Human and Veterinary Vaccines for Lyme Disease

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Abstract

Lyme disease (LD) is an emerging zoonotic infection that is increasing in incidence in North America, Europe, and Asia. With the development of safe and efficacious vaccines, LD can potentially be prevented. Vaccination offers a cost-effective and safe approach for decreasing the risk of infection. While LD vaccines have been widely used in veterinary medicine, they are not available as a preventive tool for humans. Central to the development of effective vaccines is an understanding of the enzootic cycle of LD, differential gene expression of *Borrelia burgdorferi* in response to environmental variables, and the genetic and antigenic diversity of the unique bacteria that cause this debilitating disease. Here we review these areas as they pertain to past and present efforts to develop human, veterinary, and reservoir targeting LD vaccines. In addition, we offer a brief overview of additional preventative measures that should employed in conjunction with vaccination.

Introduction

The causative agents of Lyme disease

Borrelia burgdorferi, the primary causative agent of Lyme disease (LD) in North America, was cultivated from ticks and identified in 1981 (Burgdorfer et al., 1982). A direct linkage between *B. burgdorferi* and LD was established with its isolation from the blood of human LD patients (Burgdorfer et al., 1982; Benach et al., 1983). This previously uncharacterized pathogen was classified in the genus *Borrelia* based on its shared features with the causative agents of tick-borne relapsing fever (TBRF) (Benach et al., 1983). *B. burgdorferi* and TBRF spirochetes possess a unique segmented genome comprised of a series of linear and circular DNA plasmids and prophage (Barbour, 1988, 1993; Eggers and Samuels, 1999; Zhang and Marconi, 2005). Upon denaturation, the linear plasmids transition to a single-stranded circular DNA molecule. As a consequence of extensive complementarity, the single-stranded DNA molecules can reanneal upon themselves to form double-stranded linear DNA with covalently closed hairpin termini (Barbour and Garon, 1987). While linear DNA is rare in bacteria, genetic elements with similar structure are found in some viruses including the African Swine Fever Virus, an arbovirus (Ndlovu et al., 2020). *B. burgdorferi* and the TBRF *Borrelia* have a similar spiral ultrastructure, unique mode of motility and similar nutritional requirements (Barbour, 1984; Asbrink and Hovmark, 1985).

As the number of isolates available for study increased in the 1980's, it became apparent that *B. burgdorferi* strains varied in many of their genotypic and phenotypic properties. Comparative analyses of isolates from different biological and geographic sources revealed extensive diversity in plasmid and protein profiles (Barbour, 1988; Schwan et al., 1988; Stålhammar-Carlemalm et al., 1990). Genetic relationships among isolates were subsequently assessed through DNA-DNA hybridization (LeFebrve et al., 1989), restriction fragment length polymorphism analysis (Stålhammar-Carlemalm et al., 1990; Fukunaga et al., 1993; Postic et al., 1994; Liveris et al., 1995), S1 nuclease digestion (Baranton et al., 1992) and 16S rRNA sequencing (Marconi and Garon, 1992a, b). Shortly after its initial discovery, *B. burgdorferi* was divided into three distinct species: *B. burgdorferi* (Burgdorfer et al., 1982)*, B. garinii* (Baranton et al., 1992)*,* and *B. afzelii* (previously referred to as genomic group VS461) (Marconi and Garon, 1992b; Canica et al., 1993). At last count, twenty-one distinct species associated with LD have been identified (Eisen, 2020). To reflect the diversity among *B. burgdorferi*, the taxonomic designation sensu lato was applied. *B. burgdorferi, B. garinii,* and *B. afzelii* are responsible for the majority of LD cases in humans and companion animals (Eisen, 2020). All

three of the established pathogenic species are found in Europe and Asia while *B. burgdorferi* is found in North America. *B. bavariensis* (Margos et al., 2013)*, B. valaisiana* (Wang et al., 1997)*, B. bissettii* (Postic et al., 1998)*, B. kurtenbachii* (Margos et al., 2010)*, B. speilmanii* (Maraspin et al., 2006)*, B. lusitaniae* (Collares-Pereira et al., 2004) and *B. mayonii* (Pritt et al., 2016) are considered minor contributors to disease (Eisen, 2020). *B. mayonii,* the most recent species to be identified and characterized, was discovered through PCR analyses of human diagnostic specimens by the Mayo Clinic (Pritt et al., 2016; Johnson et al., 2017). While *B. mayonii* has been recovered from both humans and ticks, its overall contribution to LD case numbers appears to be minimal. The pathogenic potential of *B. americanum* (Rudenko et al., 2009)*, B. carolinensis* (Foley et al., 2014)*, B. californiensis* (Postic et al., 2007)*, B. chilensis* (Verdugo et al., 2017)*, B. japonica* (Kawabata et al., 1993)*, B. lanei* (Margos et al., 2017)*, B. sinica* (Masuzawa et al., 2001)*, B. tanuki* (Fukunaga et al., 1996)*, B. turdii* (Fukunaga et al., 1996)*, B. andersonii* (Marconi et al., 1995), and *B. yangtzensis* (Margos et al., 2015) remains to be determined. The apparent inability of this latter group of species to infect and cause disease in humans and companion animals may be due to the feeding habits of the specific *Ixodes* tick species that serve as their vectors, as well as their sensitivity to complement. *B. andersonii*, for example, is vectored primarily by *Ixodes dentatus* ticks which do not commonly feed on humans and other large mammals (Anderson et al., 1989). In addition, *B. andersonii* is highly sensitive to human and canine complement, presumably due to the fact that it does not produce factor H binding proteins (McDowell et al., 2003b). The potential host range for many of the species listed above remains largely undefined. For convenience, henceforth we use the term LD spirochetes to refer collectively to species that cause disease in humans and companion animals.

In 2014, it was proposed that the *B. burgdorferi* sensu lato group be awarded separate genus status (Adeolu and Gupta, 2014). This reclassification was proposed based on epidemiological, biological, genotypic and clinical differences between the LD and tick-borne relapsing fever (TBRF) spirochetes. Species of the *B. burgdorferi* sensu lato complex were separated from the genus *Borrelia* and awarded a new genus designation; *Borreliella* (Adeolu and Gupta, 2014). As is often the case with taxonomic reclassification, this change was met with varying

degrees of enthusiasm and reclassification has been rigorously debated at conferences and in the literature (Adeolu and Gupta, 2014; Barbour et al., 2017; Stevenson et al., 2018). Here, we do not seek to argue for or against reclassification. However, while the use of *Borreliella* is considered optional in the literature, it is important to note that this designation has been fully integrated into the sequence and protein structure databases. Hence, for practical purposes we have adopted this new genus designation.

The enzootic cycle of Lyme disease
The *Borreliella* are obligate host-dependent bacteria maintained in nature in an enzootic cycle involving diverse vertebrate reservoir species and *Ixodes* tick vectors (Barbour and Hayes, 1986; Loss et al., 2016). The strict requirement for cycling between reservoirs and ticks reflects the absence of transovarial and horizontal transmission. While there have been isolated reports of transovarial transmission in ticks (Hauck et al., 2020), it does not appear to be significant in terms of population maintenance in nature. Tick species that are competent vectors for one or more *Borreliella* species include *I. affinis, I. angustus, I. hexagonus, I. minor, I. muris, I. pacificus, I. ricinus, I. ovatus, I. scapularis,* and *I. spinipalpis* (Eisen, 2020). The identification of *Haemaphysalis longicornis* (Asian long horned tick) in North America initially raised concerns about the potential of this invasive and rapidly spreading species to serve as a vector for *Borreliella* species (Beard et al., 2018). To date, *Borreliella* species have not been recovered from *H. longicornis* and efforts to experimentally infect these ticks with *B. burgdorferi* were unsuccessful (Breuner et al., 2020).

Diverse reservoirs for the Borreliella: implications for vaccine development
While the list of identified reservoir species for the *Borreliella* is extensive, investigations into the competence of additional species of mammals and birds to serve as reservoirs is critically important, particularly in light of ongoing efforts to develop reservoir targeting vaccines and intervention strategies. *Peromyscus leucopus* (white-footed mouse) and *Apodemus sylvacticus* (wood mouse) are among the most abundant reservoir species in North America and Europe, respectively (Anderson, 1991). However, "inconspicuous" hosts including *Tamias striatus* (eastern chipmunk), *Sorex brevicauda* (short-tailed shrew), *Sorex cinereus* (masked shrew), *Myodes glareolus* (bank vole),

Microtus agrestis (field vole), *Sorex araneus* (common shrew) and *Sorex minutus* (pygmy shrew) may be equally important (Brisson et al., 2008; Coipan et al., 2018). Birds also serve as reservoir hosts but perhaps most important is the role that birds play in the transportation and geographic dispersal of ticks (Kurtenbach et al., 1998; Hanincova et al., 2003; Ogden et al., 2008; Scott et al., 2012; Scott et al., 2018). Ground foraging, passerine birds harbor heavy tick burdens and are considered "super-carriers" (Anderson et al., 1986; Hanincova et al., 2003; Ogden et al., 2008; Scott et al., 2012; Hasle, 2013; Lommano et al., 2014; Newman et al., 2015; Scott et al., 2018). Of the 162 species of birds assessed in one study for overall tick burden, 72 species were described as "tick infested" (Loss et al., 2016). Another recent study screened 205 tissue samples from the bone marrow or spleen of 169 individual upland game birds for *B. burgdorferi* DNA. Amplicons were obtained from 12% of the samples tested with the highest prevalence in wild turkeys (*Meleagris gallopavo*) (Cleveland et al., 2020).

Predators also influence or contribute directly or indirectly to the enzootic cycle (Ostfeld et al., 2006; Levi et al., 2012). Beyond their influence on small mammal reservoir populations through predation, they may also serve as reservoirs. In Europe, red foxes (*Vulpes vulpes*) have been demonstrated to be competent reservoirs (Mysterud et al., 2019) and it has been postulated that eastern coyotes (*Canis latrans*), an apex predator, may serve as reservoirs in the northeastern United States (Izac et al., 2020a). Robust antibody titers to several *B. burgdorferi* antigens were demonstrated in 64.8% of 128 eastern coyote plasma samples collected in Pennsylvania between 2015 and 2017 (Izac et al., 2020a). In the same study, 72.7% of the samples were antibody positive for the *Anaplasma phagocytophilum* antigens P44 and P130. Notably, 51% of the samples were positive for antibodies to both *B. burgdorferi* and *A. phagocytophilum* suggesting potential coinfection. While seropositivity is not a direct indication of reservoir competence, based on the behavioral habits of eastern coyotes it is possible that they may serve as a source of infection for feeding ticks. Knowledge of the full range of competent reservoir hosts will be essential to advance wildlife and reservoir targeting vaccines, which to date have been focused primarily on targeting mice (Tsao et al., 2004; Gomes-Solecki et al., 2006; Scheckelhoff et al., 2006; Bhattacharya et al., 2011; Meirelles Richer et al., 2011; Telford et al., 2011; Voordouw et al.,

2013). It remains to be determined if an effective and practical reservoir targeting strategy can be developed that will be able to target the highly diverse reservoirs species of the *Borreliella*.

Justification for the development and use of Lyme disease vaccines
Human Lyme disease
The case for the development of a human LD vaccine is, first and foremost, supported by the failure of existing preventive strategies to reduce the incidence of disease. Current prevention strategies for tick-borne diseases, particularly in humans, are ineffective at the population level. In this section we discuss the justification for the development and use of LD vaccines and briefly review complementary, non-vaccination-based preventative strategies that are being applied in human and veterinary medicine. Prevention of tick-borne diseases will require a multi-pronged approach with vaccination as its center piece.

