Table 7. Primers used in this study.

Primer	Sequence 5'−3'	Reference
bgn wildtype allele forward for genotyping	CAGGAACATTGACCATG	(Xu et al., 1998)
bgn wildtype allele reverse for genotyping	CTTCAGTGCCATGTGTCCTTTC	(Xu et al., 1998)
bgn knock-out allele forward for genotyping	CAGGAACATTGACCATG	(Xu et al., 1998)
bgn knock-out allele forward for genotyping	CCTCGCACACATTCCACATCCA	(Xu et al., 1998)
bgn forward for RT-qPCR	ATTGCCCTACCCAGAACTTGAC	(Calmus et al., 2011)
bgn reverse for RT-qPCR	GCAGAGTATGAACCCTTTCCTG	(Calmus et al., 2011)
gapdh forward for RT-qPCR	CTCACAATTTCCATCCCAGAC	(Calmus et al., 2011)
gapdh reverse for RT-qPCR	GTTCGCTGCACCCACAAAAA	(Calmus et al., 2011)
ospA forward for qPCR	AATATTTATTGGGAATAGGTCTAA	(Ivacic et al., 2007)
ospA reverse for qPCR	CACCAGGCAAATCTACTGA	(Ivacic et al., 2007)
ospA probe for qPCR	6-FAM-TTAATAGCATGTAAGCAA AATGTTAGCA-XT-PH	(Ivacic et al., 2007)
T7 promoter #69348-3	TAATACGACTCACTATAG	Novagen
T7 terminator #69337-3	GCTAGTTATTGCTCAGCGG	Novagen

bgn ~ biglycan, *gapdh* ~ glyceraldehyde 3-phosphate dehydrogenase, *ospA* ~ outer surface protein A, *(*RT)-qPCR ~ (reverse transcriptase) quantitative polymerase chain reaction.

4.3.5 Mouse serology (Studies I and II)

The blood samples of mice were collected, centrifuged at 1000 x g (Study I) or 6000 x g (Study II) at RT for 10 minutes, and the resulting sera were stored at -20 °C. The IgG antibodies in mouse serum samples towards whole-cell sonicate antigen of *B. burgdorferi* ss B31 (ATCC 35210) (*Borrelia* WCS), and towards recombinant proteins (rBmpD, rDbpA or rDbpB of *B. burgdorferi* ss N40, *B. garinii* SBK40 or *B. afzelii* A91) were measured with in-house ELISA as described in Table 8.

Briefly, the enhanced binding 96-well microtiter plates (Thermo Fisher Scientific, Waltham, USA) were coated with *Borrelia* WCS (Viljanen & Punnonen, 1989) or recombinant proteins. After coating, the wells were washed with aqua supplemented with 0.05% Tween 20 (Aqua-T; Merck) and saturated with 1% normal sheep serum (NSS; Bio Karjalohja Oy, Karja-Lohja, Finland), and washed as above.

The mouse serum samples were added to the wells, incubated, and washed as above. The secondary antibody horseradish peroxidase (HRP) conjugated goat anti-mouse IgG (Santa Cruz Biotechnologies) was added to the wells, incubated and washed as above. The ortho-phenylenediamine (OPD) substrate solution (Kem-En-Tec Diagnostics, Taastrup, Denmark) was added to the wells, and incubated as above. The reaction of the substrate solution was stopped with H_2SO_4. Finally, the absorbance was measured at 492 nm with Multiskan EX spectrophometer (Thermo Fisher Scientific). All samples were measured in duplicates or quadruplicates. The results were expressed as OD_{492} values.

Table 8. Details of ELISA protocol for mouse serology. The abbreviations are explained in the main text.

Solutions		Concentration/ Dilution factor	Volume (µl)	Temperature (ºC)	Time
Coating	*Borrelia* WCS in PBS	20 µg/ml	120	37	overnight
	recombinant protein in PBS	10 µg/ml	120	37	overnight
Wash	Aqua-T		200	RT	3 times
Blocking	NSS-PBS	1%	150	37	1 hour
Wash	Aqua-T		200	RT	3 times
Serum sample	Mouse serum in NSS-PBS	1:100	100	37	1 hour
Wash	Aqua-T		200	RT	3 times
Secondary antibody	HRP conjugated goat anti-mouse IgG in NSS-PBS	1:8,000	100	37	1 hour
Wash	Aqua-T		200	RT	3 times
Substrate	OPD		100	37	15–45 minutes
Stop	H_2SO_4	0.5 M	100	RT	

4.3.6 Multiplex cytokine analysis (Study II)

The levels of multiple cytokines in mouse plasma samples were measured with Luminex 200 equipment (Luminex, Austin, USA) and a customized MILLIPLEX MAP Mouse Cytokine/Chemokine MCYTOMAG-70K (Merck Millipore, Billerica, USA) according to manufacturer's protocol. The results of each cytokine were calculated based on a standard curve using the xPONENT software version 3.1. (Luminex). The cytokine analytes measured were IL-1β, MCP-1/CCL2, MIP-1α/CCL3, RANTES/CCL5, TNFα and MIP-2/CXCL2.

4.4 Protein experiments

4.4.1 Cloning of recombinant proteins (Studies I and II)

A synthetic *bmpD* gene based on the sequence of *B. burgdorferi* ss B31 (Gene ID: 1195222; residues 18–323) was generated by a commercial vendor (Integrated DNA Technologies, Leuven, Belgium) with *Bgl*II and *Not*I recognition sites at both ends. The *bmpD* gene was designed not to include the signal peptide (residues 1–17), and the codons were optimized for *E. coli*. The *bmpD* gene and the pET-30 Ek/LIC -vector (Novagen) were digested with *Bgl*II and *Not*I (Thermo Scientific, Waltham, MA, USA), and ligated with the T4 DNA ligase (Novagen) according to the manufacturer's cloning protocol (Novagen). The resulting fusion construct contained a hexa-histidine tag at the N-terminal of the recombinant protein. The resulting plasmid was verified by sequencing using the T7 promoter and terminator primers (Novagen; Table 7). The plasmid was transformed into *E. coli* DH5α for plasmid amplification, and subsequently to *E. coli* BL21 (DE3)pLys (Novagen) for protein expression.

The *E. coli* BL21(DE3)pLys -strains expressing DbpA or DbpB of *B. burgdorferi* ss N40 were cloned in the pET-30 Ek/LIC -vector as described by (Salo et al., 2011). The *E. coli* M15(pREP4) strains expressing DbpA or DbpB of *B. garinii* SBK40 and *B. afzelii* A91 in the pQE-30 vector were kindly provided by Pekka Lahdenne (University of Helsinki, Finland).

4.4.2 Purification of proteins by affinity chromatography (Studies I and II)

All recombinant proteins, rBmpD, rDbpA and rDbpB of *B. burgdorferi* ss N40, *B. garinii* SBK40 and *B. afzelii* A91, were purified by affinity chromatography. For expression of recombinant proteins, an overnight *E. coli* culture was diluted 1:20 (rBmpD) or 1:50 (rDbps) in LB medium with appropriate antibiotic selection under vigorous shaking at 37°C. After reaching the OD_{600} of 0.6, the protein expression was induced by adding 1 mM isopropyl-β-D-1-thiogalactopyranoside to the bacterial culture. After induction, the culture was grown for 3–4 hours, and the bacteria were collected by centrifugation at 4000 x g for 15 minutes at 4°C. The bacterial cell pellet was stored at -80°C until purification.

The bacterial pellet was suspended in buffer A (50 mM Na_2HPO_4/NaH_2PO_4 /300 mM NaCl; pH 8.0) supplemented with 1 mM phenylmethylsulfonyl fluoride and 1 U/µl benzonase (EMD Millipore, Billerica, MA, USA), lysed by ultrasound sonication, and centrifuged by 10,000 x g at 4°C for 30 minutes. The overexpressed protein in the supernatant was allowed to adhere to a nickel-nitrilotriacetic acid (Ni-

NTA) resin column (Qiagen, Hilden, Germany) at 4°C for one hour, and the column was washed with three column volumes (CV) of buffer A containing 10 mM imidazole, one CV of buffer A containing 30 mM imidazole, and with 6 ml of buffer A containing 50 mM imidazole. The recombinant proteins were eluted with 6 ml and 12 ml buffer A containing 100 and 200 mM imidazole, respectively, at 4°C. The eluted protein fractions were dialyzed against PBS at 4°C, and concentrated with 10 kDa cut-off Amicon ultracentrifugal device (EMD Millipore, Burlington, MA, USA). The protein concentration was measured spectrophotometrically.

