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## Mechanisms of extramedullary relapse in acute lymphoblastic leukemia Reconciling biological concepts and clinical issues

J. Gaudichon, H. Jakobczyk, L. Debaize, E. Cousin, M.-D. Galibert, M.-B. Troadec, V. Gandemer

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4 **Authors:** Jérémie Gaudichon<sup>1,3</sup>, Hélène Jakobczyk<sup>1</sup>, Lydie Debaize<sup>1</sup>, Elie Cousin<sup>1,2</sup>, Marie-  
5 Dominique Galibert<sup>1</sup>, Marie-Bérengère Troadec<sup>1</sup>, and Virginie Gandemer<sup>1,2</sup>.

6 **Affiliations:** <sup>1</sup>Univ Rennes, CNRS, IGDR (Institut de Génétique et Développement de  
7 Rennes) - UMR 6290, F- 35000 Rennes, France

8 <sup>2</sup>Pediatric Hematology Department, University Hospital, Rennes, France

9 <sup>3</sup>Pediatric Hematology and Oncology Department, University Hospital, Caen, France

10

11 **Corresponding author:**

12 Prof. Virginie Gandemer,

13 Pôle Femme-Enfant

14 Service d'hémato-oncologie pédiatrique

15 CHU Hôpital Sud- 16 Bd de Bulgarie - 35203 Rennes Cedex 2

16 Email: virginie.gandemer@chu-rennes.fr

17 Telephone: (+ 33)2 23 23 70 24; Fax: (+33) 2 99 26 71 95

18 or

19 Dr Jérémie Gaudichon

20 Onco-hématologie pédiatrique

21 CHU de Caen

- 22 Avenue de la côte de nacre
- 23 14033 CAEN Cedex 9
- 24 Telephone: (+33) 2 31 06 26 81
- 25 Email: gaudichon-j@chu-caen.fr
- 26 **Email of the other authors:**
- 27 lydie.debaize@gmail.com
- 28 helenejakobczyk@gmail.com
- 29 mgaliber@univ-rennes1.fr
- 30 berengere.troadec@gmail.com

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32

33        **Abstract**

34        Long-term survival rates in childhood acute lymphoblastic leukemia (ALL) are  
35        currently above 85% due to huge improvements in treatment. However, 15-20% of children  
36        still experience relapses. Relapses can either occur in the bone marrow or at extramedullary  
37        sites, such as gonads or the central nervous system (CNS), formerly referred to as ALL-blast  
38        sanctuaries. The reason why ALL cells migrate to and stay in these sites is still unclear. In this  
39        review, we have attempted to assemble the evidence concerning the microenvironmental  
40        factors that could explain why ALL cells reside in such sites. We present criteria that make  
41        extramedullary leukemia niches and solid tumor metastatic niches comparable. Indeed,  
42        considering extramedullary leukemias as metastases could be a useful approach for proposing  
43        more effective treatments. In this context, we conclude with several examples of potential  
44        niche-based therapies which could be successfully added to current treatments of ALL.

45        **Keywords:** acute lymphoblastic leukemia; niche; cellular microenvironment; children;  
46        relapse; extramedullary; sanctuary; bone marrow; central nervous system; testis

## 47 **I. Introduction**

48 Acute lymphoblastic leukemia (ALL) is a hematological malignancy defined by the  
49 clonal expansion and accumulation of abnormal immature lymphoid precursor cells in the  
50 bone-marrow (BM) compartment. This abnormal cell population replaces normal  
51 hematopoietic cells and can spread from the BM to the entire body through systemic  
52 circulation. Relatively rare relative to other hematological malignancies in adults, ALL is the  
53 most frequent malignancy found in children and accounts for approximately 80% of  
54 childhood leukemia, with a maximal incidence rate between one and six years [1]. Over the  
55 last decades, the use of multi-agent chemotherapy protocols, better patient stratification into  
56 risk-adapted groups for treatment, and improved treatments have drastically reduced the  
57 mortality of this young population. Indeed, survival rates have risen from approximately 10%  
58 in the 1960s to above 90% for children in high-income countries in the 2000s.

59 However, approximately 15 to 20% of children and approximately 50% of adult patients  
60 still relapse [2–6]. This situation is defined as the resurgence of blast cells after the  
61 achievement of complete remission. Relapses are generally classified and stratified according  
62 to three prognostic factors: time of relapse, site of relapse, and immunophenotype B or T  
63 [7]. The time of relapse, relative to the beginning and end of treatment, is the most important  
64 prognostic factor: the earlier the relapse occurs, the worse the prognosis. The definition of  
65 relapse is slightly different depending on the classification used (Berlin-Frankfurt-Münster  
66 [BFM] or Children Oncology Group [COG]) [3,7]. The most important factor after timing is  
67 probably the anatomical sites of relapse, which are commonly divided into bone marrow  
68 (BM) alone (worst prognosis), extramedullary alone (best prognosis), and combined relapses  
69 (intermediate prognosis). Although immunophenotype T was initially associated with poor  
70 survival and is still used in tumor classification, its prognostic value appears to be less  
71 important due to better stratification [8]. Furthermore, the assessment of early minimal

72 residual disease (MRD) is also used to predict the prognosis of patients presenting a relapse,  
73 as it is for newly-diagnosed ALL [9–12]

74         Given the considerable therapeutic progress that has been made, one of the main goals  
75 is to attenuate treatment-related sequelae by reducing their toxicity, while continuing to  
76 improve current survival rates. Decreasing the incidence of relapse and identifying optimal  
77 treatments and therapeutic strategies when relapse occurs, are critical goals as well. Indeed,  
78 deciphering the underlying mechanisms that lead to relapse and understanding how and why  
79 relapses occur is a prerequisite to defining new treatment approaches.

80         In this review, we will begin with a brief overview of the principal mechanisms  
81 involved in ALL relapses. Second, we will describe where ALL cells reside within some sites  
82 of relapse and compare medullary, extramedullary, and metastatic niches, using a few  
83 examples. We will show how their microenvironments can be considered to be quite similar.  
84 Third, we will discuss the interplay between ALL cells and the microenvironments they  
85 colonize. Fourth, we will discuss the potential predisposition of some ALL cells to reside in  
86 specific niches. Finally, we will conclude with therapeutic perspectives related to  
87 microenvironment targeting.

## 88 **II. Cellular origin of relapse**

89         Multiple biological factors may contribute to the emergence of ALL relapses [13].  
90 Cumulative evidence suggests a clonal origin of ALL [14]. It has previously been shown that  
91 clonal evolution is involved in relapse, with the possible emergence of subclones derived  
92 either from the major clone found at initial diagnosis, minor subclones, or a common ancestral  
93 pre-leukemic clone (Figure 1) [15–20]. Several studies have described chemoresistance-  
94 related mutations in the BM samples of relapsed patients, such as those that affect the  
95 glucocorticoid response pathway (mutations of the glucocorticoid receptor gene *NR3C1* or

96 other genes, interfering with its function, such as *BTG1*) or nucleotide metabolism (for  
97 example, *NT5C2* or *PRSP1* genes [13,21–25]. Clonal heterogeneity is a common feature in  
98 ALL. Clonal evolution is likely to be a dynamic process in which selection plays a central  
99 role, as in the Darwinian model [14,26]. Both intrinsic and extrinsic factors, such as  
100 microenvironmental conditions or chemotherapeutic pressure, may favor a clone from which  
101 the relapse can develop.

102 Relapses may originate from a chemoresistant clone, either already present as a minor  
103 clone at diagnosis or resulting from the acquisition of secondary mutations during treatment.  
104 Aside from chemoresistance mechanisms, relapse can also be explained by the presence of  
105 dormant cells within the bulk population. These rare, non-dividing, quiescent cells are less  
106 sensitive to cytotoxic agents. They can therefore resist treatment and give rise to a new  
107 population after reentering the cell cycle, leading to relapse. Aside from the quiescent state  
108 itself, cells may also exert their resistance by residing in niches where the microenvironment  
109 confers protection from chemotherapy. Indeed, drug concentrations may be lower in niches, as  
110 suggested for brain and testicular tissues [27,28]. Local conditions, the cell matrix, cell-cell  
111 interactions, and soluble factors may also maintain quiescence. The origin of such dormant  
112 cells is still an open question, but recent studies have provided some clues to their existence at  
113 diagnosis [29,30]. Why and how they reenter the cell cycle and proliferate to induce relapse  
114 still needs to be addressed for understanding the evolution of these dormant cells. How such  
115 cells reach and invade distant favorable microenvironments from their native marrow niche is  
116 also yet to be elucidated.

117 Metastasis is the spread of cancer cells from the tissue where they first originate to  
118 another distant one. ALL relapses occur most often in the BM, whereas extramedullary  
119 relapses are less commonly reported. Nevertheless, the occurrence of relapse is generally  
120 thought to be a leukemic event, depending mainly on internal properties of the blast,



121 regardless of the type of relapse. However, the occurrence of relapse may not depend only on  
122 leukemic cell features, but also on the microenvironment. In the next section, we will describe  
123 the most frequently-reported sites of relapse and the concept of niches, proposing that  
124 extramedullary leukemia relapse is similar to that of solid tumor metastasis, anticipating a  
125 major role of the microenvironment of extramedullary sites.

### 126 **III. Sites of relapse**

127 Relapse can occur in the BM, the native niche from where the disease spreads at the  
128 onset. It can also emerge from extramedullary sites, such as the central nervous system (CNS)  
129 or gonads. Both used to be called “sanctuary sites”, because of the relative protection these  
130 sites appear to offer to blast cells from systemic chemotherapy. At these sites, the tissue-blood  
131 barrier is an obstacle to drug delivery [27,31,32]. Although CNS and testicular relapses may  
132 be more common in children, they also concern adults. These sites may represent the first  
133 extramedullary sites colonized by leukemic cells but can also be colonized secondary to other  
134 extramedullary sites that may have evaded clinical detection. Prophylactic cranial irradiation  
135 was added to systemic chemotherapy treatment in the 1970s to attempt to prevent CNS  
136 relapses [33]. Intrathecal chemotherapy and the use of high-dose methotrexate for neuro-  
137 meningeal prophylaxis have progressively replaced irradiation, with good results for  
138 diminishing neurological sequelae, showing that the blood-tissue barrier problem can be  
139 bypassed [34]. Similarly, treatment intensification, with the introduction of intermediate and  
140 high-dose methotrexate, has significantly decreased the incidence of testicular relapses, thus  
141 considerably improving the historically worse prognosis for boys [35,36]. However, extra-  
142 medullary relapses are still a problem and have been reported in many other organs. Several  
143 studies have reported that extramedullary relapses occur in approximately 40% of cases  
144 (approximately 20% combined and 20% presumably isolated) [3,8,37]. Here, we summarize  
145 the evidence that shows the role of the microenvironment in extramedullary relapses.

