Chapter 8

Summarizing discussion

This thesis covers research regarding pathogenesis as well as diagnosis and clinical presentation of an infection with *B. burgdorferi*. *B. burgdorferi* is an extracellular pathogen. It is therefore plausible that the humoral immune response in the form of complement-mediated bactericidal activity is of importance in the defense against infection and disease. It has previously been shown that distinct *B. burgdorferi* strains are able to withstand complement mediated killing in vitro.

Complement and other immune evasion strategies

The mammalian-vector cycle that Borrelia use to replicate is an elaborate lifecycle with many potential hosts. Not only is *B. burgdorferi* able to maintain itself in the vector at very low temperatures, but it is also able to withstand the relatively high temperatures of the mammalian host. Furthermore *B. burgdorferi* can infect a wide range of hosts, while all hosts have their own specific set of receptors and proteins to withstand infections. One of the proteins that *B. burgdorferi* can bind in the mammalian and avian host is complement factor H (CFH) and in humans specifically factor H like protein-1 (FHL-1). In the mammalian and avian host these proteins are responsible for inactivating the complement cascade at the C3 level *B. burgdorferi* possesses several proteins to bind CFH and FHL-1, these were previously designated as Complement Regulator-Acquiring Surface Proteins (CRASP) [74]. Several CRASPs from several species from the *B. burgdorferi* sl complex have been described and every CRASP is responsible for a specific time point in the infection cycle where complement is inactivated. CRASP-1 is thought to be responsible for inactivation of CFH and FHL-1 early in the infection [539].

In **chapter 2** of this thesis we describe two new CRASP-1 proteins of *B. bavariensis*, BGA66 and BGA71, that can bind human CFH and/or FHL-1, but we also showed that several other paralogues located on the same plasmid, and very likely under the same promoter can bind to CFH from several other mammalian and avian species. All the genes from the gbb54 paralogous family are upregulated at the same moment, namely at the time of tick feeding on the host. This means that independent of the type of host, different CFH-binding-proteins are expressed that are able to bind CFH and deactivate MAC formation on the borrelial surface in the tick midgut. Later in the infection other CFH-binding proteins are upregulated and CRASP-1 proteins are downregulated [79]. These proteins able to bind CFH later in infection, namely the Erp proteins, are a large family of similar proteins located on the cp32 plasmid of which borrelia can bear several non-similar copies [584]. It is likely that these proteins are also able to bind fluid phase complement regulators of a wide range of hosts. In

order to understand more about the mammalian-tick-lifecycle these CRASPs should be studied for host specificity.

In the **chapter 3** we studied whether infectivity from several species of *B. burgdorferi* that have been reported to be pathogenic in the human host would be altered in the absence of an effective complement system. In this study we showed that absence of complement did not lead to increased infectivity of complement sensitive strains. Studies on the role of complement in infectivity of *B. burgdorferi* have been conflicting, finding no or only a minimal effect on burden or disease severity [98, 191]. A study that used a CRASP-1 knockout *B. burgdorferi* ss strain for detecting complement resistance has shown a large negative effect on the binding of CFH and a decline in serum resistance in vitro. This strain could not be tested in vivo, because the strain itself was unable to give infection in the mammalian host [526]. In a CRASP-2 knockout *B. burgdorferi* ss strain no alteration in serum resistance was found in vitro and no effect was seen on infectivity in a mouse model [86]. Complementation of a CRASP-4 gene in a serum sensitive strain did not alter serum sensitivity [88].
An additional hurdle in unraveling the role of complement-binding proteins in *B. burgdorferi* infection is caused by differences in virulence among the strains. Reference *B. burgdorferi* ss strains are virulent as well as pathogenic in mice, in our study displaying a positive PCR as well as rapid positive cultures. On the other hand we found that both *B. afzelii and B. bavariensis* strains had positive PCR results from many organs, while we were only able to culture from heart tissue. Apparently *B. afzelii* and *B. bavariensis* were more difficult to reculture spirochetes from organs in BSK-H medium. The used *B. garinii* and *B. valaisiana* strains failed to cause murine infection altogether. A possible explanation is that these strains have lost essential plasmids and essential virulence factors during in vitro culture. The low virulence in mammals of *B. garinii* and *B. valaisiana* strains has been described before; it is likely the lack of infectivity in mice is due to the preference of these species for infection of avian hosts [24, 585].

