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**Intended category:** Systematic review

## **Unconventional diagnostic tests for Lyme borreliosis: a systematic review**

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1 **ABSTRACT (316 words)**

2 *Background:* Lyme borreliosis (LB) diagnosis currently relies mainly on serological tests and  
3 sometimes polymerase chain reaction or culture. However, other biological assays are being  
4 developed to try to improve *Borrelia*-infection diagnosis and/or monitoring.

5 *Objectives:* To analyze available data on these unconventional LB-diagnostic assays through a  
6 systematic literature review.

7 *Methods:* We searched PubMed and Cochrane Library databases according to the PRISMA-DTA  
8 method and the Cochrane Handbook for Systematic Reviews of Interventions.

9 We analyzed controlled and uncontrolled studies (published 1983–2018) on biological tests for adults  
10 to diagnose LB according to the European Study Group for Lyme Borreliosis or the Infectious  
11 Diseases Society of America definitions, or strongly suspected LB.

12 Two independent readers evaluated study eligibility and extracted data from relevant study reports; a  
13 third reader analyzed paper full-texts to resolve disagreements.

14 The quality of each included study was assessed with the QUADAS-2 evaluation scale.

15 *Results:* Forty studies were included: 2 meta-analyses, 25 prospective, controlled studies,  
16 5 prospective, uncontrolled studies, 6 retrospective, controlled studies, and 2 case reports. These  
17 biological tests assessed can be classified as: (i) proven to be effective at diagnosing LB and already  
18 in use (CXCL-13 for neuroborreliosis), but not enough yet standardized; (ii) not yet used routinely,  
19 requiring further clinical evaluation (CCL-19, OspA and interferon- $\alpha$ ); (iii) uncertain LB-diagnostic  
20 efficacy because of controversial results and/or poor methodological quality of studies evaluating them  
21 (lymphocyte transformation test, interferon- $\gamma$ , ELISPOT); (iv) unacceptably low sensitivity and/or  
22 specificity (CD57+ NK cells and rapid diagnostic tests); and (v) possible only for research purposes  
23 (microscopy and xenodiagnoses).

24 *Discussion:* QUADAS-2 quality assessment demonstrated high risk of bias in 25/40 studies and  
25 uncertainty regarding applicability for 32/40, showing that in addition to polymerase chain reaction and  
26 serology, several other LB-diagnostic assays have been developed but their sensitivities and  
27 specificities are heterogeneous and/or under-evaluated or unassessed. More studies are warranted to  
28 evaluate their performance parameters. The development of active infection biomarkers would greatly  
29 advance LB diagnosis and monitoring.

30 **Key words:** Lyme borreliosis; Diagnostic tests; *Borrelia burgdorferi*; Clinical assessment

## 31 Introduction

32

33 Lyme borreliosis (LB) is the most commonly reported vector-borne disease transmitted by ticks in  
34 the United States and Western Europe [1–3]. It is caused by spirochetes of the *Borrelia burgdorferi*  
35 sensu lato complex. The greater variety of human pathogenic genospecies in Europe (mainly  
36 *B. afzelii*, *B. garinii*, *B. burgdorferi* sensu stricto) than the United States explains the wider range of  
37 clinical pictures [4]. The most common clinical manifestations in Europe are erythema migrans (EM)  
38 and Lyme neuroborreliosis [4]. EM (early localized stage) is pathognomonic and does not require  
39 further exploration [5]. The early (<6 months) and late (>6 months) disseminated LB stages mainly  
40 affect skin, nervous system and joints, and rarely heart and eyes [4]. Some subjective symptoms  
41 (asthenia, polyalgia, cognitive complaints, etc.) may also be present at all stages and may persist after  
42 well-conducted treatment, with the latter being called post-treatment Lyme disease syndrome (PTLDS)  
43 [4,6–8]. Reliable diagnostic tests are needed to dissociate active from inactive LB, and from other  
44 diseases that share some similar clinical manifestations.

45 LB diagnosis relies mainly on serological tests and polymerase chain reaction (PCR). Their  
46 sensitivities and specificities at disease onset may be less reliable and depend on the anatomical site  
47 sampled [9]. A recent meta-analysis [10] showed that LB serological tests had heterogeneous  
48 sensitivities, depending on the disease stage: 50% (95% CI=40%–61%) for localized EM, 77% (95%  
49 CI=67%–85%) for Lyme neuroborreliosis, 97% (95% CI=94%–99%) for acrodermatitis chronica  
50 atrophicans (ACA), 96% (95% CI=93%–100%) for Lyme arthritis (LA), and 73% (95% CI=53%–87%)  
51 for unspecified LB. Specificity was ~95% for investigations with healthy controls and 80% for cross-  
52 sectional studies. All the recent European guidelines recommend a two-tier test strategy for  
53 serological testing [11–16]. *Borrelia* PCR sensitivity varies according to the disease stage and its  
54 anatomical location: 65%–90% for cutaneous biopsies of EM and ACA [12,17–20], 50%–85% for joint  
55 samples [20–22] and 10%–30% for cerebrospinal fluid (CSF) [20,23,24]. The usefulness of *Borrelia*  
56 *burgdorferi* sensu lato PCR on urine and blood has not yet been proven [4,24], even if PCR  
57 techniques are improving [25–27]. PCR is currently recommended for ambiguous LA (synovial  
58 fluid/tissue) and ACA (skin biopsies) [11–16].