146 1. Different microenvironments

147 *a) Bone marrow (Figure 2a)*

148 BM is the most frequent site of ALL relapse and is involved as an isolated or combined  
149 site of relapse in 75-90% of cases [3,8,38–41]. BM relapses are most often the unique site  
150 involved (approximately 65% of cases) [3,8,34–37]. There is no clear evidence for a  
151 privileged BM site in which lymphoblasts primarily reside. Indeed, contrary to BM aspirates,  
152 BM biopsy results are generally not given in published studies, and almost all studies  
153 presenting BM biopsy data concern patients with advanced disease. In contrast, the  
154 engraftment of leukemic cells in BM has been well-studied in murine models [42–45]. In  
155 these models, leukemic cells engraft and proliferate in specific and restricted perivascular  
156 regions, in which C-X-C motif chemokine ligand 12 (CXCL12) and E-selectin are expressed,  
157 and which overlap with perivascular niches of hematopoietic progenitors. CXCL12 appears to  
158 be required for the diapedesis of leukemic cells [42]. Leukemic cells migrate and arrest in  
159 vessels close to endosteum, where they settle. From these perivascular endosteal niches, they  
160 may proliferate and spread to the BM and beyond [44].

161 *b) Gonads (Figure 2b)*

162 Large group studies have reported that isolated testicular relapses could account for  
163 3.5-8% of ALL relapses in children (including boys and girls) followed for up to 20 years  
164 [3,8,38–41,46]. Most studies mentioning testicular biopsies in the context of ALL did not  
165 indicate the precise location of blasts [47–49]. Such information can be retrieved from rare  
166 and old clinical studies and animal models [50–56]. Animal models indicate that leukemic  
167 cells infiltrate the interstitial testicular tissue mainly at the perivascular and peritubular  
168 regions, forming small foci, notably in subcapsular areas. Despite perivascular interstitial  
169 invasion, extensive infiltration of the interstitium is rare. On the other hand, the epididymis is

170 more often massively invaded by blast cells [54,55]. Leukemic invasion seems to occur  
171 through the hematogenous route, with possible extension to the epididymis *via* the lymphatic  
172 route [55]. In these models, although seminiferous tubules were degenerated or destroyed near  
173 leukemic infiltrations, leukemic cells were never found in the tubules nor the gonadal duct  
174 [54–56]. Unlike these *in vivo* studies, testicular leukemic infiltration was mostly found to be  
175 diffuse in a postmortem series of children who died of very advanced leukemia. Leukemic  
176 cells were found in the tubules in half the patients with heavily infiltrated testes [57]. This  
177 relatively old study included children with uncontrolled leukemia at a time when outcomes  
178 were poor and probably does not reflect the route of initial invasion. Thus, it seems that  
179 leukemic cells invade the testis by the hematogenous route and first infiltrate the perivascular  
180 and peritubular spaces, where they can spread to the epididymis through the local lymphatic  
181 system. Without effective treatment, seminiferous tubules may be destroyed and invaded as  
182 well. This is concordant with the perivascular and peritubular interstitial infiltration observed  
183 in patients with testicular relapse leukemia [50–52].

184         Although less commonly involved than the testis, ovaries may also be involved in  
185 ALL [58]. Ovarian relapses are scarcely reported in clinical trials [40,59], but autopsies have  
186 provided evidence of ovarian disease in end-stage leukemia [57,60,61]. More recently,  
187 ovarian involvement was found in approximately 8% of leukemia cases in an autopsy series,  
188 regardless of age. However, the authors did not indicate whether the patients had been treated  
189 or not, nor the treatment performed [62]. The effects of chemotherapy could explain the  
190 differences observed between and within the reports mentioned above, in which ovarian  
191 involvement was found in 3% to up to 50% of cases [57,60,61]. Indeed, Pais *et al.* reported  
192 that the number of ovarian relapses dramatically decreased in their cohort from the moment  
193 they introduced intermediate-dose methotrexate in the treatment regimen (from almost 10% of  
194 all relapses before 1979 to no ovarian relapse noted afterwards, until 1991). As they assumed,

195 isolated ovarian relapse has become an exceptional event, likely representing slightly less than  
196 1% of all relapses [40,59]. However, contrary to testicular involvement, which can be  
197 clinically suspected with a simple physical examination, there is no overt clinical sign that  
198 allows suspicion of ovarian leukemic infiltration. In addition, ultrasound evaluation is not  
199 systematically performed during the course of the disease and follow-up, even if it might be  
200 [63]. However, it was possible to detect leukemic cells in up to 70% of samples by  
201 polymerase chain reaction of cryopreserved ovarian tissue retrieved at diagnosis (or shortly  
202 after), even in the absence of enlargement and with a negative histological examination  
203 (except in one patient from a study of Soares *et al.*, whose ovarian medulla was invaded) [64–  
204 67]. Most published studies concerning ovarian disease consist of case reports, which usually  
205 do not report the precise location of leukemic cells in the ovary [58]. In their autopsy series,  
206 Reid *et al.* found that leukemic invasion, which was mostly histologically mild, took place  
207 predominantly in the medulla in the perivascular spaces [57]. However, leukemic cells have  
208 also been detected in the cortex by other authors. Thus, it is difficult to say where the cells  
209 preferentially settle [64–67]. Nonetheless, follicles have never been reported to be invaded in  
210 the literature.

211 *c) Central nervous system (Figure 2c)*

212 Isolated CNS relapses represent approximately 7.5-15% of ALL relapse [3,8,38–41].  
213 The precise location of leukemic cells in the CNS is rarely indicated in published studies.  
214 Autopsy and animal model studies have allowed a better understanding of how the CNS may  
215 be invaded by leukemic cells [68,69]. Indeed, Price and Johnson studied 126 brains from  
216 children who died of ALL and developed a grading system to evaluate the severity of  
217 arachnoid involvement. They detected leukemic cells in the CNS of 55% of patients. Most  
218 showed moderate leukemic infiltration and the authors observed that infiltration of the brain  
219 parenchyma occurred only in cases of extensive and deep leukemic arachnoid involvement

220 with cerebrospinal fluid (CSF) contamination and pia-glia membrane destruction (13.4% of  
221 cases). Arachnoid invasion was most often isolated or multifocal, rather than diffuse. They  
222 concluded that leukemic cells first invade the superficial arachnoid from the vein wall, then  
223 the deep perivascular arachnoid, and finally, the brain parenchyma through disrupted pia-glia  
224 membranes [68]. *In vivo* rodent models have provided similar results and suggest that  
225 leukemic cells transit through the blood-CSF barrier to reach the leptomeninges [69,70].  
226 Although Price and Johnson only studied brain and did not consider the spinal cord or dura  
227 matter in their study, diffusion throughout the CNS *via* the CSF has been confirmed by  
228 leukemic cell DNA detection, both in the lumbar spinal cord and frontal brain of rats  
229 inoculated in the cerebellum [70]. This is consistent with neuroradiological findings, which  
230 indicate that meningeal infiltration is the most common manifestation of CNS leukemia, with  
231 most often diffuse infiltration [71,72]. However, although much rarer and mostly represented  
232 by case reports in the literature, CNS involvement may also consist of epidural masses that  
233 can compress the spinal cord [73]. Thus, leukemic cells do not colonize one specific  
234 anatomical region within the brain or other CNS sites, but rather appear to preferentially  
235 localize to the subarachnoid space, likely in perivascular areas. A very recent study, using a  
236 mouse model, has shown that meningeal invasion occurs through the diapedesis of leukemic  
237 cells from cranial or spinal bone marrow of adjacent bones. The authors reported that  
238 leukemic cells transit through blood vessels, the emissary vessels that bridge the CSF space  
239 from the brain or spinal cord to the calvarium or vertebral bodies. They also discovered that  
240 this mechanism involves an interaction between  $\alpha 6$  integrin and laminin and that it can be  
241 blocked by specific PI3K $\delta$  inhibitors [74].

#### 242 *d) Others*

243 Because leukemic cells spread throughout the organism via systemic circulation, other  
244 sites of relapse may be considered. Other extramedullary sites have been little studied and

245 published data consist mostly of case reports [75]. Although no site can be defined as  
246 privileged, since ALL can settle in many tissues, breast involvement may be a privileged site  
247 of extramedullary leukemia, mostly described by Isabel Cunningham [75–79].

## 248 2. Similarities between sanctuary sites

249 We chose not to provide an exhaustive comparison between metastatic niches but  
250 rather to focus on three representative common actors: the CXCL12/C-X-C chemokine  
251 receptor type 4 (CXCR4) axis, hypoxia, and stem cell factor (SCF).

### 252 a) *The concept of “niche”*

253 The BM microenvironment has been, and is still, extensively studied [80–84]. First  
254 postulated by Schofield in 1978, it is now accepted that hematopoietic stem cells (HSC) reside  
255 in specialized compartments, called “niches”, within the BM [85]. Such niches allow the  
256 maintenance and regulation of HSCs by providing specific local conditions, cytokines,  
257 chemokines, and other soluble factors, as well as various types of specialized stroma cells.  
258 Different types of HSC niches have been described in the literature and may be named  
259 differently, depending on the authors, which can be confusing [80,81,86,87]. Our  
260 understanding of the BM microenvironment is constantly evolving and is still a subject of  
261 debate. For example, some studies have provided evidence for the existence of an osteoblastic  
262 niche [86], but this has been more recently questioned. Indeed, the existence of an authentic  
263 osteoblastic HSC niche has been ruled out, even if osteoblastic lineage cells have an indirect  
264 role in the regulation of HSCs and appear to be part of a specialized niche for lymphoid  
265 progenitors [81,84]. Rather than viewing the BM as a suite of separated niches, it is more  
266 likely a unique continuous niche in which some components are shared (and some others not)  
267 between more specialized compartments within this niche, as proposed by Hira *et al.* [88].

268 HSCs localize mainly to perivascular zones, as do leukemic cells, with most found close to  
269 sinusoids, whereas approximately 10% are found near small arterioles.

270 The microenvironments of the testes and CNS also have niches that maintain and  
271 regulate spermatogonia and neural or glial stem cells, respectively[89–93]. It has been shown  
272 in several cancer models that cancer cells can migrate from the tissue they originate to  
273 predisposed distant niches, which they can then remodel to suit their needs [94,95]. Such  
274 metastatic, BM, testicular, and neural niches may share characteristics that attract leukemic  
275 cells and offer them a favorable microenvironment for maintenance and growth. We focus on  
276 some of these shared components that have been found to play a major role in these  
277 microenvironments.

#### 278 *b) CXCL12/CXCR4*

279 CXCL12, also known as stromal derived factor 1 alpha (SDF1 $\alpha$ ), is one of the most  
280 important chemokines in the BM niche [84]. Through binding to its receptor, CXCR4,  
281 CXCL12 favors HSC homing to the BM and participates in the maintenance of these cells in  
282 the niche. CXCR4 is a seven-transmembrane G-protein-coupled receptor that is expressed on  
283 multiple cell types, including, notably, hematopoietic cells [96]. The inhibition of CXCR4 is  
284 frequently used clinically to mobilize HSCs into the peripheral blood as a source of stem cells  
285 for transplantation [84,97–99]. CXCL12 is highly expressed by specialized cells close to  
286 sinusoid vessels within the BM, called CXCL12 abundant reticular cells , and favors HSC  
287 retention in the BM [84,97].

