**Leukemic stem cells in AML: where are we now? An update on recent findings and detection.**

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**Summary**

Ample evidence was provided these past decades that leukemic stem cells (LSC) play a role in the outcome of adult and pediatric AML patients. Although it is generally accepted that the CD34+/CD38- compartment is most LSC-enriched, novel data have emerged illustrating a distinct biology between CD34+ and CD34- AML. In this review, we discuss the main LSC phenotypes in CD34+ and CD34- AML, as they are of utmost importance for the development of broadly applicable LSC-targeted strategies. The leukemia-initiating capacity of these cells upon xenografting is still considered to be the gold standard for LSC detection. However, more feasible techniques have been researched to allow the implementation of LSC measurements into clinical practice. Here, we summarize the current state-of-the-art methodologies using flow cytometry and molecular detection.

**Key messages for clinical practice**

* Evidence has been provided that, in contrast to CD34+ AML where a CD34+/CD38- LSC population can give rise to a CD34+/CD38+ LSC population, in CD34- AML, both CD34+ and CD34- LSC populations co-exist and are non-hierarchically organised.
* Flow cytometric detection of LSC benefits from the ability to detect multiple LSC-specific characteristics, i.e. the expression of leukemia-associated immunophenotypic (LAIP) markers, aberrant light scatter properties and increased chemoresistance (side population assay and aldehyde dehydrogenase assay).
* The search for novel LSC-specific molecular markers, i.e. the ERG gene enhancer+85 (ERG+85) region, is ongoing.
* Future LSC-targeted therapy will benefit from a combinatorial strategy that integrates flow cytometric and molecular markers.

# Introduction

Acute myeloid leukemia (AML) is a heterogeneous haematological clonal disorder, characterized by the accumulation of immature myeloid cells in the bone marrow (BM). AML accounts for 80% of adult[1](#_ENREF_1) and 20% of pediatric[2](#_ENREF_2) leukemias. Despite good initial clinical remission rate, patients are prone to relapse, resulting in a 5-year overall survival rate of 30% in adults[3](#_ENREF_3) and 70% in children[4](#_ENREF_4). This high relapse rate is thought to partly arise from a chemotherapy-resistant cell fraction with unlimited self-renewal capacities, denominated as leukemic stem cells (LSC).

In this review, we give an overview of the advances made in understanding the involvement of LSC in AML biology and the state-of-the-art detection methods.

# Conceptualisation of the (non-)hierarchical leukemic stem cell model

A century ago, hematopathologists envisioned that leukemic cells, similar to their healthy counterparts, are hierarchically structured[5](#_ENREF_5) **(Fig. 1)**. *In vitro* support for this hypothesis was provided by the outgrowth of leukemic blast colonies in semi-solid media, defined as colony-forming cells (CFC)[6](#_ENREF_6),[7](#_ENREF_7). The presence of a CFC population with higher survival capacity, long-term culture initiating cells (LTC-ICs)[8](#_ENREF_8), was confirmed by serial xenotransplantation experiments and led to the definition of a leukemia-initiating cell (LIC)[9](#_ENREF_9). In 1997, Bonnet and Dick[10](#_ENREF_10) postulated the concept of AML being hierarchically organised with LSC residing at the apex. Their theory is still today the cornerstone of our AML model and defines LSC as most enriched within the CD34+/CD38- cell compartment. These cells are able to serial engraft and give rise to leukemia, based on their self-renewal capacities and proliferation/differentiation capacities, respectively. In addition, LSC were endowed with multiple characteristics that are biologically crucial for disease propagation and chemotherapy resistance, e.g. relative quiescence, drug efflux and apoptosis-resistance mechanisms[11](#_ENREF_11).