59 Beyond those recommended tests, researchers have tried to develop alternative assays to  
60 serology, PCR and culture to improve diagnosis and monitoring of early and late LB, even for cases

61 with non-specific symptoms. Several studies on these alternative tests have been published and it is  
62 often difficult to make a well-thought-out decision about them.

63 This literature review was undertaken to analyze available data on unconventional tests to  
64 diagnose LB, and to make physicians aware of their characteristics and limitations.

65

## 66 **Methods**

67

68 This literature review was based on screening of the PubMed and Cochrane Library databases,  
69 according to the PRISMA-DTA method and the Cochrane Handbook for Systematic Reviews of  
70 Interventions [28–32]. Two independent readers (content-area experts AR and AG) assessed study  
71 eligibility by reviewing titles and abstracts first, and, if relevant, by examining the full-texts. AR and AG  
72 extracted data from retained reports. They knew the authors' names, institutions, publication journal  
73 and results when they applied the eligibility criteria. When they disagreed about a study's relevance, a  
74 third expert (BJ) was asked to analyze the full-text. The quality of each included study was assessed  
75 with the QUADAS-2 evaluation scale (Fig. 3 and 4, Suppl table 4) [33].

76 We included studies published from 1983 to 2018, conducted on adult humans, with control  
77 groups or not, randomized or not and in which LB cases were defined according to the European  
78 Study Group for Lyme Borreliosis (ESGBOR) or the Infectious Diseases Society of America (IDSA)  
79 criteria [4,34], or cases reported as strongly clinically suspected LB but without microbiological  
80 documentation. The latter choice was made to avoid excluding studies describing diagnostic tools for  
81 ambiguous LB cases with no microbiological documentation. Meta-analyses and case series were  
82 included; reports with only abstracts available, congress communications and letters were excluded,  
83 as were studies evaluating serological tests and PCR. When Cochrane Handbook for Systematic  
84 Reviews of Interventions were closely respected, studies already included in those meta-analyses  
85 were excluded and all subsequently published studies were included. No language restriction was  
86 applied.

87 First, we conducted a free search to get an overview of all the available diagnostic tests using  
88 the following terms: "Lyme borreliosis" or "Lyme disease" and "diagnostic tests" or "diagnosis". Then,  
89 the following MeSH terms were used: "Lyme borreliosis" or "Lyme disease", and "diagnostic tests",  
90 "CXCL-13", "apolipoprotein B-100", "CCL-19", "lymphocyte transformation test", "interferon", "CD57",

91 “xenodiagnosis”, “membrane protein OspA”, “rapid diagnostic test”, “focus-floating microscopy”, “dark-  
92 field microscopy”, “silver-stain microscopy” and “microscopy”. We could not conduct a meta-analysis  
93 for any of the tests detailed below because study quality was too heterogeneous and too few  
94 publications reported test performances (sensitivity/specificity).

95

96

97

## 98 **Results**

99

100 Forty studies were included: 2 meta-analyses, 25 prospective, controlled studies, 5 prospective,  
101 uncontrolled studies, 6 retrospective, controlled studies, and 2 case reports (Fig. 1).

102

### 103 ***Tests exploring inflammatory or autoimmune responses***

104

#### 105 *CXCL-13*

106

107 The C-X-C motif chemokine ligand-13 (CXCL-13), produced by antigen-presenting cells,  
108 selectively attracts B lymphocytes [35–37]. During Lyme neuroborreliosis, CXCL-13 released into the  
109 CSF by resident mononuclear cells [38] triggers B-cell migration into the CSF, resulting in the  
110 characteristic B-cell–enriched CSF pleocytosis [39].

111 Database searches identified 117 reports. After removing duplicates, 52 publications were  
112 screened. Nine full-text articles were assessed for eligibility. Four studies were finally retained: two  
113 meta-analyses [40,41], one retrospective, controlled study [42] and one prospective, uncontrolled  
114 study [43]. QUADAS-2 quality assessment demonstrated low risk of bias and low applicability  
115 concerns for both meta-analysis [40–41], unclear risk of bias and uncertainty regarding applicability for  
116 the retrospective controlled study [42], and high risk of bias and high concerns regarding applicability  
117 for the prospective uncontrolled study [43].

118 As demonstrated previously for Lyme neuroborreliosis, the CSF CXCL-13 concentration is high  
119 [40–44]. CXCL-13 is detectable from days to weeks before antibodies in CSF and its level rapidly  
120 declines after antibiotics, suggesting its potential role as a therapeutic marker [40–43,45,46]. For the

121 meta-analyses, including 961 Lyme neuroborreliosis patients and 3,282 controls, the pooled CSF  
122 CXCL-13 sensitivity range was 89%–97% and its pooled specificity was 96% (95% CI=92%–98%)  
123 [40,41]. The specific antibody index recommended to diagnose definite Lyme neuroborreliosis had  
124 86% (95% CI=63%–95%) sensitivity and 94% (95% CI=85%–97%) specificity [47,48].