The human health toll and socioeconomic impact of LD cannot be understated. LD is the most common tick-borne disease in North America, Europe, and Asia. In the United States a total of 33,666 confirmed and probable cases of LD were reported to the CDC in 2018 (www.cdc.gov/lyme/index.html). While surveillance for LD and other tick-borne disease has provided important information about the incidence and spread of diseases, these approaches are almost entirely passive. It is widely recognized that passive surveillance data are compromised by low compliance, non-mandated reporting, misdiagnosis, and under and over reporting (Meek et al., 1996; Yound, 1998; Bacon et al., 2008). Studies suggest that the true incidence of LD in humans in some regions of the United States is 3 to 12 times greater than that inferred from the reported case numbers (Meek et al., 1996; Yound, 1998; Sykes and Makiello, 2017)). Between 1992 and 1998, a total of 88,967 cases of human LD were reported to the CDC by state departments of health (www.cdc.gov). Between 2008 and 2015, this number rose to 275,000 cases. A CDC based analysis of clinician diagnosed cases from 2005 through 2010 estimated the annual incidence of LD in the United States at 106.6 cases per 100,000 persons with 296,000 to 376,000 new cases each year (95% credible interval) (Nelson et al., 2015). A separate CDC study that analyzed insurance records and diagnostic codes also concluded that the actual number of cases is considerably higher than that inferred from passive

surveillance (Tseng et al., 2015). Data from the National Notifiable Disease Surveillance System indicates that the geographic distribution of high-incidence areas for LD is expanding. The number of counties with an incidence of ≥10 confirmed cases per 100,000 persons increased from 324 in 2008 to 415 in 2018. It is clear that the LD threat is increasing.

Consistent with the geographic expansion of high-incidence zones for LD, the rapid and ongoing geographic spread of *I. scapularis* and *I. pacificus* ticks is well documented (Eisen et al., 2016; Eisen et al., 2017). *I. scapularis* and *I. pacificus* have been documented in 43 states and in 1,531 of the 3,110 continental United States counties (49.2%). This represents a 44.7% increase since an earlier assessment conducted in 1998. The number of counties in which *I. scapularis* is considered established (i.e., six or more individual ticks or one or more life stages identified in a single year) has more than doubled over the past two decades. The majority of county status changes have occurred in the North-Central and Northeastern states. It is anticipated that the two previously distinct foci for *I. scapularis* in the Northeast and North-Central states will merge in the upper Ohio River Valley resulting in the formation of a single contiguous "tick zone" encompassing much of the eastern half of the United States. Endemic regions for *Ixodes* ticks, and the incidence of LD, are also increasing in Canada, Europe, and Asia as well (Gasmi et al., 2017; Levy, 2017; Sykes and Makiello, 2017; Lloyd and Hawkins, 2018; Ogden et al., 2019).

The socioeconomic costs of LD in the United States are staggering. A study conducted in 1998, based on an incidence of 4.73 cases per 100,000 persons and year 1996 dollars, estimated costs at $500 million in the United States alone (Maes et al., 1998). A study published in 2015 estimated the direct costs of LD and post-treatment LD syndrome in the United States at approximately $712 million and $1.3 billion a year, respectively (Adrion et al., 2015). The most recent analysis of the inpatient costs of LD-related hospitalizations assessed data collected between 2005-2014 from the Truven Health Analytics MarketScan Commercial Claims and Encounters Databases (Schwartz et al., 2020). Of 20,983,165 admission records assessed, 2,823 (0.01%) met inclusion criteria for LD-related hospitalizations. Over half of the identified records contained an ICD-9-CM code for meningitis (n = 614), carditis (n = 429), facial palsy (n = 400) or arthritis (n = 377). The median cost per LD-related hospitalization was $11,688 (range: $140-$323,613). The median costs per stay based on clinical manifestations were $17,461 for carditis, $15,177 for meningitis, $13,012 for arthritis, and $10,491 for facial palsy. Extrapolation of the data to the U.S. population suggests that there are 2,196 LD-related hospitalizations in persons under 65 years of age annually with an estimated annual cost of $25,826,237. The estimated costs for vaccination of individuals at risk for LD is estimated to be approximately $100 a year per vaccinated individual. The potential cost-effectiveness of vaccination for LD is clear (Mac et al., 2019; Behler et al., 2020). The increasing incidence and expansion of endemic regions for LD, coupled with the real or perceived controversies surrounding diagnosis and treatment, highlight the need for aggressive prevention strategies. It follows that an effective and publicly accepted vaccine would address and lessen some of the most controversial aspects of LD diagnosis and treatment and dramatically reduce the costs associated with LD.

LD in companion animals
LD was first described in dogs in the mid 1980's (Lissman et al., 1984; Kornblatt et al., 1985; Magnarelli et al., 1985) and is now the most common tick-borne disease of companion animals. Point-of-care tests that allow for rapid serological screening of dogs for antibodies to *B. burgdorferi, Anaplasma phagocytophilum, Anaplasma platys, Ehrlichia canis* and *Ehrlichia ewingii* are widely used in veterinary medicine (Little et al., 2010; Littman et al., 2018). There is no equivalent screening strategy in place in human medicine. The data obtained from yearly screening informs our understanding of disease spread and exposure risk in humans (Mead et al., 2011; Yancey et al., 2014; Bowser and Anderson, 2018) . The Companion Animal Parasite Council (CAPC; https://capcvet.org/articles/parasite-prevalence-maps/), an independent non-profit organization that is focused on increasing awareness of infectious diseases in companion animals, gathers and reports antibody test results for tick-borne diseases that affect canines in the United States and Canada. In 2019, the results from 7,057,815 LD and tick-borne pathogen serological screening tests were catalogued by the CAPC. Approximately, 5.0% of the tests run were antibody positive for *B. burgdorferi*. The second most prevalent tick-borne disease in North America is anaplasmosis (*Anaplasma phagocytophilum*) with over 221,000 (3.1%) positive

tests reported in 2019. It is important to note that the CAPC captures results for less than ~30% of the total number of serological tests conducted each year. Hence, the probable number of positive *B. burgdorferi* antibody tests in dogs may be three-fold greater and most likely exceeds 1,000,000. In Canada, serological screening of dogs suggests that endemic regions for LD currently exist as isolated foci. Defined risk areas with increasing incidence are found in Manitoba, Ontario, New Brunswick, Quebec, and Nova Scotia. In Nova Scotia, 19.5% of the serological tests for *B. burgdorferi* in dogs reported in 2019 were antibody positive. Significant case numbers have also been reported in British Columbia on Canada's western coast (Mak et al., 2000). With time, the now isolated and defined foci are expected to expand and merge. As in the United States, vaccination is widely employed in veterinary medicine in Canada. It is possible that with the implementation of an aggressive vaccination strategy, the growing threat of LD in dogs can be contained.

The exposure risk for LD in humans is associated with occupation and with the frequency and duration of outdoor activities (hiking, outdoor recreation, gardening etc.). Central tenets of the CDC's recommendations for prevention of tick-borne diseases are to minimize exposure to tick habitats (grassy, brushy and wooded areas) and to perform diligent tick checks (physical body inspection) after engaging in at risk activities. In both human and veterinary medicine, acaricides are available for use. The application of permethrin (0.5%) (Connally et al., 2018) to clothing is one preventative strategy recommended by the CDC. Permethrin is long-lasting and has been demonstrated to convey significant protection against bites from *I. scapularis* in laboratory studies and sustained protection against bites by *Amblyomma americanum* ticks in field tests. Recently a two-year randomized, placebo-controlled, double-blinded trial of 82 outdoor workers in Rhode Island and southern Massachusetts was completed (Mitchell et al., 2020). Study participants in the treatment-arm wore factory-impregnated permethrin clothing while the control group wore sham-treated clothing. Factory-impregnated clothing reduced tick bites by 65% in the first study year and by 50% in the second year. No significant treatment-related adverse outcomes were reported. Additional tick repellents registered with the Environmental Protection Agency are also available, but some, such as oil of lemon eucalyptus and para-methane-diol, should not be used on children under three years old. While the

rationale behind the CDC recommendations listed above are obvious, the practicality or likelihood of widespread voluntary compliance with some of these practices at the population level seems unlikely.

The risk for tick-borne diseases is inherently greater for companion animals (Littman et al., 2018; Divers et al., 2019) than it is for humans due to their greater risk of exposure. As discussed above, veterinary medicine has several tools available to screen for and aid in the prevention of tick-borne diseases. The American College of Veterinary Internal Medicine 2018 consensus statement on LD (Littman et al., 2018) recommends screening of all healthy dogs that live in, live near, or travel to LD endemic areas in North America for antibody to *B. burgdorferi*. Furthermore, it is recommended that *B. burgdorferi* antibody positive dogs be tested for proteinuria due to concerns about the development of potentially fatal kidney disease (Lyme nephritis). Acaricides, including fluralaner and afoxolaner (isooxazoline family), are widely used on dogs and have good efficacy and safety profiles (Allen et al., 2020; Holzmer et al., 2020; Kryda et al., 2020; Petersen et al., 2020). While varying with the product, acaricides can be delivered orally as chewables, topically (so called "spot on" application) or through the use of treated collars. Tick checks should also be performed on dogs after outdoor activity but they can be difficult to thoroughly conduct due to the dense coat and skin tone of most common dog breeds. Several bacterin and subunit vaccines have been approved by the USDA and are commercially available. The composition, mechanism of action, safety, and efficacy of canine LD vaccines are discussed in detail below. For further information on the socioeconomic burden of human LD and the cost-effectiveness of treatment, general prevention and vaccination, readers are directed to a recent and comprehensive review of the existing literature (Mac et al., 2019).

Outer surface proteins and LD vaccine development
The *Borreliella* genome encodes a vast repertoire of surface-exposed proteins (Fraser et al., 1997; Casjens et al., 2000; Glockner et al., 2004; Schutzer et al., 2011). Many are differentially expressed in response to environmental variables (Fingerle et al., 2000; Revel et al., 2002; Roberts et al., 2002; Crandall et al., 2006; Caimano et al., 2007; Rogers et al., 2009b; Iyer et al., 2015; Caimano et al., 2016). To date, only two surface proteins have been successfully developed as subunit vaccine antigens in human or veterinary LD vaccines: outer surface

protein A (OspA) and a laboratory designed outer surface protein C (OspC)-based recombinant chimeric epitope protein.

Outer surface protein A: transcriptional expression, biological function and diversity

Outer surface protein (Osp) A was among the first *B. burgdorferi* proteins to be identified and characterized (Howe et al., 1985). Early analyses of OspA were prompted in part by the simple observation that it was one of the most abundant *B. burgdorferi* proteins produced during *in vitro* cultivation. OspA is a 31 kDa surface lipoprotein encoded by a linear plasmid of 54 kb (lp54) (Barbour, 1988). It is cotranscribed as a bicistronic operon with *ospB* (Howe et al., 1986; Bergström et al., 1989). While *B.* burgdorferi strains that lack lp54 have been obtained upon prolonged *in vitro* cultivation (Hughes et al., 1993; Sadziene et al., 1995), lp54 is universal among isolates collected from nature (Casjens et al., 2012) and is essential for completion of the enzootic cycle. OspA is selectively produced during spirochete residence in ticks (Fikrig et al., 1990) with a transcriptional expression rank relative to all other detectable transcripts of 8th during cultivation and 18th in unfed larval ticks (Caimano et al., 2019). Upon ingestion of the bloodmeal, *ospA* transcription is downregulated (Schwan and Piesman, 2000) before terminating at the tick-tissue interface (Caimano et al., 2019). Gene deletion studies demonstrated that OspA is required for survival in ticks (Yang et al., 2004; Battisti et al., 2008). In the tick midgut, OspA functions as an adhesin and binds to the tick protein TROSPA (tick receptor for OspA) (Pal et al., 2000; Pal et al., 2004a). The crystal structure of recombinant OspA in complex with the Fab fragment of monoclonal antibody 184.1 was determined at a resolution of 1.9 A (PDB: 1OSP) (Li et al., 1997). A unique structural organization was revealed that consists of repetitive antiparallel beta sheets with a nonglobular region connecting the globular N- and C-terminal domains.