Genetic tools for transformation of strains of the *B. burgdorferi* sl complex have been described, but transformation of low passage strains has proven to be tedious. Transformation of higher passage plasmids is possible, but these strains often lack plasmids that are essential for natural infection [586, 587]. This complicates the ability to study the effect of alteration of CRASPs on the infectivity in vivo. Knock-in of genes in *B. garinii* strains has been successfully performed, albeit not in an infective strain [88]. We were not successful in producing knockout CRASP-1 *B. afzelii*, *B. bavariensis* or *B. garinii*. Therefore it was impossible to do additional studies on knockout mutants of *B. afzelii*, *B. bavariensis* and *B. garinii* strains.

In our model we studied complement resistance by syringe inoculating the cultured *B. burgdorferi*. We now know that the system of up and downregulating proteins in the tick-host cycle is a complex and meticulous system involving many proteins that are able to alter serum sensitivity. The CRASPs for inactivating complement by binding to CFH, but also OspC and very likely many other outer surface proteins can bind tick derived salivary proteins that can aid entry and persistence of *B. burgdorferi* in the vertebrate host. Furthermore the wide scala of mammalian hosts and the specificity of specific complement altering proteins for specific hosts further complicate studying these proteins. This complex combination of expression of proteins makes it difficult to study the individual roles of the different proteins in the entire infective process. One can argue that perhaps specific proteins in the cycle are not essential but all contribute small pieces that cumulatively ensure adequate transmission of *B. burgdorferi* in the large group of potential hosts.

Is the laboratory and clinical diagnosis of Lyme disease really so complicated?

The difficulties observed in the laboratory diagnosis of *B. burgdorferi* strikingly contrast with the broad introduction of many rapidly evolving techniques for detection of microorganisms in modern clinical practice. Until now no good gold standard for detection of active infection with *B. burgdorferi* sl has been developed. What makes *B. burgdorferi* sl so special that the laboratory diagnosis seems to be so difficult?

Direct detection of *B. burgdorferi*

The most reliable way of diagnosing an infection with a microorganism is cultivating the organism which causes the disease from blood or the site of infection. Cultivating the pathogenic organism from a diseased individual proves that viable organisms can replicate and can cause infection. Bacterial culture is widely applied in the field of diagnostic microbiology. *B. burgdorferi* sl is a highly fastidious organism, some species of *B. burgdorferi* sl are more difficult to culture, which we have shown in **chapter 3** in an in vivo experiment. *B. burgdorferi* sl culture is laborious and can take up to several weeks and the sensitivity of culture in an active infection is too low to use it in a routine diagnostic setting [235].

Other ways of direct detection of a pathogenic microorganism can be direct visualization, for example by IFA. This generally has a very low sensitivity due to

the low load of Borrelia in the host [235]. This has also been demonstrated in **chapter 3** of this thesis.
Nucleic acid amplification techniques have been widely applied during the last two decades for detection of pathogens. In general they are considered to be very sensitive diagnostic tools for diagnosing infection with fastidious or uncultivable organisms. Most infections have a high load of microorganisms in tissue or blood (up to 10^9 copies/ml blood), but *B. burgdorferi*, specifically in disseminated infection, are present only at very low levels. The low load of *B. burgdorferi* in tissue may lead to false negative PCR results during active infection. However, as is demonstrated in **chapter 3** of this thesis, PCR on infected tissue does give the highest sensitivity of the abovementioned techniques. Direct detection of *B. burgdorferi* can have an additive effect in a specific individual, but the general conclusions is that currently available direct detection methods for *B. burgdorferi* are insufficiently sensitive to in- or exclude active *B. burgdorferi* infection [257].