125 Optimal cut-off values were determined to be 162 pg/mL in one meta-analysis [41] and 131  
126 pg/mL using the Luminex platform versus 259 pg/mL for enzyme-linked immunosorbent assay (ELISA)  
127 in a retrospective, controlled study [42]. Cut-off determination is a major issue for CXCL-13 use to  
128 diagnose Lyme neuroborreliosis, since no official recommendation has yet been advanced. In  
129 addition, few available kits are marked European Community-in vitro diagnosis (European conformity  
130 authorization). Each laboratory has to determine its own threshold, which might lead to different  
131 interpretations.

132 Moreover, the CSF CXCL-13 level is elevated in other central nervous system (CNS) disorders,  
133 like neurosyphilis, cryptococcosis, trypanosomiasis, viral meningitides, meningoencephalitides and  
134 CNS lymphoma [41,42]. Attention should also be paid to those pathologies when interpreting the  
135 results.

136 CXCL-13 seems to be a good add-on marker, with high sensitivity and specificity to diagnose  
137 untreated, acute Lyme neuroborreliosis. It could be useful for patients with early typical clinical  
138 symptoms, CSF pleocytosis and a negative specific antibody index, and for cases of reinfection. It  
139 remains to be evaluated for late Lyme neuroborreliosis.

140

141 *CCL-19*

142

143 In their recent prospective controlled study, Aucott *et al.* described the T-cell chemokine CCL-19  
144 as a potential immunological risk factor of PTLDS [49]. Seventy-six patients with physician-  
145 documented EM were followed for 1 year post-antibiotics: 11/76 (14.5%) developed PTLDS-  
146 compatible symptoms [49]. Persistently high 1-year CCL-19 levels were only observed in PTLDS  
147 patients.

148 QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty regarding  
149 applicability in this study [49].

150 CCL-19 could be of potential interest to screen patients at risk of PTLDS. More studies are

151 warranted.

152

153 *Apolipoprotein B-100 (ApoB-100)*

154

155 We identified four papers through database searches. After removing duplicates, two prospective  
156 controlled studies were screened, their full-texts assessed for eligibility and, finally, included for the  
157 review [50,51]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty  
158 regarding applicability in these two studies [50,51]. Crowley *et al.* identified ApoB-100 as a target of T-  
159 cell responses in 14/37 (38%) LA patients [50]. Moreover, 5/37 (13%) LA patients had autoantibodies  
160 directed against ApoB-100, an autoantigen involved in refractory LA [50,52,53].

161 To date, no study has used ApoB-100 as a diagnostic tool for LB. It cannot be used in routine  
162 practice.

163

#### 164 ***Tests exploring cellular immunity***

165

166 *Lymphocytes-transformation test*

167

168 A lymphocyte-transformation test evaluates the lymphoproliferative response of peripheral blood  
169 mononuclear cells to *B. burgdorferi* antigens. The results are expressed as a stimulation index (SI),  
170 with SI>10 considered positive and SI<10 negative [54].

171 Among 38 publications identified through database searches, 24 were screened, after removing  
172 duplicates, and 14 full-texts were assessed for eligibility. Ten studies were finally retained for the  
173 review: eight prospective, controlled studies [54–61], one prospective, uncontrolled study [62] and  
174 one retrospective, controlled study [63]. QUADAS-2 quality assessment demonstrated low risk of bias  
175 and uncertainty regarding applicability for two prospective controlled studies [54,61], unclear risk of  
176 bias and uncertainty regarding applicability for six prospective studies [55-60], and high risk of bias  
177 and high applicability concerns for the two others [62,63].

178 Only four provided sensitivities and specificities for any stage with combined respective ranges of  
179 45%–89.4% and 33%–98.7% [54,59–61]. Only one study reported data on late LB with 45% (95%  
180 CI=30–60) sensitivity and 95% (95% CI=87–99) specificity [54].



181        Among the 10 studies retained, for early LB, mean SIs were >10 in two [60,61], <10 in one [58]  
182 and not mentioned in seven [54–57,59,62-63]; while for late LB, mean SIs were >10 in five  
183 [54,55,57,60,61], <10 in two [58,63] and not mentioned in three [56,59,62]. For controls (healthy  
184 subjects or other diseases), mean SIs were <10 in five studies [54,55,57,58,63], >10 in one [61], and  
185 not mentioned in four [56,59,60,62]. Exposed healthy subjects (forest workers, *Borrelia* laboratory  
186 workers) had high seroprevalence, with mean SI>10 in one report, <10 in two [54,59] and not given in  
187 seven [55–58,61–63]. Fig. 2 summarizes these findings.

188        After antimicrobials, SI declined in three studies [56,59,60] and was not reported in the other  
189 seven. Zoschke *et al.* noted the late persistence of lymphocyte proliferation post-antibiotics [61].

190        Concordance with serological tests was found in four studies, two each performed on late LB or  
191 at all stages [55,57,60,63], and discordance in the six others conducted at all stages combined  
192 [54,56,58,59,61,62].