288 CXCL12 has been shown to be involved in metastasis in many cancer models, such as  
289 breast, prostate, and lung cancer, pancreatic adenocarcinoma, rhabdomyosarcoma, and others  
290 [100]. For example, it was shown in an ovarian cancer model that the most advanced stages of  
291 the disease and most invasive phenotypes are associated with increased CXCR4 expression at

292 the cancer cell surface. More importantly, ascites samples from patients with advanced  
293 ovarian cancer showed significantly higher CXCL12 levels [101]. The role of the  
294 CXCL12/CXCR4 axis in the dissemination of peritoneal cancer has been clarified more  
295 recently in a study using a primarily murine colon cancer model. The authors found that  
296 CXCL12-expressing cells, similar to CXCL12 abundant reticular cells and distributed  
297 throughout the perivascular zones of adipose tissue, were a privileged site from which cancer  
298 cells could disseminate [102]. In another example, Ogawa *et al.* showed that CXCL12  
299 expression increased in the regional lymph nodes of mice with intrapulmonary transplanted  
300 cancer cells in a Lewis lung carcinoma model, and participated in the formation of a pre-  
301 metastatic niche [103].

302         The CXCL12-CXCR4 axis is also involved in the pathogenesis of leukemia, as it favors  
303 not only ALL cell homing in the BM, but also their survival and proliferation [42,43,104,105].  
304 Some evidence indicates that, in the human testis, CXCL12 is expressed solely by Sertoli  
305 cells to promote spermatogonial stem-cell migration through the seminiferous tubules during  
306 spermatogenesis [106–109]. Sertoli cells reside in seminiferous tubules, where leukemic cells  
307 are generally not found, or only very late during testicular involvement. However, the use of  
308 transwell migration assays showed that ALL cells can also migrate towards mouse testis  
309 conditioned medium and that such migration was reduced when the cells were treated with  
310 plerixafor, a CXCR4 chemical inhibitor [110]. This result suggests that leukemic-cell  
311 migration towards the testis might be at least partially mediated (or not exclusively) by the  
312 CXCL12/CXCR4 axis. Moreover, McIver *et al.* have shown that although CXCL12  
313 expression is restricted to Sertoli cells in the normal testis, it is expressed in the tumor stroma  
314 of seminoma samples [107]. Pathological conditions may induce CXCL12 expression in the  
315 interstitium of the testis [111]. Thus, it is possible that leukemic cells could be attracted and  
316 settle in the testis.



317 The CXCL12/CXCR4 axis is also known to play an important role in neurogenesis and  
318 neuron migration during development [112–116] and CXCL12 is constitutively expressed in  
319 the adult CNS, where it participates in neuromodulatory functions and acts as a major player  
320 in neuro-inflammation by recruiting leukocytes across the blood-brain barrier [112,117–120].  
321 McCandless *et al.* showed that normal expression of CXCL12 on the basolateral side (*i.e.*  
322 away from vessel lumen) of CNS endothelial cells retains leukocytes in the perivascular  
323 space, thus preventing their egress to the CNS parenchyma [119,120]. Leukemic cells may  
324 therefore be attracted into perivascular areas by such a CXCL12 expression pattern, where  
325 they could stay, possibly in a quiescent state. Indeed, recent studies in a T-ALL model have  
326 shown that the CXCL12/CXCR4 axis plays an important role in leukemic-cell migration into  
327 the CNS [121,122]. Nevertheless, although treating xenografted mice with plerixafor (a  
328 CXCR4 antagonist) reduced engraftment into the BM and liver in a B-ALL model, it failed to  
329 prevent blast infiltration of the CNS, suggesting that CNS infiltration may not be mediated by  
330 the CXCL12/CXCR4 axis in B-ALL [69]. Other studies indicate that the CXCL12/CXCR4  
331 axis is also involved in leukemic-cell infiltration into other extramedullary tissues. Indeed,  
332 Crazzolaro *et al.* found that high CXCR4 expression at the surface of ALL cells was  
333 associated with more pronounced infiltration of extramedullary tissues, such as liver and  
334 spleen, which was confirmed later in another study using a NOD/SCID immunodeficient  
335 mouse model [123,124]. Consistent with the latter studies, Kato *et al.* demonstrated that the  
336 liver constitutes a favorable niche promoting ALL cell engraftment, survival, and  
337 chemoresistance through the CXCL12/CXCR4 axis [125]. Overall, there is evidence in the  
338 literature showing that the CXCL12/CXCR4 axis plays a major role in ALL cell homing, both  
339 to the BM and extramedullary niches. Thus, CXCL12 represents a common feature of  
340 hematopoietic, extramedullary, and solid tumor niches, which may reconcile ALL and solid  
341 tumor models.

342                    *c) Oxygen content*

343            Improperly termed hypoxia, low oxygen concentrations are physiologically found in  
344 specific areas in mammalian organisms [126]. Indeed, the BM is likely to be one of the tissues  
345 in which the oxygen concentration is the lowest [127]. Ishikawa and Ito measured oxygen  
346 tension in BM samples from 4.9% to 8.8%, but it likely poorly reflects the true oxygen level  
347 found in the BM [128]. Later, mathematical modeling predicted the existence of an oxygen  
348 gradient in the BM, with oxygen tension decreasing progressively, starting from the sinus  
349 [129,130]. Since then, experimental evidence has confirmed very low oxygen concentrations  
350 in the BM, with the lowest value measured at 0.6% [131,132]. Increasing evidence suggests  
351 that the niches in which HSCs reside are hypoxic and that low oxygen concentrations may be  
352 needed to maintain their stemness [132–137].

353            Low oxygen concentrations have been highlighted during cancerogenesis in solid  
354 tumors. As reviewed elsewhere, hypoxia in cancer cells and in the tumor microenvironment  
355 promotes aberrant angiogenesis, epithelial-mesenchymal transition, radio- and  
356 chemoresistance, and metastasis [138]. For example, it has recently been shown in a breast  
357 cancer model that hypoxia can upregulate pro-metastatic genes and promote metastasis,  
358 particularly under conditions of intermittent hypoxia, [139]. Moreover, the authors found that  
359 breast-cancer cells cultured under hypoxic conditions secreted pro-tumorigenic cytokines.  
360 Hypoxia was similarly shown to enhance the invasiveness of melanoma cell lines *in vitro* and  
361 promote metastasis *in vivo* [140].

362            More importantly, low oxygen concentrations are thought to be an important component  
363 of leukemic niches in the BM and recent results have shown that targeting this hypoxic  
364 microenvironment may be promising [141–148]. Indeed, hypoxia has been shown to enhance  
365 the chemoresistance of ALL cells and appears to confer stem-cell properties, such as

366 quiescence [141,149,150]. Drugs active only under conditions of hypoxia were shown to  
367 reduce the leukemia burden in the BM when administrated three days after ALL cell injection  
368 into NOD/SCID mice, suggesting that hypoxia may favor leukemic-cell engraftment  
369 [141,147].

370 The testes are also thought to be one of the most hypoxic tissues. Direct measurements  
371 in a rat model established the interstitial testicular oxygen concentration between 1.5 and  
372 3.5% [151,152]. Another study using pimonidazole staining, commonly used as a hypoxic  
373 marker, confirmed these results in a mouse model and suggested an even lower intratesticular  
374 oxygen tension, especially in the seminiferous tubules, as well as the interstitium [153].  
375 Nevertheless, there is no evidence of an association between the hypoxic features of the testis  
376 and testicular leukemia infiltration as it has not been directly studied.

377 Low oxygen concentrations are also found in the CNS, from 0.5% to 7%, both in  
378 humans and animal models [126]. There are few clues in the literature to estimate the oxygen  
379 tension of subarachnoidal tissue, where ALL cells preferentially settle. Some studies have  
380 evaluated the oxygen tension within the CSF, with estimated values of approximately 5 to 6%  
381 [154,155]. More recently, Sharan *et al.* showed that oxygen tension decreases rapidly from  
382 pial vessels to the extravascular space in a rodent model, with oxygen levels as low as 3% 30  
383  $\mu\text{M}$  from the vessels, especially the narrowest ones [156]. Thus, although oxygen  
384 concentrations have not been precisely measured in the subarachnoidal space, they are likely  
385 to be low. Recent published data have indeed suggested a role for low oxygen concentrations  
386 in leukemic cell CNS infiltration [157,158]. In these two independent studies, the authors  
387 found that leukemic cells retrieved from the CNS of xenografted mice had a hypoxia-related  
388 gene set profile and expressed a particularly high level of the transcript of the vascular  
389 endothelial growth factor A (VEGFA) gene, which is a principal target of the hypoxia master  
390 transcription factor, hypoxia inducible factor 1 (HIF1). More importantly, Münch *et al.* further

391 showed that VEGFA mediated the migration of ALL cells through a monolayer of a brain-  
392 microvasculature cell line [158]. The results from these studies are in accordance with those  
393 of a previous one showing that patients with CNS leukemia had elevated levels of VEGF in  
394 the CSF [159]. Thus, the role of hypoxia in extramedullary leukemia merits exploration and  
395 may offer new therapeutic perspectives, as outlined by Izraeli and Eckert [160].

396 *d) Stem cell factor*

397 Stem cell factor (SCF), also known as steel factor or mast cell growth factor, is one of  
398 the main growth factors that regulate the proliferation and survival of HSCs and  
399 hematopoietic progenitors in the BM, through binding to its receptor, c-KIT [161–163].  
400 Indeed, insertion of the *Egfp* gene into the endogenous locus of the *Scf* gene in C57BL mice  
401 results in perinatal death with severe anemia. GFP expression patterns within the BM have  
402 suggested that SCF is produced in the BM by perivascular stromal and endothelial cells,  
403 which was further demonstrated using a conditional deletion model in the same mice [164].  
404 Moreover, Ding *et al.* demonstrated that SCF is necessary to maintain adult HSCs, using the  
405 same conditional deletion model. Along with its effects on progenitor cell survival and  
406 proliferation, SCF has also been shown to have a chemotactic function in these cells [162].  
407 Proteolytic cleavage and alternative splicing lead to various tissue-specific isoforms of SCF,  
408 which can be produced as soluble or membrane-bound forms [161–163,165,166]. The  
409 membrane-bound form of SCF appears to be essential [162]. The membrane-bound form of  
410 SCF has notably been shown to enhance human HSC engraftment in murine xenografts. It has  
411 also been shown to promote cell adhesion *in vitro* and HSC residence in the BM niche *in vivo*  
412 [162,167–169].

