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Review

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

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ABSTRACT

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

1. Introduction

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

However, approximately 15 to 20% of children and approximately 50% of adult patients still relapse [2–6]. This situation is defined as the resurgence of blast cells after the achievement of complete remission. Relapses are generally classified and stratified according to three prognostic factors: time of relapse, site of relapse, and immunophenotype B or T [7].The time of relapse, relative to the beginning and end of treatment, is the most important prognostic factor: the earlier the relapse occurs, the worse the prognosis. The definition of relapse is slightly different depending on the classification used (Berlin-Frankfurt-Münster [BFM] or Children Oncology Group [COG]) [3,7]. The most important factor after timing is probably the anatomical sites of relapse, which are commonly divided into bone marrow (BM) alone (worst prognosis), extramedullary alone (best prognosis), and combined relapses (intermediate prognosis). Although immunophenotype T was initially associated with poor survival and is still used in tumor classification, its prognostic value appears to be less important due to better stratification [8]. Furthermore, the assessment of early minimal residual disease (MRD) is also used to predict the prognosis of patients presenting a relapse, as it is for newly-diagnosed ALL [9–12].

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

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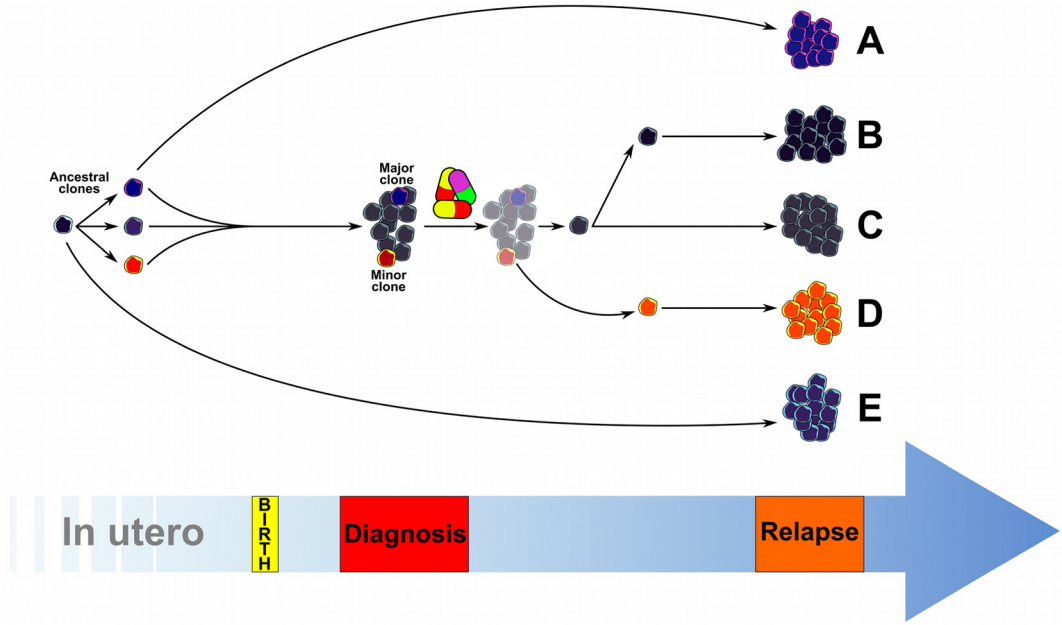

Fig. 1. Clonal evolution of leukemic cells from preleukemic stage to relapse. A first genetic hit, such as an ETV6-RUNX1 rearrangement, in a hematopoietic progenitor leads to the apparition of a preleukemic clone, which can subsequently accumulate other genetic alterations to give rise to leukemic "ancestral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

prerequisite to defining new treatment approaches.

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

2. Cellular origin of relapse

Multiple biological factors may contribute to the emergence of ALL relapses [13]. Cumulative evidence suggests a clonal origin of ALL [14]. It has previously been shown that clonal evolution is involved in relapse, with the possible emergence of subclones derived either from the major clone found at initial diagnosis, minor subclones, or a common ancestral pre-leukemic clone (Fig. 1) [15–20]. Several studies have described chemoresistance-related mutations in the BM samples of relapsed patients, such as those that affect the glucocorticoid response pathway (mutations of the glucocorticoid receptor gene *NR3C1* or other genes, interfering with its function, such as *BTG1*) or nucleotide metabolism (for example, *NT5C2* or *PRSP1* genes [13,21–25]. Clonal heterogeneity is a common feature in ALL. Clonal evolution is likely to be a dynamic process in which selection plays a central role, as in the Darwinian model [14,26]. Both intrinsic and extrinsic factors, such as microenvironmental conditions or chemotherapeutic pressure, may favor a clone from which the relapse can develop.

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

Metastasis is the spread of cancer cells from the tissue where they first originate to another distant one. ALL relapses occur most often in the BM, whereas extramedullary relapses are less commonly reported. Nevertheless, the occurrence of relapse is generally thought to be a leukemic event, depending mainly on internal properties of the blast, regardless of the type of relapse. However, the occurrence of relapse may not depend only on leukemic cell features, but also on the microenvironment. In the next section, we will describe the most frequently-reported sites of relapse and the concept of niches, proposing that extramedullary leukemia relapse is similar to that of solid tumor metastasis, anticipating a major role of the microenvironment of extramedullary sites.

3. Sites of relapse

Relapse can occur in the BM, the native niche from where the disease spreads at the onset. It can also emerge from extramedullary sites, such as the central nervous system (CNS) or gonads. Both used to be

called “sanctuary sites”, because of the relative protection these sites appear to offer to blast cells from systemic chemotherapy. At these sites, the tissue-blood barrier is an obstacle to drug delivery [27,31,32]. Although CNS and testicular relapses may be more common in children, they also concern adults. These sites may represent the first extramedullary sites colonized by leukemic cells but can also be colonized secondary to other extramedullary sites that may have evaded clinical detection. Prophylactic cranial irradiation was added to systemic chemotherapy treatment in the 1970s to attempt to prevent CNS relapses [33]. Intrathecal chemotherapy and the use of high-dose methotrexate for neuro-meningeal prophylaxis have progressively replaced irradiation, with good results for diminishing neurological sequelae, showing that the blood-tissue barrier problem can be bypassed [34]. Similarly, treatment intensification, with the introduction of intermediate and high-dose methotrexate, has significantly decreased the incidence of testicular relapses, thus considerably improving the