4.4.3 Purification of the ligand-free protein (Study I)

In order to remove the endogenously bound ligand, rBmpD was denaturated and refolded (Deka et al., 2006). rBmpD was first processed by affinity chromatography as described above, but instead of elution, the Ni-NTA bound protein was incubated with 10 ml of denaturation buffer B (8 M urea/ 100 mM Tris-HCl; pH 8.5) at RT for one hour. The Ni-NTA column was washed with 20 ml of buffer B and with buffer B diluted 1:1 and 1:3 with buffer C (20 mM Tris-HCl/ 20 mM NaCl/ 20 mM imidazole, pH 8.5), and with 20 ml buffer C. The resin was incubated with 10 ml of buffer C at RT for one hour. The refolded protein was eluted with 5 ml of buffer D (20 mM Tris-HCl/ 20 mM NaCl/ 200 mM imidazole, pH 8.5), concentrated, and purified by size exclusion chromatography (SEC) as described below.

4.4.4 Purification by size exclusion chromatography (Study I)

To exclude *E. coli* derived impurities from the native and ligand-free rBmpD solutions, size exclusion chromatography (SEC), an additional purification step, was performed before the crystallization and ligand-binding assays. The rBmpD was purified with ÄKTA pure chromatography system (GE Healthcare Life Sciences, Chicago, USA) and SuperdexTM 75 10/300 GL column (GE Healthcare Life Sciences). The protein buffer was exchanged to 50 mM Tris-HCl (pH 8.0). The fractions of the elution peaks were analyzed by SDS-PAGE stained with Simply blue as described below.

4.4.5 Crystallization, X-ray data collection, and refinement (Study I)

The rBmpD formed crystals by the sitting drop vapor diffusion method in 2:1 reservoir solution of 0.2 M sodium chloride, 0.1 M Tris, 20% (w/v) PEG 6000 (pH 8.0) supplemented with 15% MPD (2-methyl-2,4-pentanediol) as cryoprotectant. The crystals diffracted to 1.43 Ångström (Å) resolution at the beamline ID30A-3

European Synchrotron Radiation Facility (ESRF, Grenoble, France). Datasets were collected and processed with XDS, and the structure was solved by molecular replacement with Phaser using *T. pallidum* PnrA (PDB ID: 2FQY) as search model. The model building of the amino acid residues corresponding to BmpD was performed in Coot and refined by *phenix.refine*. The structure of adenosine was fitted into the electron density observed in the substrate-binding cleft in the initial BmpD model. The refinement statistics for the final refined model are listed in Study I, Table 1.

4.4.6 SDS-PAGE and Western blot (Study I)

The protein or bacterial lysate samples were boiled at 95°C for 8 minutes with sample buffer before running the samples on a 10% Bis-Tris polyacrylamide gel (PAGE, NuPage®, Life Technologies, Carlsbad, CA, USA) with MES buffer containing sodium dodecyl sulfate (SDS) at 200 V for 50 minutes. For protein detectetion, the SDS-PAGE was stained with Simply blue (Invitrogen, Carlsbad, CA, USA) at RT for one hour. The stained gel was imaged with LicorOdyssey imaging system (Licor Biotechnology, Bad Homburg, Germany).

For Western blotting, the samples on SDS-PAGE were blotted to a 0.2 µm nitrocellulose membrane (Whatman, Dassel, Germany) at 30 V for one hour. The membranes were blocked with 5% skim milk in PBS at 4°C overnight. The membranes were incubated either with serum samples of LB positive (n=3) or non-LB patients (n=3), with polyclonal rabbit anti-BmpD serum, polyclonal rabbit anti-DbpB serum, polyclonal p41 antibody or with monoclonal mouse anti-OspA antibody at RT for one hour. After rinsing the membrane with PBS supplemented with 0.05% Tween 20 (PBST; Merck), the membranes were incubated with an appropriate secondary antibody listed in Table 9 at RT for one hour. The bound antibodies were detected with WesternBright ECL HRP substrate (Advansta, San Jose, USA) and Odyssey Fc imaging system (Licor Biotechnology). The serum samples and antibodies were diluted in PBST supplemented with 5% skim milk with dilution factors listed in Table 9.

Table 9. Antibodies used in Western blots in this study. The dilution factors are indicated in the parentheses.

Sample	Primary antibody	Manufacturer	Secondary antibody	Manufacturer
rBmpD	Human serum sample (1:100)		HRP[a] conjugated rabbit-anti-human IgG (1:1,000)	DAKO Agilent Technologies, Santa Clara, CA, USA
rBmpD or *Borrelia* lysate	Rabbit polyclonal anti-BmpD serum (1:1,000)	Custom made by Harlan Laboratories, Leicester, UK	HRP conjugated goat-anti-rabbit IgG (1:5,000)	Santa Cruz Biotechnology, Santa Cruz, CA, USA
Borrelia lysate	Rabbit polyclonal anti-DbpB serum (1:1,000)	Custom made by Medprobe, Oslo, Norway	HRP conjugated goat-anti-rabbit IgG (1:5,000)	Santa Cruz Biotechnology
Borrelia lysate	Rabbit polyclonal p41 (1:1,000)	Aviva Systems Biology, San Diego, CA, USA	HRP conjugated goat-anti-rabbit IgG (1:5,000)	Santa Cruz Biotechnology
Borrelia lysate	Mouse monoclonal OspA (1:2,500)	H5332; kindly provided by Dr. Sven Bergström, University of Umeå, Sweden	HRP conjugated goat anti-mouse IgG (1:5,000)	Santa Cruz Biotechnology

[a]HRP~ Horseradish peroxidase.

4.4.7 Microscale thermophoresis (Study I)

Microscale thermophoresis (MST) (Seidel et al., 2013) was used to study the binding of ligands to the ligand-free rBmpD using the label-free approach. Adenosine, inosine, guanosine, ribose, and xanthosine (Sigma-Aldrich) were dissolved in 50 mM Tris-HCl (pH 8.0). A 24-point serial dilution of a ligand was mixed with LF-rBmpD (final concentration 500 nM). The concentration of the ligands ranged from 5 mM to 1.2 nM. Samples were filled into Zero Background Treated Standard Capillaries (MO-AZ002, NanoTemper Technologies, Munich, Germany) and measured with Monolith.NT115 LabelFree equipment (NanoTemper Technologies) using 60% LED-power and medium MST-power. The data were analyzed by MO.affinity software (Nanotemper Technologies) and GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA).

4.4.8 Liquid chromatography-mass spectrometry (Study I)

Liquid chromatography-mass spectrometry (LC-MS) was used to determine the small molecule bound to rBmpD with the modified analytical method of (Ren et al., 2008). First, rBmpD and for comparison LF-rBmpD samples were heated at 95 ºC for 10 minutes, and centrifuged with 21,000 x g for 30 minutes.

The resulting supernatant was directly used in the Agilent 1100 series liquid chromatography system (Agilent Technologies, Santa Clara, USA). Separations were conducted using gradient elution on a SunFire™ C18 analytical column (2.1 × 150 mm, particle size 3.5 µm, Waters, USA). The mobile phases were 0.1% formic acid in water (A) and 0.1% formic acid in methanol (B). The gradient conditions were 5% of B (0–12 min), from 5% to 80% (12–13 min), 80% (13–18 min), from 80% to 5% (18–18.5 min) and 5% (18.5–25 min). The flow rate was 0.25 ml/min. Retention times for adenosine and inosine were 4.7 and 7.7 min, respectively (Study I, Figure 4A).