193        Those 10 studies had numerous biases. First, lymphocytes-transformation tests were  
194 standardized only in two investigations that used lymphocytes-transformation test– memory  
195 lymphocyte immunostimulation assay (MELISA) [59,62]. Second, LB was defined with criteria other  
196 than those of ESGBOR or IDSA in six [4,54–56,58,59,62]. Third, the international SI cut-off to interpret  
197 results was not respected in four [59,60,62,63] and not mentioned in five [55–58,61], meaning that  
198 nine studies do not enable any conclusion to be drawn. Finally, the numbers of patients with SI>10  
199 were not given in six studies [55–57,59,62,63] that reported only mean SIs.

200        At present, no guidelines recommend using lymphocytes-transformation tests because of their  
201 lack of specificity [11,13,14,16,64,65]. Studies on lymphocytes-transformation tests yielded  
202 heterogeneous results and mixed quality, without clear sensitivity and specificity. They do not permit  
203 conclusions to be drawn about the usefulness of lymphocytes-transformation tests to diagnose LB,  
204 including for rare seronegative patients.

205

#### 206 *Interferon-γ (IFN-γ) ELISPOT*

207

208        IFN-γ release into the blood is assessed to explore T-cell activation after *B. burgdorferi* sensu lato  
209 stimulation.

210        Among 73 publications identified by database searches, 39 were screened, after removing

211 duplicates, and 16 full-texts were assessed for eligibility. Eight studies were finally retained for the  
212 review: seven prospective, controlled studies [66–72] and one retrospective, controlled study [73]. All  
213 but one of them [73] respected ESGBOR or IDSA clinical and microbiological definitions of LB.  
214 QUADAS-2 quality assessment demonstrated low risk of bias and low applicability concerns for one  
215 study [67], low risk of bias and uncertainty regarding applicability for 3 studies [69,71,72], unclear risk  
216 of bias and unclear applicability concerns for one study [70], unclear risk of bias and high applicability  
217 concerns for 2 studies [66,68], and high risk of bias and high applicability concerns for one  
218 retrospective controlled study [73].

219 All eight studies showed that IFN- $\gamma$  was associated with exposure to *Borrelia*. Circulating IFN- $\gamma$   
220 was elevated in patients with early LB, late LB or PTLDS and seropositive, asymptomatic subjects. It  
221 was significantly less elevated in healthy seronegative subjects in all studies ( $p < 0.01$ ). Moreover, IFN- $\gamma$   
222 concentrations did not parallel *Borrelia* activity in six studies [66–68,73]. Sensitivities during early LB  
223 ranged from 36% to 69% in 2/3 studies [69,70] and only one study provided specificity (~82%) [70].  
224 Callister *et al.* demonstrated that combining positive *Borrelia*-specific C6-peptide–ELISA and IFN- $\gamma$   
225 results increased sensitivity to 83% [69]. Only one study on late LB reported 84% sensitivity and 96%  
226 specificity for the iSpot Lyme [68]; four studies failed to mention any test performance [66,71–73].

227 Only one study found an IFN- $\gamma$  decrease after antibiotics [69]. No study demonstrated any  
228 concordance between serological test results and circulating IFN- $\gamma$ .

229 The main limitation of IFN- $\gamma$  ELISPOT appears to be its wide variability. First, the cut-off to  
230 interpret results was not given in 6/8 studies. In the two studies indicating a threshold, only two  
231 patients with anaplasmosis served as controls [69] or a receiver operating characteristics curve with  
232 105 controls was used [68]. Second, specificity varied markedly according to the control population  
233 chosen. Third, among the studies, different *Borrelia* species were used to assess the performance of  
234 the circulating IFN- $\gamma$  level: two did not mention the species [68,69], three used a *B. afzelii* strain (ACA-  
235 I) [66,71,73], two used a *B. garinii* strain (Ip90) [70,72], and the last used the *B. burgdorferi* strain B31  
236 combined with antigens specific to each of 4 species (*B. afzelii*, *B. garinii*, *B. burgdorferi sensu stricto*,  
237 *B. bavariensis*) [67]. Pertinently, the *B. burgdorferi* B31 lysate cross-reacts with other spirochetes  
238 [67,71]. Finally, no standardized test has been validated at present: notably, three studies had  
239 demonstrated lack of reproducibility for LB [67–69].

240 IFN- $\gamma$  secretion has been also studied in other tissues/biological fluids (skin biopsies, synovial

241 fluid, CSF) to assess its role in the inflammatory response to *Borrelia* but it was not considered a  
242 diagnostic tool [66,71,72,74–78].

243 At present, IFN- $\gamma$  ELISPOT cannot be used in routine practice because its role has only been  
244 established as a witness of *Borrelia* exposure. Specificity, cut-offs and reproducibility require more  
245 investigations.

246

247 *IFN- $\alpha$*

248

249 Among 24 articles identified through database searches, 12 were screened, after removing  
250 duplicates, and three full-texts were assessed for eligibility. Finally, only one prospective comparative  
251 study was included [79]. QUADAS-2 quality assessment demonstrated unclear risk of bias and  
252 uncertainty regarding applicability for this study [79].