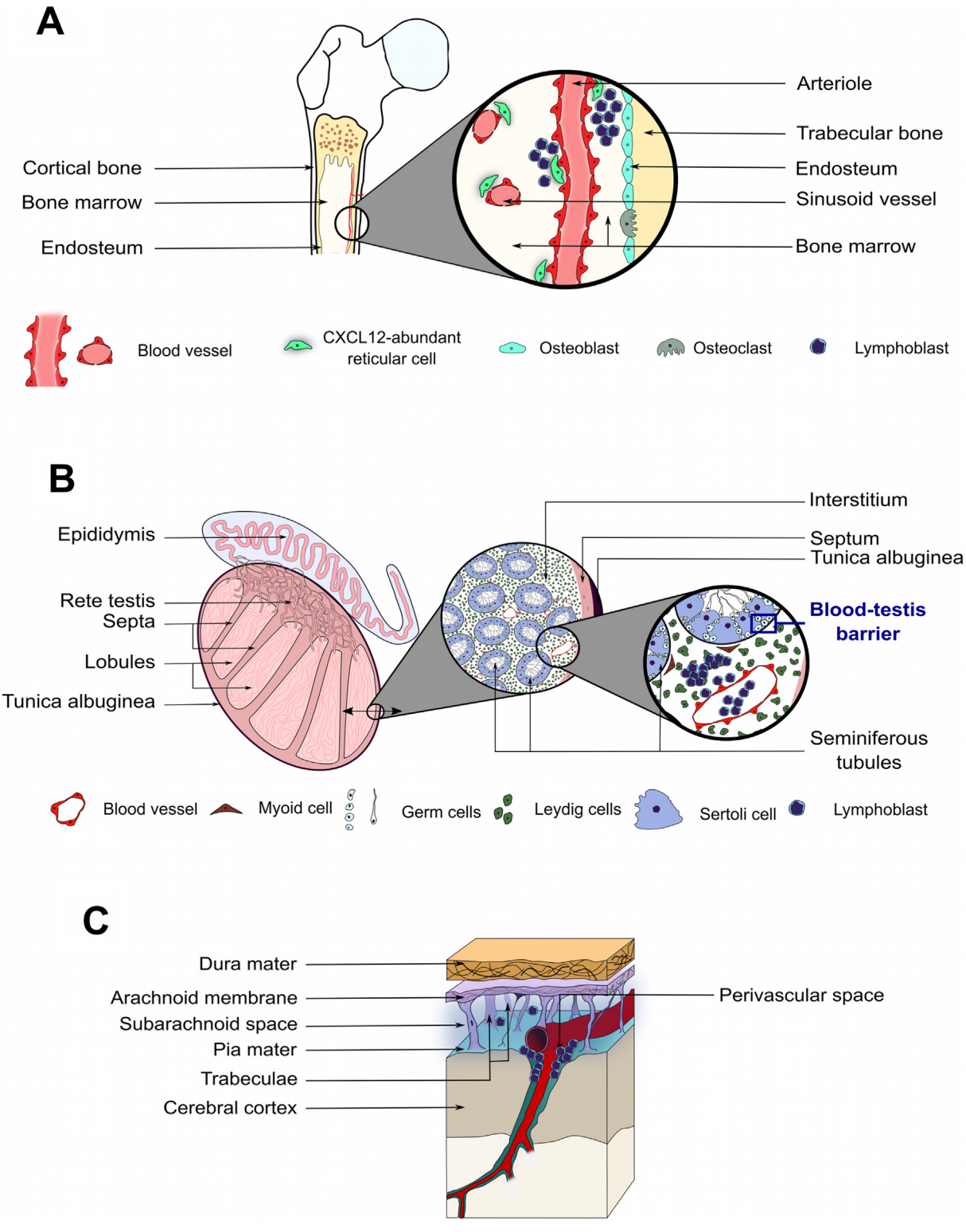

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Fig. 2. Main sites of acute lymphoblastic leukemia (ALL) relapse. (A) Bone marrow. This extensively simplified schematic view of the bone marrow (BM) illustrates where ALL cells are mainly found in the perivascular spaces close to the endosteum. The endosteum corresponds to a thin layer of conjunctive tissue delineating BM and covered with osteoblasts and osteoclasts, which participate in balancing the renewal and remodeling of bone tissue. CXCL12-abundant reticular cells, specialized stromal cells near vessels, produce CXCL12, which appears to be necessary for ALL cell engraftment in BM. (B) Testis. Testis is the organ in which male gametes are produced. Germ-cell maturation takes place in specialized structures called seminiferous tubules, which are surrounded by an interstitial tissue and regrouped into lobules. The lobules are separated by fibrous septa which are in continuity with the capsular region. The testis is encapsulated in a fibrous covering called the tunica albuginea. The production of germ cells begins at the basement membrane, which surrounds the seminiferous tubule and where germ cells are the most immature. The germ cells mature as they progress from the basal part of the tubule to the lumen, where fully mature sperm cells are found. Throughout the maturation process, germ cells are enclosed by Sertoli cells, which form tight junctions between themselves to protect the germ cells and compartmentalize the maturation steps. The role of the blood-testis barrier is to protect germ cells from potentially deleterious molecules or cells. The blood-testis barrier is anatomically composed of the basement membrane, Sertoli cells, and their tight junctions, but does not encompass blood vessels, making the term "blood-testis barrier" inaccurate. Between the seminiferous tubules, the interstitial tissue, or interstitium, is composed mainly of supportive cells, called Leydig cells, of which one of the main roles is to produce testosterone. During testicular leukemia, ALL cells are mainly found in the interstitial perivascular spaces, and occasionally in seminiferous tubules, but only at very advanced stages of the disease, following inappropriate treatment. (C) Central nervous system. The central nervous system (CNS) consists of the brain, cerebellum, and spine. CNS tissue is surrounded by three meningeal layers: the thick dura mater, the arachnoid membrane, from which trabeculae fibers cross the subarachnoid space to reach the third thin layer, the pia mater, which is in direct contact with CNS tissue. Cerebrospinal fluid circulates in the subarachnoid space, where blood vessels are found. When the CNS is involved, ALL cells localize mainly in the subarachnoid space, close to vessels, whereas some cells may circulate freely in the cerebrospinal fluid.

historically worse prognosis for boys [35,36]. However, extra-medullary relapses are still a problem and have been reported in many other organs. Several studies have reported that extramedullary relapses occur in approximately 40% of cases (approximately 20% combined and 20% presumably isolated) [3,8,37]. Here, we summarize the evidence that shows the role of the microenvironment in extramedullary relapses.