Mass spectrometric detection was performed in selected-ion monitoring mode (SIM) with a single quadrupole mass spectrometer (HP 1100 LC/MSD). Ionization was based on electrospray ionization in positive mode. The capillary voltage was 4.0 kV and the drying gas temperature 350 °C. The selected ions for adenosine and inosine were m/z 268.0 and 269.0, respectively (Study I, Figure 4A). These masses correspond to their protonated molecules $[M+H]^+$. Adenosine was also detected as m/z 269.0 due to its isotopic distribution.

4.4.9 Thermofluor (Study I)

Thermofluor is a fluorescence-based thermal stability assay for biophysical ligand screening (Boivin et al., 2013; Bai et al., 2019). 25 µM of LF-rBmpD was mixed with 50 µM of ligand (adenosine, inosine, guanosine, ribose and xanthosine) and SYPRO Orange (1:55, Life Technologies, Carlsbad, USA). The protein-ligand -mix was heated from 20 to 98 °C with stepwise increments of 0.01 °C per second. The resulting fluorescence signals were measured in triplicates with LightCycler 480 equipment (Roche) at 465 nm excitation and 580 nm emission wavelengths.

4.5 Analyses of human serum samples

4.5.1 Study samples and ethics (Study III)

Serum samples (n=994) collected during the Finnish Mobile Clinic Health Survey (FMC) from 1968 to 1972 were used for the serological study. The FMC was a cross-sectional health survey of over 50,000 voluntary study participants aged 15 years or

older where data was collected in 31 municipalities in different parts of Finland from 1966 to 1972 (Knekt et al., 2017).

As the study was conducted in the time preceding the current legislation on ethics in medical research, agreeing to participate in the study was taken to denote as informed consent form (Knekt et al., 2017). From the study participants, a wide-ranging health questionnaire, physical examinations, X-ray examinations, and electrocardiograms were included in the FMC survey data as well as urine and serum samples were collected.

4.5.2 Quality check of the historical serum sample collection (Study III)

The historical human serum samples were stored in -20°C for 50 years. Before analysis, serum samples were thawed at RT and visually inspected. All samples contained yellowish serum with white precipitate. Hence, serum samples were always vortexed before pipetting to well plates. For initial sample quality check, the presence of IgG levels in 41 randomly selected sera was checked using an in-house varicella zoster virus (VZV) ELISA (Alanen et al., 2005), and 50 serum samples determined negative by the *Borrelia* WCS screening test were tested with recomBead IgG 2.0.

4.5.3 Serology (Study III)

The human serum samples were screened for *Borrelia* antibodies with ELISA. The ELISA protocol was similar to the one used for the mouse samples described above. Briefly, *Borrelia* WCS coated well plates were incubated, washed, blocked as described above. Human serum samples diluted to 1:100 in NSS-PBS and standard samples of 0 and 100 enzyme immunoassay units (EIU) were added to the wells. The IgG levels in the human samples were detected with secondary antibody goat anti-human IgG (Calbiochem, Darmstadt, Germany) diluted 1:20,000 in NSS-PBS and the p-nitrophenyl phosphate substrate (Reagena, Toivakka, Finland). The reaction was stopped with 1 M NaOH. All samples with IgG levels of ≥20 EIU were further tested with the second-tier test C6 Lyme ELISA (Immunetics, Boston, MA, USA).

The screening-positive serum samples were added to wells coated with synthetic C6 peptide (a conserved region of the VlsE protein of *Borrelia*) and the ELISA was performed according to the protocol of the manufacturer (Immunetics, Oxford, UK). The serum samples were determined with the resulting Lyme index (LI) as negative (LI ≤0.9), borderline (LI = 0.91–1.09) or positive (LI ≥1.10). All samples with borderline or positive result (LI ≥0.91) were further tested with a third tier test.

All samples with positive C6 peptide result, or samples with negative by C6 peptide ELISA, but with *Borrelia* WCS IgG result ≥40 EIU, were tested with recomBead IgG 2.0 immunoassay (Mikrogen, Neuried, Germany) according to the protocol of the manufacturer.

The IgG levels in serum samples towards thirteen different antigens (p100, VlsE, p58, p39, OspA and OspC) of five *Borrelia* genospecies (*B. burgdorferi* ss, *B. garinii*, *B. afzelii*, *B. bavariensis*, and *B. spielmanii*) were detected using magnetic polystyrene beads (recomBead *Borrelia* beads) coated with the individual antigens, the MAGPIX System with Luminex® xPONENT software, and the Mikrogen recomQuant evaluation software. Using the test result, serum samples were determined as negative (test results 0–2 points), borderline (3 points) or positive (> 3 points).

4.6 Statistics (Studies I–III)

In Studies I and II, data with continuous variables of non-normality were analyzed with Kruskal-Wallis test or Mann Whitney-U test, and the results were corrected with Steel-Dwass or Sidak's post hoc test. Data with categorical variables were analyzed with Fisher's Exact Test.

In Study III, the serology results were combined with background data of the study participants to estimate the overall seroprevalence and the risk factors with univariate and multivariable logistic regression. Further, the self-reported diseases, symptoms and health-related questions associated with LB seropositivity were analyzed with univariate logistic regression and with single variable logistic regression adjusted for sex, age groups, and regions. The results are presented as odds ratio (OR) with 95% confidence intervals (CI).

5 Results

5.1 BmpD – A novel purine nucleoside-binding protein identified (Study I)

5.1.1 BmpD is not an outer surface protein

The cellular localization of BmpD in *Borrelia* was detected by enzymatic degradation with proteinase K, which cleaves bacterial surface proteins. The signals of the well-characterized outer membrane proteins OspA and DbpB decreased with increasing concentration of proteinase K, whereas the signals of BmpD and of the known periplasmic flagella remained visible at equal levels despite the addition of proteinase K (Figure 10). Thus, BmpD is not expressed on the outer surface of *Borrelia*.

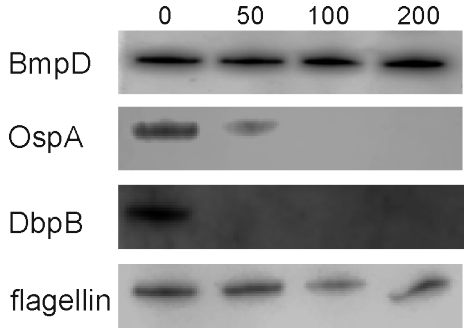

Figure 10. Cellular localization of BmpD in *B. burgdorferi* ss B31. Detection of BmpD, Outer surface protein A (OspA), Decorin binding protein B (DbpB) and flagellin by Western blot after incubating bacterial cells with proteinase K at concentrations of 0, 50, 100, or 200 µg/ml.

5.1.2 Anti-BmpD do not protect from *Borrelia* infection

We showed that *Borrelia* expresses BmpD during infection in the mammalian host as antibodies towards BmpD were detected in sera of LB patients, but not in serum samples negative for LB (Study I, Figure 7A). Also, mice generated antibodies against BmpD (Study I, Figure 7B). However, our results indicated that the BmpD antibodies were not protective against *Borrelia* infection (Table 10). All mice immunized with BmpD antibodies became infected after *B. burgdorferi* ss B31 challenge as evidenced by *Borrelia* positive tissue cultures (Table 10), significant

joint swelling, detection of *Borrelia* DNA in the investigated tissues, and by significant antibody response against *Borrelia* (Study I, Figure 8A-C). The infection was similar in the study groups of mock immunized mice and positive control mice. The mice injected with PBS only remained uninfected.

Table 10. Results of the mouse immunization study. Number of *Borrelia* positive mice analyzed by tissue culturing.

Study group (n)	*Borrelia* positive mice at 28 days post-infection
BmpD immunized (12)	12/12
Mock immunized (12)	12/12
Positive control (10)	10/10
Negative control (4)	0/4

5.1.3 Crystal structure of BmpD

Our study revealed the crystal structure and function of BmpD derived from *B. burgdorferi* ss B31 (Figure 11). The rBmpD crystallized in 50 mM Tris-HCl (pH 8.0) buffer. The 3D crystal structure of BmpD was successfully solved after computational refinement of the X-ray diffraction data (Study I, Figure 3 and Table 1). The structure of BmpD consisted of two domains with a ligand-binding cleft between the two domains (Figure 11). The residues 8–115 and 243–269 formed the N-terminal domain with six beta strands and four alpha helices, whereas residues 116–242 and 270–323 formed the C-terminal domain with six beta strands and six alpha helices. The two domains were linked together with three connecting loops.