Antigenic determinants of OspA

Based on reactivity with monoclonal antibodies and comparative DNA sequence analyses, distinct variants or serotypes of OspA have been identified (discussed below) (Wilske et al., 1993a; Will et al., 1995; Wilske et al., 1996). OspA serotype 1 is the dominant variant produced by *B. burgdorferi* isolates from North America. OspA diversity is greater among *Borreliella* isolates from Europe where several serotypes have been identified (Wilske et al., 1993a; Wilske et al., 1995; Marconi et al., 1999). OspA

serotype 2 is associated with *B. afzelii,* serotypes 3 and 5 with *B. garinii*, serotype 6 with *B. garinii* and serotype 4 with *B. bavariensis*. Serotype 4 has been postulated to correlate with a neuroinvasive phenotype, but since OspA is not produced in mammals, the potential correlation would be an indirect one (Marconi et al., 1999).

The protective efficacy of OspA was first demonstrated in the C3H/HeJ mouse model (Fikrig et al., 1990). Mice were vaccinated with either live *E. coli* expressing OspA (strain N40; 5×10^6 cells; three doses one week apart) or purified recombinant strain N40 OspA fused to GST. It is not indicated in the original publication if the formulation was adjuvanted. Both vaccine formulations provided short term protection. The live *E. coli* formulation expressing OspA protected against strains N40, B31 and CD16. The recombinant GST-OspA fusion protein was only tested for efficacy using strain N40. In a follow up study, mice were immunized with recombinant OspA (10 µg; 3 doses using Freund's complete and incomplete adjuvants) and protection against different spirochete challenge doses was assessed (Fikrig et al., 1992b). Mice were protected against 1×10^4 laboratory cultured *B. burgdorferi* cells but not 1×10^7. In the same study, the protective efficacy of OspB and flagellin were assessed. OspB protected mice from infection with an inoculum of 10^3 spirochetes but not a dose of 10^4. Consistent with its periplasmic localization, flagellin did not convey protection against any challenge dose. OspA has proven to be a protective vaccinogen in dogs, rhesus macaques (Probert and LeFebvre, 1994; Straubinger et al., 1995; Philipp et al., 1997) and humans (discussed in detail below). A caveat to OspA mediated protective antibody responses is that they are relatively short-lived and an aggressive booster schedule is required. The necessity for frequent boosts stems from the unique mechanism of action of OspA vaccinogens. Since OspA is produced only in ticks, antibody can only target *B. burgdorferi* prior to transmission into mammals. Hence, it is essential to maintain high levels of circulating antibody since an immune memory response will not develop following natural infection by tick inoculation.

Several studies have localized protective epitopes of OspA within its C-terminal half (Huang et al., 1998; Ding et al., 2000; Legros et al., 2000; Kharitonenkov et al., 2002; Izac et al., 2017; Shandilya et al., 2017). Using a protective and well-characterized monoclonal antibody designated as LA-2, the corresponding LA-2

epitope was identified (Huang et al., 1998; Ding et al., 2000). The LA-2 epitope is discontinuous and formed by residues contained within three surface-exposed loops present in the C-terminal domain of OspA (Ding et al., 2000). Based on this finding, and epitope mapping studies with other monoclonal antibodies, it has been suggested that maintenance of OspA structure is essential for maximizing productive antibody responses. Efforts have been taken to generate N-terminally truncated OspA proteins that still retain the native LA-2 epitope conformation (Koide et al., 2005). A lipidated OspA fragment consisting of residues 130-273 (*B. burgdorferi* B31; ORF designation, BBA_15) fused to the IgG binding domain of staphylococcal protein A (Koide et al., 2005) retained native conformation and bound monoclonal antibody LA-2. However, the protein proved to be unstable and it exhibited reduced efficacy in challenge studies in C3H-HeJ mice (Koide et al., 2005). To stabilize the fragment, residues involved in buried salt-bridge interactions were replaced with residues that promote hydrophobic interactions. Specifically, substitution of amino acids R139, E160 and K189 with M, Y and M, respectively, increased the stability of the fragment and restored protective efficacy. The vaccination protocol consisted of three doses of immunogen in alum at two-week intervals. Challenge was administered by feeding ten field collected ticks (infectivity percentage of 30%) on each mouse. To our knowledge, there have been no additional reports indicating that this protein has been pursued further as a vaccine antigen.

A recent study identified a continuous, linear epitope in OspA that is localized within amino acids spanning residues 221-240 (*B. burgdorferi* B31 OspA-serotype 1) referred to as the OspA$_{221-240}$ epitope (Izac et al., 2017). IFA assays using antibodies raised against OspA$_{221-240}$ peptide conjugated to keyhole limpet hemocyanin (KLH) surface labeled intact *B. burgdorferi*. The hyperimmune sera displayed potent complement-dependent bactericidal activity that killed *B. burgdorferi* at a level equivalent to that of full length OspA. The bactericidal activity of the antisera against *B. afzelii, B. bavariensis* and *B. garinii* was significant but reduced relative to that seen against *B. burgdorferi*. BLAST searches using the OspA$_{221-240}$ amino acid sequence revealed regions of conservation and variation within this epitope containing span of OspA. Interestingly, an amino acid segment of OspB spanning residues 244-260 displayed significant homology with the OspA

221-240 peptide. The corresponding OspB sequence was found to be well-conserved among *B. afzelii, B. bavariensis* and *B. garinii* isolates. Consistent with the decreased antibody mediated killing of *B. bavariensis* and *B. afzelii* isolates by the hyperimmune sera relative to that of *B. burgdorferi* isolates, the variable amino acid residues are centrally located within the OspB$_{244-260}$ sequence. The positioning of these mismatches most likely accounts for the lower level of bactericidal activity. Based on sequence similarity between the *B. burgdorferi* OspA$_{221-240}$ epitope and OspB$_{244-260}$, it is possible that antibody raised against variants of the OspA$_{221-240}$ domain may be able to target both OspA and OspB. It remains to be determined if OspA peptides or fragments consisting of the multiple variants of the 221-240 peptide can elicit protective antibodies in a tick-mouse challenge model.

Outer surface protein C (OspC) expression, structure and function
OspC is an approximate 22kDa surface lipoprotein encoded by a circular plasmid of 26 kb (cp26). Cp26 is genetically stable and ubiquitous among *Borreliella* species and isolates (Marconi et al., 1993a; Sadziene et al., 1993; Tilly et al., 1997). Orthologs of OspC have been identified in some TBRF species including *Borrelia hermsii* (Marconi et al., 1993b). *OspC* is the most highly expressed *B. burgdorferi* gene during early stage infection in mice and the 8th most abundant transcript in fed *I. scapularis* nymphs (Caimano et al., 2019). Antibody to OspC develops early and can be detected in the serum of mice within two weeks of syringe inoculation of *B. burgdorferi* (Oliver Jr et al., 2016). Consistent with this, non-targeted mass spectrometry of skin biopsies collected from mice seven days after delivery of *B. burgdorferi* by syringe inoculation also identified OspC as one of the most frequently detected *B. burgdorferi* proteins (Talagrand-Reboul et al., 2020). In contrast, during laboratory cultivation *ospC* is expressed at low and varying levels with a transcriptional ranking of 816 (Iyer et al., 2015). The transcriptional regulation of OspC is complex and has been detailed in several studies (Marconi et al., 1993a; Hubner et al., 2001; Alverson et al., 2003; Yang et al., 2005; Gilbert et al., 2007; Hayes et al., 2014).

The structure of OspC (PDB: 1GGQ, 1F1M, 1G5Z) offers significant insight into its potential functional domains as well as its antigenic determinants (Eicken et al., 2001; Kumaran et al., 2001a; Kumaran et al.,

2001b). In contrast to OspA, which is largely β-sheet and monomeric, OspC consists of five α-helices connected by loops. OspC forms a homodimer with an extensive buried interface suggesting that the biologically active form of the protein is the dimer. Gene deletion analyses demonstrated that OspC is required for *B. burgdorferi* to infect mice but not required to infect ticks or be transmitted by ticks (Grimm et al., 2004; Pal et al., 2004b; Dunham-Ems et al., 2012). Specific amino acids required for the *in vivo* function were identified through the introduction of amino acid substitution mutants into *B. burgdorferi* B31 *ospC* using allelic exchange replacement (Earnhart et al., 2010). The substitutions were targeted within a putative small ligand binding pocket (designated as LBD1) predicted using the ConCavity ligand analysis algorithm. LBD1 is formed near the membrane proximal end of the OspC dimer and lined by residues K60, E61, E63 and A64 of each monomer. The mutated proteins were expressed in *B. burgdorferi* and presented on the cell surface in a manner consistent with wild-type OspC. A single amino acid substitution in which E61 was replaced with Q rendered *B. burgdorferi* B31 non-infectious in C3H/HeJ mice (dose of 1×10^4 syringe administered spirochetes) (Earnhart et al., 2010). Substitution of K60 with Y did not affect infectivity but attenuated colonization of the heart relative to the parental B31 strain. The use of site-directed mutagenesis allelic exchange replacement is a powerful approach that can be used to identify the determinants of OspC that are involved in its different and distinct functions.

Evidence suggests that OspC is a multi-functional protein. Several tick or mammalian derived ligands have been demonstrated to bind to OspC including Salp15 (tick salivary protein) (Anguita et al., 2002; Ramamoorthi et al., 2005), plasminogen (Lagal et al., 2006; Onder et al., 2012) and complement C4b (Caine et al., 2017). Salp15 is a potential immune modulatory protein that may interfere with CD4+ T-lymphocyte function (Anguita et al., 2002). The binding of plasminogen has been postulated to facilitate tissue invasion (Lagal et al., 2006; Onder et al., 2012), while the binding of C4b has been hypothesized to inhibit the classical and lectin complement pathways and compete with complement protein C2 for binding to C4b (Caine et al., 2017). OspC has also been reported to have anti-phagocytic properties (Carrasco et al., 2015). Additional research is needed to demonstrate the biological significance of the individual ligand interactions summarized above.

OspC phylogeny

OspC is among the most variable LD spirochete proteins (Jauris-Heipke et al., 1993; Theisen et al., 1993; Wilske et al., 1993b). Phylogenetic analyses have delineated approximately 38 distinct OspC phyletic clusters or "types" (Seinost et al., 1999a; Wang et al., 1999; Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c). Within a phyletic type, OspC sequences are highly conserved (>95% amino acid identity) while at the inter-type level, amino acid identity values range from 55 to 80% (Wang et al., 1999; Eicken et al., 2001; Kumaran et al., 2001b; Earnhart and Marconi, 2007c). OspC variants are referred to as OspC types; letter based, isolate of origin, or other designations are used to differentiate them (i.e., OspC type A, OspC type B, OspC type Pko, etc.,). In spite of extended regions of sequence conservation, antibody responses to OspC during natural infection or upon immunization with bacterin vaccines or recombinant OspC proteins are largely OspC type-specific indicating that the immuno-dominant epitopes are localized within variable domains of the protein (Earnhart et al., 2005; Earnhart et al., 2007; Oliver Jr et al., 2016; Izac et al., 2019). Considerable OspC diversity has been demonstrated among LD spirochete strains isolated from tightly defined geographic regions (Wang et al., 1999; Lin et al., 2002; Alghaferi et al., 2005; Earnhart et al., 2005). Twenty-one OspC types have been demonstrated in a single tick through deep sequencing (Di et al., 2018). *B. burgdorferi* isolates recovered from naturally infected animals (Seinost et al., 1999b; Oliver Jr et al., 2016; Izac et al., 2019) or from the tissues of dogs experimentally infected with field collected ticks (Rhodes et al., 2013) are highly heterogenous with regard to the OspC types represented. The existence of stable and distinct OspC types in nature may be essential for population maintenance and may facilitate the ability of the LD spirochetes to infect an immunologically primed, OspC antibody positive host (Seinost et al., 1999a; Wang et al., 1999). OspC diversity has long been a significant obstacle in the development of broadly protective OspC based vaccines. The recent development of recombinant proteins consisting of linear epitopes derived from numerous OspC types has effectively addressed the OspC diversity issue (Izac et al., 2020b). These unique proteins, referred to as chimeritopes, have been demonstrated to elicit potent bactericidal antibodies against a wide range of OspC types and to convey protection against the diverse strains presented during challenge with field-collected ticks (Ball, 2016; Marconi, 2020b). The

construction and analysis of OspC chimeritopes is described in detail below.