3.1. Different microenvironments

3.1.1. Bone marrow (Fig. 2a)

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

3.1.2. Gonads (Fig. 2b)

Large group studies have reported that isolated testicular relapses could account for 3.5-8% of ALL relapses in children (including boys and girls) followed for up to 20 years [3,8,38–41,46]. Most studies mentioning testicular biopsies in the context of ALL did not indicate the precise location of blasts [47–49]. Such information can be retrieved from rare and old clinical studies and animal models [50–56]. Animal models indicate that leukemic cells infiltrate the interstitial testicular tissue mainly at the perivascular and peritubular regions, forming small foci, notably in subcapsular areas. Despite perivascular interstitial invasion, extensive infiltration of the interstitium is rare. On the other hand, the epididymis is more often massively invaded by blast cells [54,55]. Leukemic invasion seems to occur through the hematogenous route, with possible extension to the epididymis *via* the lymphatic route [55]. In these models, although seminiferous tubules were degenerated or destroyed near leukemic infiltrations, leukemic cells were never found in the tubules nor the gonadal duct [54–56]. Unlike these *in vivo* studies, testicular leukemic infiltration was mostly found to be diffuse in a postmortem series of children who died of very advanced leukemia. Leukemic cells were found in the tubules in half the patients with heavily infiltrated testes [57]. This relatively old study included children with uncontrolled leukemia at a time when outcomes were poor and probably does not reflect the route of initial invasion. Thus, it seems that leukemic cells invade the testis by the hematogenous route and first infiltrate the perivascular and peritubular spaces, where they can spread to the epididymis through the local lymphatic system. Without effective treatment, seminiferous tubules may be destroyed and invaded as well. This is concordant with the perivascular and peritubular interstitial infiltration observed in patients with testicular relapse leukemia [50–52].

Although less commonly involved than the testis, ovaries may also be involved in ALL [58]. Ovarian relapses are scarcely reported in clinical trials [40,59], but autopsies have provided evidence of ovarian disease in end-stage leukemia [57,60,61]. More recently, ovarian involvement was found in approximately 8% of leukemia cases in an autopsy series, regardless of age. However, the authors did not indicate whether the patients had been treated or not, nor the treatment performed [62]. The effects of chemotherapy could explain the differences observed between and within the reports mentioned above, in which ovarian involvement was found in 3% to up to 50% of cases [57,60,61]. Indeed, Pais et al. reported that the number of ovarian relapses dramatically decreased in their cohort from the moment they introduced intermediate-dose methotrexate in the treatment regimen (from almost 10% of all relapses before 1979 to no ovarian relapse noted afterwards, until 1991). As they assumed, isolated ovarian relapse has become an exceptional event, likely representing slightly less than 1% of all relapses [40,59]. However, contrary to testicular involvement, which can be clinically suspected with a simple physical examination, there is no overt clinical sign that allows suspicion of ovarian leukemic infiltration. In addition, ultrasound evaluation is not systematically performed during the course of the disease and follow-up, even if it might be [63]. However, it was possible to detect leukemic cells in up to 70% of samples by polymerase chain reaction of cryopreserved ovarian tissue retrieved at diagnosis (or shortly after), even in the absence of enlargement and with a negative histological examination (except in one patient from a study of Soares et al., whose ovarian medulla was invaded) [64–67]. Most published studies concerning ovarian disease consist of case reports, which usually do not report the precise location of leukemic cells in the ovary [58]. In their autopsy series, Reid et al. found that leukemic invasion, which was mostly histologically mild, took place predominantly in the medulla in the perivascular spaces [57]. However, leukemic cells have also been detected in the cortex by other authors. Thus, it is difficult to say where the cells preferentially settle [64–67]. Nonetheless, follicles have never been reported to be invaded in the literature.

3.1.3. Central nervous system (Fig. 2c)

Isolated CNS relapses represent approximately 7.5-15% of ALL relapse [3,8,38–41]. The precise location of leukemic cells in the CNS is rarely indicated in published studies. Autopsy and animal model studies have allowed a better understanding of how the CNS may be invaded by leukemic cells [68,69]. Indeed, Price and Johnson studied 126 brains from children who died of ALL and developed a grading system to evaluate the severity of arachnoid involvement. They detected leukemic cells in the CNS of 55% of patients. Most showed moderate leukemic infiltration and the authors observed that infiltration of the brain parenchyma occurred only in cases of extensive and deep leukemic arachnoid involvement with cerebrospinal fluid (CSF) contamination and pia-glial membrane destruction (13.4% of cases). Arachnoid invasion was most often isolated or multifocal, rather than diffuse. They concluded that leukemic cells first invade the superficial arachnoid from the vein wall, then the deep perivascular arachnoid, and finally, the brain parenchyma through disrupted pia-glial membranes [68]. *In vivo* rodent models have provided similar results and suggest that leukemic cells transit through the blood-CSF barrier to reach the leptomeninges [69,70]. Although Price and Johnson only studied brain and did not consider the spinal cord or dura matter in their study, diffusion throughout the CNS *via* the CSF has been confirmed by leukemic cell DNA detection, both in the lumbar spinal cord and frontal brain of rats inoculated in the cerebellum [70]. This is consistent with neuroradiological findings, which indicate that meningeal infiltration is the most common manifestation of CNS leukemia, with most often diffuse infiltration [71,72]. However, although much rarer and mostly represented by case reports in the literature, CNS involvement may also consist of epidural masses that can compress the spinal cord [73]. Thus, leukemic cells do not colonize one specific anatomical region within the brain or other CNS sites, but rather appear to preferentially localize to the subarachnoid space, likely in perivascular areas. A very recent study, using a mouse model, has shown that meningeal invasion occurs through the diapedesis of leukemic cells from cranial or spinal bone marrow of adjacent bones. The authors reported that leukemic cells transit through blood vessels, the emissary vessels that bridge the CSF space from the brain or spinal cord to the calvarium or vertebral bodies. They also discovered that this mechanism involves an interaction between a6 integrin and laminin and that it can be blocked by specific PI3Kδ inhibitors [74].