Indirect detection of *B. burgdorferi*

Indirect detection of recent or past infection by serology is a common diagnostic tool for fastidious or uncultivable microorganisms. In the diagnosis of *B. burgdorferi* infection it is the most frequently used technique, because of the abovementioned reasons. In the development of serological tests it is logical to start with whole cell lysates. Development of serologic tests of bacteria has some major complications, because bacteria share many homologous proteins with other bacteria. Antibodies induced against major structural proteins can cross react with antibodies against other, similar, bacteria. Using either a whole bacterium in an immunofluorescent assay (IFA) or a whole-cell bacterial sonicate in an enzyme immunoassay (EIA) can easily give false positive results because of the abovementioned reasons. Serology for bacterial diseases can therefore be more compromised than for many clinically relevant viruses that contain a relatively unique and more limited amount of different proteins.

In viral serology often virus-specific conserved proteins have been isolated and used for specific serology, like gp41 or gp120 in HIV infection [588]. Nowadays, in virological serology many virus EIAs are built with recombinant antigens. In *B. burgdorferi* sl some specific recombinant antigens have been identified that elicit good antibody responses in many infected individuals, but are uncommon in other bacteria. Examples of these are VlsE (or C6-peptide), OspA, OspC, DbpA and flagellin internal fragment. The setback of using these antigens is that the species in the *B. burgdorferi* sl complex that are able to cause disease

in humans are so different that sometimes only a mix of an antigen like OspC from several species is warranted to obtain positive serology in all affected individuals. As the *B. burgdorferi* sl complex in North America only consists of *B. burgdorferi* ss this is mainly a problem in the Eurasian continent [589].

In **chapter 4** we show a high sensitivity of anti-C6-peptide serology in serum and CSF in the manifestation of Lyme neuroborreliosis in Dutch patients. VlsE or C6-peptide based assays are now the most commonly used commercial diagnostic strategy for screening in the Netherlands. It can be debated whether a screening assay solely based on one peptide will detect all different species of *B. burgdorferi* sl. If we compare this to commercial EIAs for other pathogens often at least two different antigens are used in the screening EIA. Primarily C6-peptide is conserved between species, while VlsE is less conserved between species. From literature however there is little reason to presume that the VlsE or C6-peptide based assays lack sensitivity to detect patients with later manifestations of Lyme borreliosis [314, 345, 590, 591]. It has been demonstrated however that species specific C6-peptide has a higher sensitivity especially in early manifestations of LB [345]. For early manifestations of the illness additional antigens can improve sensitivity, for instance OspC, being a good candidate in the IgM EIA [592].

A more specific way to look at antibody responses is a (native) western immunoblot. Antibodies against a specific subset of bacterial proteins can be measured, but the interpretation of the immunoblot is highly dependent on the algorithm that is used for determining positivity. It was previously shown that the different species throughout Europe of *B. burgdorferi* sl can have different serological profiles. A previously mentioned suggestion was that perhaps different algorithms for interpretation of the immunoblot for different region with prevalence of other *B. burgdorferi* sl species should be used [303]. In this setting and considering that a specific species of the *B. burgdorferi* sl complex can cause specific clinical manifestations it could be useful to define different interpretation algorithms for specific clinical manifestations. In a routine laboratory setting this is difficult to implement, because the clinical microbiologist does not always have the clinical data at hand.

The presence of anti-pathogen IgM generally is an indicator of early or active infection. However, in Lyme disease it has already been demonstrated that IgM can stay positive after effective treatment for many years [317, 326-330]. Furthermore, IgM is not an indicator of active disease. In late manifestations of Lyme disease IgM can often be absent, while there is still an active infection. This combination can easily lead to misinterpretation of serologic results. For

late manifestations of Lyme disease anti-Borrelia IgM results should play no role in the interpretation of serological results. An example of a proposed algorithm to interpret *B. burgdorferi* serology is shown in figure 1.