Several studies have reported on the identification of conformationally defined OspC protective epitopes (Gilmore et al., 1996; Gilmore and Mbow, 1999; Norek and Janda, 2017). Gilmore et al (1996) vaccinated specific-pathogen-free outbred mice intradermally with recombinant non-denatured OspC excised from a gel and with denatured OspC (adjuvanted with alum). Three doses were delivered each two weeks apart. The mice vaccinated with non-denatured recombinant OspC were protected upon challenge with laboratory reared-ticks infected with *B. burgdorferi* strain B31 (ten nymphal ticks per mouse). In contrast, mice immunized with denatured OspC were not protected.

To further investigate the influence of conformation on protective efficacy, recombinant OspC preparations were denatured using heat or chemical treatment prior to animal immunization (Gilmore and Mbow, 1999). Additional groups of mice received soluble or insoluble non-denatured recombinant OspC. All proteins were adjuvanted with TiterMax (oil in immersion). After confirming seroconversion, the mice were challenged with *B. burgdorferi* B31 infected ticks. Mice immunized with soluble, nondenatured OspC were fully protected, whereas mice that received insoluble or denatured OspC, were not. Protective epitope localization experiments were then conducted. OspC fragments were tested for their ability to bind the protective OspC monoclonal antibody B5. The fragments did not bind prompting the suggestion that the amino or carboxy end of OspC is required to bind monoclonal antibody B5. Immunization of mice with truncated forms of OspC also failed to provide protection against tick challenge. The authors postulated that the N and C-terminal domains of OspC, which are in close physical proximity to one another in the OspC structure (Eicken et al., 2001; Kumaran et al., 2001b), interact to present a conformational epitope. A potential caveat of this study is that some of the recombinant OspC proteins were produced with a 38 kDa T7 gene 10 fusion at the N-terminus of OspC. It is possible that effects of denaturation, and the results obtained with truncated OspC proteins, were influenced by the large N-terminal tag.

The conserved C-terminal ten amino acids of OspC (amino acid sequence PVVAESPKKP) have also been reported to constitute an immunodominant,

continuous linear epitope that elicits protective antibody responses (Rousselle et al., 1998; Jobe et al., 2003; Lovrich et al., 2005). However, this suggestion is difficult to reconcile since a single OspC protein elicits only strain-specific protection (Bockenstedt et al., 1993; Earnhart et al., 2007; Izac and Marconi, 2019). The conserved C-terminal motif of OspC has been referred to as the C7, C10, and pepC10 motifs (Mathiesen et al., 1998a; Mathiesen et al., 1998b; Porwancher et al., 2011; Earnhart et al., 2014; Izac and Marconi, 2019). It stands to reason that if this conserved C10 motif, which is shared by all OspC types, is in fact immunodominant, then antibody elicited by any OspC type protein should be immunoreactive with all OspC type proteins. To assess this further, the relative contribution of the C10 domain to OspC antibody responses and to OspC function was assessed by allelic exchange replacement of *B. burgdorferi* B31 wild-type *ospC* type A with an *ospC* gene truncated to eliminate the C10 domain (Earnhart et al., 2014). The truncated protein (OspCΔ10) was expressed and presented on the cell surface in a manner similar to that of the parental strain, and the B31::*ospC*Δ10 strain retained the ability to infect mice (syringe inoculation) and to elicit anti-OspC IgG titers equivalent to the B31 parental strain. Additional evidence that the C10 motif is not immunodominant came from a study in which OspCΔ10 truncations were generated for 20 different OspC type proteins (Izac et al., 2019). The OspC protein panel was screened by immunoblot and dot-blot with sera from experimentally infected C3H-HeJ mice and non-human primates (Rhesus macaques) and naturally infected dogs, horses and humans (Izac et al., 2019). The truncated proteins retained their type specific immunoreactivity. Finally, sera harvested from rats (Sprague-Dawley) immunized with recombinant OspC proteins (types I, T or F), and their corresponding OspCΔ10 proteins (Freund's adjuvant), displayed equivalent levels of complement-dependent bactericidal activity. It can be concluded that the C10 domain does not contribute to an essential *in vivo* function, is not an immunodominant epitope, and is not required for the presentation of epitopes that elicit protective antibody responses.

While conformational and discontinuous epitopes undoubtedly contribute to the antibody responses elicited by OspC (Pulzova et al., 2016; Norek and Janda, 2017), linear epitopes that elicit antibody responses have been identified (Buckles et al., 2006). Immunoscreening of overlapping OspC

peptides and OspC fragments using sera from experimentally infected mice (C3H-HeJ) and naturally infected humans identified two distinct linear epitope containing sequences (Earnhart et al., 2005; Buckles et al., 2006). These epitopes, which reside within the C-terminal half of OspC, have been designated as the helix 5 (H5) and loop 5 (L5) epitopes. The L5 epitope maps within the C-terminal end of helix 3 and the N-terminal end of loop 5 while H5 epitope is contained within a region encompassing the 5th alpha helix and a few residues C-terminal to this helix. Both epitopes reside within membrane proximal surface-exposed segments of OspC. The L5 and H5 epitopes elicit OspC type-specific bactericidal antibody that kills through a complement dependent mechanism (Buckles et al., 2006). The specificity of the OspC antibody response elicited by the variable domain epitopes of OspC explains the narrow protective efficacy obtained upon vaccination with a single OspC type protein (Bockenstedt et al., 1997; Probert et al., 1997). It is noteworthy that type-specific antibody responses are also elicited upon vaccination with recombinant OspC proteins. Rabbits vaccinated individually with different recombinant OspC type proteins developed type-specific antibody responses (Oliver Jr et al., 2016). The membrane proximal location of the L5 and H5 epitopes may enhance the bactericidal activity of antibodies directed at these epitopes by fixing complement near the cell surface.

Important considerations in LD vaccine development
Approaches for assessing potential vaccine efficacy in vitro
Prior to advancing vaccine candidates to testing in animal models, *in vitro* assays can be used to assess correlates of protection. However, since a significant number of promising vaccine candidates are selectively expressed *in vivo* and not produced during laboratory cultivation, *in vitro* analyses can pose unique challenges. OspC serves as an example. The level of OspC expression during cultivation can vary considerably among strains (Xiang et al., 2017) and among cells in a population (Oliver Jr et al., 2016). IFA analyses in which *B. burgdorferi* B31 cells were screened with anti-OspC type A antisera revealed that less than 10% of cells produced detectable levels of OspC. The non-uniform production of OspC has in some cases compromised efforts to use wild-type strains in bactericidal assays. However, the expression levels of OspC and other antigens can be enhanced *in vitro* by several approaches. Adjusting the pH of the growth media or temperature-shifting cultures has been demonstrated to upregulate the expression of

some *in vivo* antigens including OspC, OspE and OspF (Ramamoorthy and Philipp, 1998; Carroll et al., 1999; Ramamoorthy and Scholl-Meeker, 2001). However, it has proven difficult to achieve consistent levels of expression by manipulation of culture conditions. The dialysis membrane chamber implant model developed by Akins and colleagues was a key step forward in efforts to study *B. burgdorferi* in a host-adapted state (Akins et al., 1998). In the dialysis membrane chamber implant model, actively growing spirochetes are introduced into the peritoneal cavity of rats or mice within the confines of dialysis membrane chambers (5 kDa MW cutoff). After allowing for growth and adaptation to the mammalian environment, the chambers are removed from the peritoneal cavity and the host-adapted spirochetes harvested for analysis. Since cultures volumes as high as 10 mL can be recovered from a single rat, this approach allowed for the direct analysis of host-adapted without *in vitro* cultivation. The transcriptional and proteome profiles of host-adapted spirochetes are consistent with that reported for the expression of individual genes in mammals (Brooks et al., 2003; Tokarz et al., 2004; Caimano et al., 2007; Iyer et al., 2015). Perhaps the most efficient approach for mimicking the *in vivo* expression of an individual antigen is through the application of genetic manipulation methodologies (Samuels et al., 1994). The development of genetic manipulation approaches for *B. burgdorferi* was a significant breakthrough in LD research. Strains can be engineered to constitutively produce proteins such as OspC (Tilly et al., 1997) that are not expressed at steady levels in wild-type strains but that are dominant antigens *in vivo*. A *B. burgdorferi* B31 derived strain that constitutively produces OspC has been used in several studies to measure the antibody-dependent bactericidal activity of OspC derived recombinant vaccine antigens *in vitro* (Earnhart et al., 2014; Izac et al., 2019; Izac et al., 2020b).

Challenge studies: the approach matters
When conducting challenge studies, the route by which the challenge is delivered is important to consider. Studies of decorin binding protein A (DbpA) serve as an example as they demonstrated that protection is dependent upon route of administration (i.e., tick versus syringe). Vaccination of C3H/HeJ mice with recombinant DbpA in Freund's adjuvant induced high-titer antibody responses that were protective against syringe challenge with host-adapted *B. burgdorferi* (Cassatt et al., 1998).

However, when the challenge was delivered via tick bite, the mice were not protected (Hagman et al., 2000). While needle challenge is an important first step in efficacy studies, it is important to recognize its limitations and eventually progress to tick challenge. Naïve ticks or egg masses are now commercially available, and their availability has made an essential research tool more accessible. One approach to infect ticks with the desired strain is through immersion feeding (Policastro and Schwan, 2003). It is a simple approach whereby ticks (typically larval ticks) are immersed in a dense and actively growing culture. The ticks will imbibe the culture thus allowing the spirochetes to enter the midgut. The percentage of ticks that became infected was reported to be 65% after immersion for 45 minutes in a dense culture of *B. burgdorferi* B31. Ticks can also be infected by microinjection (Kariu et al., 2011; Smith et al., 2018). The process takes just a few hours and allows for the delivery of specific numbers of spirochetes into each tick. Microinjection can also be used to infect ticks with genetically modified strains that are unable to infect mice and thus cannot be introduced into ticks by the natural route, namely feeding of larvae on infected mice. It would be possible using either immersion feeding or microinjection to infect ticks with a mixture of strains. However, to our knowledge, this has not yet been reported. Lastly, large numbers of ticks can be infected by feeding on laboratory infected mice. While tick challenge studies are widely used for assessing vaccine efficacy, the overwhelming majority of studies published to date have used ticks infected in the laboratory with a single LD spirochete strain. As noted above, field collected ticks typically are infected with a heterogenous population of LD spirochetes and, as such, the use of field collected ticks in challenge studies most closely resembles natural challenge. A caveat of using field collected ticks is the potential for the ticks to be carrying multiple pathogens. With all of the approaches described above, it is critical to determine the percentage of infected ticks. This information is essential for determining the number of ticks that should be fed on an individual animal in order to ensure delivery of a robust challenge dose. In contrast to studies with mice, canine vaccine challenge studies have routinely used field collected ticks (Baum et al., 2014; Wagner et al., 2015; Stillman et al., 2019; Marconi, 2020b). Lastly, the mouse strain or dog breed used in challenge studies is also of great importance and consequence. Some dog breeds are more likely to develop symptoms of infection than others (Dambach et al., 1997;

Goldstein et al., 2007; Littman, 2013; Preyß-Jägeler et al., 2020). Age is also an important variable and sex as a biological variable should be considered. These topics are covered elsewhere (Barthold et al., 1990; Moody and Barthold, 1998) and thus are not discussed here.

First generation human LD vaccines
Two investigational LD vaccines intended for use in humans (ImuLyme, Pasteur-Merieux-Connaught; LYMErix, SmithKline Beecham) were developed in the 1990's and advanced to clinical trials (Schoen et al., 1995; Sigal et al., 1998; Schoen et al., 2003). Both vaccines consisted of recombinant lipidated OspA derived from North American isolates of *B. burgdorferi* (Erdile et al., 1993; Sigal et al., 1998).