3.1.4. Others

Because leukemic cells spread throughout the organism via systemic circulation, other sites of relapse may be considered. Other extramedullary sites have been little studied and published data consist mostly of case reports [75]. Although no site can be defined as privileged, since ALL can settle in many tissues, breast involvement may be a privileged site of extramedullary leukemia, mostly described by Isabel Cunningham [75–79].

3.2. Similarities between sanctuary sites

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

3.2.1. The concept of “niche”

The BM microenvironment has been, and is still, extensively studied [80–84]. First postulated by Schofield in 1978, it is now accepted that hematopoietic stem cells (HSC) reside in specialized compartments, called “niches”, within the BM [85]. Such niches allow the maintenance and regulation of HSCs by providing specific local conditions, cytokines, chemokines, and other soluble factors, as well as various types of specialized stroma cells. Different types of HSC niches have been described in the literature and may be named differently, depending on the authors, which can be confusing [80,81,86,87]. Our understanding of the BM microenvironment is constantly evolving and is still a subject of debate. For example, some studies have provided evidence for the existence of an osteoblastic niche [86], but this has been more recently questioned. Indeed, the existence of an authentic osteoblastic HSC niche has been ruled out, even if osteoblastic lineage cells have an indirect role in the regulation of HSCs and appear to be part of a specialized niche for lymphoid progenitors [81,84]. Rather than viewing the BM as a suite of separated niches, it is more likely a unique continuous niche in which some components are shared (and some others not) between more specialized compartments within this niche, as proposed by Hira et al. [88]. HSCs localize mainly to perivascular zones, as do leukemic cells, with most found close to sinusoids, whereas approximately 10% are found near small arterioles.

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

3.2.2. CXCL12/CXCR4

CXCL12, also known as stromal derived factor 1 alpha (SDF1α), is one of the most important chemokines in the BM niche [84]. Through binding to its receptor, CXCR4, CXCL12 favors HSC homing to the BM and participates in the maintenance of these cells in the niche. CXCR4 is a seven-transmembrane G-protein-coupled receptor that is expressed on multiple cell types, including, notably, hematopoietic cells [96]. The inhibition of CXCR4 is frequently used clinically to mobilize HSCs into the peripheral blood as a source of stem cells for transplantation [84,97–99]. CXCL12 is highly expressed by specialized cells close to sinusoid vessels within the BM, called CXCL12 abundant reticular cells, and favors HSC retention in the BM [84,97].

CXCL12 has been shown to be involved in metastasis in many cancer models, such as breast, prostate, and lung cancer, pancreatic adenocarcinoma, rhabdomyosarcoma, and others [100]. For example, it was shown in an ovarian cancer model that the most advanced stages of the disease and most invasive phenotypes are associated with increased CXCR4 expression at the cancer cell surface. More importantly, ascites samples from patients with advanced ovarian cancer showed significantly higher CXCL12 levels [101]. The role of the CXCL12/CXCR4 axis in the dissemination of peritoneal cancer has been clarified more recently in a study using a primarily murine colon cancer model. The authors found that CXCL12-expressing cells, similar to CXCL12 abundant reticular cells and distributed throughout the perivascular zones of adipose tissue, were a privileged site from which cancer cells could disseminate [102]. In another example, Ogawa et al. showed that CXCL12 expression increased in the regional lymph nodes of mice with intrapulmonary transplanted cancer cells in a Lewis lung carcinoma model, and participated in the formation of a pre-metastatic niche [103].

The CXCL12-CXCR4 axis is also involved in the pathogenesis of leukemia, as it favors not only ALL cell homing in the BM, but also their survival and proliferation [42,43,104,105]. Some evidence indicates that, in the human testis, CXCL12 is expressed solely by Sertoli cells to promote spermatogonial stem-cell migration through the seminiferous tubules during spermatogenesis [106–109]. Sertoli cells reside in seminiferous tubules, where leukemic cells are generally not found, or only very late during testicular involvement. However, the use of transwell migration assays showed that ALL cells can also migrate towards mouse testis conditioned medium and that such migration was reduced when the cells were treated with plerixafor, a CXCR4 chemical

inhibitor [110]. This result suggests that leukemic-cell migration towards the testis might be at least partially mediated (or not exclusively) by the CXCL12/CXCR4 axis. Moreover, McIver et al. have shown that although CXCL12 expression is restricted to Sertoli cells in the normal testis, it is expressed in the tumor stroma of seminoma samples [107]. Pathological conditions may induce CXCL12 expression in the interstitium of the testis [111]. Thus, it is possible that leukemic cells could be attracted and settle in the testis.

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

3.2.3. Oxygen content

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

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

More importantly, low oxygen concentrations are thought to be an important component of leukemic niches in the BM and recent results have shown that targeting this hypoxic microenvironment may be promising [141–148]. Indeed, hypoxia has been shown to enhance the chemoresistance of ALL cells and appears to confer stem-cell properties, such as quiescence [141,149,150]. Drugs active only under conditions of hypoxia were shown to reduce the leukemia burden in the BM when administrated three days after ALL cell injection into NOD/SCID mice, suggesting that hypoxia may favor leukemic-cell engraftment [141,147].

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

Low oxygen concentrations are also found in the CNS, from 0.5% to 7%, both in humans and animal models [126]. There are few clues in the literature to estimate the oxygen tension of subarachnoidal tissue, where ALL cells preferentially settle. Some studies have evaluated the oxygen tension within the CSF, with estimated values of approximately 5 to 6% [154,155]. More recently, Sharan et al. showed that oxygen tension decreases rapidly from pial vessels to the extravascular space in a rodent model, with oxygen levels as low as 3% 30 μM from the vessels, especially the narrowest ones [156]. Thus, although oxygen concentrations have not been precisely measured in the subarachnoidal space, they are likely to be low. Recent published data have indeed suggested a role for low oxygen concentrations in leukemic cell CNS infiltration [157,158]. In these two independent studies, the authors found that leukemic cells retrieved from the CNS of xenografted mice had a hypoxia-related gene set profile and expressed a particularly high level of the transcript of the vascular endothelial growth factor A (VEGFA) gene, which is a principal target of the hypoxia master transcription factor, hypoxia inducible factor 1 (HIF1). More importantly, Münch et al. further showed that VEGFA mediated the migration of ALL cells through a monolayer of a brain-microvasculature cell line [158]. The results from these studies are in accordance with those of a previous one showing that patients with CNS leukemia had elevated levels of VEGF in the CSF [159]. Thus, the role of hypoxia in extramedullary leukemia merits exploration and may offer new therapeutic perspectives, as outlined by Izraeli and Eckert [160].