For some microorganisms antibodies against early and late expressed proteins are detected in combination to distinguish past from recent infection. In this case Epstein Barr Virus (EBV) would be an excellent example. Anti-EBV-Viral Capsid Antigen (EBV-VCA) IgG is used as a marker for EBV infection, and presence of EBV-Early Antigen (EBV-EA) IgG and absence of EBV-Nuclear Antigen (EBV-NA) IgG generally indicate recent infection [593]. In the case of EBV-infection the most common clinical manifestation is a recent primary infection. It is known that in *B. burgdorferi* infection certain proteins are expressed early during transmission to the mammalian hosts. Examples are OspC, OspA and several CRASPs. Although anti-OspC is a good marker for early infection, antibodies against OspC can not distinguish past from present infection. To date no conserved *B. burgdorferi* antigen has been described that can distinguish cleared from active infection. The conserved C6-peptide seemed to be a promising antigen for detecting active infection because antibody titers are highly elevated and can drop after treatment. The seroprevalence of anti-C6-peptide antibodies is lower than for VlsE, making it likely that anti-C6-peptide would be a more suitable marker of active disease [591]. This is also clearly demonstrated in figure 1 of **chapter 4** of this thesis; the patients with recent or active LB have higher anti-C6-peptide values than the controls. However, this phenomenon is clearly measurable on a population level, but will often not lead to a solid conclusion in an individual case in a single serum sample. An infection with *B. burgdorferi* is dependent on many factors, including host response. The combination of the heterogeneity of *B. burgdorferi* sl, with the heterogeneity in clinical presentation and the duration from infection to clinical presentation make the search for biomarkers, antigens or serological profiles that can distinguish cleared infection from active present infection an almost impossible quest.

Other surrogate markers in serum are sometimes used for detecting infection with spirochetes. One example is the anti-cardiolipin antibodies that are found during active *T. pallidum* infections [594, 595]. It is well known that anti-cardiolipin are not specific and also detectable in a number of other inflammatory diseases but for example also during pregnancy [596]. Detection of these antibodies has a solid status in the detection of clinical response to treatment for syphilis. The test strategy consists of detecting of specific anti-*T. pallidum* antibodies together with anti-cardiolipin antibodies. The titers against anti-cardiolipin are followed over prolonged time intervals to detect treatment response, failure

and perhaps reinfection. During infection with *B. burgdorferi* anti-cardiolipin (IgM) antibodies can be positive but they are usually negative in patients with active infection [597]. Screening for anti-cardiolipin as it is used in *T. pallidum* serology is not a suitable option in Lyme patients.

One of the additional problems in Lyme disease serology is that residual complaints after diagnosis and treatment of Lyme disease have been described, but that no serological test is able to discriminate between persistent infection and residual antibody response after successful treatment. Again, anti-C6-peptide antibodies in many patients decline over time. Although it will not be a marker that can give a black or white answer, a significant decline in titers, or even becoming anti-C6 seronegative after treatment is a strong clue that the original antigen has been eliminated. In specific cases this can perhaps aid the clinician in the choice between treatment directed on reducing symptoms instead of an antimicrobial treatment. Other markers of infection have been studied; a biomarker of recent interest is CXCL13. In serum this is not a good predictor of active *B. burgdorferi* infection [383, 390]. A complicating factor is that treatment failure measurable by objective parameters is extremely rare, so serological follow up of patients with persistent complaints can never be compared to a worthy gold standard.

Diagnosing Lyme neuroborreliosis

For the diagnosis of Lyme neuroborreliosis (LNB) the gold standard is the antibody index for anti-Borrelia antibodies. The problem with this assay is that not all routine clinical laboratories have the capacity to perform this assay. Also there is a lack of expertise in interpreting the results as LNB is a relatively rare diagnosis, while the request for LNB diagnostics is fairly high. There is a need for a screenings assay on CSF without having to implement new assays in routine laboratories.