413 In solid tumors, SCF/c-KIT signaling is also frequently thought to participate in  
414 tumorigenesis, tumor progression, and metastasis. Soluble SCF is produced by cancer cells

415 within tumor tissues in different cancer models [170]. c-KIT has been found to be  
416 overexpressed, abnormally expressed, or associated with an aggressive phenotype in several  
417 types of tumors, including melanoma, lung cancer, renal carcinoma, and seminoma  
418 [163,171,172]. Activating mutations of c-KIT can be found in some tumor types, especially  
419 gastrointestinal stromal tumors, and may be involved in their pathogenesis. The SCF/c-KIT  
420 pathway may also be involved in the pathogenesis of such tumors through its effect on mast  
421 cells [163] and the mast cell accumulation has been associated with a worse prognosis in  
422 several cancer models.

423 The SCF/c-KIT signaling pathway has also been found to be deregulated mostly in  
424 AML models rather than in ALL models. However, although c-KIT is little expressed in ALL  
425 relative to AML, RNA-seq data indicate that some ALLs overexpress c-KIT, such as ETS  
426 variant 6-runt related transcription factor 1 (ETV6-RUNX1) B-ALL or mixed lineage  
427 leukemia (MLL) rearranged leukemias [173]. Consistent with the expression of c-KIT in these  
428 malignancies, SCF participates in early B-cell development in association with interleukin-7  
429 (IL7) [161]. Moreover, it was shown that ALL cells can modify their microenvironment by  
430 producing SCF [43]. Thus, the involvement of SCF signaling in the homing and  
431 pathophysiology of ALL may merit exploration, as suggested by a recent report [174].

432 The SCF/c-KIT axis plays also an important role in male reproduction, as suggested by  
433 the infertility of mice with mutations affecting both the *W* (encoding c-KIT) or *Sl* (encoding  
434 SCF) loci [161,163]. In the testis, SCF is produced by Sertoli cells and c-KIT is expressed on  
435 the surface of germ cells and Leydig cells, raising the possibility that Sertoli cells secrete a  
436 soluble form of SCF [171,175,176]. Indeed, a higher percentage of mRNA for the soluble  
437 than membrane form of SCF correlated with higher serum testosterone levels in infertile  
438 patients [177]. These results suggest that Sertoli cells may produce soluble SCF that reaches  
439 Leydig cells and thus enhance testosterone production. Moreover, SCF expression in normal

440 adult interstitial testicular tissue has been reported both in mice and humans in several  
441 immunohistochemistry studies, even if several others did not [178–181]. Thus, leukemia cells  
442 may be attracted to or retained in testicular interstitial tissue at least partially through  
443 activation of the SCF/c-KIT pathway.

444 SCF is also expressed in the adult CNS, as well as in CSF, and is involved in CNS  
445 development during embryogenesis [182–184]. There is no direct evidence in the literature for  
446 a role of the SCF/c-KIT axis in the attraction of leukemia cells to the CNS or their subsequent  
447 survival. However, SCF is one of the specific cytokines expressed by endothelial cells of the  
448 blood-brain barrier [183]. Kallman *et al.* compared gene expression profiles of human  
449 umbilical vein endothelial cells (HUVEC) and primary human cerebral endothelial cells using  
450 a cDNA array technique and found that 35 genes were differentially expressed in cerebral  
451 endothelial cells, including those for interleukin-6 (*IL-6*), transforming growth factor  $\beta$ 2  
452 (*TGF- $\beta$ 2*), *VEGFA*, and *SCF* [183]. An enzyme-linked immunosorbent assay (ELISA)  
453 confirmed that the supernatant from these cells contained SCF. Thus, endothelial cells of the  
454 CNS can produce SCF that may attract and/or promote the survival of leukemia cells that  
455 express c-KIT.

456 Overall, these examples suggest that the SCF/c-KIT axis may be an attractive  
457 therapeutic target for leukemia relapse, both at medullary and extramedullary sites, as well as  
458 for various solid tumors.

#### 459 *d) Other shared factors*

460 The extracellular matrix protein osteopontin, which is physiologically produced in the  
461 BM, participates in the pathophysiology of various cancers, including hematological  
462 malignancies, with a potential role in leukemic cell CNS infiltration [185,186]. Colony  
463 stimulating factor 1 (CSF1), a cytokine involved in macrophage differentiation and

464 constitutively expressed by osteoblasts, is expressed in prepubertal and adult testicular  
465 interstitial tissue, notably by macrophages, and has a role in regulating spermatogonial stem  
466 cells and their niche[187–189]. CSF1 is also expressed in the CNS, where it maintains  
467 microglia and participates in CNS development [190]. CSF1 deficiency impairs both  
468 reproductive function and integrity of the CNS. Genetic alterations involving the CSF1  
469 receptor, including the recurrent SSBP2-CSF1R fusion, have been identified in B-ALL and  
470 have been found to lead to a functional fusion, suggesting that targeting CSF1 signaling could  
471 be promising [191–193]. Another cytokine, transforming growth factor  $\beta$ 1 (TGF $\beta$ 1), regulates  
472 Leydig cell function in the testis, but is also secreted by the choroid plexus in the CSF, where  
473 it contributes to the maintenance of the choroid plexus itself and appears to be involved in the  
474 protection and repair of nervous tissue. TGF $\beta$ 1 has been shown to be involved in cancer  
475 stemness, metastasis, and epithelial-mesenchymal transition in several cancer models.  
476 Recently, TGF $\beta$ 1 was found to promote the escape of leukemic cells from natural killer (NK)  
477 immune surveillance in an ALL model.

478 All these factors and other components, such as the various cell types found within the  
479 niche, may also contribute to therapy resistance by preventing treatments from reaching their  
480 targets. BM and extramedullary sites may therefore contain pharmacological niches, where  
481 leukemic cells are protected from the effects of chemotherapy. For example, mesenchymal  
482 stem cells have been shown to participate in the resistance of leukemic cells to asparaginase  
483 treatment by providing them with asparagine [194]. Interestingly, it was further found that  
484 vincristine treatment administered prior to asparaginase can affect mesenchymal cells, thus  
485 blocking the secretion of asparagine and overcoming this resistance mechanism [195]. This  
486 indicates that treatments may exert their effect not only by affecting leukemic cells but also  
487 their microenvironment.

488 We have shown that leukemic cells, like other cancer cells, exploit several identical  
489 features of the microenvironment to reach distant sites. We now discuss whether blast cells  
490 may prepare their distant niches for engraftment and proliferation.

#### 491 **IV. The interplay between leukemic cells and the microenvironment**

##### 492 1. Leukemic and cancer cells model their niche

493 More than one century ago, Stephen Paget noted that breast cancer metastases occur  
494 more often in some tissues than others and, refuting that this could hardly occur by chance or  
495 solely by embolism, formulated his famous “seed and soil” hypothesis [196]. He compared  
496 cancer cells to plant seeds that can only grow in favorable soil, that is, in a tissue somewhat  
497 predisposed to welcome them. At that time, he already recognized that not only cancer cells  
498 but also their “soil” would be worth studying. Since then, a large amount of information has  
499 accumulated on cancer cell biology and the organotropism phenomenon has been  
500 experimentally explored and demonstrated [197,198]. We illustrated above that some  
501 leukemic cells, like some cancer cells, may be prone to home to and remain in certain  
502 microenvironments that resemble that from which they originated. However, such a remote  
503 and different microenvironment will not perfectly match the original niche and will probably  
504 not meet all the needs of the leukemic cells.

505 Indeed, there is evidence that leukemic cells can remodel their microenvironment [199].  
506 Colmone *et al.* used an *in vivo* microscopy imaging approach and found that ALL cells  
507 remodeled the BM microenvironment they colonized, notably through SCF signaling, leading  
508 to an abnormal niche that recruited and somehow trapped normal hematopoietic progenitors  
509 [43]. More recently, Boyerinas *et al.* xenografted the leukemic Nalm6 cell line in a mouse  
510 model and showed that the leukemic cells secreted osteopontin into the invaded  
511 microenvironment, which in turn also produced osteopontin [200]. It was also shown using *ex*



512 *in vivo* co-cultures of primary mesenchymal stromal cells and either the Nalm6 cell line or  
513 primary ALL cells that although the CXCL12/CXCR4 axis was involved in leukemic cell  
514 migration, other chemokines, such as C-C motif chemokine ligand 2 (CCL2) and CXCL8 may  
515 be involved in leukemic niche formation, independently of the CXCL12/CXCR4 axis.  
516 Transwell assays also demonstrated that leukemic cells could recruit mesenchymal stromal  
517 cells to initiate the leukemic niche, in which secreted chemokines might attract other leukemic  
518 cells while inhibiting that of healthy hematopoietic progenitors and mesenchymal stromal  
519 cells [201]. These results are consistent with those of other studies including that of Colmone  
520 *et al.* in which Nalm6 cells colonized CXCL12 niches in the BM of xenografted mice, which  
521 subsequently resulted in marked downregulation [43,202]. A recent report showed that  
522 leukemic cells can promote osteoclast-mediated bone resorption through the production of  
523 receptor activator of nuclear factor  $\kappa$ B ligand (RANKL) [203]. This is in accordance with a  
524 previous report that demonstrated in a T-ALL mouse model that ALL cell engraftment in the  
525 BM of mice was associated with bone microenvironment remodeling due to a marked loss of  
526 osteoblastic cells [204]. Duan *et al.* used *ex vivo* imaging of xenografted mice to confirm that  
527 engrafting ALL cells alters the BM microenvironment, resulting in damage to the vasculature  
528 and endosteal lining. This report also showed that, following chemotherapy, leukemic cells  
529 secreted cytokines, namely CCL3 and TGF $\beta$ , inducing phenotypic changes in the surrounding  
530 mesenchymal stem cells. These cells provided a protective microenvironment for the  
531 leukemic cells by converting a growth differentiation factor 15 (GDF-15) proform secreted by  
532 the leukemic cells into a mature form, which led to the activation of TGF $\beta$  signaling, thereby  
533 conferring chemoresistance [44].

534         Recent studies on solid tumors have led to a better understanding of cancer-cell  
535 organotropism and metastatic niche formation. Kaplan *et al.* first demonstrated, in 2005, that  
536 cancer cells can remotely prepare an appropriate niche before reaching their metastatic site

537 [198,205]. The authors demonstrated that cancer cells secrete certain factors that may  
538 predispose them to metastasize toward a specific location. They first suggested cytokines, but  
539 discovered several years later that the cancer cells produced exosomes that could direct  
540 metastatic cancer cells to specific tissues [198,206]. Exosomes are small extracellular vesicles  
541 (30-100 nm of diameter) that can be transferred to other distant cells to deliver functional  
542 molecules, such as peptides or nucleic acids. Thus, exosomes are a means of intercellular  
543 communication suitable for remote cells. Exosomes derived from different cancer cell lines  
544 specifically target different tissues. An elegant experiment showed that exosomes can  
545 “educate” cancer cells to reach a specific tissue. Mice were injected with exosomes from cells  
546 that usually metastasize to the lungs before being transplanted with cells that do not usually  
547 metastasize to the lungs, but to bones. This experiment resulted in a large significant increase  
548 in lung metastases. Going further, the authors found that exosomal organotropism was  
549 determined by their integrins, which allowed exosome uptake by specific tissue-resident  
550 stromal cells within target tissues. Exosome uptake by these cells was followed by  
551 upregulation of the expression of *S100* family pro-migratory and pro-inflammatory genes,  
552 which could favor tumor metastasis. Importantly, specific exosomal integrins recovered from  
553 plasma samples of patients with pancreatic or breast cancer correlated with the location of the  
554 metastases, and may even likely predict future metastatic sites at the time diagnosis.