253 Jacek *et al.* [79] demonstrated higher IFN- $\alpha$  activity in PTLDS patients, which suggests the  
254 existence of an immune-related disease process in patients with persistent symptoms, perhaps  
255 contributing to ongoing symptoms. The authors also reported that  $\beta$ -lactam therapy did not modulate  
256 the activated immune response.

257 More studies are warranted to assess the reliability of this test.

258

259 *CD57+ natural killer (NK) cells*

260

261 CD57 is an NK-cell marker but is also expressed on other cells. The method is based on an  
262 immunophenotyping technique.

263 Among 10 articles identified through database searches, five were screened, after removing  
264 duplicates, and three full-texts were evaluated for eligibility. Two prospective controlled studies were  
265 finally retained for the review [80,81]. QUADAS-2 quality assessment demonstrated unclear risk of  
266 bias and uncertainty regarding applicability in these two studies [80,81].

267 Stricker *et al.* reported lower CD57+ lymphocyte counts (mean=30 $\pm$ 16 cells/ $\mu$ L) in 73 patients  
268 with late LB before starting antibiotics; the lymphocyte count increased after treatment (mean=66 $\pm$ 39  
269 cells/ $\mu$ L) [80]. That study had several biases: low numbers of patients included, bias of presentation of  
270 the results, no repeated test on the same patients to follow CD57-expression kinetics, no validated

271 control groups, and no clear case definition and treatment. Marques *et al.* found no significant  
272 difference ( $p=0.68$ ) among nine PTLDS patients, the 12 cured of LB and nine healthy volunteers [81].  
273 The authors did not report CD57 results before antibiotics (all were already treated or healthy), and  
274 very few patients and controls were tested. CD57-marker specificity for LB is probably low, because  
275 low CD57 levels were also found in patients with numerous autoimmune diseases, infections or  
276 cancers [82].

277 CD57 measurement does not seem useful, even after antibiotics or for persistent symptoms. To  
278 our knowledge, no study has provided this test's performance (sensitivity/specificity).

279

## 280 **Microbiological tests**

281

### 282 *Xenodiagnosis*

283

284 Xenodiagnosis, an experimental test used in animal models of LB, uses uninfected ticks to detect  
285 low-level infection, even after antibiotics.

286 Our database searches identified 22 papers, among which 11 were screened, after removing  
287 duplicates, and five full-texts were assessed for eligibility. Only one prospective, controlled study was  
288 included [83]. QUADAS-2 quality assessment demonstrated unclear risk of bias and uncertainty  
289 regarding applicability in this study [83].

290 This study investigates the feasibility of xenodiagnosis in humans: 2/26 patients treated for LB had  
291 positive xenodiagnoses post-treatment (one control EM subject and one PTLDS). Xenodiagnosis was  
292 positive only by DNA detection after PCR of tick cultures or the tick itself. No spirochetes were  
293 detected. Bockenstedt and Radolf questioned whether *Borrelia*-DNA detection was sufficient to  
294 confirm the diagnosis [84].

295 Currently available xenodiagnosis performance is insufficient to assess its diagnostic usefulness.

296

### 297 *Outer surface protein-A (OspA) detection*

298

299 OspA, a *Borrelia burgdorferi* lipoprotein that binds to its receptor TROSPA in the tick midgut, is  
300 required to infect the tick.

301 The two identified studies were included in this review [85,86]. QUADAS-2 quality assessment  
302 demonstrated low risk of bias and uncertainty regarding applicability in one study [85] and unclear risk  
303 of bias and uncertainty regarding applicability in the other study [86].

304 In their prospective, uncontrolled study on only three patients, Cheung *et al.* described a liquid  
305 chromatography–tandem mass-spectroscopy method to directly detect OspA in sera from early-  
306 infected patients (EM) [85]. That technique detected low OspA levels in the three patients' sera.  
307 Although the proof-of-concept merits further examination, neither sensitivity nor specificity was  
308 reported. In a prospective controlled study on 140 patients, Magni *et al.* described a nanotrap  
309 technology-based method to detect the OspA carboxyl-terminus domain in urine samples [86].  
310 Although the authors reported excellent sensitivity (100%) and specificity (100%) before antibiotics,  
311 those results must be confirmed by other studies.

312 Upcoming investigations on this technique should be of interest.

313

#### 314 *Direct microscopy of human tissues*

315

316 Database searches identified 81 studies. After removing duplicates, 29 publications were  
317 identified, on “dark-field microscopy”, 18 on “silver staining” and 2 on “focus-floating microscopy”. All  
318 papers were screened. Nine full-texts were assessed for eligibility and seven were retained: two  
319 prospective, controlled studies [87,88], two prospective, uncontrolled studies [89,90], one  
320 retrospective, controlled study [91] and two case reports [92,93]. QUADAS-2 quality assessment  
321 demonstrated low risk of bias and unclear applicability concerns for one study [87], unclear risk of bias  
322 and uncertainty regarding applicability for two studies [88,91], and unclear risk of bias and high  
323 applicability concerns in four studies [89,90,92,93].