The existing ligand-binding site between the domains was occupied with a bound ligand (close-up in Figure 11). The endogenously bound ligand was probably obtained from the cytoplasm of the protein expression host *E. coli* as no ligand was added to the growth medium. The electron density of the ligand-binding site indicated that the bound ligand was a purine nucleoside, either adenosine or inosine. Thus, the BmpD crystal structure revealed the function of BmpD as a substrate-binding protein of a bacterial ABC transporter. The structure of *B. burgdorferi* ss B31 BmpD is deposited with the accession code 6SHU into the Protein Data Bank (http://www.rcsb.org/).

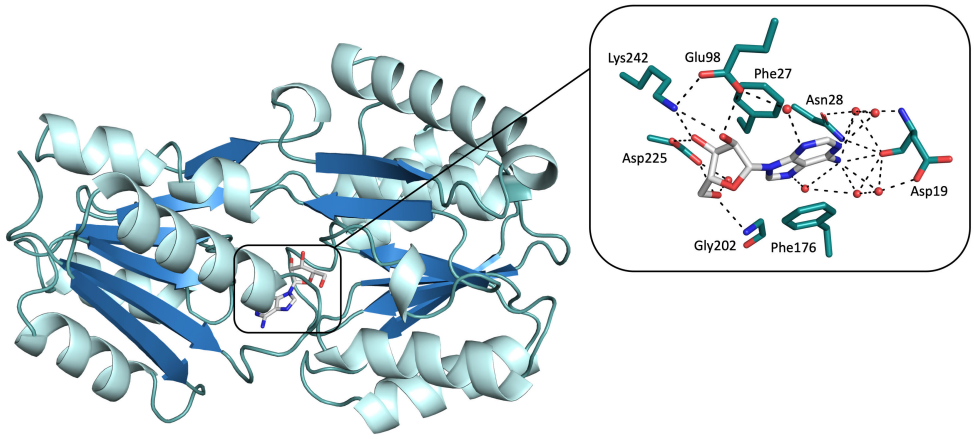

Figure 11. The crystal structure of *B. burgdorferi* ss B31 BmpD bound to adenosine (white sticks). The interactions between BmpD and adenosine are shown in the close-up, with the interacting residues shown as green sticks and water molecules as red spheres. Figure by Mia Åstrand.

5.1.4 BmpD binds to purine nucleosides

Further, we discovered that BmpD binds to purine nucleosides. First, the LC-MS analysis revealed that BmpD bound to adenosine by (Figure 11 and Study I, Figure 4B). The amount of adenosine was significantly decreased after denaturing and refolding of BmpD (Study I, Figure 4C). Later, the MST results showed that BmpD also bound to inosine (Study I, Figure 5).

Also, the aforementioned results were supported by a ligand-screening assay with thermofluor (Figure 12). LF-rBmpD combined with adenosine lead to a melting temperature shift of approximately 10 ºC compared to LF-rBmpD alone. The thermofluor results also suggested that inosine and guanosine could bind to LF-rBmpD as the melting temperature shifted approximately 5 ºC compared to the LF-rBmpD alone. However, BmpD binding to guanosine could not be detected by MST as guanosine exhibited autofluorescence. Xanthosine and, as expected, the negative control ligand ribose did not change the melting temperature of LF-rBmpD.

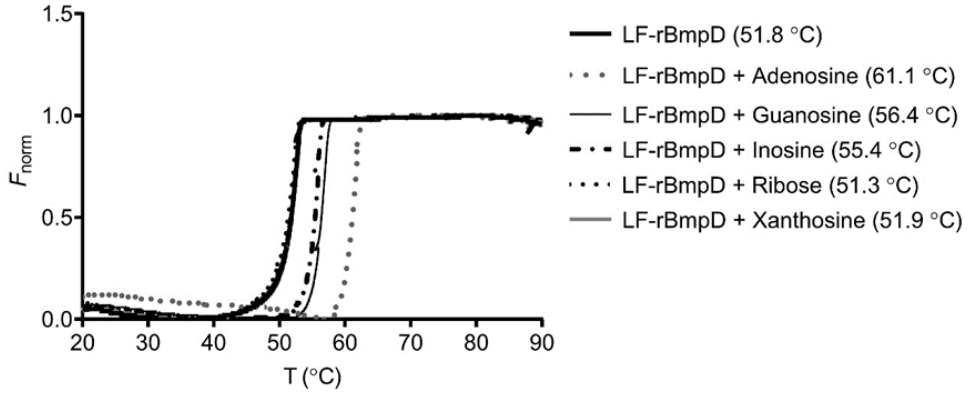

Figure 12. Ligand-binding assay with thermofluor. The melting curves of ligand-free recombinant BmpD (LF-rBmpD) alone and combined with purine nucleoside ligands and ribose are shown with the respective melting temperature in the parentheses. The mean melting temperature was calculated from three independent binding curves. *F*norm ~ normalized fluorescence.

5.2 Biglycan has a role in *Borrelia* infection (Study II)

5.2.1 *Borrelia* infection is genospecies-dependent

First, we generated a biglycan KO mice in the *Borrelia* infection susceptible C3H/HeNHSd background (Barthold et al., 1990). The genotype and phenotype of the novel KO mouse model was verified by RT-qPCR and immunohistochemistry. The KO mice were smaller and lightweight compared to the WT mice (Study II, Figure 1A). On the mRNA and tissue level, biglycan was neither produced in the lung tissues nor detected in the blood vessel walls of KO mice (Study II, Figure 1B-C). Then, the KO and WT mice were similarly infected either with representative strains of *B. burgdorferi* ss, *B. garinii* or *B. afzelii*. The infection and dissemination was followed up to 56 days.

The *B. burgdorferi* ss was infectious in KO and WT mice (Table 11). *Borrelia* were detected and quantified in all tissues of both mouse genotypes by culture and qPCR (Study II, Table 1). The loads of *Borrelia* were at equal levels in the following tissues of KO and WT mice; in the ear biopsy samples (at days 7, 10, 14, 21, 28, 42, and 56), in the heart and bladder (at days 28 and 56), and in the joints (at day 28) (Study II, Figure 3A-B). The bacterial load was significantly higher in the joints of KO mice than in WT mice at day 56 (Study II, Figure 3A-B). Further, *B. burgdorferi* ss infected KO and WT mice generated high antibody levels towards *Borrelia* WCS, rDbpA, and rDbpB, and developed visible joint swelling (Study II, Figures 4A-C and 5A-B).

Table 11. Comparison of the results of *Borrelia* interaction to biglycan *in vitro* (Salo et al., 2016) and *in vivo* (Study II). The qualitative analysis shows the intensity of *Borrelia* binding to biglycan in cell adhesion assays and the virulence of *Borrelia* in biglycan knock-out (KO) and wildtype (WT) mice indicated as plus and minus the brackets.The quantitative analysis shows the number of *Borrelia* positive mice analyzed by tissue culturing.

	***In vitro* study (Salo et al., 2016)**	***In vivo* study (Study II)**	
Borrelia strain	cells expressing biglycan	biglycan KO mice	WT mice
B. burgdorferi ss	(++)	15/15 (++)	15/15 (++)
B. garinii	(++)	7/7 (++)	7/8 (++)
B. afzelii	(-)	0/8 (-)	5/8 (+)
Negative control	(-)	0/12 (-)	0/13 (-)

++ ~ strong binding/ high virulence, + ~ weak binding/ low virulence, - ~ no binding/ avirulent.