## 555 2. Means of communication between ALL cells and supportive cells

556 Several means of communication are available to ALL cells to interact and  
557 communicate with their surrounding microenvironment and they may be used simultaneously.  
558 The role of exosomes in the pathophysiology of several cancers is becoming increasingly  
559 understood but has been little studied, to date, in the context of ALL [83,207]. However,  
560 recent findings have shown that large extracellular vesicles (EVs), released through plasma  
561 membrane budding, may be of particular importance for the communication between ALL

562 cells and their microenvironment [208,209]. Indeed, the group of Vaskar Saha showed that  
563 ALL cells can produce EVs of varying size, some of which could be transferred horizontally  
564 to other ALL cells, as well as BM stromal cells, resulting in phenotypic changes in the  
565 recipient cells [209]. The transfer of large EVs to BM stromal cells induced a metabolic  
566 change, favoring glycolysis in these cells, which increased the lactate concentration in the  
567 medium, thereby providing the leukemic cells with their preferred fuel, as hypothesized by the  
568 authors [208]. These large EVs were released into the systemic circulation of xenografted  
569 mice. It is thus possible that EVs participate in similar metabolic reprogramming and/or other  
570 phenotypic changes in remote stromal cells to promote ALL-cell migration to the secondary  
571 sites. Although more compelling evidence has yet to be provided, it seems likely that both  
572 exosomes and large EVs are involved in preparing a remote suitable niche for ALL cells. In  
573 addition, large EVs may also contain intact organelles that could be transferred to recipient  
574 cells [209]. Not all cells can generate EVs. Thus, as discussed by the authors, such  
575 mechanisms may allow EV-producing resistant leukemic cells to promote chemoresistance, or  
576 even resistance to targeted therapies, by transferring pro-resistant cellular content to other  
577 sensitive cells.

578         The transfer of organelles, such as mitochondria, could also allow recipient cells to cope  
579 with acute stress [210]. Another recent study showed that ALL cells also communicate with  
580 mesenchymal stromal cells using tunneling nanotubes to set up their niche [211]. Tunneling  
581 nanotubes are thin plasma membrane protrusions that connect one cell to another and allow  
582 the exchange of cellular content. This means of intercellular communication is independent of  
583 others and can promote leukemic cell survival, chemoresistance, and the release of cytokines  
584 into the microenvironment. The use of fluorescent dyes has made it possible to visualize the  
585 exchange of cellular content in both directions, from leukemic cells to mesenchymal stromal

586 cells and *vice versa*, confirming that ALL cells establish crosstalk with the surrounding  
587 microenvironment to protect themselves and promote their survival [211].

588         Direct contact of leukemic cells with stromal cells has also been previously  
589 highlighted in the pathogenesis of leukemia. Indeed, direct contact with stromal cells favors  
590 ALL cell survival, offers them a protective environment, and may minimize the effects of  
591 cytotoxic drugs [212–215]. Such cell-cell contact was found to be mediated, notably, by the  
592 binding of integrin  $\alpha 4\beta 1$ , also called very late antigen-4 (VLA4), expressed on the surface of  
593 ALL cells, to the vascular adhesion molecule-1 (VCAM1), expressed on the stromal cell  
594 surface [216]. Integrin  $\alpha 4\beta 1$  can also bind to extracellular matrix proteins, such as  
595 osteopontin and fibronectin, and has been shown to play an important role in ALL cell homing  
596 in mice [200,217,218]. In addition to its role in adhesion to extracellular matrix and/or stroma,  
597 it is likely that integrin  $\alpha 4\beta 1$  binding to its receptor induces signaling cascades to promote  
598 leukemic cell survival, proliferation, and/or chemoresistance [218]. Gap junctions are also  
599 involved in cell-cell interactions within the leukemic niche. Gap junctions allow the direct  
600 exchange of small molecules between two adjacent cells by creating a channel, most often  
601 through the alignment and docking of two connexons (consisting of hexamers of specialized  
602 proteins called connexins) of each cell. Several studies have suggested that leukemic cells can  
603 communicate with stromal cells through gap junctions formed with connexin 43 (Cx43) [219–  
604 223]. Although the mechanisms are still unclear, it appears that Cx43 gap junctions are  
605 dysregulated in leukemic BM. Another connexin, Cx25, has recently been thought to be  
606 involved in the pathogenesis of leukemia through gap junctions [224]. The authors showed,  
607 using both T-ALL and AML cells, that inhibiting gap junctions by selective Cx25 knockdown  
608 sensitized the cells to chemotherapy, suggesting that concomitant Cx25-targeted treatment  
609 could allow reducing the dose of cytotoxic agents in clinical practice, thereby reducing the  
610 side effects of chemotherapy while conserving its efficacy [224].

611 Direct communication of leukemic cells with the surrounding supportive cells,  
612 probably as well as distant communication from the original to the secondary niche, appears  
613 to be of crucial importance. Once in the secondary niche, leukemic cells must deal with the  
614 local cells, which must be suitable surrogates that sufficiently resemble BM stromal cells. In  
615 the CNS, for example, Akers *et al.* found, *in vitro*, that ALL cell lines were attracted to  
616 astrocytes, choroid plexus epithelial cells, and even more strongly to meningeal cells, to  
617 which they could strongly adhere. The authors showed that these cells from the CNS secreted  
618 amounts of CXCL12 equivalent to those of BM stromal cells and osteoblasts and that CXCR4  
619 blockade with plerixafor could inhibit ALL cell migration toward meningeal and plexus  
620 choroid epithelial cells. They also observed that co-culture of both ALL cell lines and primary  
621 ALL cells with any of these CNS cells decreased the effects of commonly used chemotherapy  
622 drugs on leukemic cell viability. The provided chemoprotection was likely the result of the  
623 combined effects of soluble factors and cell-cell contacts between the ALL cells and the CNS  
624 cells [225]. This study illustrates that ALL cells can interact with supportive cells other than  
625 those which belong to the niche from which they originate. Such cells may therefore be  
626 similar to those of the BM niche and ALL cells can communicate with them to enhance their  
627 leukemic-supportive properties and adapt themselves to better settle into secondary niches.

628 The interconnections involved in this interplay are complex, but the methods used in  
629 studies usually only focus on one aspect of one component of the leukemic  
630 microenvironment. For example, cell-cell interactions have mostly been studied through co-  
631 culture systems consisting of only leukemic cells and one type of stromal cell, although  
632 leukemic cells probably interact not only with supportive stromal cells but also other cells  
633 within the microenvironment. Similarly, hypoxia can induce CXCL12 and CXCR4  
634 expression, as well as that of SCF, and SCF itself has been found to increase HIF1 $\alpha$   
635 expression [226]. Leukemic cells may escape the effects of therapy by taking advantage of

636 this system, of which the complexity is even more pronounced if we consider other  
637 parameters, such as temporality.

### 638 3. The chicken or the egg causality dilemma in ALL relapses

639 An intriguing question is whether the cells found far from their original niche are the  
640 result of the development of a resistant clone that reached its secondary niche in response to  
641 treatment, or whether these cells were already there as leukemic or preleukemic clones, even  
642 before diagnosis. Recently, Williams *et al.* addressed this question in the context of B-ALL  
643 and found that most leukemic cells had the capacity to infiltrate the CNS and were not  
644 restricted to a specific population [69]. They demonstrated, in clonal tracking experiments,  
645 that the populations engrafting the CNS were polyclonal, and that the clones found in the  
646 CNS were also found in the spleen or BM of xenografted mice. The authors concluded that  
647 CNS invasion is a “generic” capacity of leukemic cells but, as they also discussed, their  
648 results do not rule out the possibility of the existence of specific clones particularly well-  
649 adapted for invading the CNS microenvironment and surviving there. They also did not  
650 discuss whether the cells already possessed this capacity to invade the CNS at a preleukemic  
651 stage. Also, one cannot exclude that a leukemic cell may have been generated during  
652 embryonic or fetal hematopoiesis and would stay in a dormant state in the CNS.

653 Similarly, solid tumor cells can settle at tissues distant from their original site early  
654 during the course of the disease, even before detection of the primary tumor [227,228]. Solid  
655 cancers, as well as leukemia, must be viewed as a systemic disease, in which cancer cells  
656 spread and engraft early at predisposed tissues that provide protection against insults, such as  
657 chemotherapy, notably through the induction and maintenance of a dormant state [227,229].  
658 However, as metastasis is an inefficient process, only a few cells may have the ability to  
659 survive and grow out of their original niche [230]. Such rare cells would be able to adapt

660 themselves to a new microenvironment, where they could become quiescent or grow slowly,  
661 explaining why some patients experience very late relapses in distant tissues. The existence of  
662 leukemic stems cells (LSCs) that would be responsible for late relapses may also be evoked.  
663 However, these LSCs are not well characterized, especially in B-ALL, and several reports  
664 indicate that ALL cells with the capacity to initiate leukemia are relatively frequent [231, Le  
665 viseur 2008]. We have therefore chosen to focus on the role of the microenvironment, which  
666 appears to play an important part in the leukemia-initiating ability of ALL cells.

667 Ebinger *et al.* recently used an ingenious cell labeling system in patient-derived  
668 xenografts to provide evidence that such dormant cells exist in ALL and provided a robust  
669 preclinical model [45]. The authors found that these dormant cells shared a similar  
670 transcriptional program with leukemic cells found at MRD level in patients. Moreover, these  
671 dormant cells were resistant to conventional chemotherapy *in vivo*, but became sensitive to  
672 drugs when retrieved from their microenvironment and treated *ex vivo* [45]. This result has  
673 two principal clinically relevant implications: first, the dormant phenotype that appears to be  
674 harbored by MRD cells is reversible and second, the microenvironment appears to play a  
675 critical role, strongly suggesting that targeting the microenvironment could be a promising  
676 strategy to kill the disease and prevent relapse and treatment failure. As the authors state, it  
677 may also explain why children treated for ALL benefit from standard maintenance therapy  
678 that continues for two years from diagnosis: daily doses of low-dose chemotherapy might help  
679 to progressively eradicate all ALL cells, including the dormant cells that may reenter the cell  
680 cycle after the intensive phase of treatment. Although the authors showed that dormant ALL  
681 cells were preferentially found close to the BM endosteum, they did not explore engraftment  
682 in other tissues. It would be informative to use their model to verify whether such dormant  
683 cells can also be found in the extramedullary tissues frequently invaded in clinical practice,  
684 especially the perivascular areas described above. This study also argues in favor of an

685 extramedullary origin for combined ALL relapses, especially late relapses [59], as the  
686 dormant ALL cells were shown to have leukemia-initiating properties.