ImuLyme: a first generation OspA investigational vaccine
The safety of recombinant lipidated OspA (ImuLyme) in humans was investigated using a randomized, double-blinded, placebo-controlled trial (36 adult volunteers; aged 18 to 65 years) (Keller et al., 1994). Alum adsorbed and non-adsorbed OspA formulations were tested and both were found to induce bactericidal antibody responses that inhibited growth of *B. burgdorferi in vitro*. It is noteworthy that the authors reported that adsorption to alum did not influence antibody titers. Adverse health events (AHEs) after the first vaccine dose were minor and consisted primarily of local pain and tenderness at the injection site. An increase in AHEs was not observed following the second or third dose. It was concluded that a two or three dose series of the OspA Lyme vaccine was safe in adults.

The efficacy of the ImuLyme formulation was assessed in a double-blind trial (10,305 participants; 18 years of age or older) at 14 sites in endemic areas of the United States (Sigal et al., 1998). Participants were randomly assigned to receive either placebo (5149 subjects) or 30 µg of lipidated OspA vaccine (5156 subjects). Two doses of the non-adjuvanted vaccine were delivered 1 month apart with 7515 subjects receiving a booster at 12 months. The subjects were monitored for two seasons with the primary end point being the number of new clinically and serologically confirmed cases of LD. During the first year there were 49 confirmed cases of LD with 37 in the placebo group and 12 in the vaccine group (68% efficacy; 95 percent confidence interval - 36 to 85 %). The efficacy rate was highest among participants under the age of 60. In the second year, 35 cases of LD were confirmed with 28 in the

placebo group and 7 in the vaccine group. Efficacy among those participants that received the 12-month booster was 92% (95 percent confidence interval - 69 to 97%). In the absence of the booster dose, there was little demonstrable effect of vaccination during the second year of the study. It is noteworthy that in this study, the percentage of subjects with definite LD was higher in the placebo group than in the vaccinates (1.3 percent vs. 0.4 percent, P<0.001). In addition, the percentage of participants that reported symptoms of LD was higher in the placebo group than in vaccinates (5.5% vs. 4.2 %, P=0.002). This was also the case for confirmed seroconversion (1.5% vs. 0.6%, P<0.001). While this study highlighted the efficacy of OspA in humans, it also revealed the need for booster vaccinations.

LYMErix: the only human LD vaccine to advance to market
Prior to the final formulation of the LYMErix vaccine, the safety, reactogenicity and immunogenicity of three precursor candidate OspA based vaccine formulations were assessed (Van Hoecke et al., 1996). Two different study designs were employed. The first was an open, randomized pilot study with 60 volunteers (20 per group; 18 to 50 years of age). The second, which was initiated 14 days after the first study began, was double-blinded and randomized with respect to vaccine formulation. Two-hundred and forty volunteers were enrolled and randomly assigned to three groups. The first formulation consisted of lipidated OspA (10 µg in alum) fused at its single cysteine residue to the N-terminal 81 amino acids of non-structural protein 1 (NS1) from influenza virus. The second formulation consisted of 10 µg of NS1-OspA in alum combined with the experimental adjuvant, 3-0-deacylated monophosphoryl lipid A (MPL). The MPL was derived from the lipid A moiety of *Salmonella minnesota* lipopolysaccharide (Ribi Immunochem Research). The third formulation consisted of lipidated OspA lacking the NS-1 sequence in alum. The vaccines were administered intramuscularly at monthly intervals. After each immunization, volunteers were monitored for AHEs. Only minor local and general reactions were noted. Serum was collected from each study participant prior to vaccination and then one month after each vaccine dose the sera were evaluated for anti-OspA IgG antibody levels. Of the three vaccine formulations tested the lipidated OspA in alum elicited the highest anti-OspA IgG levels and LA-2 equivalent geometric mean titers (GMTs). Volunteers that received this formulation remained seropositive

for anti-OspA IgG for the longest period of time. Based on the results of this study, the lipidated OspA in alum formulation was pursued for further development.

The safety and efficacy of the recombinant lipidated OspA vaccine, LYMErix, was then assessed in 956 volunteers (17 to 72 years of age) at three LD endemic sites in the United States (Schoen et al., 2000). Anti-OspA IgG titers were measured in blood collected at 0, 2, 3, 12 and 13 months. The study compared the tolerability of a two-dose schedule (0 and 1 months; intramuscular administration) with a three-dose schedule (0, 1, and 2 months). AHEs were mild to moderate and transient. Three doses of the vaccine proved more immunogenic than two and conveyed a higher probability of protection. Based on the GMTs determined, an antibody kinetics model used to assess duration of immunity predicted that protection would only last for a single typical tick-transmission cycle.

Using an intention-to treat analysis, the efficacy of LYMErix in humans was measured in terms of its ability to protect against "definite" LD (Steere et al., 1998). Definite LD was defined as the presentation of erythema migrans or objective neurologic, musculoskeletal, or cardiovascular manifestations consistent with LD plus laboratory confirmation via culture, PCR positivity or seroconversion (immunoblot). The phase III-randomized controlled trial included 10,936 subjects (15 to 70 years of age) from endemic regions of the United States. A two-dose vaccination series (30 µg lipidated OspA; alum adjuvant) provided 49% efficacy (95% confidence interval (CI) = 15%-69%) while a three-dose series delivered over 13 months provided 76% efficacy (95% CI=58-68%). The minimum protective titer was determined by measuring anti-OspA antibody titers in a subset of the study participants at 2, 12, 13 and 20 months. The GMTs at one month and 10 months after the second vaccine dose were 1227 ELISA units/mL and 116 ELISA units/mL, respectively. One month after a third vaccine dose (month 13), the GMT rose to 6006 ELISA units/mL indicative of a vaccination induced amnestic response. The anti-OspA GMTs at 20 months declined to 1991 ELISA units/mL. The minimum protective titer was determined to be ≥1200 ELISA U/mL (CDC, 1999). This value serves as a comparator for measuring immune responses to new OspA vaccines in development. It should be noted that individuals or animals that are vaccinated with OspA, and then

naturally exposed to infected ticks, do not develop an exposure induced amnestic response because OspA is not produced during infection.

The safety of LYMErix was assessed by following study participants for twenty months post-delivery of the first vaccine dose. Injection site soreness was the most frequent vaccination induced AHE and was reported by 24.1% of vaccine recipients (7.6% of placebo recipients; *p* <0.001). Vaccine recipients were more likely to report arthralgia and myalgia than placebo vaccinated individuals (*p* < 0.05). Potential AHEs were not reported in vaccine recipients with previous LD (Schoen et al., 1995). LYMErix was approved for use on December 21, 1998. The Advisory Committee on Immunization Practices (ACIP) suggested the LYMErix™ vaccine for persons who live in an endemic area and who engage in activities that result in frequent or prolonged exposure to ticks. The ACIP did not recommend Lyme vaccination for individuals in non-endemic areas or for those with low exposure risk or individuals under 15 years or older than 70 years of age. This rather lukewarm recommendation did not serve to further the cause for the widespread use of LYMErix.

LYMErix advanced to the market in 1998 and sales were initially robust. However, shortly after its introduction, reports emerged of potentially serious AHEs (Zundorf and Dingermann, 2008). The possible associations between the vaccine and putative AHEs were well-publicized and widely disseminated by some LD advocacy groups and the media. Class action lawsuits alleging vaccine-related harm were first filed in 1999. It was speculated that OspA may be associated with the development of antibiotic refractory arthritis with a reported linkage to particular MHC II alleles (HLA-DR4) (Steere et al., 1990; Kalish et al., 1993; Gross et al., 1998; Steere et al., 2011). Reports then emerged implicating an OspA T-cell epitope with sequence similarity to lymphocyte function antigen 1 as a contributor to autoimmune arthritis (Trollmo et al., 2001). In spite of these concerns, Phase IV studies were initiated by the manufacturer. These studies were designed to assess 25,000 vaccine recipients and 75,000 age and sex-matched unvaccinated controls. However, by November of 2000, only 10% of the planned enrollment had been met. In January of 2001, the results obtained up to that point were presented to an FDA advisory panel. A correlation between vaccination and autoimmunity was not identified. The

CDC and FDA also evaluated data from the Vaccine Adverse Event Reporting System collected between December 1998 and July 2000. After the distribution of 1.4 million vaccine doses, 905 AHEs were reported and of these 102 were coded as arthritis, arthrosis, or rheumatoid arthritis and 12 were facial paralysis. However, the reported rate of both arthritis and facial paralysis were below what would be expected as background. This rigorous multi-agency review concluded that the reported AHEs were not related to the product and thus did not recommend removal of LYMErix from the market. Nonetheless, the commercial viability of the vaccine was irreparably damaged, and sales declined dramatically resulting in the voluntary removal of LYMErix from distribution in 2002.

Unsubstantiated safety issues aside, the vaccine was also compromised by evidence-based limitations including its booster schedule and moderate efficacy (Zundorf and Dingermann, 2008). The need for repeated boosters with LYMErix and most likely with OspA based vaccines that are currently in development, is a direct reflection of the mechanism of action of these vaccines. As noted above, OspA is not produced by the LD spirochetes during their residence in mammals. Hence, vaccination induced antibody decreases the risk of LD by targeting spirochetes in ticks upon ingestion of the bloodmeal (Fikrig et al., 1992a). Vaccination induced antibody is not effective at targeting spirochetes that have successfully transmitted into a vaccinated individual. Efficacy of OspA based vaccines is therefore strictly dependent on the maintenance of high circulating antibody titers at a level above a specific threshold. A second consequence of the absence of OspA expression in mammals is that when vaccinated individuals are naturally exposed to *B. burgdorferi* via tick bite they will not mount an amnestic response. It is important to point out that this does not negate the positive contributions that OspA can provide in a vaccine formulation, especially if coupled with an antigen that can lead to memory immune responses. As detailed below, the canine LD vaccine VANGUARD®crLyme, which consists of OspA and an OspC based chimeric-epitope protein (chimeritope) (Izac et al., 2020b) designated as Ch14, was designed with this in mind (Marconi, 2020b).

Next generation Lyme disease vaccines
Several efforts are underway to develop modified OspA based vaccines for use in humans. In a study supported by Sanofi, a prototype recombinant OspA-

ferritin nanoparticle was generated by fusing non-lipidated OspA (serotype 1; *B. burgdorferi* B31) to the amino terminus of ferritin derived from *Helicobacter pylori* (Kamp et al., 2020). As detailed by the authors, the OspA-ferritin fusion was designed so as to allow for self-assembly of 24 ferritin units into a hollow spherical nanoparticle that presents the C-terminal domain of OspA outwards from the interior. The efficacy of this experimental vaccinogen was assessed in mice using the canine LD OspA based RECOMBITEK® Lyme vaccine (Boehringer-Ingelheim) as a comparator. The OspA-ferritin nanoparticles induced endpoint antibody titers 4.4-fold greater than the non-adjuvanted OspA of the comparator ($p < 0.001$). In addition, OspA-ferritin induced antibody persisted for a longer duration than antibody induced by the comparator vaccine lipidated OspA. Protective efficacy was assessed in C3H/HeN mice vaccinated intramuscularly with Addavax (Invivogen) adjuvanted OspA-ferritin nanoparticles in a two-dose series (four weeks apart). Mice were challenged two weeks after the final vaccine dose with 5 to 6 nymphal ticks infected with *B. burgdorferi* B31. The ticks were fed to repletion, collected, and efficacy assessed by culture of ear, ankle and heart biopsies. While sample size was relatively limited (4 mice per group), the OspA-ferritin formulation and the comparator vaccine protected all vaccinated mice from infection. The authors then tested an OspA-ferritin nanoparticle cocktail that included OspA serotypes 1, 2, 3, 4, 5 and 7. Robust antibody titers to each component OspA protein were demonstrated and the cocktail proved to be protective against challenge with *I. ricinus* ticks infected with *B. afzelii* (OspA serotype 2). The immunogenicity of the hexavalent formulation was compared with the comparator vaccine in non-human primates using the squalene-based adjuvant, AF03. The hexavalent formulation elicited an 11 to 200-fold higher antibody titer than the comparator. The durability of the antibody response, although relatively short (6 months), was also higher than RECOMBITEK® Lyme.