324 Electron microscopy, silver staining with light microscopy and focus-floating microscopy enabled  
325 spirochete detection in various samples [87,91-94]. Nevertheless, it has limited clinical utility because  
326 of the apparent *Borrelia* scarcity in mammalian tissues and its 41% sensitivity [90], and the time  
327 required for analysis [95,96]. False-positive results have also been reported [88].

328 Laane *et al.* published an uncontrolled study describing a modified dark-field microscopy  
329 technique, called LM-method, which identified structures claimed to be *Borrelia* in 21/32 (66%)  
330 patients' blood samples among those with non-specific symptoms [97]. Using the same methodology

331 and including a control group, Aase *et al.*'s findings refuted Laane *et al.*'s results, i.e., the LM-method  
332 was positive for 85% of the 41 healthy controls and was unable to detect *Borrelia* in the 21 positive  
333 controls with five *B. afzelii* per 100 red cells [98]. The LM-method should not be used for LB diagnosis.

334 At present, *Borrelia* detection by microscopy can only be used for research purposes. Inclusion of  
335 positive/negative controls and the microscopy reader's expertise are necessary.

336

### 337 *Rapid diagnosis tests (RDTs)*

338

339 Among the 32 articles identified by database searches, 16 were screened, after removing  
340 duplicates, and two full-texts were assessed for eligibility. Two retrospective, controlled studies were  
341 included in the review [99,100]. QUADAS-2 quality assessment demonstrated unclear risk of bias and  
342 uncertainty regarding applicability in these two studies [99,100].

343 Two RDT categories are available: immunochromatography-based methods and microfluidic-  
344 based point-of-care tests.

345 Immunochromatography-based methods are commercially available at pharmacies throughout  
346 Europe for patient self-use. These RDTs require a drop of blood and can be done with minimal  
347 training. Only one study assessed RDT performances and no regulations are available [99]. Smit *et al.*  
348 used two commercially available RDTs with sensitivities of 26% and 32%, and specificities of 85% and  
349 88%, values that are much lower than those of laboratory-based diagnostic tools [99]. Their results do  
350 not support RDT use for diagnostic strategies. Neither European nor American guidelines recommend  
351 using RDTs.

352 A microfluidic-based point-of-care test that can be performed in 15 minutes is not yet  
353 commercially available [100]. This test's performance was comparable to that of the laboratory-based  
354 C6-peptide–ELISA with 84% sensitivity and 92% specificity. However, it was shown to be significantly  
355 less sensitive than a recombinant (VlsE+DbpA+OspC) ELISA [100].

356 RDTs for LB remain experimental, requiring further investigation.

357

## 358 **Discussion**

### 359 **Summary of evidence**

360 In addition to polymerase chain reaction and serology, several other LB-diagnostic assays have been

361 developed but their sensitivities and specificities are heterogeneous and/or under-evaluated or  
362 unassessed.

### 363 **Study Strengths and weaknesses**

#### 364 *Included studies*

365 We included studies defining LB cases according to ESGBOR or IDSA criteria [4,34] and avoided  
366 excluding studies describing diagnostic tools for ambiguous case lacking microbiological  
367 documentation. That choice represents a strength of our study as it attempts to identify which test  
368 might perform well at diagnosing such ambiguous cases.

369 QUADAS-2 quality assessment demonstrated high risk of bias in 25/40 studies and uncertainty  
370 regarding applicability for 32/40 (cf. Fig. 3, Fig. 4, and Supplementary Table S4) [30,33]. Most authors  
371 failed to report the sensitivities or specificities of the evaluated diagnostic tests, indicating that the  
372 studies on alternative diagnostic tests were not methodologically well-conducted.

373

#### 374 *Review process*

375 Two databases were screened, without search filters or language restriction. Consensus was  
376 reached for the selection of included studies. We report all the QUADAS-2 domains to evaluate each  
377 included study (Supplementary Table S4, Fig. 3 and Fig. 4). For all the included studies,  
378 Supplementary Tables S1 to S3 report: study design, patient-inclusion criteria, stage, controls, other  
379 tests; main results including reported sensitivities and/or specificities and limitations. A meta-analysis  
380 could not be undertaken because of too extensive heterogeneity among included studies and the  
381 small numbers of studies for some tests.

382

### 383 **Conclusion**

384

385 Studies on unconventional biological LB-diagnostic strategies can be classified as follows: (i)  
386 tests proven to diagnose LB accurately and already used routinely (e.g. CXCL-13 for Lyme  
387 neuroborreliosis), which, nonetheless, remain to be standardized; (ii) tests not yet used in routine  
388 practice requiring clinical evaluation (e.g. CCL-19, OspA and IFN- $\alpha$ ); (iii) tests with uncertain abilities  
389 to diagnose LB because of controversial results and/or poor methodological quality of the studies (e.g.  
390 lymphocytes-transformation tests, IFN- $\gamma$  ELISPOT); (iv) tests with unacceptably low sensitivity and/or

391 specificity (e.g., CD57+ NK-cells and RDTs); and (v) tests possible only for research purposes at  
392 present (e.g., microscopy and xenodiagnoses).