## 687 **V. From bench to bedside**

688 Leukemic cells interact dynamically with their microenvironment. Indeed, intravital  
689 microscopy experiments, which allowed the high-resolution observation of leukemic cells in  
690 live animals, showed the leukemic cells to be motile without any objective preference for a  
691 specific location or supportive cells [204]. Treatment of the mice with drugs commonly used  
692 in clinical practice (vincristine, dexamethasone, and asparaginase) reduced the leukemic  
693 burden and induced an increase in the proportion of quiescent cells, but did not modify the  
694 patterns of cell distribution and the cells kept moving, even faster than those of untreated mice  
695 [204]. These results suggest that leukemic engraftment was randomly driven, in contrast with  
696 other reports and commonly held beliefs. Thus, as stated by Williams *et al.*, engraftment  
697 potential may not be restricted to a specific cell population but may be shared by most ALL  
698 cells. This was recently corroborated by two independent studies [69,231,232]. In these  
699 studies, the authors used a cellular barcoding strategy to track leukemic clone engraftment in  
700 the BM of different bones and several extramedullary tissues of xenografted mice [231,232].  
701 Both studies showed that the engrafting cell populations were polyclonal, similarly to solid  
702 tumors, suggesting that all founder cells were able to reconstitute a leukemic bulk population.  
703 However, the authors found that the clonal distribution differed depending on the tissues  
704 analyzed: a clone could be more highly represented in one tissue, whereas another could be  
705 predominant in another tissue. The authors stated that this asymmetric clonal distribution was  
706 presumably stochastic and probably not due to clonal preference or a predisposition to settle  
707 in one site over another. As the authors discussed, the specific microenvironment may confer  
708 an advantage to arriving leukemic cells that favors settlement and/or the proliferation,  
709 explaining the observed asymmetric distribution. Notwithstanding the evidence provided by



710 the intravital microscopy experiment of Hawkins *et al.*, some cells from the founder  
711 population exposed to such a niche may become quiescent and be the ancestor of a subsequent  
712 relapse [204].

713         Aside from these studies, there is evidence in the literature suggesting that leukemic  
714 cells could themselves carry specific features that may direct their engraftment to one or  
715 another tissue. Van der Velden *et al.* recently identified a protein profile that could potentially  
716 help predict CNS relapse at the initial diagnosis. A comparison of the gene expression profiles  
717 between leukemic cells from the BM and CNS of patients allowed the identification of genes  
718 differentially expressed by leukemic cells from the CNS. Among these genes, *SSP1* (coding  
719 for osteopontin) and *SCD* (coding for stearyl-CoA desaturase) were found to be upregulated,  
720 which was further confirmed both at the mRNA and protein levels. Retrospective flow  
721 cytometry analysis of BM samples from patients who experienced a CNS relapse showed that  
722 both SPP1 and SCD proteins were elevated in a small subpopulation of cells, suggesting that  
723 this expression profile might be used in a clinical setting to predict CNS relapse [233]. As  
724 Frishman-Levy and Izraeli discussed in a recent review, in which they summarized current  
725 knowledge concerning CNS leukemia, the contradictory nature of these results with those of  
726 Williams *et al.* could be partially explained by methodological discrepancies (mouse  
727 xenograft model *versus* a human model). The idea that CNS tropism may be linked to a  
728 specific ALL population is also supported by the association of several recurrent genetic  
729 alterations with the incidence of CNS relapse. Indeed, the chromosomal rearrangement  
730 t(1;19), leading to the *TCF3-PBX1* fusion transcript, has been associated with an increased  
731 risk of ALL relapse in the CNS [234,235]. This clinical finding has recently been supported  
732 by a biological explanation. It was shown that primary leukemic cells expressing the *TCF3-*  
733 *PBX1* transcript expressed high levels of Mer tyrosine kinase, for which the signaling

734 pathway was further shown to be involved in ALL cell survival and chemoresistance in the  
735 CNS microenvironment [236].

736 Leukemia with the t(12;21) chromosomal rearrangement, which leads to the ETV6-  
737 RUNX1 fusion protein, is associated with an increased risk of testicular relapse [4]. We  
738 previously compared the expression profiles of ETV6-RUNX1 with those of other B-ALL and  
739 found that fourteen genes were differentially expressed in this type of leukemia [237]. Among  
740 these genes, *CD9* (tetraspanin) was found to be underexpressed, which was further confirmed  
741 by flow cytometry of patient samples [238]. We reasoned that CD9 may be involved in the  
742 pathogenesis of this particular B-ALL subtype [239]. We found that CD9 has a role in  
743 leukemic cell dissemination through the Ras-related C3 botulinum toxin substrate 1 (RAC1)  
744 signaling pathway. Importantly, we showed that the more CD9 was expressed at the surface of  
745 ALL cells, the more cells were able to migrate into the testes in a mouse xenograft model  
746 [110]. Thus, ETV6-RUNX1 ALL relapses may be partially explained by enhanced CD9  
747 expression, which favors blast dissemination and engraftment into the testis.

748 We postulate that ALL relapses occurring at specific sites could be explained by the  
749 association of both extrinsic and intrinsic factors, thus reconciling biological and clinical  
750 research findings. Specific genetic alterations may modify cell signaling pathways that could  
751 render the cells stochastically exposed to a specific microenvironment to take advantage of it  
752 and durably settle in it.

753 The role of the microenvironment in the pathogenesis of ALL has also been suggested  
754 by the good results obtained by treating or preventing extramedullary relapses with  
755 specifically directed local treatments. Indeed, aside from the introduction of high-dose  
756 methotrexate or aracytine, successful treatment of testicular or meningeal involvement has  
757 been specifically directed at the tumor: surgery/radiation therapy for the testis and intrathecal  
758 chemotherapy or radiation therapy for the meninges. This may explain the relatively good

759 prognosis for relapses at these sites and is another argument for an important role of the  
760 microenvironment, which is removed by surgery and radiotherapy. The role of the intrinsic  
761 properties of leukemic cells concerning their interaction with their surrounding  
762 microenvironment may be illustrated by the worse prognosis seen in Ph-like and Ph+ B-  
763 ALLs. Ph+ B-ALL and Ph-like B-ALLs share similar genetic alterations and a poor response  
764 to chemotherapy relative to other B-ALL subtypes. Within these subtypes, alteration of the  
765 Ikaros gene is frequently found. This alteration was shown to alter the adhesive properties of  
766 leukemic cells and may influence their settlement in their niche.[240].

## 767 **VI. Therapeutic challenges**

768 Therapeutic efforts in the past 15 years have mainly focused on the development of  
769 new molecules and immunological approaches directed against specific targets expressed on  
770 leukemic cells. Treatment intensification in patients with the most unfavorable prognostic  
771 factors has not only improved their survival, but also allowed to efficiently treat  
772 extramedullary leukemia without using local treatments. As mentioned earlier, the use of high  
773 doses of certain drugs, such as methotrexate, reduced the incidence of extramedullary  
774 involvement. Apart from these leukemic cell-oriented targeted therapies, therapeutic strategies  
775 oriented toward leukemic niches are also attracting increasing interest [241]. Figure 3  
776 illustrates a few examples of strategies that could be used to target leukemic cells in their  
777 microenvironment and Table 1 shows several examples of molecules that could be used or  
778 tested in clinical practice during the coming years. These strategies may also be applied to  
779 reach cancer cells in solid tumor metastatic niches.

780 First, preventing cell migration and engraftment into the niche or, conversely, the  
781 mobilization of niched cells could be attempted, for example by disruption of the  
782 CXCL12/CXCR4 axis using CXCR4 inhibitors, such as plerixafor [105,124,242,243].

783 Targeting cell adhesion to the extracellular matrix or supportive cells may also be a useful  
784 strategy, for example, by inhibiting  $\alpha 4\beta 1$  integrin-mediated adhesion with a specific  
785 monoclonal antibody, such as natalizumab, or a chemical inhibitor, such as TCB3486  
786 [244,245]. Another possible niche-oriented therapeutic strategy could be to target integrins,  
787 since exosomes home to future metastatic sites via integrins in melanoma and lung cancer  
788 models. Directly targeting integrins could thus interrupt cellular communication with the  
789 secondary niches [206]. Targeting niche features may also be a beneficial strategy, for  
790 example by using hypoxia-activated prodrugs, such as PR104 and TH-302 [141,145–  
791 147,246]. In ALL, modifying the leukemic microenvironment to reverse the phenotype of  
792 dormant blast cells could be also a viable option [45]. This could be achieved by restoring a  
793 healthy microenvironment, for example by using bisphosphonates that could prevent bone  
794 degradation, which is observed in leukemia pathogenesis [203,204]. Another possible strategy  
795 could target signaling pathways that are upregulated upon the interaction of malignant cells  
796 with their microenvironment, such as the TGF $\beta$  and HIF1 $\alpha$  pathways, for which several  
797 preclinical or early clinical studies have been published [246,247]. The immune system could  
798 also be manipulated, as seen in Hodgkin lymphoma, a hematological malignancy with a rich  
799 microenvironment, with impressive results from recent early clinical trials testing specific  
800 anti-programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) antibodies [248,249].

801 Many challenges have yet to be addressed concerning these and other strategies that  
802 could target malignant cells in their niches in hematological and solid cancers. Given the  
803 complexity of the microenvironment, successful treatment will probably require a  
804 combinatory approach, by associating these therapies both between themselves and with  
805 conventional chemotherapies. It is likely that such treatments would exert their full potential  
806 by adding them to chemotherapy after the initial diagnosis to prevent the occurrence of

807 relapse. They should probably be reserved for refractory patients or those with the highest risk  
808 of relapse.

## 809 **VII. Conclusion and future directions**

810 Leukemic niches and their interplay with leukemic cells are of tremendous complexity,  
811 for which we have only limited information. The aim of this review was to illustrate this  
812 concept by giving a few examples, rather than being exhaustive, to describe potential niche-  
813 targeted therapeutic strategies that could be considered in the near future. We chose to  
814 consider leukemia relapses like solid tumor metastases. We described several features of the  
815 microenvironments of medullary, extramedullary, and metastatic niches, focusing on their  
816 similarities, suggesting that ALL relapses might be considered more like leukemic metastases.  
817 We provide lines of evidence showing that ALL cells, as well as other cancer cells, can  
818 communicate with and remodel their microenvironment. Similar to solid tumors, ALL cells  
819 can reach secondary sites and settle down, where they can remain dormant for years before  
820 initiating a relapse. Although some intrinsic factors may predispose cells to invade one tissue  
821 over another, the microenvironment undoubtedly plays an important role in leukemic cell  
822 engraftment, making niche-based targeted therapies promising and worth developing.  
823 However, given the complexity of the niches and the dynamic interconnections between each  
824 microenvironmental actor, it will be a considerable challenge to elaborate therapies that can  
825 efficiently and durably overcome resistance. Determining the appropriate placement of such  
826 therapies in current treatment strategies is also an open question that will need to be  
827 addressed.