Baxter BioScience has also pursued a multi-valent OspA based vaccine that consists of three recombinantly engineered OspA based proteins (Wressnigg et al., 2013; Wressnigg et al., 2014). The three OspA antigens were designed to harbor protective epitopes from two different OspA serotypes. While the details on the molecular design of these proteins and the identity of the protective epitopes employed is not in the public domain, one

protein consists of epitopes from OspA serotypes 1 (*B. burgdorferi*) and 2 (*B. afzelii*); the second of OspA serotypes 3 and 5 (*B. garinii*) and the third of OspA serotypes 6 (*B. garinii*) and 4 (*B. bavariensis*). For each recombinant protein, the proximal end of one OspA serotype was fused to the distal portion of another OspA serotype. The strategy was to retain the overall structure of native OspA so as to maintain potentially conformational epitopes. All three of the unique recombinant antigens were lipidated. A double blinded, randomized, dose escalation, phase1/2 study was conducted at four sites in Austria and Germany. Study participants (age 18-70; n=300) were administered 30, 60, or 90 ug of the antigen formulated with or without aluminum hydroxide. The dosing schedule consisted of three intramuscular vaccinations 28 days apart with a fourth dose delivered nine to twelve months after the initial immunization. The primary immunization endpoint was 28 days after the third dose, at which time the IgG titer to each OspA serotype was measured. Individual recombinant OspA proteins representing each serotype served as the detection antigens. A dose response was not evident in participants that received the adjuvanted formulation, yet an inverse dose response was noted after the fourth vaccine dose. A similar observation was noted in clinical trials of two viral vaccines (Ehrlich et al., 2008; Aichinger et al., 2011). It is also noteworthy that the adjuvanted formulation of the OspA cocktail was better tolerated than the non-adjuvanted formulation. The authors suggest that alum may mask or shield the lipidation motif thereby inhibiting its interaction with Toll-like receptors that are mediators of inflammatory responses (Hirschfeld et al., 1999). The 30 μg adjuvanted vaccine was determined to be the best dose and formulation. The bactericidal activity of vaccinal antibody was assessed by incubating hyperimmune sera with strains producing each OspA serotype. While vaccination induced bactericidal antibody, the killing activity dropped to near baseline prior to the booster vaccination. This is consistent with the relatively short-lived bactericidal activity elicited by other OspA based vaccines. In a follow up study, the safety of the OspA vaccine cocktail was assessed in an adult population that had been previously diagnosed with LD. The vaccine was well-tolerated and immunogenic (Wressnigg et al., 2014). Further development was not pursued by Baxter. The rights to the vaccine ultimately went to Shire which did not pursue further development.

Another engineered OspA based vaccine formulation, VLA15 (Comstedt et al., 2014), is currently in development by Valneva. VLA15 consists of three engineered recombinant proteins that harbor the C-terminal domain of OspA serotypes 1, 3, 4, 5, and 6. To stabilize the recombinant proteins and allow for heterodimerization, molecular modeling was conducted to identify amino acid positions that could be substituted with cysteines and thus allow for inter-monomer disulfide bond formation. Both the monomeric and heterodimeric proteins displayed increased thermal stability. A cocktail of the three resulting heterodimers (referred to in the original study as the "LB vaccine") was assessed for immunogenicity and protective efficacy using the mouse model for LD. LB-vaccine was delivered with or without aluminum hydroxide in a three-dose series. Ten-fold greater anti-OspA IgG titers were generated with the adjuvanted vaccine. A series of challenge experiments were conducted by sub-cutaneous injection of *B. burgdorferi* ZS7 (serotype 1), *B. garinii* PHei (serotype 5) or *B.* afzelii (serotype 2) individually. The challenge dose of spirochetes was stated to be between 5x and 10x of the ID_{50}. However, since the ID_{50} was not detailed in the study, the number of spirochetes in the challenge dose is not known. Challenge was delivered two weeks after the third dose of vaccine and efficacy assessed with cultivation, PCR, seroconversion, and bactericidal growth inhibition titers serving as readouts. For the tick challenge experiments, *I. ricinus* ticks infected with *B. afzelii* IS1 (serotype 2) were used. In contrast to most challenge analyses that feed 5 or more ticks on each animal, only 2 ticks were fed on each mouse. While the challenge inoculum might be considered low, the percentage of placebo mice that became infected in each experiment was at least 87%. The protective efficacy varied slightly for each experiment but was >85% in all cases. In a follow up study, protection against strains expressing OspA serotypes 1, 2, 4, 5 and 6 was demonstrated (Comstedt et al., 2017). Protection against infection with serotype 3 was inferred from *in vitro* bactericidal assays.

Phase 1 studies of VLA15 have been conducted in Europe. After initiating a phase 1 study in which immunogenicity and safety were evaluated, Valneva amended its Phase 1 study protocol to determine if a booster vaccination administered to a sub-cohort of the Phase 1 study population was warranted. VLA15 demonstrated a favorable safety profile and no significant AHEs were noted. In addition, the final

Phase 1 immunogenicity results indicated that the alum-adjuvanted formulations elicit higher immune-responses at all time-points than the non-adjuvanted formulation. However, antibody titers declined post Day 84 across all groups, trending towards baseline at approximately one year post initial vaccination.

To evaluate the benefit of a booster dose, 64 subjects across the two higher-dose groups (48μg and 90μg; with and without alum) from the Phase 1 study received a booster 12 to 15 months after the primary immunization. The booster resulted in anti-OspA antibody titers that ranged from 2.7 to 5.8-fold higher than the titers observed at Day 84. In 2020, Valneva and Pfizer partnered to advance the VLA15 vaccine. The planned Phase 2 study will consist of 250 subjects that will receive one of two dosage levels of alum adjuvanted VLA15 (100 subjects each) or placebo (50 subjects). The investigational vaccine will be administered intramuscularly in three-doses on Days 1, 57 and 180. Study participants will be followed for 18 months with final immunogenicity readouts assessed on day 208. At the time of this writing, the initiation of the trial was pending. It remains to be determined if the dose vaccination schedule of this, and other potential OspA based vaccines, will be embraced by the public.

Chimeric epitope based OspC vaccinogens
As noted above, a significant challenge in the development of broadly protective OspC based vaccinogens is the type specificity of the antibody response (Wang et al., 1999; Brisson and Dykhuizen, 2004; Earnhart and Marconi, 2007c). With the identification of the antigenic determinants of OspC, recombinant proteins consisting of OspC linear epitopes derived from multiple OspC types were constructed (Earnhart et al., 2007; Earnhart and Marconi, 2007a, b). These unique epitope-based proteins are referred to as chimeritopes (Izac et al., 2020b). The original prototype chimeritope harbored L5 and H5 epitopes from OspC types A, B, K and D (Earnhart et al., 2007). The "ABKD" construct was immunogenic in mice and elicited antibodies that recognized all OspC types represented in the construct. Anti-ABKD antibodies readily surface-labeled intact spirochetes and displayed potent complement-dependent bactericidal activity. However, it was noted that the titers induced by immunization were lower for the C-terminal OspC type D epitope than observed for epitopes at the N-terminus of the chimeritope. The immunogenicity of the C-terminal epitopes was increased through

epitope reiteration, epitope reordering, and by increasing the solubility of the construct (Earnhart and Marconi, 2007a). The tetravalent construct was then expanded to an octavalent construct through the addition of L5 and H5 epitopes derived from OspC types E, N, I and C (Earnhart and Marconi, 2007b). When delivered to mice, the OspC-A8.1 construct (adjuvanted with alum) elicited high-titer antibody to each component epitope that efficiently surface-labeled diverse *B. burgdorferi* strains. The antibody response was skewed to IgG2a and IgG2b isotypes, both of which efficiently fix complement. As efforts continued to introduce additional epitopes into the chimeritope so as to expand OspC type representation, the increasing molecular weight of the chimeritope hampered expression of 'clean' full-length protein. The chimeritopes that had been produced up to this point harbored linkers between the individual epitopes to maintain protein flexibility. However, the linkers and sequences shared by some H5 and L5 epitopes from different ospC types accounted for nearly 30% of the molecular weight of the proteins. To determine if the linkers and redundant sequences could be eliminated or minimized without adversely affecting immunogenicity, three chimeritopes designated as Chv1, Chv2, and Chv3, were constructed and comparatively assessed (Izac et al., 2020b). All three Chv proteins possess the same set of 18 linear epitopes derived from 9 OspC type proteins but differ in the physical ordering of epitopes and by the presence or absence of linkers. The Chv proteins were immunogenic in mice, rats and dogs, eliciting similar antibody titers. Immunoblot and ELISA assays demonstrated that the Chv proteins elicit IgG that recognizes a diverse array of OspC type proteins including North American and European strains of the LD spirochetes. This important observation indicates that some of the L5 and H5 epitope-containing domains share identity with OspC types that are not directly represented in their entirety in the Chv proteins. Thus, it appears that the 18 epitopes present in the Chv proteins collectively elicit antibodies that can recognize the majority of OspC types. Anti-Chv antisera surface-labeled intact *B. burgdorferi* demonstrating that vaccinal antibody can bind to epitopes as they are presented on the cell surface. Immunization induced antibody also displayed potent complement dependent-antibody mediated killing activity. This study demonstrated that high-valance OspC chimeritopes can potentially elicit broadly protective antibody responses. OspC

chimeritopes have not yet advanced to a phase 1 clinical trial.

Other vaccine candidates (past and present)
A comprehensive assessment of all proteins that have been investigated as potential vaccine candidates is beyond the scope of this writing. Here we focus on those for which significant information was available at the time of submission. To begin, it is worthy of note that while numerous immunogenic proteins have been assessed as vaccine candidates, the majority do not induce bactericidal or protective antibody responses. Examples of immunogenic but non-protective antigens include OspD (Probert and LeFebvre, 1994), BmpA (also known as P39) (Gilmore et al., 2003), OspE (paralogs BBN38 and BBL39), CspA (McDowell et al., 2006), CspZ (Rogers et al., 2009a), and VlsE (Liang et al., 2001). Examples of immunogenic proteins that convey partial protective immunity include P35, P37 (Fikrig et al., 1997), BBA52 (Kumar et al., 2011), BB0405 (Kung et al., 2016), BBI39 (Singh et al., 2017), P66 (oms66) (Exner et al., 2000) and BB0172 (Small et al., 2014; Hassan et al., 2019). Studies conducted on a subset of these proteins are summarized below.

The outer membrane proteins BBA52, BBI39, and BBI36, have been reported to play possible roles in spirochete survival within fed ticks or during transmission to mammals (Kumar et al., 2010; Kumar et al., 2011; Kung et al., 2016; Singh et al., 2017). Each protein has been assessed for its potential to induce antibody that can block spirochete transmission. While each protein was immunogenic in mice (Freund's adjuvant), only limited protection against challenge by *B. burgdorferi* infected ticks was observed. Serum harvested from immunized mice also lacked significant *in vitro* bactericidal activity (Kumar et al., 2010; Kumar et al., 2011; Kung et al., 2016; Singh et al., 2017). Anti-BBI39 antisera was reported to have bacteriostatic properties against *in vitro* cultivated *B. burgdorferi* (Singh et al., 2017). The utility of a vaccine antigen that induces bacteriostatic immune responses remains to be determined.