In **chapter 4** we developed and tested a protocol for CSF using a commonly used antigen for the screening EIA, namely C6-peptide. We found a high sensitivity of the anti-C6-peptide serology for detection of antibodies in the CSF in case of a LNB. Therefore the anti-C6 peptide assays can be used to screen for LNB. Complete absence of anti-C6-peptide antibodies in the CSF makes a LNB highly unlikely. As is often the case with a screening test the specificity of the C6-peptide total Ig assay is not so high, but 93% of all non-neuroborreliosis patients could be excluded with just one test on the CSF. Confirmation with the gold standard for detecting LNB, calculating the AI, is less sensitive, but has a higher specificity. It is advisable that the confirmation assay is performed in a

laboratory that has clinical expertise in the field of LNB and interpreting the results from the calculated Ig-index assays. This means also taking into account the duration of illness, (multiple) treatment before the lumbar puncture and cell count together with other abnormalities in the CSF.

It is clear that in the case of LNB gold standard for diagnosis should be comprised of multiple parameters. All parameters that are being used to diagnose acute active disease have exceptions that have been elaborately described. The clinical manifestation is often typical, but some anecdotal atypical manifestations have been described [188, 598]. Serum serology is usually positive, but especially in early LNB this can be negative. The latter is infrequently seen in children who present with a facial nerve paralysis (FNP) very early after infection [599]. The a priori chance that a FNP in children is caused by *B. burgdorferi* is high in endemic countries, so a clinician should always perform follow up serology or do a lumbar puncture at presentation. The intrathecal white blood cell count is usually elevated, but in anecdotal cases no elevation has been found [114]. The anti-Borrelia antibody index is specific, but not very sensitive, especially in early LNB [366, 368, 369]. PCR on CSF can help confirm the diagnosis, but is not sensitive enough and certainly cannot exclude LNB.

Furthermore, after treatment the abovementioned parameters can all stay positive after adequate antimicrobial treatment. Many patients however suffer from residual complaints where the abovementioned parameters can not beyond a doubt demonstrate or disprove persistent infection in all individuals (See table 1). There is a need for a marker that can differentiate between persistent active and past infection.

In **chapter 5** we show a high sensitivity and reasonable specificity of intrathecal CXCL13 levels in LNB patients compared to patients with other inflammatory and infectious neurologic illnesses. It was shown in this study that most inflammatory illnesses have no or only minimal amounts of intrathecal CXCL13. In particular, longstanding infections like cryptococcal meningitis or *T. pallidum* especially during HIV co-infection, also resulted in elevated levels of intrathecal CXCL13. These specific groups of patients can usually be eliminated in the differential diagnosis. Furthermore, in our study several autoimmune diseases, where B-cell dysregulation or dysfunction plays a role, can also present with increased levels of intrathecal CXCL13. These findings underscore the nature of the chemokine CXCL13: CXCL13 is only a marker of inflammation and merely semi-specific for LNB due to the slow, protracted infection *B. burgdorferi* can cause [600]. It is another marker in the already widespread field of *B. burgdorferi* diagnostics that cannot be interpreted on its own, like serology, medical history

and clinical presentation. In a diagnostic strategy for LNB it can take a strong position, because elevated levels of CXCL13 are highly sensitive compared to the AI and far more specific than intrathecal leucocytosis. An algorithm in which the parameters from table 1 are classified into major and minor criteria should be studied prospectively in LNB patients presenting pre-treatment on a regional basis in order to develop two diagnostic algorithms; one algorithm with a high sensitivity in order to diagnose all individual LNB patients and start antimicrobial treatment, and one algorithm for European international multicenter studies with high specificity in order to select patients for new diagnostic or treatment studies on Lyme disease. The latter is important in future research to reduce the background noise of inclusion of patients without an active infection with *B. burgdorferi*.