393 For early LB, CSF CXCL-13 is the only test whose performances allow its development for  
394 routine practice. For late LB, satisfactory performance has not yet been demonstrated for any test.  
395 IFN- $\gamma$  was only associated with *Borrelia* exposure. CCL-19, ApoB-100 and IFN- $\alpha$  might be of potential  
396 interest to screen patients at risk for PTLDS.

397 In addition to PCR and serology, several other diagnostic assays have been developed for LB but  
398 their performances are heterogeneous, underevaluated and/or unassessed. More studies are  
399 warranted to evaluate their sensitivities, specificities and reproducibilities. Identification of biomarkers  
400 for active *Borrelia* infection would represent a major advancement in LB diagnosis and monitoring.

401

402

403 **Conflicts of interest:** The authors declare no conflict of interest related to this work.



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## **Figure Legends**

**Fig.1.** Flow chart of article selection for the systematic review.

**Fig.2.** Pooled Lymphocyte-transformation-test results of the included studies for Lyme borreliosis (LB), according to different groups of patients. SI, stimulation index.

**Fig.3.** Proportions of studies with low, high or unclear risk of bias (%), according to the QUADAS-2 domains (Quality Assessment of Diagnostic Accuracy Studies-2)

**Fig.4.** Proportions of studies with low, high or unclear concerns regarding applicability (%), according to the QUADAS-2 domains (Quality Assessment of Diagnostic Accuracy Studies-2)