3.2.4. Stem cell factor

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

In solid tumors, SCF/c-KIT signaling is also frequently thought to participate in tumorigenesis, tumor progression, and metastasis. Soluble SCF is produced by cancer cells within tumor tissues in different cancer models [170]. c-KIT has been found to be overexpressed, abnormally expressed, or associated with an aggressive phenotype in several types of tumors, including melanoma, lung cancer, renal carcinoma, and seminoma [163,171,172]. Activating mutations of c-KIT can be found in some tumor types, especially gastrointestinal stromal tumors, and may be involved in their pathogenesis. The SCF/c-KIT pathway may also be involved in the pathogenesis of such tumors through its effect on mast cells [163] and the mast cell accumulation has been associated with a worse prognosis in several cancer models.

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

The SCF/c-KIT axis plays also an important role in male reproduction, as suggested by the infertility of mice with mutations affecting both the *W* (encoding c-KIT) or *Sl* (encoding SCF) loci [161,163]. In the testis, SCF is produced by Sertoli cells and c-KIT is expressed on the surface of germ cells and Leydig cells, raising the possibility that Sertoli cells secrete a soluble form of SCF [171,175,176]. Indeed, a higher percentage of mRNA for the soluble than membrane form of SCF correlated with higher serum testosterone levels in infertile patients [177]. These results suggest that Sertoli cells may produce soluble SCF that reaches Leydig cells and thus enhance testosterone production. Moreover, SCF expression in normal adult interstitial testicular tissue has been reported both in mice and humans in several immunohistochemistry studies, even if several others did not [178–181]. Thus, leukemia cells may be attracted to or retained in testicular interstitial tissue at least partially through activation of the SCF/c-KIT pathway.

SCF is also expressed in the adult CNS, as well as in CSF, and is involved in CNS development during embryogenesis [182–184]. There is no direct evidence in the literature for a role of the SCF/c-KIT axis in the attraction of leukemia cells to the CNS or their subsequent survival. However, SCF is one of the specific cytokines expressed by endothelial cells of the blood-brain barrier [183]. Kallman et al. compared gene expression profiles of human umbilical vein endothelial cells (HUVEC) and primary human cerebral endothelial cells using a cDNA array technique and found that 35 genes were differentially expressed in cerebral endothelial cells, including those for interleukin-6 *(IL-6)*, transforming growth factor β2 (*TGF-β2*), *VEGFA,* and *SCF* [183]. An enzyme-linked immunosorbent assay (ELISA) confirmed that the supernatant from these cells contained SCF. Thus, endothelial cells of the CNS can produce SCF that may attract and/or promote the survival of leukemia cells that express c-KIT.

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

3.2.5. Other shared factors

The extracellular matrix protein osteopontin, which is physiologically produced in the BM, participates in the pathophysiology of various cancers, including hematological malignancies, with a potential role in leukemic cell CNS infiltration [185,186]. Colony stimulating factor 1 (CSF1), a cytokine involved in macrophage differentiation and constitutively expressed by osteoblasts, is expressed in prepubertal and adult testicular interstitial tissue, notably by macrophages, and has a role in regulating spermatogonial stem cells and their niche[187–189]. CSF1 is also expressed in the CNS, where it maintains microglia and participates in CNS development [190]. CSF1 deficiency impairs both reproductive function and integrity of the CNS. Genetic alterations involving the CSF1 receptor, including the recurrent SSBP2-CSF1R fusion, have been identified in B-ALL and have been found to lead to a functional fusion, suggesting that targeting CSF1 signaling could be promising [191–193]. Another cytokine, transforming growth factor β1 (TGFβ1), regulates Leydig cell function in the testis, but is also secreted by the choroid plexus in the CSF, where it contributes to the maintenance of the choroid plexus itself and appears to be involved in the protection and repair of nervous tissue. TGFβ1 has been shown to be involved in cancer stemness, metastasis, and epithelial-mesenchymal transition in several cancer models. Recently, TGFβ1 was found to promote the escape of leukemic cells from natural killer (NK) immune surveillance in an ALL model.

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

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

4. The interplay between leukemic cells and the microenvironment

4.1. Leukemic and cancer cells model their niche

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

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

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

Recent studies on solid tumors have led to a better understanding of cancer-cell organotropism and metastatic niche formation. Kaplan et al. first demonstrated, in 2005, that cancer cells can remotely prepare an appropriate niche before reaching their metastatic site [198,205]. The authors demonstrated that cancer cells secrete certain factors that may predispose them to metastasize toward a specific location. They first suggested cytokines, but discovered several years later that the cancer cells produced exosomes that could direct metastatic cancer cells to specific tissues [198,206]. Exosomes are small extracellular vesicles (30-100 nm of diameter) that can be transferred to other distant cells to deliver functional molecules, such as peptides or nucleic acids. Thus, exosomes are a means of intercellular communication suitable for remote cells. Exosomes derived from different cancer cell lines specifically target different tissues. An elegant experiment showed that exosomes can “educate” cancer cells to reach a specific tissue. Mice were injected with exosomes from cells that usually metastasize to the lungs before being transplanted with cells that do not usually metastasize to the lungs, but to bones. This experiment resulted in a large significant increase in lung metastases. Going further, the authors found that exosomal organotropism was determined by their integrins, which allowed exosome uptake by specific tissue-resident stromal cells within target tissues. Exosome uptake by these cells was followed by upregulation of the expression of *S100* family pro-migratory and pro-inflammatory genes, which could favor tumor metastasis. Importantly, specific exosomal integrins recovered from plasma samples of patients with pancreatic or breast cancer correlated with the location of the metastases, and may even likely predict future metastatic sites at the time diagnosis.