## 828 **VIII. Practice points**

- 829 • ALL cells appear to localize preferentially in perivascular regions of the tissues they  
830 colonize.

- 831 • Testicular, CNS and BM leukemic niches and solid tumor metastatic niches display  
832 similarities potentially accounting for the residence of leukemic cells in these  
833 preferred microenvironments.
- 834 • The interplay between leukemic niches and ALL cells is complex, dynamic and  
835 bidirectional.
- 836 • Relapses in specific tissues can be explained by both the microenvironment in these  
837 tissues and the intrinsic properties of ALL cells.
- 838 • Niche-based targeted therapies are currently being developed and may effectively  
839 prevent ALL relapses.
- 840 • Systemic treatments such as chemotherapy remain the best option to successfully treat  
841 extramedullary leukemia.

842

## 843 **IX. Research agenda**

- 844 • Further efforts are required to determine why leukemic cells settle preferentially in  
845 specific niches, to identify the most relevant microenvironmental features for  
846 therapeutic targeting.
- 847 • The combination of niche-based targeted treatments and conventional chemotherapy  
848 may prevent relapse.
- 849 • Clinicians should define the categories of patients most likely to benefit from such  
850 treatments.

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1585 **Conflict of Interest Statement**

1586 All authors declare to have no conflict of interest to disclaim.

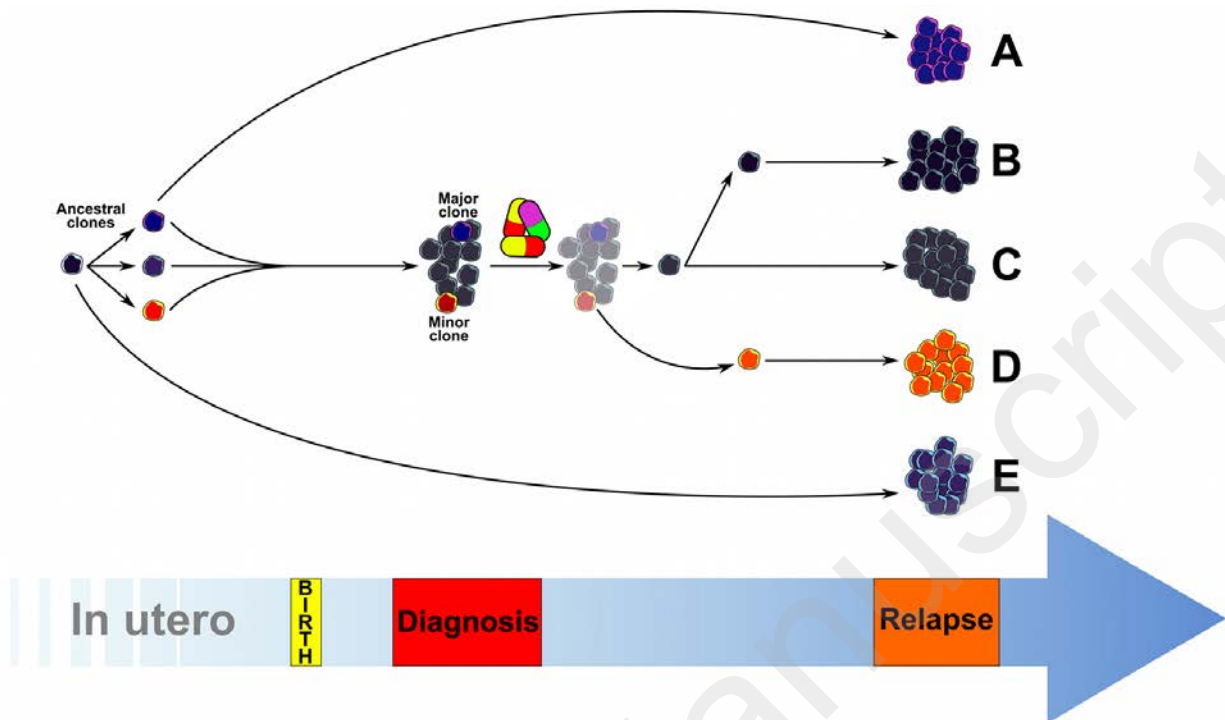
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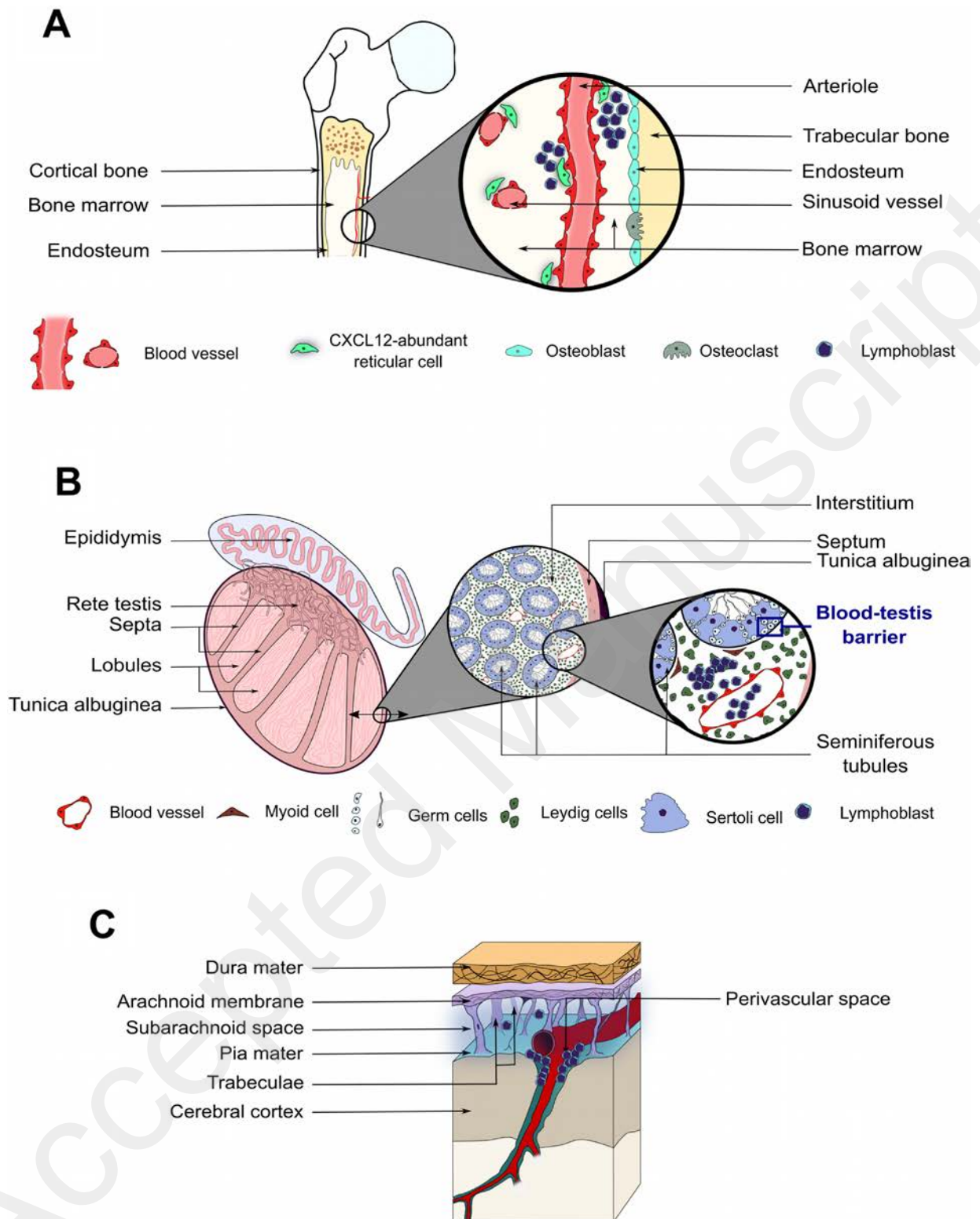
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1591 **FIGURE LEGENDS**



1592

1593 **Figure 1. Clonal evolution of leukemic cells from preleukemic stage to relapse.** A first  
 1594 genetic hit, such as an ETV6-RUNX1 rearrangement, in a hematopoietic progenitor leads to  
 1595 the apparition of a preleukemic clone, which can subsequently accumulate other genetic  
 1596 alterations to give rise to leukemic “ancestral clones”. Such clones may be present before  
 1597 birth, as notably shown for ETV6-RUNX1. Further genetic hits favor the emergence of clones  
 1598 that give rise to an overt acute lymphoblastic leukemia (ALL), which may be composed of a  
 1599 major clone and minor clones. After diagnosis, treatment of ALL with chemotherapy can  
 1600 eradicate all or almost all leukemic cells. Some cells from the bulk population may survive  
 1601 and give rise to a relapse with the same major clone found at diagnosis (situation C).  
 1602 Alternatively, cells derived from either major or minor clones may undergo other genetic  
 1603 alterations, leading to a different type of relapse, as shown in situations B and D. Relapsed  
 1604 leukemia may also derive from another ancestral clone or even directly from the preleukemic  
 1605 clone, which may have evolved on their own (situations A and E).