The BB0172 protein is a von Willebrand factor A (vWFA) domain-containing protein that potentially functions as a metal-dependent, integrin binding protein (Wood et al., 2013). Proteinase K digestion and triton X-114 extraction and phase partitioning analyses demonstrated that BB0172 is an outer membrane protein (Wood et al., 2013). RT-PCR

analyses did not detect BB0172 transcript in cells cultivated under standard conditions. However, transcript was detected in cells recovered from cultures maintained at pH of 6.8 and 37 °C. Based on this observation and the absence of an antibody to BB0172 during mammalian infection, it was postulated that BB0172 is produced specifically in fed ticks. Peptides derived from B0172 protein were subsequently tested as transmission blocking vaccine antigens (Small et al., 2014). Four BB0172 derived peptides, designated as peptides A, B, C and D, were synthesized, conjugated to Keyhole Limpet Hemocyanin, formulated in Titer Max® adjuvant, and used to immunize C3H/HeN mice (3 dose immunization series). Vaccinated mice were syringe-challenged 28 days after immunization with 10^3 or 10^5 *B. burgdorferi* cells. Peptide B, but not peptides A, C, and D, elicited protection against a challenge dose of 10^3 spirochetes but not against a dose of 10^5 spirochetes. In addition, immunized mice were only partially protected when challenged with infected ticks. In a follow up study, the 12 amino acid PepB peptide was conjugated to Cross-Reacting Material 197 (CRM197) or to Tetanus Toxoid heavy chain (TTHc). C3H/HeN mice were immunized with each vaccinogen in alum and challenged with 10^5 spirochetes or with infected ticks. While CRM197:PepB was not protective, TTHc:PepB conveyed induced protective responses in a subset of mice. Follow up studies on BB0172 or PepB have not been published.

Factor H binding proteins as vaccine antigens
Numerous microbial pathogens including several *Borrelia, Borreliella,* and *Treponema* species, evade complement-mediated killing in part by binding to the cell surface negative regulators of complement. The contribution of members of the factor H protein family to complement evasion has been intensively studied (Hellwage et al., 2001; Alitalo et al., 2002; Kraiczy et al., 2003; McDowell et al., 2003a; McDowell et al., 2003b; McDowell et al., 2009; Rogers et al., 2009a; McDowell et al., 2012). Factor H binding proteins have been investigated as potential vaccine candidates. The fHbp protein of *Neisseria meningitidis* (Beernink and Granoff, 2009), has been successfully developed as a vaccine for meningococcal disease caused by type B *Neisseria meningitidis* (Beeslaar et al., 2020a; Beeslaar et al., 2020b; Borrow et al., 2020). FHbp is the main vaccinogen in two FDA approved and licensed vaccines: Bexsero® (GlaxoSmithKline) and Trumenba®(Pfizer). Studies of fHbp demonstrated

that the immunogenicity of the protein can be enhanced through the introduction of site-directed mutations that attenuate its ability to bind FH (Beernink et al., 2011; Beernink et al., 2012). The potential protective efficacy of two *Borreliella* factor H binding proteins, OspE and CspZ, have also been investigated (FHBP). While both proteins were immunogenic in mice (Hefty et al., 2002; Rogers et al., 2009a), they did not elicit bactericidal or protective antibody responses (Rogers et al., 2009a; Marcinkiewicz et al., 2018). To prevent masking of potentially protective epitopes by bound FH, CspZ was modified at two amino acid positions through site-directed mutagenesis to abolish factor H binding (Marcinkiewicz et al., 2020). The mutated protein was conjugated to virus-like particles and assessed for *in vitro* bactericidal activity and protective efficacy. Bactericidal activity was significantly enhanced using the mutated protein and immunization studies demonstrated protection in mice against both needle and tick delivered *B. burgdorferi* (Marcinkiewicz et al., 2020). FH binding proteins in general appear to be attractive candidates for vaccine development.

Veterinary Lyme disease Vaccines
Bacterin vaccines
The first USDA approved canine LD vaccines were formalin inactivated, *B. burgdorferi* cell lysate formulations (bacterins), produced by Fort Dodge Labs (Lyme Vax) and Schering-Plough Animal Health (Galaxy Lyme) (Chu et al., 1992; Levy et al., 1993). The licenses for these vaccines have since been transferred among companies over the past few decades and the original formulations modified to an unknown degree to decrease AHEs. The utility of one of the earliest canine LD vaccines was demonstrated in a study that compared the C6 seropositivity of client owned dogs vaccinated with Lyme Vax (Fort Dodge) with dogs that had not been vaccinated (Levy, 2002). Antibody responses to the C6 peptide of VlsE served as a readout of protective efficacy. Approximately 5% (8 of 163) of vaccinated dogs became C6 antibody positive overtime whereas 64% (25 of 39) of unvaccinated dogs seroconverted and were C6 antibody positive. These early studies established the health benefits and value of vaccination of dogs at risk for LD.

Currently, three LD bacterin vaccines are available on the North American market: NOVIBAC[7] Lyme (Merck; USDA approval, 03/19/2007) and Ultra™ Duramune® Lyme (Elanco; USDA approval 12/6/1995) and Duramune® Lyme (Elanco;

12/6/1995). Duramune® Lyme and Ultra™Duramune® Lyme differ primarily in the vaccine dose volume: 1.0 mL versus 0.5mL, respectively. It has not been demonstrated in the literature if the reduction in dose volume conveys added protection or decreases the potential for AHEs. All LD bacterin vaccines distributed in the United States consist of two strains of *B. burgdorferi*. Information provided by the manufacturers indicate that the rationale for the bivalent formulations is to include a strain that expresses OspA and a second strain that produces elevated levels of OspC. Since OspA is typically produced at high levels by wild-type laboratory cultivated strains, anti-OspA antibodies are indeed likely to be elicited upon vaccination and contribute to the neutralization of *B. burgdorferi* in ticks. However, claims regarding elevated OspC expression by the second strain in these vaccine formulations have not been rigorously substantiated by the manufacturers, or by independent peer reviewed publications. While NOVIBAC® Lyme has a USDA approved 12-month duration of immunity label, duration of immunity information is not available for Duramune® Lyme or Ultra™ Duramune® Lyme. Combination vaccines have been developed in which LD bacterin formulations were combined with *Leptospira* bacterins and antigens from parvovirus type b, adenovirus type 2, coronavirus, and canine distemper. There is limited information in the public domain regarding the efficacy of combination vaccines and hence, they are not discussed here. In Europe, a trivalent bacterin vaccine consisting of *B. afzelii, B. garinii,* and *B. burgdorferi* strains is available (MeriLym3; Merial). The rationale behind the trivalent formulation is to provide broad protective efficacy against the three primary *Borreliella* species associated with canine LD in Europe. While this appears conceptually sound, to our knowledge, evidence demonstrating specific protection against each species is not in the public domain.

While bacterin vaccines have played an important role in the ongoing development of LD vaccines for companion animals, a shift to vaccines of defined antigenic composition is desirable for several reasons. First, bacterins consist of all proteins produced by a LD spirochete during laboratory cultivation. Sequence analyses have revealed that the genome of a typical *Borreliella* strain harbors approximately 1500 open reading frames (Fraser et al., 1997; Casjens et al., 2000; Casjens, 2003; Casjens et al., 2011). Transcriptional profiling analyses have demonstrated that cultivated *B.*

burgdorferi produce over 1000 different proteins (Iyer et al., 2015), the majority of which are not presented on the cell surface. This leads to non-productive antibody responses since intracellular proteins cannot be targeted by vaccinal antibody. Secondly, some *Borreliella* proteins that are selectively produced in the mammalian environment are absent from these vaccine formulations. Proteins expressed in mammals are advantageous as vaccinogens as they have the potential to elicit immune memory responses upon exposure of a vaccinated dog to *B. burgdorferi* infected ticks.

Recombinant protein-based subunit vaccines
The second general class of canine LD vaccines are subunit vaccines. LD subunit vaccines differ from bacterins in that they are of defined composition and contain well-characterized proteins that have been demonstrated to elicit protective antibody responses (reviewed in (Earnhart and Marconi, 2009; Izac and Marconi, 2019)). The use of vaccines with low complexity antigen content is important in the veterinary setting since multiple vaccines are typically delivered during a single visit. By eliminating extraneous antigens, the potential for vaccine induced AHEs is theoretically reduced. The first canine subunit vaccine to advance to market, RECOMBITEK® Lyme (Boehringer-Ingelheim; USDA approval; 03/27/1995), consists of lipidated OspA (90 µg) in a non-adjuvanted formulation. Efficacy and other important study data for RECOMBITEK® Lyme are not available on the USDA website because manufacturers were not required to compile and publish the data for vaccines evaluated by, and approved by USDA-APHIS, prior to 01/01/2007. However, post-licensure efficacy studies have been conducted (Conlon et al., 2000). In a blinded, controlled study using purpose-bred, mixed-breed dogs (10 to 12 weeks of age), vaccinate dogs received two-doses of vaccine, three weeks apart (Conlon et al., 2000). Sentinel dogs (not exposed to ticks, *B. burgdorferi,* or vaccine), which can provide information regarding the frequency with which random events may occur, were not included in the study. Three weeks after challenge with field collected ticks, the dogs were assessed for *B. burgdorferi* antibody by ELISA and immunoblot. Biopsies were also obtained for cultivation and PCR analyses. The vaccinate dogs were seronegative except for vaccinal antibody to OspA, and none were culture or PCR positive. Vaccinates did not present with clinical signs indicative of LD. In contrast, all placebo dogs were antibody and culture positive and

nine of ten were PCR positive for *B. burgdorferi* DNA. Twenty percent of the placebo dogs developed symptoms indicative of LD. Vaccinates were protected from challenge delivered three weeks after the last boost. Long-term protective immunity was not assessed.

A subsequent study, conducted at a private veterinary hospital in Maine, assessed the ability of the vaccine to prevent reinfection in vaccine compliant dogs over a five year period (Eschner and Mugnai, 2015). The authors reported that 1% of the fully compliant dogs, defined as receiving all intended vaccine doses, developed serological responses indicative of LD. Of the dogs that were incompletely vaccinated (defined as missing one or more booster doses), 357 positive C6 antibody tests were reported (the number of dogs that this corresponds to was not indicated). The vaccination protocol used in this study did not follow the manufacturers recommended protocol. Label instructions call for a two-dose series, three weeks apart, and an annual booster thereafter. In the above referenced study, three vaccine doses were delivered during the first year of use (0, 1 and 6 months), followed by a single vaccine dose each year thereafter. The modified protocol was adopted based on a study that demonstrated enhanced antibody responses when a third dose of vaccine was delivered in year 1 (Topfer and Straubinger, 2007). This study, as well as the human OspA vaccines studies detailed above, speak to the utility of OspA as a vaccine component. However, a common underlying issue with all OspA alone based vaccines is the need to deliver frequent boosters.

VANGUARD®crLyme (Zoetis), the most recent canine LD vaccine to be approved by the USDA (12/23/2015), is a subunit vaccine consisting of OspA and an OspC chimeritope (Marconi, 2020b) designated as Ch14. The OspA component of VANGUARD®crLyme is non-lipidated and delivered at a 10µg antigen dose whereas the OspA in RECOMBITEK®crLyme is lipidated and delivered at a 90µg antigen dose. A recent study compared the IgG titers elicited by the OspA in these subunit vaccines in purpose bred dogs (Grosenbaugh et al., 2018). After a two-dose series the IgG titers elicited by lipidated OspA versus non-lipidated OspA were similar and differences were not statistically significant. Hence, lipidation and dose do not appear to influence anti-OspA IgG titer. As alluded to above, the OspC chimeritope in VANGUARD®crLyme, Ch14, is single recombinant protein that harbors at least 14

different linear epitopes derived from several antigenically distinct OspC proteins. The epitopes selected for inclusion included some that are predicted to elicit antibodies that can recognize multiple diverse OspC types. The efficacy of VANGUARD®crLyme was demonstrated in purpose bred dogs (7.1 to 7.7 weeks of age; 18 males; 18 females). Vaccinates received two-doses of vaccine (three weeks apart) by subcutaneous injection in the left dorsal scapular area. Three weeks after challenge, the vaccinates and placebo dogs were challenged with 30 ticks (15 males and 15 females; field collected from Rhode Island). Blood and tissue biopsies were collected at defined timepoints post-challenge. The dogs were euthanized approximately four months after challenge and necropsied for histopathology analyses. The serum samples collected over the course of the study were tested for antibody to the VlsE derived C6 peptide. Sentinel dogs, which were not exposed to *B. burgdorferi,* vaccine or ticks at any point during the study, were C6 antibody negative throughout. All placebo dogs (16/16) were C6 antibody positive on two or more occasions post-challenge. Fifteen of sixteen vaccinates were C6 antibody negative at all timepoints. A single vaccinate was C6 antibody positive 35 days after initiation of challenge but was antibody negative thereafter. Histopathological analyses of tissue from at or near the tick bite site identified nodular lymphoplasmacytic inflammation in the hypodermis/subcutis typical of *B. burgdorferi* infection in 14 of 16 placebo dogs, but not in the vaccinate or sentinel dogs. Within and around joints, histopathological changes observed in placebo dogs included synovial hypertrophy and hyperplasia (including papillary projections), mononuclear and neutrophilic inflammation within the joint and local subcutis, and fibrin deposition. Intra-articular lymphoplasmacytic inflammation was identified in 13 of 16 placebo dogs, 0 of 16 vaccinates, and 0 of 4 sentinel dogs. Inflammation was identified in more than one joint of each placebo dog. It can be concluded that vaccination resulted in a significant difference in the prevention of *B. burgdorferi* infection compared to placebo ($P < 0.0001$). Vaccination also prevented the synovitis and dermatitis typically associated with *B. burgdorferi* infection (p<0.0001). In 2017, VANGUARD®crLyme was approved for a 15-month duration of immunity label by the USDA. This is the longest duration of immunity label for any LD vaccine on the market (Littman et al., 2018).