4.2. Means of communication between ALL cells and supportive cells

Several means of communication are available to ALL cells to interact and communicate with their surrounding microenvironment and they may be used simultaneously. The role of exosomes in the pathophysiology of several cancers is becoming increasingly understood but has been little studied, to date, in the context of ALL [83,207]. However, recent findings have shown that large extracellular vesicles (EVs), released through plasma membrane budding, may be of particular importance for the communication between ALL cells and their microenvironment [208,209]. Indeed, the group of Vaskar Saha showed that ALL cells can produce EVs of varying size, some of which could be transferred horizontally to other ALL cells, as well as BM stromal cells, resulting in phenotypic changes in the recipient cells [209]. The transfer of large EVs to BM stromal cells induced a metabolic change, favoring glycolysis in these cells, which increased the lactate concentration in the medium, thereby providing the leukemic cells with their preferred fuel, as hypothesized by the authors [208]. These large EVs were released into the systemic circulation of xenografted mice. It is thus possible that EVs participate in similar metabolic reprogramming and/or other phenotypic changes in remote stromal cells to promote ALL-cell migration to the secondary sites. Although more compelling evidence has yet to be provided, it seems likely that both exosomes and large EVs are involved in preparing a remote suitable niche for ALL cells. In addition, large EVs may also contain intact organelles that could be transferred to recipient cells [209]. Not all cells can generate EVs. Thus, as discussed by the authors, such mechanisms may allow EV-producing resistant leukemic cells to promote chemoresistance, or even resistance to targeted therapies, by transferring pro-resistant cellular content to other sensitive cells.

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

Direct contact of leukemic cells with stromal cells has also been previously highlighted in the pathogenesis of leukemia. Indeed, direct contact with stromal cells favors ALL cell survival, offers them a protective environment, and may minimize the effects of cytotoxic drugs [212–215]. Such cell-cell contact was found to be mediated, notably, by the binding of integrin α4β1, also called very late antigen-4 (VLA4), expressed on the surface of ALL cells, to the vascular adhesion molecule-1 (VCAM1), expressed on the stromal cell surface [216]. Integrin α4β1 can also bind to extracellular matrix proteins, such as osteopontin and fibronectin, and has been shown to play an important role in ALL cell homing in mice [200,217,218]. In addition to its role in adhesion to extracellular matrix and/or stroma, it is likely that integrin α4β1 binding to its receptor induces signaling cascades to promote leukemic cell survival, proliferation, and/or chemoresistance [218]. Gap junctions are also involved in cell-cell interactions within the leukemic niche. Gap junctions allow the direct exchange of small molecules between two adjacent cells by creating a channel, most often through the alignment and docking of two connexons (consisting of hexamers of specialized proteins called connexins) of each cell. Several studies have suggested that leukemic cells can communicate with stromal cells through gap junctions formed with connexin 43 (Cx43) [219–223]. Although the mechanisms are still unclear, it appears that Cx43 gap junctions are dysregulated in leukemic BM. Another connexin, Cx25, has recently been thought to be involved in the pathogenesis of leukemia through gap junctions [224]. The authors showed, using both T-ALL and AML cells, that inhibiting gap junctions by selective Cx25 knockdown sensitized the cells to chemotherapy, suggesting that concomitant Cx25-targeted treatment could allow reducing the dose of cytotoxic agents in clinical practice, thereby reducing the side effects of chemotherapy while conserving its efficacy [224].

Direct communication of leukemic cells with the surrounding supportive cells, probably as well as distant communication from the original to the secondary niche, appears to be of crucial importance. Once

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

The interconnections involved in this interplay are complex, but the methods used in studies usually only focus on one aspect of one component of the leukemic microenvironment. For example, cell-cell interactions have mostly been studied through co-culture systems consisting of only leukemic cells and one type of stromal cell, although leukemic cells probably interact not only with supportive stromal cells but also other cells within the microenvironment. Similarly, hypoxia can induce CXCL12 and CXCR4 expression, as well as that of SCF, and SCF itself has been found to increase HIF1α expression [226]. Leukemic cells may escape the effects of therapy by taking advantage of this system, of which the complexity is even more pronounced if we consider other parameters, such as temporality.

4.3. The chicken or the egg causality dilemma in ALL relapses

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

Similarly, solid tumor cells can settle at tissues distant from their original site early during the course of the disease, even before detection of the primary tumor [227,228]. Solid cancers, as well as leukemia, must be viewed as a systemic disease, in which cancer cells spread and engraft early at predisposed tissues that provide protection against insults, such as chemotherapy, notably through the induction and maintenance of a dormant state [227,229]. However, as metastasis is an inefficient process, only a few cells may have the ability to survive and grow out of their original niche [230]. Such rare cells would be able to adapt themselves to a new microenvironment, where they could become quiescent or grow slowly, explaining why some patients experience very late relapses in distant tissues. The existence of leukemic stems cells (LSCs) that would be responsible for late relapses may also be evoked. However, these LSCs are not well characterized, especially in B-ALL, and several reports indicate that ALL cells with the capacity to initiate leukemia are relatively frequent [231,232]. We have therefore chosen to focus on the role of the microenvironment, which appears to play an important part in the leukemia-initiating ability of ALL cells.

Ebinger et al. recently used an ingenious cell labeling system in patient-derived xenografts to provide evidence that such dormant cells exist in ALL and provided a robust preclinical model [45]. The authors found that these dormant cells shared a similar transcriptional program with leukemic cells found at MRD level in patients. Moreover, these dormant cells were resistant to conventional chemotherapy *in vivo*, but became sensitive to drugs when retrieved from their microenvironment and treated *ex vivo* [45]. This result has two principal clinically relevant implications: first, the dormant phenotype that appears to be harbored by MRD cells is reversible and second, the microenvironment appears to play a critical role, strongly suggesting that targeting the microenvironment could be a promising strategy to kill the disease and prevent relapse and treatment failure. As the authors state, it may also explain why children treated for ALL benefit from standard maintenance therapy that continues for two years from diagnosis: daily doses of low-dose chemotherapy might help to progressively eradicate all ALL cells, including the dormant cells that may reenter the cell cycle after the intensive phase of treatment. Although the authors showed that dormant ALL cells were preferentially found close to the BM endosteum, they did not explore engraftment in other tissues. It would be informative to use their model to verify whether such dormant cells can also be found in the extramedullary tissues frequently invaded in clinical practice, especially the perivascular areas described above. This study also argues in favor of an extramedullary origin for combined ALL relapses, especially late relapses [59], as the dormant ALL cells were shown to have leukemia-initiating properties.