Parameter	Pre-treatment	Post-treatment
Clinical presentation	Typical presentation to anecdotal atypical reports	Often: residual neurological damage or aspecific complaints
Blood serology (Several Ags)	Early LNB: can be negative	Early treatment: serology can stay negative, or become positive
	Late LNB: positive	Late treatment: serology can stay positive or become negative.
CSF white blood cell count	Usually elevated	Decline over weeks
Antibody-index CSF	~75% positive AI	Can stay positive for years,
		Can become negative,
	~25% negative AI*	Can stay negative.
PCR	Early: low sensitivity	Usually negative, but anecdotal reports describe a positive PCR without residual complaints**.
	Late: low sensitivity	
CXCL13	Usually highly elevated	Rapid decline after treatment

Table 1: Parameters for diagnosing Lyme neuroborreliosis pre-treatment and post-treatment. (Ags: Antigens, CSF: cerebrospinal fluid, AI: antibody index) * Mostly early, but also late infections; **Reference [259]

It has also been shown that CXCL13 shows a rapid decline after start of treatment in literature and in **chapter 5** of this thesis [382]. This is most likely due to the rapid deterioration of antigen and the subsequent lack of production of CXCL13 in antigen presenting cells. This potentially makes CXCL13 an interesting marker in the follow up of patients with residual complaints after treatment for a LNB. If CXCL13 levels drop and residual complaints are present a treatment strategy focused on symptom reduction by anti-inflammatory agents, pain relief medication or cognitive behavioral therapy instead of antimicrobial

therapy are more warranted. In this sense intrathecal CXCL13 can be used comparably to the serum anti-cardiolipin antibodies in *T. pallidum* infection where a non-specific marker is followed over time to have insight in the treatment response. This specific application for routine diagnostics of LNB will very likely play an important role in a diagnostic algorithm in the near future.

Preselection of patients eligible for Lyme disease testing.

In clinical practice it is important to select patients eligible for Lyme disease testing in order to have a higher pretest probability. In the general healthy population in the Netherlands the seroprevalence of antibodies against *B. burgdorferi* sl is about 5-10%, depending on the assay used and the region [111, 319-321, 599]. The seroprevalence can even be up to 45% in persons working in professions where they are at risk for contracting a tick bite [320, 322, 322, 324, 601, 602].
In **chapter 6** of this thesis we show in a large early arthritis cohort that testing for Lyme disease should only be done in a selected population with a clinical presentation that is compatible with Lyme disease. The pretest probability for Lyme disease in this cohort was only 0.5-1.1%. The positive predictive value of randomly performed *B. burgdorferi* serology in patients presenting with arthritis is disturbingly low. Clinicians should never underestimate the influence of positive serology on the mental state of the patient. Doctors might be able to see the relativity of positive serology, but to the patient a positive serology can be equal to actually having the active disease. This psychological burden underscores the importance of clinically preselecting patients eligible for *B. burgdorferi* serologic testing.

On the other hand testing for Lyme disease should be performed in patients with a clinical picture that is compatible with *B. burgdorferi* infection. One problem is the many clinical pictures Lyme disease can mimic, especially in immunocompromised patients that already have a more elaborate differential diagnosis.
In **chapter 7** we describe a case of an HIV patient with a meningoencephalitis caused by *B. burgdorferi*. The progression of disease was unusually fast and after treatment there were elaborate sequelae. We hypothesized that *B. burgdorferi* can have a more severe course in HIV co-infected patients. This has previously been described for *T. pallidum* and *Leptospira* spp. infection [579-581].
The increased pathogenesis of spirochetes in HIV infected patients is not well understood. In **chapter 5** we found highly elevated levels of intrathecal CXCL13 in LNB patients. It is known that B-cells of HIV infected patients have downregulated CXCR5 and inadequately respond to CXCL13 produced by antigen presenting cells [384, 567]. This specific dysfunction might be a first clue in