A comprehensive field safety study using two serials of prelicensed VANGUARD®crLyme demonstrated an excellent safety profile (Marconi, 2020a). The vaccine was tested in its target population (dogs) under the conditions of its intended use. Six-hundred and twenty dogs, from three distinct geographic regions of the United States, were enrolled in this study with each receiving two-doses of vaccine 3 to 4 weeks apart by subcutaneous injection. Approximately, one-third of the dogs were ≤8 weeks of age as required to meet regulatory requirements. Safety was evaluated by observation of local and systemic reactions for at least 10 days after each vaccination. Minor AHEs occurred at low frequencies and serious AHEs were not observed. The study results demonstrated that VANGUARD®crLyme is safe for use in healthy dogs 8 weeks of age or older.

DNA based vaccine candidates

Two of the first studies to investigate DNA based LD vaccines assessed the protective potential of OspA encoded by different delivery plasmids (Simon et al., 1996; Luke et al., 1997). O*spA* was inserted into the pEF-BOS plasmid which carries the promoter/enhancer region of human elongation factor 1-alpha (EF-1alpha). A similar construct was generated that the lacked the EF-1alpha promoter enhancer region. One to four doses of each plasmid were delivered to BALB/c and AKR/N mice via intramuscular injection. Antibody to OspA was readily detected even after a single dose and the IgG titer increased in a dose dependent manner. Efficient antibody production was determined to not be dependent on the presence of the EF-1alpha promoter/enhancer element. The dominant IgG isotypes induced by vaccination were IgG2a, IgG2b and IgG1. Immunized mice were challenged with *B. burgdorferi* ZS7 (10^4 cells) and deemed to be fully protected. Passive transfer of the sera from vaccinated mice to SCID mice also provided protection.

An *ospA* based plasmid construct has also been tested. The DNA backbone of the plasmid was derived from the expression plasmid pVR2210. The construct carries *ospA* fused to the leader peptide of human tissue plasminogen activator (htpA) (Luke et al., 1997). A similar plasmid was constructed with *ospB* fused to the htpA leader peptide. Mice were administered three-doses of the constructs, two weeks apart. Sera were harvested from the vaccinated mice and assessed for OspA specific IgG antibody titer. However, the titer was determined to be 3 to 4-fold lower than that obtained using a

comparator vaccine consisting of lipidated, recombinant OspA. *In vitro* growth inhibition assays demonstrated that sera from the *ospA* DNA vaccinated mice was inhibitory to growth. Mice that received the *ospA* DNA construct were protected against syringe challenge with 10^4 spirochetes delivered 76 days after the last vaccine dose. *OspB* was also investigated but it did not elicit protective antibody responses.

In a recent study, the efficacy of a proprietary DNA vaccine (pLD1; Inovio Pharmaceuticals) comprised of a synthetic consensus (SynConR) OspA combined with CELLECTRAR *in vivo* delivery technology was assessed. VANGUARD®crLyme served as a comparator (Guibinga et al., 2020). Results from both bacterial tissue burden and bacterial growth inhibition assays demonstrated equivalent protective efficacy of pLD1 and the comparator vaccine.

OspC based DNA vaccines also have been tested and demonstrated to elicit protective antibody responses (Scheiblhofer et al., 2003). *OspC* was fused to the htpA leader sequence to yield pCMV-TPA/ZS7. BALB/c and C3H/HeJ mice were immunized with the plasmid or with recombinant OspC as a comparator. Vaccine was administered by gene gun or syringe injection. In both mouse strains, purified protein as well as gene-gun delivered plasmid DNA skewed to Th2 type responses. In contrast, plasmid delivered by needle injection resulted in Th1 type antibody production. Mice challenged with 10^4 *B. burgdorferi* cells were protected with an efficacy range of 80% to 100%. This was the first study to demonstrate that a DNA vaccine encoding OspC induced protection against LD. OspC DNA constructs also have been delivered by DNA tattooing (Wagemakers et al., 2014). DNA tattooing is a novel vaccination approach that can be applied with an accelerated vaccination schedule (0, 2 and 4 weeks) (Wagemakers et al., 2014). The codon optimized *ospC* of *B. afzelii* PKo was assessed for efficacy with the corresponding recombinant protein serving as the comparator. The IgG responses induced by both approaches were comparable except that that IgG1/IgG2a ratio was lower when administration of the vaccine by DNA tattoo. Two weeks after syringe-challenge with 5 × 10^5 *B. afzelii* Pko spirochetes, most vaccinated mice were negative for *B. afzelii* DNA in tissues and all biopsies were culture negative. However, the DNA vaccine was not protective against heterologous strains including *B. burgdorferi* N40. Protection

against tick challenge and long-term protective efficacy were not assessed. As described elsewhere in this review, the strain-specific protection reported in this study is not surprising in light of OspC diversity. Additional studies investigating DNA based LD vaccines have also been conducted, and varying levels of protection have been described (Wallich et al., 2001). As reported for vaccination studies using recombinant proteins, a multi-valent approach will most likely be required for DNA vaccines to elicit broadly protective antibody responses.

Reservoir targeting vaccines
Considerable research has focused on devising vaccination-based strategies to reduce the carriage rate of the LD spirochetes in specific reservoir populations. It follows that if carriage in reservoirs can be reduced, there will be a concomitant reduction in the percentage of ticks that are infected. In Europe, the yellow-necked mouse (*Apodemus flavicollis*) is an important reservoir for *Borreliella* species. In one study, yellow-necked mice were immunized with recombinant lipidated OspA (Kurtenbach et al., 1997) adjuvanted with ABM3. Three doses (10 µg protein) were delivered at 13-day intervals. Consistent with studies in laboratory mice, all immunized mice developed robust antibody responses to OspA including antibody to the epitope recognized by the protective monoclonal antibody LA-2. Infected *I. ricinus* ticks were cleared of infection after feeding on the vaccinated mice. This study was among the first to raise the possibility that natural mouse reservoir hosts are competent to generate transmission-blocking antibodies after vaccination that could potentially decrease carriage of *Borreliella* species in mice (Kurtenbach et al., 1997).

In another study, *Peromyscus leucopus* were infected with *B. burgdorferi* and then vaccinated with an OspA-GST fusion protein (Tsao et al., 2001). Using xenodiagnoses, it was demonstrated that ticks that fed on the vaccinated mice had a significantly reduced capacity to become infected with *B. burgdorferi*. The authors concluded that vaccination of *P. leucopus* with OspA could reduce transmission of *B. burgdorferi* to the tick vector, even in mice with prior infection.

In a controlled field study conducted in Connecticut, 1000 wild *P. leucopus* mice were captured, tagged, vaccinated with non-lipidated, recombinant OspA (N-terminal GST fusion) adjuvanted with Freund's and released. Follow up analyses at the defined field sites

revealed that vaccination reduced the prevalence of *B. burgdorferi* in nymphal *I. scapularis* ticks. However, the study also revealed that non-mouse hosts played a more significant role than expected as a source for infecting ticks. The authors concluded that a wildlife vaccination strategy focused only on mice would be insufficient to decrease carriage rates overall and that vaccination of additional reservoir species would be required (Tsao et al., 2004). This is consistent with studies detailed above that revealed that inconspicuous hosts and birds are significant reservoirs for the LD spirochetes (Anderson et al., 1986; Brisson and Dykhuizen, 2004; Scott et al., 2018).

Parenteral vaccination of wildlife at a level sufficient enough to significantly reduce carriage of LD in reservoirs is arguably not a feasible and sustainable strategy. Hence, others have turned their efforts to vaccination using bait vaccines or reservoir targeting vaccines. An ideal vector would be one that is environmentally stable and able to elicit protection after a single dose. In addition, it would need to be non-toxic to both targeted and non-targeted wildlife species. Vaccinia virus has been suggested to meet these criteria and to be desirable due to its high levels of protein synthesis and ability to accept foreign DNA without losing infectivity. Precedent for the use of Vaccinia virus can be found in the oral rabies vaccine, Raboral™, which is targeted at raccoons and foxes (Pastoret et al., 1995). Oral gavage vaccination of C3H/HeN mice with Vaccinia virus encoding *ospA* resulted in protection of vaccinated mice against *B. burgdorferi* and significant clearance of *B. burgdorferi* from ticks that were fed on the vaccinated animals (Scheckelhoff et al., 2006). This approach was extended by delivery of the vaccinia virus-based vaccine to *P. leucopus* by oral gavage (Bhattacharya et al., 2011). The vaccine was found to be efficacious in decreasing the acquisition of *B. burgdorferi* by larval ticks and in decreasing transmission from ticks to *P. leucopus*. In another study, an OspA bait vaccine was developed using *E. coli* expressing OspA from a plasmid (Meirelles Richer et al., 2011). Lyophilized cells (200mg) were mixed with rolled oats and provided ad libitum. Mice that consumed the vaccine over periods of 1 or 4 months developed a neutralizing IgG response that persisted for 1 year. Based on the outcome of this study, the authors postulated that delivery of the vaccine twice a week for a period of 4 months from mid-April until mid-August could provide protection to young mice that are born throughout

two seasons. In a prospective 5-year field trial, oral vaccination of wild white-footed mice resulted in reductions of 23% and 76% in the nymphal infection prevalence in a time-dependent manner (Richer et al., 2014). Significant decreases in infection prevalence were observed after 3 years of vaccine deployment. Hence, it is possible that a long-term bait vaccine strategy could be employed to reduce LD exposure risk. However, due to the limited migration range of mice and other small mammal reservoirs, wide and high-density distribution of bait vaccines over expansive geographic areas would be required. It remains to be determined if the costs of such an approach are feasible and sustainable.

Closing comments

Vaccination offers the most cost-efficient and reliable approach to address the expanding LD problem in both humans and companion animals. There have been advances made in the development of LD vaccines over the past 20 years that offer significant hope for those at risk for this debilitating disease. New technologies and innovative approaches have been employed that offer great promise and next generation LD vaccines for humans are undergoing clinical trials. However, as a cautionary note we must not forget or ignore the lessons learned from the rise and fall of LYMErix, the only human vaccine to advance to market. The perceived safety concerns that led to the voluntary removal of the vaccine from the market had a damaging effect on subsequent efforts to advance a human LD vaccine. Research on LD vaccines slowed and the interest of big pharma to take up LD vaccine development programs essentially came to a halt. Fortunately, interest in LD vaccines for humans has been renewed. It has become increasingly apparent that a vaccine that can recapture public interest and trust will need to be safe and convey long-lasting protection without the requirement for an aggressive booster schedule. As detailed above, it remains to be determined if a vaccine consisting only of OspA will meet these criteria. Vaccines that elicit protective antibodies that can work synergistically to attack multiple targets on the LD spirochete surface, and that have broad protective efficacy, are, in this authors opinion, our best, and potentially last opportunity to bring a successful vaccine to market.

Acknowledgements

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