5. From bench to bedside

Leukemic cells interact dynamically with their microenvironment. Indeed, intravital microscopy experiments, which allowed the high-resolution observation of leukemic cells in live animals, showed the leukemic cells to be motile without any objective preference for a specific location or supportive cells [204]. Treatment of the mice with drugs commonly used in clinical practice (vincristine, dexamethasone, and asparaginase) reduced the leukemic burden and induced an increase in the proportion of quiescent cells, but did not modify the patterns of cell distribution and the cells kept moving, even faster than those of untreated mice [204]. These results suggest that leukemic engraftment was randomly driven, in contrast with other reports and commonly held beliefs. Thus, as stated by Williams et al., engraftment potential may not be restricted to a specific cell population but may be shared by most ALL cells. This was recently corroborated by two independent studies [69,231,233]. In these studies, the authors used a cellular barcoding strategy to track leukemic clone engraftment in the BM of different bones and several extramedullary tissues of xenografted mice [231,233]. Both studies showed that the engrafting cell populations were polyclonal, similarly to solid tumors, suggesting that all founder cells were able to reconstitute a leukemic bulk population. However, the authors found that the clonal distribution differed depending on the tissues analyzed: a clone could be more highly represented in one tissue, whereas another could be predominant in another tissue. The authors stated that this asymmetric clonal distribution was presumably stochastic and probably not due to clonal preference or a predisposition to settle in one site over another. As the authors discussed, the specific microenvironment may confer an advantage to arriving leukemic cells that favors settlement and/or the proliferation, explaining the observed asymmetric distribution. Notwithstanding the evidence provided by the intravital microscopy experiment of Hawkins et al., some cells from the founder population exposed to such a niche may become quiescent and be the ancestor of a subsequent relapse

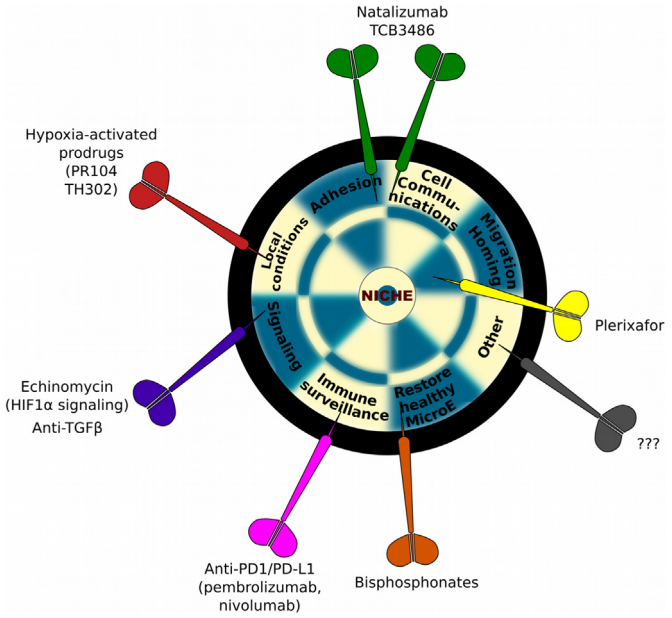

Fig. 3. Potential therapeutic strategies to target the leukemic niche. The leukemic niche is a highly complex microenvironment made of many components or aspects that could be targeted. Potential niche-based treatments are represented by the darts, which could be used simultaneously to target different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

[204].

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Aside from these studies, there is evidence in the literature suggesting that leukemic cells could themselves carry specific features that may direct their engraftment to one or another tissue. Van der Velden et al. recently identified a protein profile that could potentially help predict CNS relapse at the initial diagnosis. A comparison of the gene expression profiles between leukemic cells from the BM and CNS of patients allowed the identification of genes differentially expressed by leukemic cells from the CNS. Among these genes, *SSP1* (coding for osteopontin) and *SCD* (coding for stearoyl-CoA desaturase) were found to be upregulated, which was further confirmed both at the mRNA and protein levels. Retrospective flow cytometry analysis of BM samples from patients who experienced a CNS relapse showed that both SPP1 and SCD proteins were elevated in a small subpopulation of cells, suggesting that this expression profile might be used in a clinical setting to predict CNS relapse [234]. As Frishman-Levy and Izraeli discussed in a recent review, in which they summarized current knowledge concerning CNS leukemia, the contradictory nature of these results with those of Williams et al. could be partially explained by methodological discrepancies (mouse xenograft model *versus* a human model). The idea that CNS tropism may be linked to a specific ALL population is also supported by the association of several recurrent genetic alterations with the incidence of CNS relapse. Indeed, the chromosomal rearrangement t(1,19), leading to the *TCF3-PBX1* fusion transcript, has been associated with an increased risk of ALL relapse in the CNS [235,236]. This clinical finding has recently been supported by a biological explanation. It was shown that primary leukemic cells expressing the *TCF3-PBX1* transcript expressed high levels of Mer tyrosine kinase, for which the signaling pathway was further shown to be involved in ALL cell survival and chemoresistance in the CNS microenvironment [237].

Leukemia with the t(12,21) chromosomal rearrangement, which leads to the ETV6-RUNX1 fusion protein, is associated with an increased risk of testicular relapse [4]. We previously compared the expression profiles of ETV6-RUNX1 with those of other B-ALL and found that fourteen genes were differentially expressed in this type of leukemia [238]. Among these genes, *CD9* (tetraspanin) was found to be underexpressed, which was further confirmed by flow cytometry of patient samples [239]. We reasoned that CD9 may be involved in the pathogenesis of this particular B-ALL subtype [240]. We found that CD9 has a role in leukemic cell dissemination through the Ras-related C3 botulinum toxin substrate 1 (RAC1) signaling pathway. Importantly, we showed that the more CD9 was expressed at the surface of ALL cells, the more cells were able to migrate into the testes in a mouse xenograft model [110]. Thus, ETV6-RUNX1 ALL relapses may be partially explained by enhanced CD9 expression, which favors blast dissemination and engraftment into the testis.