1606

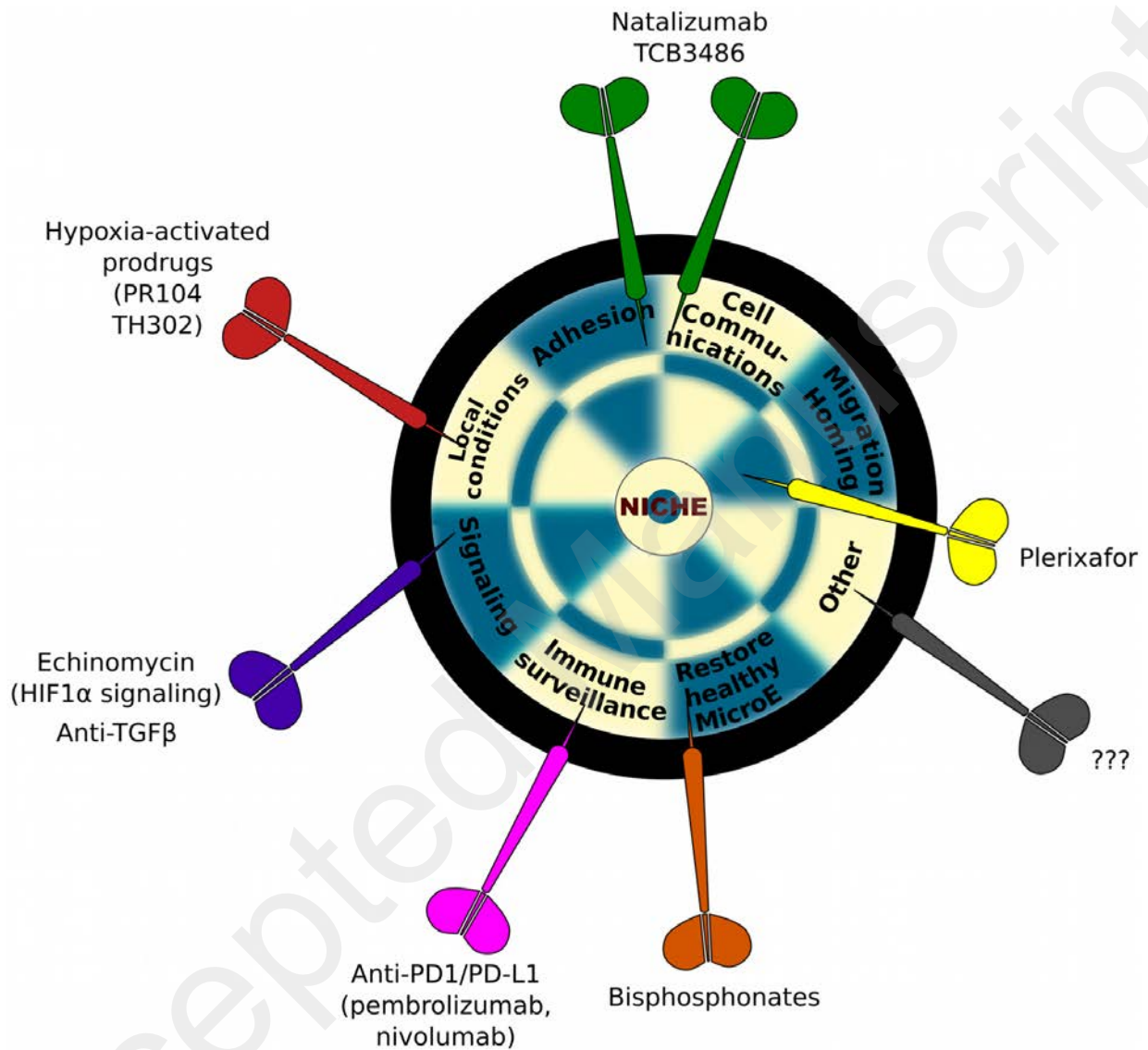
1607 **Figure 2. Main sites of acute lymphoblastic leukemia (ALL) relapse. (A) Bone marrow.**

1608 This extensively simplified schematic view of the bone marrow (BM) illustrates where ALL

1609 cells are mainly found in the perivascular spaces close to the endosteum. The endosteum

1610 corresponds to a thin layer of conjunctive tissue delineating BM and covered with osteoblasts  
1611 and osteoclasts, which participate in balancing the renewal and remodeling of bone tissue.  
1612 CXCL12-abundant reticular cells, specialized stromal cells near vessels, produce CXCL12,  
1613 which appears to be necessary for ALL cell engraftment in BM. (B) *Testis*. Testis is the organ  
1614 in which male gametes are produced. Germ-cell maturation takes place in specialized  
1615 structures called seminiferous tubules, which are surrounded by an interstitial tissue and  
1616 regrouped into lobules. The lobules are separated by fibrous septa which are in continuity  
1617 with the capsular region. The testis is encapsulated in a fibrous covering called the tunica  
1618 albuginea. The production of germ cells begins at the basement membrane, which surrounds  
1619 the seminiferous tubule and where germ cells are the most immature. The germ cells mature  
1620 as they progress from the basal part of the tubule to the lumen, where fully mature sperm cells  
1621 are found. Throughout the maturation process, germ cells are enclosed by Sertoli cells, which  
1622 form tight junctions between themselves to protect the germ cells and compartmentalize the  
1623 maturation steps. The role of the blood-testis barrier is to protect germ cells from potentially  
1624 deleterious molecules or cells. The blood-testis barrier is anatomically composed of the  
1625 basement membrane, Sertoli cells, and their tight junctions, but does not encompass blood  
1626 vessels, making the term “blood-testis barrier” inaccurate. Between the seminiferous tubules,  
1627 the interstitial tissue, or interstitium, is composed mainly of supportive cells, called Leydig  
1628 cells, of which one of the main roles is to produce testosterone. During testicular leukemia,  
1629 ALL cells are mainly found in the interstitial perivascular spaces, and occasionally in  
1630 seminiferous tubules, but only at very advanced stages of the disease, following inappropriate  
1631 treatment. (C) *Central nervous system*. The central nervous system (CNS) consists of the  
1632 brain, cerebellum, and spine. CNS tissue is surrounded by three meningeal layers: the thick  
1633 dura mater, the arachnoid membrane, from which trabeculae fibers cross the subarachnoid  
1634 space to reach the third thin layer, the pia mater, which is in direct contact with CNS tissue.

1635 Cerebrospinal fluid circulates in the subarachnoid space, where blood vessels are found.  
 1636 When the CNS is involved, ALL cells localize mainly in the subarachnoid space, close to  
 1637 vessels, whereas some cells may circulate freely in the cerebrospinal fluid.



1638

1639 **Figure 3. Potential therapeutic strategies to target the leukemic niche.** The leukemic  
 1640 niche is a highly complex microenvironment made of many components or aspects that could  
 1641 be targeted. Potential niche-based treatments are represented by the darts, which could be  
 1642 used simultaneously to target different components, as symbolized by the blurry delineations  
 1643 on the target.

1645 Table 1. Examples of studies using molecules that target components of the leukemic niche.

Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
CXCL12/CXR4 axis	Cooper et al., 2017 [243]	Phase I	Plerixafor	20 (5 ALL, 12 AML, 1 AML/MDS)	Maximum dose 15 g/m <sup>2</sup> without DLT Heavily pretreated patients Plerixafor administrated prior to chemotherapy (HD-araC + VP16) Mobilization of lymphoblasts achieved No clinical response in ALL patients (4 SD, 1 PD)
	Juarez et al., 2007 [124]	Preclinical (animal study)	Plerixafor, TC14012, AMD3465	NA	Reduced extramedullary engraftment of ALL cells in xenografted mice Mobilization of ALL cells in PB and spleens of xenografted mice Continuous administration equivalent to intermittent administration
	Ranhawa et al, 2016 [244]	Preclinical ( <i>in vitro</i> and animal study)	Plerixafor, BKT140, CXCR4 genetic deletion	NA	Reduced chemotaxis of B-ALL cells Restoration of drug sensitivity of resistant B-ALL cells co-cultured with BMSCs Delayed engraftment and prolonged survival of mice xenografted with CXCR4-deleted B-ALL cells
	Juarez et al. 2003, [105]	Preclinical ( <i>in vitro</i> study)	Plerixafor, TC14012	NA	Reduced chemotaxis of B-ALL cells Reduced proliferation of B-ALL cells cultured on stromal BM cells

Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
					Augmentation of inhibitory effects of chemotherapy
Integrin alpha4	Hsieh et al. 2013 [245]	Preclinical ( <i>in vitro</i> and animal study)	Natalizumab	NA	Prolonged survival of xenografted mice Synergistic effect with chemotherapy (marked prolonged survival) Reduced engraftment in liver, spleen, and BM of xenografted mice and enhanced engraftment in lungs.
	Hsieh et al. 2014 [246]	Preclinical ( <i>in vitro</i> and animal study)	TBC3486	NA	Reduced chemoresistance of B-ALL cells Prolonged survival of xenografted mice Synergistic effects with chemotherapy <i>in vivo</i>
HIF1a	Badar et al., 2016 [146]	Phase I	TH-302 (evofosfamide)	49 (39 AML, 9 ALL, 1 CML)	Heavily pretreated patients 5 days of administration over 21-day cycles Maximum tolerated dose 460 mg/m <sup>2</sup> as intermittent administration and 330 mg/m <sup>2</sup> as continuous infusion. 1 patient with T-ALL with PR
	Benito et al. 2016 [147]	Preclinical ( <i>in vitro</i> and animal study)	TH-302 (evofosfamide)	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions Not tested in an <i>in vivo</i> ALL model
	Konopleva et al. 2015 [145]	Phase I/II	PR104	50 (40 AML, 10 ALL[3 T-ALL, 7 B-ALL])	Heavily pretreated patients Chosen doses for phase II: 3 g/m <sup>2</sup> and 4g/m <sup>2</sup> 2 responses (1 CR and 1 MLFS) in 7 B-ALL
	Benito et al. 2011 [141]	Preclinical ( <i>in vitro</i> and animal	PR104	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions

Target	Study [ref]	Type of study	Tested molecule	Number of patients	Main results/comments
		study)			Delayed engraftment of mice xenografted with B-ALL cells Prolonged survival of xenografted mice with maintained complete response at 550 mg/kg
Restoring normal microenvironment	Cheung et al, 2018 [203]	Preclinical ( <i>in vitro</i> and animal study)	Zoledronic acid	NA	Reduced leukemia burden in xenografted mice with B-ALL cells Prolonged survival of mice xenografted with B-ALL cells
Immune system	Chen et al. 2017, [250]	Case report	Nivolumab	2	Heavily pretreated patients with one or two prior HSCTs Off-label use Low-dose nivolumab (40-80 mg/course) administered to prevent GVHD Clinical response with the 2 patients alive 13 months and 4 months after relapse, but with persistent disease
	Boekstegers et al. 2017 [251]	Case report	Pembrolizumab	1	Refractory T-ALL treated with HSCT followed by 4 DLIs Fast clinical response with MRD decrease Severe acute GVHD, leading to immunosuppressive treatment with subsequent re-increase of MRD, multi-organ failure, and death

1646

1647 **Table 1. Examples of studies using molecules that target components of the leukemic**

1648 **niche.** The apparently disappointing results from clinical studies may be explained by the use

1649 of new treatments mostly alone in heavily pretreated patients. The association of each of these



1650 molecules with each other and/or conventional chemotherapy or immunotherapy might lead to  
1651 better outcomes.

1652 Abbreviations: AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; BM:  
1653 bone marrow; BMSC: bone marrow stem-cell; CML: chronic myeloid leukemia; CR:  
1654 complete remission; DLI: donor lymphocyte infusion; DLT: dose-limiting toxicity; GVHD:  
1655 graft versus host disease; HD-AraC: high-dose aracytine; HSCT: hematopoietic stem cell  
1656 transplantation; MLFS: morphological leukemia-free state; MRD: minimal residual disease;  
1657 NA: not applicable; PB: peripheral blood; PD: progressive disease; PR: partial response; SD:  
1658 stable disease