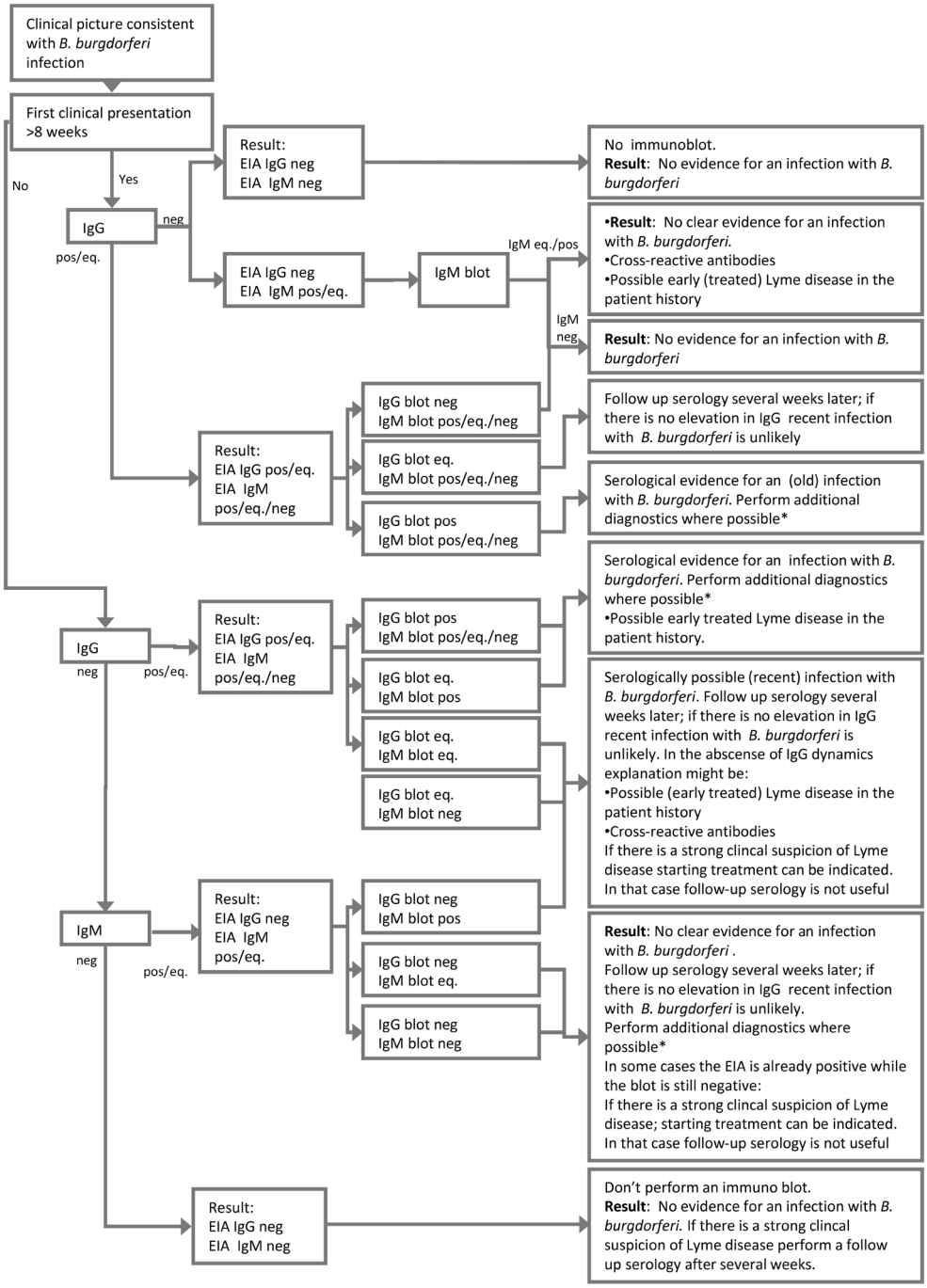
unraveling the altered and more severe pathogenic course in spirochetal infections in HIV patients.