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

The role of the microenvironment in the pathogenesis of ALL has also been suggested by the good results obtained by treating or preventing extramedullary relapses with specifically directed local treatments. Indeed, aside from the introduction of high-dose methotrexate or aracytine, successful treatment of testicular or meningeal involvement has been specifically directed at the tumor: surgery/radiation therapy for the testis and intrathecal chemotherapy or radiation therapy for the meninges. This may explain the relatively good prognosis for relapses at these sites and is another argument for an important role of the microenvironment, which is removed by surgery and radiotherapy. The role of the intrinsic properties of leukemic cells concerning their interaction with their surrounding microenvironment may be illustrated by the worse prognosis seen in Ph-like and Ph+ B-ALLs. Ph+ B-ALL and Ph-like B-ALLs share similar genetic alterations and a poor response to chemotherapy relative to other B-ALL subtypes. Within these subtypes, alteration of the Ikaros gene is frequently found. This alteration was shown to alter the adhesive properties of leukemic cells and may influence their settlement in their niche.[241].

6. Therapeutic challenges

Therapeutic efforts in the past 15 years have mainly focused on the development of new molecules and immunological approaches directed against specific targets expressed on leukemic cells. Treatment intensification in patients with the most unfavorable prognostic factors has not only improved their survival, but also allowed to efficiently treat extramedullary leukemia without using local treatments. As mentioned earlier, the use of high doses of certain drugs, such as methotrexate, reduced the incidence of extramedullary involvement. Apart from these leukemic cell-oriented targeted therapies, therapeutic strategies oriented toward leukemic niches are also attracting increasing interest [242]. Fig. 3 illustrates a few examples of strategies that could be used to target leukemic cells in their microenvironment and Table 1 shows several examples of molecules that could be used or tested in clinical practice during the coming years. These strategies may also be applied to reach cancer cells in solid tumor metastatic niches.

First, preventing cell migration and engraftment into the niche or, conversely, the mobilization of niched cells could be attempted, for example by disruption of the CXCL12/CXCR4 axis using CXCR4 inhibitors, such as plerixafor [105,124,243,244]. Targeting cell adhesion to the extracellular matrix or supportive cells may also be a useful strategy, for example, by inhibiting α4β1 integrin-mediated adhesion with a specific monoclonal antibody, such as natalizumab, or a chemical inhibitor, such as TCB3486 [245,246]. Another possible niche-oriented therapeutic strategy could be to target integrins, since exosomes home to future metastatic sites via integrins in melanoma and lung cancer models. Directly targeting integrins could thus interrupt cellular communication with the secondary niches [206]. Targeting niche features may also be a beneficial strategy, for example by using hypoxia-activated prodrugs, such as PR104 and TH-302

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^2 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 (*in vitro* 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 (*in vitro* study)	Plerixafor, TC14012	NA	Reduced chemotaxis of B-ALL cells Reduced proliferation of B-ALL cells cultured on stromal BM cells Augmentation of inhibitory effects of chemotherapy
Integrin alpha4	Hsieh et al. 2013 [245]	Preclinical (*in vitro* 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 (*in vitro* and animal study)	TBC3486	NA	Reduced chemoresistance of B-ALL cells Prolonged survival of xenografted mice Synergistic effects with chemotherapy *in vivo*
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^2 as intermittent administration and 330 mg/m^2 as continuous infusion. 1 patient with T-ALL with PR
	Benito et al. 2016 [147]	Preclinical (*in vitro* and animal study)	TH-302 (evofosfamide)	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions Not tested in an *in vivo* 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^2 and 4g/m^2 2 responses (1 CR and 1 MLFS) in 7 B-ALL
	Benito et al. 2011 [141]	Preclinical (*in vitro* and animal study)	PR104	NA	Cytotoxicity on B-ALL cells in hypoxic culture conditions 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 (*in vitro* and animal study)	Zolendronic 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) administrated 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

The apparently disappointing results from clinical studies may be explained by the use of new treatments mostly alone in heavily pretreated patients. The association of each of these molecules with each other and/or conventional chemotherapy or immunotherapy might lead to better outcomes.
Abbreviations: AML: acute myeloid leukemia; ALL: acute lymphoblastic leukemia; BM: bone marrow; BMSC: bone marrow stem-cell; CML: chronic myeloid leukemia; CR: complete remission; DLI: donor lymphocyte infusion; DLT: dose-limiting toxicity; GVHD: graft versus host disease; HD-AraC: high-dose aracytine; HSCT: hematopoietic stem cell transplantation; MLFS: morphological leukemia-free state; MRD: minimal residual disease; NA: not applicable; PB: peripheral blood; PD: progressive disease; PR: partial response; SD: stable disease.

[141,145–147,247]. In ALL, modifying the leukemic microenvironment to reverse the phenotype of dormant blast cells could be also a viable option [45]. This could be achieved by restoring a healthy microenvironment, for example by using bisphosphonates that could prevent bone degradation, which is observed in leukemia pathogenesis [203,204]. Another possible strategy could target signaling pathways that are upregulated upon the interaction of malignant cells with their microenvironment, such as the TGFβ and HIF1α pathways, for which several preclinical or early clinical studies have been published [247,248]. The immune system could also be manipulated, as seen in Hodgkin lymphoma, a hematological malignancy with a rich microenvironment, with impressive results from recent early clinical trials testing specific anti-programmed cell death 1 (PD-1)/PD-1 ligand 1 (PD-L1) antibodies [249,250].

Many challenges have yet to be addressed concerning these and other strategies that could target malignant cells in their niches in hematological and solid cancers. Given the complexity of the microenvironment, successful treatment will probably require a combinatory approach, by associating these therapies both between themselves and with conventional chemotherapies. It is likely that such treatments would exert their full potential by adding them to chemotherapy after the initial diagnosis to prevent the occurrence of relapse. They should probably be reserved for refractory patients or those with the highest risk of relapse.

7. Conclusion and future directions

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

Practice points

- ALL cells appear to localize preferentially in perivascular regions of the tissues they colonize.
- Testicular, CNS and BM leukemic niches and solid tumor metastatic niches display similarities potentially accounting for the residence of leukemic cells in these preferred microenvironments.
- The interplay between leukemic niches and ALL cells is complex, dynamic and bidirectional.
- Relapses in specific tissues can be explained by both the microenvironment in these tissues and the intrinsic properties of ALL cells.
- Niche-based targeted therapies are currently being developed and may effectively prevent ALL relapses.
- Systemic treatments such as chemotherapy remain the best option to successfully treat extramedullary leukemia.

Research agenda

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

Conflict of interest statement

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

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