Similarly to *B. burgdorferi* ss, *B. garinii* also infected and disseminated in the KO and WT mice (Table 11). Starting from day 28, *Borrelia* were detected and quantified by culture and qPCR in all tissues of both mouse genotypes, except for one KO mouse (Study II, Table 1). The loads of *Borrelia* were at equal levels in the following tissues of KO and WT mice; in the ear biopsy samples (at days 28, 42, and 56), in the heart, bladder, and joints (at days 28 and 56) (Study II, Figure 3C). At day 28, the KO mice harbored significantly higher amount of *Borrelia* in the ear than the WT mice (Study II, Figure 3C). Further, *B. garinii* infected KO and WT mice generated moderate antibody levels towards *Borrelia* WCS, and high antibody levels towards the rDbpA and rDbpB (Study II, Figure 4A-C). The *B. garinii* caused only moderate joint swelling in both genotypes with moderate and high joint inflammation in KO and WT mice, respectively (Study II, Figures 5C and 6A).

In contrast to the results of *B. burgdorferi* ss and *B. garinii*, *B. afzelii* was not infectious in KO mice (Table 11). All tissues of the *B. afzelii* infected KO mice collected throughout and at the end of the study remained negative by *Borrelia* culture and no bacterial genomes were detected by qPCR (Study II, Table 1 and Figure 3D). Further, no antibodies towards *Borrelia* WCS, rDbpA or rDbpB were detected in the KO mice (Study II, Figure 4A-C).

Also, *B. afzelii* displayed low virulence in WT mice as only 5 of 8 became infected as investigated by tissue culture and qPCR (Study II, Table 1). The bacterial loads in the ear biopsy samples (at days 28 and 56) and in the heart, bladder and joint of WT mice were significantly higher than in the negative tissues of KO mice (Study II, Table 1 and Figure 3D). The *B. afzelii* infected WT mice had moderate antibody levels towards *Borrelia* WCS and rDbpB, and high levels towards rDbpA (Study II,

Figure 4A-C). Only a small increase of joint swelling and no joint inflammation were detected in the KO and WT mice (Study II, Figures 5D and 6A).

In summary, the infection study revealed that *B. burgdorferi* ss and *B. garinii* were able to establish infection and disseminate in biglycan KO mice, although the *in vitro* results indicated that *B. burgdorferi* ss and *B. garinii* bound strongly to biglycan (Table 11). Unexpectedly, *B. afzelii* was non-infectious in the biglycan KO mice, while the *in vitro* results suggested that *B. afzelii* did not bind to biglycan (Table 11). Thus, we concluded that the infection and dissemination of *Borrelia* in the biglycan deficient mice were depended on the *Borrelia* genospecies.

5.2.2 Host inflammatory reaction is mouse genotype-dependent

Interestingly, the infection study also revealed that the *Borrelia* infection resulted in different host inflammatory responses depending on the mouse genotype.
The joint inflammation of *B. burgdorferi* ss infected biglycan KO mice resulted in a high scoring at day 28 and remained continuously elevated at day 56 (Study II, Figure 6A and C). Meanwhile, the WT mice displayed visible arthritis at day 28, but the arthritis score had statistically significantly lowered at day 56. The heart inflammation of *B. burgdorferi* ss infected KO and WT mice was at equal levels at days 28 and 56 (Study II, Figure 6B).

Further, *B. garinii* infection in WT mice induced up-regulation of biglycan mRNA expression in the aorta and spleen compared to the uninfected WT mice (Study II, Supplementary Figure 1). In addition, the expression of multiple cytokines in the *B. burgdorferi* ss, *B. garinii*, and *B. afzelii* infected KO and WT mice showed no difference between the mouse genotypes (Study II, Supplementary Figure 2).

5.3 LB seroprevalence in Finland 50 years ago (Study III)

5.3.1 The good-quality sera after 50 years

To check the quality of the 50-year-old serum samples, we evaluated the presence of antibodies by measuring the VZV IgG antibodies in a random subset of 41 sera (Figure 13). Indeed, 38 of 41 serum samples contained detectable levels of VZV IgG antibodies corresponding to the expected VZV seroprevalence. Further, to check the immunoassay performance, 50 serum samples determined negative by the *Borrelia* WCS screening test were tested with recomBead IgG 2.0. All samples remained clear negative (data not shown). Thus, we assessed that the historical serum samples were of good-quality after long-term storage.

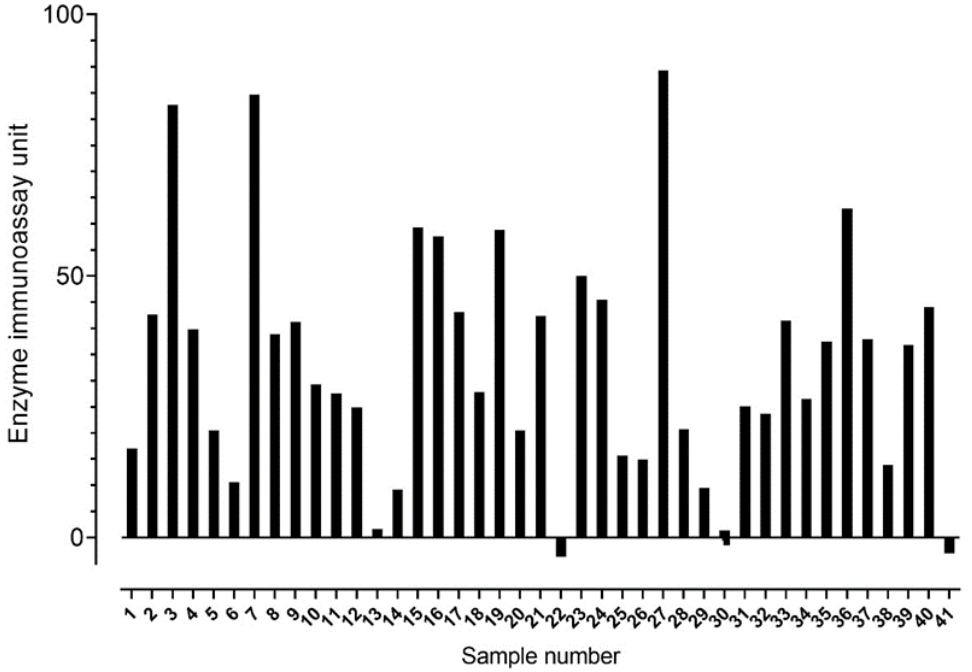

Figure 13. IgG antibody levels towards varicella zoster virus in a subset of 41 historical serum samples as measured by enzyme immunoassay.

5.3.2 LB seroprevalence and the associated risk factors

We analyzed 994 serum samples of the FMC survey for antibodies towards *Borrelia* (Figure 14). The serum samples were collected from 546 men and 448 women aged 15 to 86 years from 24 locations in different parts of Finland during the years 1968 to 1972.

In total 199 of 994 (20.0%) sera were tested as *Borrelia* IgG positive. Hence, the unweighted LB seroprevalence among the healthy general population was 20.0% (95% Confidence Intervals (CI): 17.6–22.6). The association of gender, age, field of work, employment status, sport/exercise activities and regions of Finland with LB seroprevalence was analyzed (Study III, Table 1). The LB seroprevalence was slightly higher among males than females (21.8% vs 17.9%). The age of 50 years and older, unemployment, and regions of South and Central Finland were statistically significantly associated with LB seroprevalence (Study III, Table 1). However, the effect of migration of the study participants was not analyzed.

Interestingly, the LB seroprevalence was about 20% in persons working in the field of agriculture and forestry, industry and mining, transportation and logistics, and service as well as among housewives and pensioners without statistical significance between the occupational groups (Study III, Table 1). Further, the self-

reported perception of not feeling healthy, previous heart failure, and current heart valvular disease were statistically significantly associated with *Borrelia* seropositivity (Study III, Supplementary Table 1).