Fig. 1

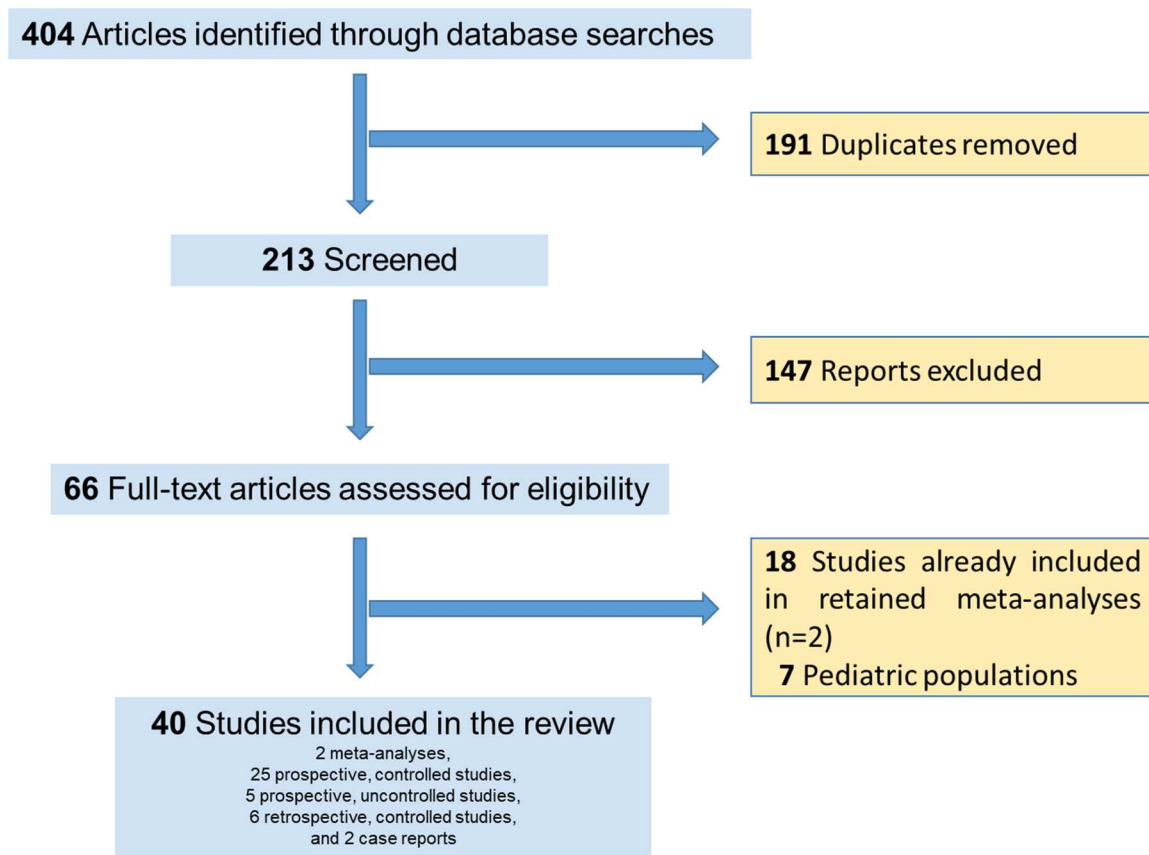
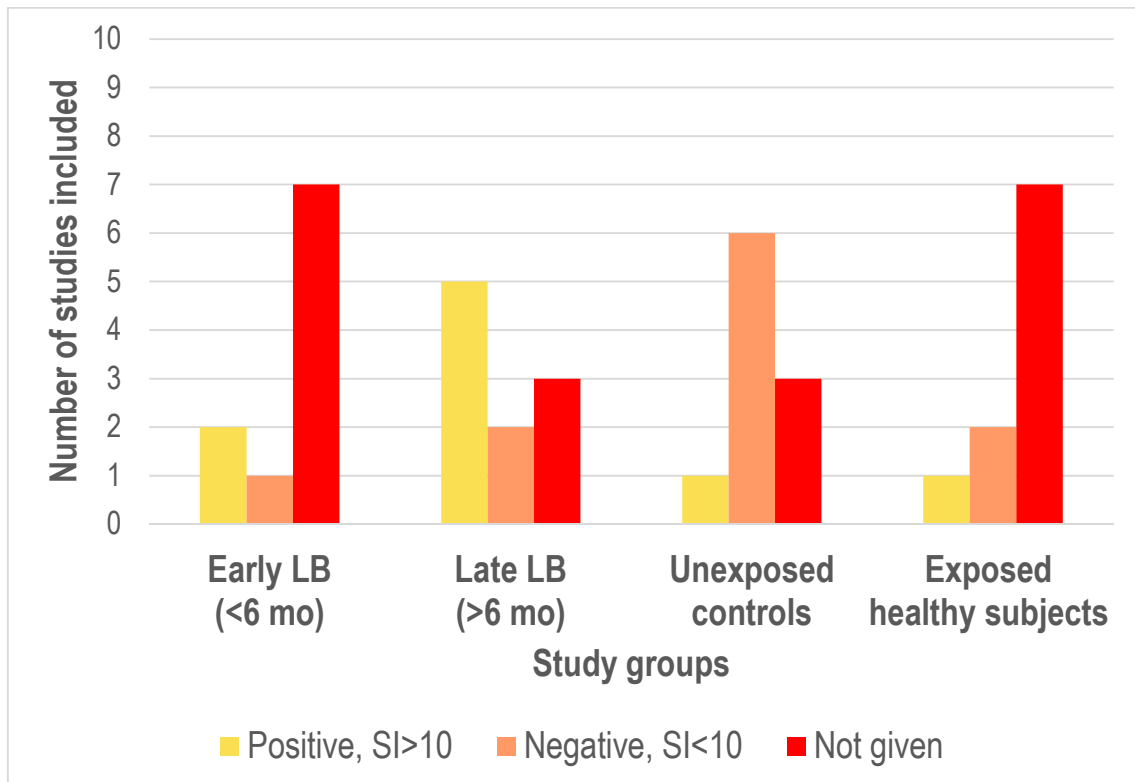
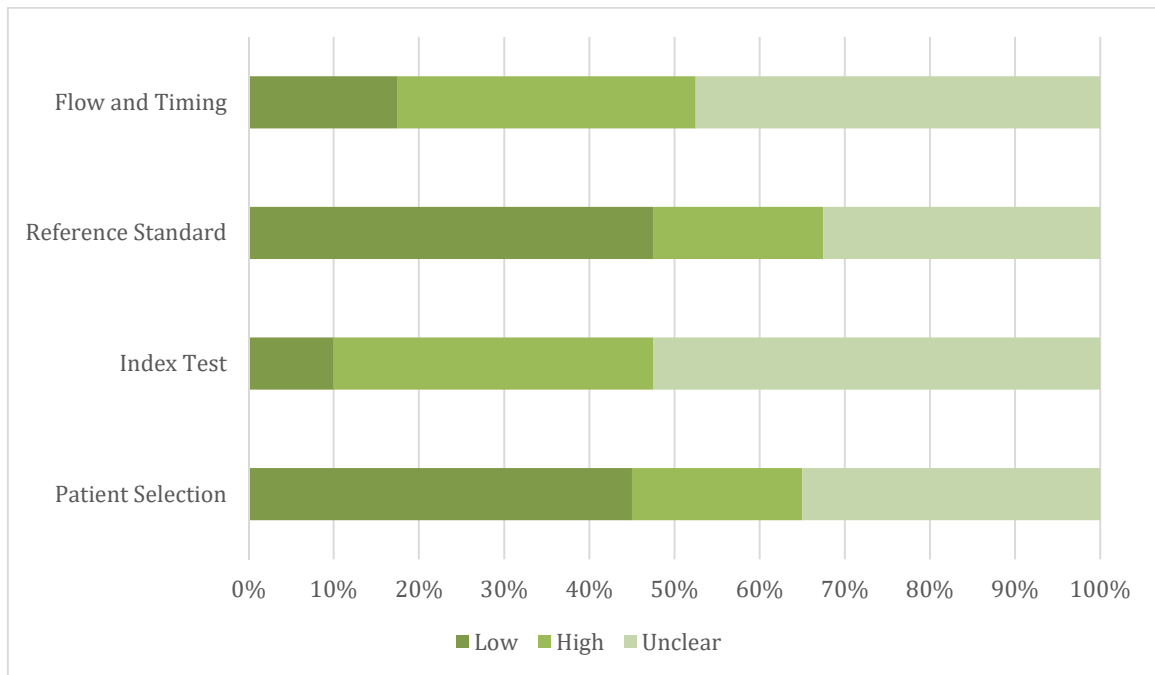


Fig. 2



**Fig. 3**



**Fig. 4**