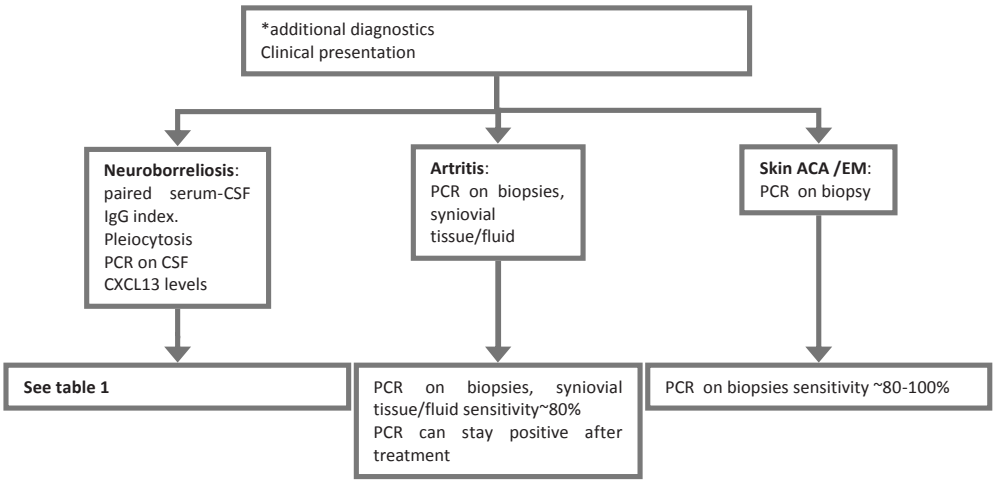
Concluding remarks

Lyme disease can have a significant impact on a patient's quality of life and correctly making the diagnosis is essential for proper treatment. It is important to test for *B. burgdorferi* infection in the presence of a clinical picture compatible with Lyme disease.
As serological tests are only surrogate markers of a disease, no single marker can prove or disprove active infection. It is of the highest importance to take into account all available clinical data from a patient in order to correctly interpret serological results in combination with non-specific biomarkers such as CSF white blood cell count and CXCL13 levels in CSF. For *B. burgdorferi* infection this is becoming a rapidly expanding field of tests and tests strategies with all their own specific pitfalls, leading to confusing discrepancies of test results for clinicians as well as patients. Identifying the optimal test strategy in an international setting, combined with quality control and recognition of the available commercial assays by a European panel of experts is desirable.

Perhaps *B. burgdorferi* sl is not so special in the world of diagnostics, as was stated earlier in this chapter. However, *B. burgdorferi* sl has an unfortunate combination of factors which makes it an infection that needs some special attention in correctly interpreting the tests at hand. The question at the start was: Is the laboratory and clinical diagnosis of Lyme disease really so complicated? The correct answer is that it is difficult, but not more complicated than in other infectious diseases involving uncultivable or fastidious organisms. In a field where one is forced to use an indirect strategy such as serology, the interpretation of the tests should be an interdisciplinary discussion between a treating physician and a medical microbiologist. If the medical microbiologist and clinician can properly utilize their expertise simultaneously for the benefit of the patient then perhaps the diagnosis is not so difficult after all.

Figure 1: An algorithm to interpret *B. burgdorferi* serology and an indication of when to perform additional diagnostics with their specific pitfalls. (Published in revised version [603]).




*additional diagnostics
Clinical presentation
Neuroborreliosis: paired serum-CSF IgG index. Pleiocytosis PCR on CSF CXCL13 levels
Artritis: PCR on biopsies, syniovial tissue/fluid
Skin ACA /EM: PCR on biopsy
See table 1
PCR on biopsies, syniovial tissue/fluid sensitivity~80% PCR can stay positive after treatment
PCR on biopsies sensitivity ~80-100%