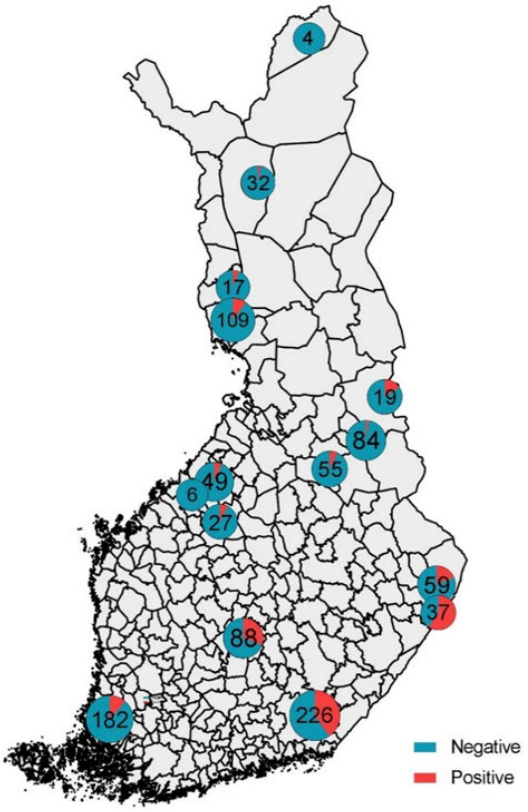

Figure 14. The locations of the collected serum samples of the Finnish Mobile Clinic Study during 1968–72 analyzed for antibodies towards *Borrelia*. The number on the pie chart indicate the total number of sera collected at a certain location. The blue and red colours indicate the percentages of the serum samples negative or positive for *Borrelia* antibodies.

6 Discussion

6.1 The role of BmpD in the purine nucleoside salvage in *Borrelia*

Previous studies have suggested that the chromosomal encoded BmpA-D are important proteins for *Borrelia* survival and host infection (Ramamoorthy et al., 1996; Bryksin et al., 2005; Pal et al., 2008). However, the localization and function of the Bmps have not been completely characterized. In Study I, the function of one representative Bmp protein, BmpD, was characterized and its potential as a vaccine antigen evaluated.

The BmpA-D are lipoproteins with a typical signal sequence of about twenty amino acids from a methionine to a cysteine residue (Zückert, 2014; Åstrand et al., 2019). The signal sequence is cleaved by the signal peptidase II (Zückert, 2014). Lipids, such as N-palmitoyl or S-diacylglycerol, are covalently attached to the sulfur atom of the cysteine. The lipid modification destinies the export of the protein to the exterior of the cytoplasm, either to the inner membrane or to the outer membrane of *Borrelia*. BmpA (also known as P39) was shown to be expressed on the outer surface (Bryksin et al., 2010). In contrast, BmpB, C, and D were found to be periplasmic proteins tethered to the inner membrane (Dowdell et al., 2017). In accordance, the results of the proteinase K-assay in Study I strongly suggests that BmpD is, indeed, a periplasmic protein.

Despite the periplasmic localization of BmpD, LB patients had antibodies recognizing BmpD in Study I which is in line with earlier result by Bryksin and colleagues (Bryksin et al., 2005). However, the anti-BmpD antibodies did not prevent *Borrelia* infection since all BmpD immunized mice became infected in Study I. Accordingly, the antibodies against BmpA and BmpB did not protect mice from *Borrelia* infection (Gilmore et al., 1996; Pal et al., 2008). On the other hand, mice immunized with outer surface proteins, such as OspA and OspC, were protected from *Borrelia* infection (de Silva et al., 1996; Gilmore et al., 1996).

Evidently, the host immune system can produce antibodies against proteins located both on the outer surface and in the periplasmic space of *Borrelia*. The proteins expressed on the outer surface of *Borrelia* are rapidly detected by host innate immune cells due to easy accession, leading to phagocytosis of *Borrelia*. After

phagocytosis, also the intracellular and periplasmic proteins are exposed, and can be presented to B cells for antibody production. However, Modolell and colleagues showed that antibodies against the periplasmic flagella did not contribute to *Borrelia* clearance (Modolell et al., 1994). In contrast, antibodies against outer surface proteins, OspA and OspB, were important for *Borrelia* clearance as anti-OspA and anti-OspB opsonized the spirochetes enhancing the phagocytosis of *Borrelia* (Modolell et al., 1994). The antibodies against periplasmic proteins, such as BmpD and flagella, do not detect their intracellular target antigen in intact *Borrelia*. Thus, no opsonization of *Borrelia* occurs which is one explanation why BmpD immunization did not protect mice from *Borrelia* infection.

Yet, the LB vaccine development has clearly shown that the *Borrelia* protein localization is only one of multiple factors determing the efficacy of a vaccine antigen. For example, the first human LB vaccine antigen, OspA, prevented *Borrelia* infection only in 80% of the study participants (Steere et al., 1998).

After solving the crystal structure of BmpD, we characterized the first purine nucleoside-binding protein in *Borrelia*. Previously, BmpD has been described as a laminin-binding protein (Verma et al., 2009), as an endothelium-adhering protein (Antonara et al., 2007), and as a sugar transporter (Gherardini et al., 2010).
However, the periplasmic localization combined with the results from the crystal structure presented in Study I revealed the function of BmpD as a substrate-binding protein of an ABC transporter for purine nucleosides. The substrate-binding proteins are important components of the ABC transporter complex as they bind to specific nutrients essential for the bacterial survival (Maqbool et al., 2015). Other known *Borrelia* substrate-binding proteins of an ABC transporter complex are the riboflavin-, phosphate-, and oligopeptide-binding proteins (Deka et al., 2013; Brautigam et al., 2014; Groshong et al., 2017). Recent *in silico* modelling results predicted the function of BmpA-D as purine nucleoside-binding proteins (Åstrand et al., 2019), which we now confirmed by solving the crystal structure of BmpD.

Further, we showed that BmpD binds at least to two different purine nucleosides, adenosine and its derivative inosine as analyzed by LC-MS and MST. Both purine nucleosides are essential for *Borrelia* to scavenge from the host environment as *Borrelia* lacks the enzymes for *de novo* purine nucleoside biosynthesis (Fraser et al., 1997; Pettersson et al., 2007). In the *Borrelia* cytoplasm, the BmpD-imported inosine can directly be converted to IMP, which is further converted to GMP, GDP, and GTP for RNA incorporation. The enzymes, adenylosuccinate synthase and lyase catalyzing the formation of IMP to AMP, are lacking in *Borrelia* (Pettersson et al., 2007). Yet, the missing enzymes can be compensated by the uptake of adenosine via BmpD leading to the direct formation of AMP, ADP, and ATP for RNA incorporation.

As the yield of the ligand-free rBmpD was low due to the heavy three-step purification protocol, we chose the label-free MST approach for the ligand-binding assays. Possibly, further purine nucleosides could have been identified with other biomolecular binding assays, such as isothermal calorimetry (ITC) and surface plasmon resonance (SPR). The MST requires protein sample in the nanomolar range, whereas ITC and SPR consume large amount of highly concentrated sample. The binding affinities of *T. pallidum* PnrA to various nucleosides was studied by ITC (Deka et al., 2006). The methodological differences might explain why PnrA bound to adenosine (dissociation constant (K_D)=0.3 µM), inosine (K_D=0.2 µM), and guanosine (K_D=0.07 µM) with 50 to 200-fold higher affinity than BmpD bound to inosine (K_D=15 µM; SD 1µM). Different batches of purified LF-rBmpD were used in the label-free MST assay. Thus, some of batches probably contained residual bound adenosine even after the denaturation process which might explain why the K_D of adenosine could not be calculated. For K_D calculation, at least three reproducible binding curves should have been obtained by label-free MST assay. In addition, the label-free MST was not suitable to detect binding of BmpD to guanosine as guanosine emits autofluorescence.

Interestingly, the binding of *T. pallidum* PnrA to pyrimidines was also measured (Deka et al., 2006). However, the binding affinities of pyrimidines (K_D=6–13 µM) were considerably lower than the binding affinities of purines which were explained by structural differences. The pyrimidines have a base moiety of only one nitrogenous ring whereas purines have two (Figure 3). The two-ring base moiety of the purine nucleosides is sandwiched between two phenylalanines of PnrA forming hydrogen bonds with the aspartic acid residues, while the pyrimidine one ring moiety is smaller and cannot form similar hydrogen bonds. Further, the methyl group of thymidine hinders sterically the binding to glycine residue. Hence, *T. pallidum* PnrA was determined to transport purine nucleosides and not pyrimidines (Deka et al., 2006). The ligand bound to BmpD was similarly stacked between the two phenylalanines and formed similarly hydrogen bonds with aspartic acid residues and glycine (close-up in Figure 11) as the purine nucleosides bound to PnrA. One can therefore suggest that BmpD also prefers purine nucleosides over pyrimidine nucleosides.

In summary, the function of BmpD was identified as a periplasmic substrate-binding protein of an ABC transporter for purine nucleosides. However, *in vitro* and *in vivo* studies with *bmpD* knock-out and complemented *Borrelia* strains would reveal the role of BmpD as a purine nucleoside-binding protein for survival and infectivity of *Borrelia* in the host. Further, the functions of BmpA, B, and C need to be investigated in more detail. For example, BmpA might present multifunctional roles as a laminin-binding and a purine nucleoside-binding protein (Verma et al.,

2009; Caine & Coburn, 2016; Åstrand et al., 2019). These studies remain to be conducted in the future.

6.2 The role of host molecule biglycan in *Borrelia* dissemination

The virulence and infectivity of *Borrelia* is dependent on the dissemination and ability to persist in the host. The structure of host tissues contains multiple adhesion targets which *Borrelia* exploits for the purpose of dissemination and persistence. Biglycan is one proteoglycan expressed in the ECM of multiple tissue types and on the endothelium lining the blood vessels (Bianco et al., 1990; Salo et al., 2016). Recent *in vitro* studies showed that the DbpA and B adhesins of *Borrelia* bound to biglycan with different affinities depending on the genospecies (Salo et al., 2016). Therefore, we studied the infection of same three *Borrelia* genospecies in biglycan KO mice. Originally, the biglycan deficient mice were generated as animal disease model for osteoporosis studies (Xu et al., 1998). Hence, we needed to generate a novel biglycan KO mouse model which was susceptible for *Borrelia* infection. The results of Study II revealed that the three *Borrelia* genospecies behaved differently in the biglycan KO mice.

The *in vitro* assays showed that *B. burgdorferi* ss and *B. garinii* adhered strongly to biglycan expressed on endothelial cells (Salo et al., 2016) suggesting that dissemination of these two *Borrelia* genospecies could be impaired in biglycan KO mice. However, the infectivity of *B. burgdorferi* ss and *B. garinii* was not affected by the lack of biglycan. The biglycan deficient and wildtype mice became equally infected as evidenced by detection of *Borrelia* in tissues and significant antibody response towards *Borrelia* in both mouse genotypes. Similarly, the *B. burgdorferi* ss was able to infect and disseminate in mice deficient of decorin, a close relative of biglycan (Brown et al., 2001). *Borrelia* expresses a plethora of adhesion proteins with overlapping adhesion targets in the host tissues (Caine & Coburn, 2016). One missing adhesion molecule can be compensated with multiple other adhesion targets. Thus, the results imply that *Borrelia*-biglycan interaction is not necessary for *B. burgdorferi* ss and *B. garinii*.

In contrary, the *in vitro* results suggested that biglycan is not an adhesion target in the host tissues for *B. afzelii* as the DbpA and B adhesins of *B. afzelii* did not bind to biglycan (Salo et al., 2016). Unexpectedly, *B. afzelii* was unable to establish infection in the biglycan deficient mice. The non-infectivity of *B. afzelii* might be explained by the poor binding to biglycan and decorin host molecules, while *B. burgdorferi* ss and *B. garinii* bound strongly or moderately to biglycan and decorin (Salo et al., 2011; Salo et al., 2016). Possibly, *B. afzelii* needs both adhesion molecules DbpA and DbpB as well as both adhesion targets in the host to establish

infection. On the other hand, *B. afzelii* might express uncharacterized adhesion proteins essential for mediating binding to biglycan, which have yet to be identified. Further, *B. afzelii* presented low virulence in the wildtype mice suggesting that the needle-inoculated load of bacteria (10^5) was too low. The *B. afzelii* A91 strain was isolated from skin biopsy samples of Finnish LB patients (Panelius et al., 2001). In Study II, this strain was used for first time in mouse infection studies. Probably, with higher inoculation load, the *B. afzelii* A91 strain would have presented higher virulence in the wildtype mice. Generally, *B. afzelii* displays high virulence in mammalians as it is the most common genospecies causing LB in Finland (Carlsson et al., 2003).

In addition to the structural role of biglycan, the proteoglycan promotes the host inflammatory reactions in sterile inflammation caused by tissue stress, injury or cell death, which are common features in myocardial infarctions, atherosclerosis, autoimmune diseases, and cancer (Schaefer, 2014; Frevert et al., 2018). However, biglycan affects also the host inflammatory reaction against bacterial infections. In Study II, *B. burgdorferi* ss infected biglycan deficient mice had persisting joint inflammation and higher number of spirochetes in the joints compared to the wildtype mice. In contrast to *B. burgdorferi* ss, the joint inflammations in the biglycan deficient mice caused by *B. garinii* were moderate.

The variation in the intensity of the joint inflammation might be accounted by the different genospecies as *B. burgdorferi* ss presents greater inflammatory potential than *B. garinii* and *B. afzelii* (Strle et al., 2009; Cerar et al., 2016). *B. burgdorferi* ss induced significantly higher levels of chemokines and cytokines than *B. garinii* and *B. afzelii* in stimulated macrophages of healthy donors (Strle et al., 2009). Further, patients infected with *B. burgdorferi* ss experienced a more severe form of LB associated with following symptoms, such as fever, neck stiffness, malaise, and fatigue, than patients infected with *B. garinii* and *B. afzelii* (Cerar et al., 2016).

On the other hand, similarly to Study II, *B. burgdorferi* ss infected mice deficient in other proinflammatory molecules, 5-lipoxygenase, MyD88, and TLR2, had more severe and persistent arthritis including high levels of spirochetemia in tissues despite normal antibody production (Wooten et al., 2002; Bolz et al., 2004; Blaho et al., 2011).

Biglycan, 5-lipoxygenase, MyD88, and TLR2 are not only involved in the host proinflammatory reaction to clear the invading bacteria, but are also involded in the rapid resolution of the inflammatory reaction (Basil & Levy, 2016). Without the initial proinflammatory reaction induced by the aforementioned molecules, there is also no host induced resolution of the host inflammatory reaction. Thus, the host inflammatory reaction continues in the bacterial infection sites as manifested by the long-term arthritis reaction in the *B. burgdorferi* ss infected mice deficient of the proinflammatory molecules production (Wooten et al., 2002; Bolz et al., 2004; Blaho

et al., 2011). The proinflammatory effects of biglycan in other bacterial infection have been under investigation. For example, the presence of biglycan enhanced the complement-mediated killing of the respiratory pathogen *Moraxella catarrhalis* (Laabei et al., 2018).

In summary, Study II revealed that the host molecule biglycan has a dual role in *Borrelia* infection. On one hand, biglycan alters the infectivity and dissemination of *Borrelia* depending on the genospecies. On the other hand, biglycan affects also the host proinflammatory reaction.

6.3 LB seroprevalence in Finland 50 years ago

Virtually all LB patients develop *Borrelia*-specific IgG antibodies (Dessau et al., 2018). The antibodies towards *Borrelia* persist for decades after the initial infection (Kalish et al., 2001), and thus, are useful for seroprevalence studies.

The first LB seroprevalence studies using human serum samples were conducted in the late 1980s and in the beginning of 1990s (Smith et al., 1988; Gustafson et al., 1990). However, no LB seroprevalence studies had been conducted during the decades before the description of the LB disease entity in the late 1970s and early 1980s (Steere et al., 1977b; Burgdorfer et al., 1982). Study III is the first study shedding light on the exposure to LB infection in a European population half a century ago. The analyzed human serum samples were a subset of samples collected during the Finnish Mobile Clinic study in the years 1968–72.

Besides Study III, there is one another LB seroprevalence study using historical serum samples of American gray wolves in the years 1972–98 (Thieking et al., 1992), the same time period as the FMC study was conducted. *Borrelia* antibodies were detected in 3% of the historical wolf serum samples (Thieking et al., 1992). The presence of *Borrelia* antibodies in the historical wolf serum samples, and the historical case reports of patients with skin or neurological conditions after a tick bite (Afzelius, 1910; Garin & Bujadoux, 1922) led to the assumption that infections caused by *Borrelia* were not uncommon in Europe and North America in the late 19th and early 20th centuries. At that time, the European, especially the Northern European, economies were still heavily depending on agriculture and forestry which further supports the expectation of *Borrelia* seropositive people in the 1970s. Even today, the farmers and forestry workers are high-risk occupational groups for LB (Dessau et al., 2018).

The overall LB seroprevalence in Finland in 1968–72 was 20.0% which was five times higher than the recent seroprevalence of 3.9% observed in serum samples collected in 2011 (van Beek et al., 2018). Generally, comparisons between two epidemiological studies need to be considered carefully as there is no “gold standard” for serological methods to detect *Borrelia* antibodies. However, in this

case, both Finnish seroprevalence studies were conducted with the same serological test algorithm. Hence, the comparison is reasonable. The five times higher seroprevalence could be partly explained by the agrarian society 50 ago compared to the service-based society of Finland in the 21st century.

Yet, the considerably high LB seroprevalence was surprisingly not associated with the occupation of the study subjects (Study III, Table 1). High LB seroprevelance was distributed evenly among those working in agriculture and forestry, industry and mining, transportation and logistics, and in service. Further, unemployment and age of 50 and older were risk factors associated with LB seroprevalence. The unemployed included housewives, school children and students who possibly spent more time gardening, berry picking and doing sport activities, which could have led to more frequent encounters with ticks. The aforementioned explanation could also be applied to the increased risk of LB in study subjects with older age. Finally, the regions of Central and South Finland were significantly associated with LB seroprevalence in 1970s, whereas today, Southwest Finland is a region with probably the highest tick density (Laaksonen et al., 2017; Laaksonen et al., 2018) and LB incidences (Sajanti et al., 2017).

Another interesting finding in Study III was the analysis of the self-reported symptoms, diseases and perceptions of health of the study subjects after adjusting the statistical analyses for sex, age-groups, and regions (Study III, Supplementary table 1). Logically, the LB seropositive study participants reported more frequently of not feeling healthy compared to the LB seronegative persons suggesting that, indeed, the study subjects suffered from unspecified symptoms affecting their well-being. Surprisingly, the difference was even statistically significant. In contrast, the self-reported diseases, previous heart failure and current heart valvular disease, were also statistically significantly associated with LB seropositivity, but clinically these conditions are not associated with LB (Steere et al., 2016).

In summary, the results of Study III revealed the considerably high LB seroprevalence in Finland 50 years ago. The historical seroprevalence is important for understanding that today the LB risk is relatively low. Also, the historical seroprevalence strongly suggests that the hysteria around ticks and tick-borne diseases created by (social) media is overrated. On the other hand, the knowledge on ticks and tick-borne diseases by medical professionals and citizens, diagnostics and efficient treatments with antibiotics have probably led to the low LB seroprevalence today.

6.4 Strengths and weaknesses of the study

In Study I, we evaluated the role of BmpD in *Borrelia* from the perspective of infection biology and structural biology. Often, studies on bacterial proteins are

conducted either from the infection biological (Brooks et al., 2006; Kung et al., 2016), or from the structural biological perspective (Deka et al., 2006; Brautigam et al., 2014). However, in Study I, we combined data from both perspectives. On one hand, we analyzed the structure and function of BmpD as a purine nucleoside-binding protein by solving the crystal structure of BmpD, LC-MS, and ligand-binding assays. On the other hand, we studied the cellular localization of BmpD in *Borrelia*. Importantly, with the mouse immunization study, we also analyzed whether BmpD would be a novel LB vaccine candidate.

Yet, from the infection biological perspective, there are limitations. *Borrelia* strains lacking *bmpD* and complemented with *bmpD* should have been generated to evaluate the role of BmpD in the survival of *Borrelia in vitro*. Further, assays to determine the purine nucleoside transportation could have been assessed with these strains. Moreover, mouse infection studies with these *Borrelia* strains would have revealed the effect of BmpD on the infectivity and dissemination of *Borrelia* in the host.

In Study II, we generated a novel mouse model which lacked biglycan and was susceptible for *Borrelia* infection. There are multiple mouse models deficient of different proteoglycans, such as biglycan, decorin, fibromodulin, and lumican, which are all expressed in the ECM of connective tissues (Ameye & Young, 2002). *Borrelia* infection has been only studied in the decorin deficient mouse model (Brown et al., 2001), although *Borrelia* adheres to multiple ECM targets (Caine & Coburn, 2016). Thus, Study II, is the second study to analyze the effect of a proteoglycan on *Borrelia* infection. Here, we analyzed the effect of biglycan on the infection and dissemination of three different *Borrelia* genospecies which represent the three most common human pathogens. We showed that biglycan affects the course of infection depending on the *Borrelia* genospecies, but we also showed that biglycan affected the host proinflammatory reaction.

Furthermore, usually, *Borrelia* infection studies are only assessed with *B. burgdorferi* ss, the common human pathogen in the USA. In contrast, in Study II, we used three different genospecies. Hence, the study results are applicable to Europe and Asia, where *B. afzelii* and *B. garinii* are common pathogens. However, repetition of the mouse infection study with *B. afzelii* could have been conducted. Thus, we could have determined whether the particular strain, *B. afzelii* A91, is truly non-infectious or only dissemination-defective in the biglycan KO mice.

Study III is a unique study set-up as a 50-year-old collection of serum samples of Finnish healthy population was available for serological studies. National health surveys have been conducted regularly in Finland since the first survey, FMC, in 1968 to 1972. The serum sample collection of the FMC is probably one of the oldest and largest collection worldwide, which is available for scholars. Importantly, for every serum sample, we could link the corresponding data about the background,

such as age, sex, region, occupation, outdoor activities, and answers of health questionnaire, such as symptoms, previous and current diseases, from the study participants. However, the effect of migration was not assessed. Although, we could show that the highest seroprevalence rates were in South and Central Finland, but we do not know if the same regions are also the locations of the acquired *Borrelia*-infected ticks.

Moreover, the three-step serological test algorithm used in Study III was the same as in the previous study (van Beek et al., 2018). Hence, the comparison of the seroprevalence rates 20% in 1968–72 vs 3.9% in 2011 was possible. Yet, we cannot analyse the exact time of first *Borrelia* infection and whether recurrent *Borrelia* infections had occurred as the *Borrelia* IgG antibodies remain elevated for decades.

7 Conclusions

The *Borrelia* spirochetes transmitted by ticks to humans are causing the infectious disease Lyme borreliosis, which was discovered only 40 years ago. Since then, the research on *Borrelia* cell biology, pathogenesis, and diagnostics has been ongoing. Still today, the function of the majority of *Borrelia* proteins has not been thoroughly characterized.

Study I revealed the structure and function of one protein of *Borrelia*, Basic membrane protein D, as a substrate-binding protein for purine nucleosides involved in the purine salvage pathway of *Borrelia*. Further, the role of the host molecule biglycan in *Borrelia* pathogenesis was characterized in Study II. Importantly, the determination of structure and function of bacterial proteins and of host binding molecules are the basic requirements to discover new bactericidal therapies. Novel drug molecules targeting BmpD could interfere with the metabolism of *Borrelia*. Hence, the DNA and RNA synthesis in *Borrelia* would be disrupted, which would, in turn, lead to killing of the bacteria. Also, drug molecules mimicking the structure of biglycan could result in anti-adhesion based treatment.

The historical LB seroprevalence study brought to light that LB has been a common infectious disease in Finland 50 years ago. Further, the observed high seroprevalence is a scientific argument to proportionate the current scare on ticks and tick-borne diseases.

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I wish you all a new decade with less worries and more hope!

Turku, January 2020

Julia Cuellar

Julia Cuellar

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