**Fig. 1. Timeline describing the conceptualisation and evolution of the leukemic model in CD34+ and CD34- AML.**



At first, it was believed that LSC arise from the leukemic transformation of normal hematopoietic stem cells (HSC)[10](#_ENREF_10). This view was however challenged multiple times[11-15](#_ENREF_11), with a major breakthrough provided by Goardon and colleagues[16](#_ENREF_16). This group demonstrated that in the vast majority of AML patients (87%), LSC carry a lymphoid-primed multipotential progenitor ‘LMPP-like’ (CD34+/CD38-/CD90-/CD45RA+) phenotype, and the CD34+/CD38+ populations in those patients carried a ‘GMP-like’ (CD34+/CD38+/CD123+low/CD110-/CD45RA+) phenotype **(Fig. 2)**. Both LMPP- and GMP-like populations were able to serial engraft. However, LMPP-like LSC were more potent, more immature and illustrated distinct gene expression profiles (GEPs). Interestingly, LMPP cells were capable of converting into GMP-like LSC, whilst the opposite versatility could not be demonstrated. The fact that LSC phenotypically and molecularly incline more towards progenitor cells than towards HSC, implies that they potentially originate from progenitors that re-gain self-renewal capacities upon leukemic transformation.

Although the CD34+/CD38- compartment is most commonly associated with stem cell activity, *in vitro* and *in vivo* xenograft experiments showed that LSC activity also resides in CD34+/CD38+ and CD34- compartments, especially in CD34- AML[16-18](#_ENREF_16). Of utmost importance was the discovery by Quek et al.[19](#_ENREF_19)who showed that, in contrast to CD34+ AML, CD34+ and CD34- LSCs are non-hierarchically organised, co-exist next to each other, and can be regarded as nearly equivalent in terms of transcriptional and engraftment features. Hence, they proposed two models in CD34- AML: (i) CD34- leukemic blasts arise from CD34+ stem or progenitor cells (~CD34+ LSC) that lose CD34 expression through oncogenic events or induced by the micro-environment, and (ii) CD34- leukemic blasts derive from CD34- myeloid cells that have gained self-renewal potential (~CD34- LSC). Furthermore, they illustrated that CD34- LSC are concentrated in the CD34-/CD117+ compartment, showing variable CD244 expression **(Fig. 2)**.

**Fig. 2. Cartoon illustrating the distinct LSC phenotypes in CD34+ and CD34- AML.**



Bulk leukemic population for CD34- and CD34+ AML are depicted on the left side, LSC populations for each group separately on the right side. CD34+ AML is characterised by distinct hierarchically organised LSC phenotypes, among which the most dominant CD34+/CD38- LMPP-like LSC population can give rise to GMP-like LSCs, but not vice versa. CD34- AML is characterised by multiple CD34+ and CD34- LSC populations and CD34- LSCs are characterised by CD117 expression and versatile CD244 expression.

# Detection and characterization of leukemic stem cells

## Xenografts

Providing evidence of serial engraftment in immunocompromised mice is still considered the most reliable technique to demonstrate LSC activity. Four different mouse models have been developed with variable success rates between 0-70%[20](#_ENREF_20). Collectively, it can be stated that 0.1% human leukemic chimerism is not reached in 50% of the primary AML cases. Apart from the disappointing engraftment successes, significant heterogeneity was noticed in the phenotype and frequency of LSC amongst patients and mice[21](#_ENREF_21). It should be acknowledged that current murine models do not sufficiently encompass the genomic complexity of human leukemias. In addition, uncertainty exists on whether engrafted populations truly represent founder clones and whether they are responsible for relapse.

*In vitro* culture systems have been developed that mimic the micro-environment and maintain LSC sufficiently long enough to allow self-renewal [22](#_ENREF_22),[23](#_ENREF_23). Although these novel niche-like *ex vivo* models do seem promising, they are still at an early stage. Therefore, more pragmatic surrogate LSC definitions, based on the immunophenotype, functional characteristics or molecular profile, are currently in use.

## Flow cytometric detection

### Membrane markers

The CD34+/CD38- compartment is widely accepted to contain the highest LSC fraction, especially in CD34+ AML, but is also acknowledged to contain HSC[24](#_ENREF_24). In order to discriminate between leukemic and normal stem cells, a plethora of membrane proteins have been proposed. They are commonly referred to as leukemia-associated immunophenotype (LAIP) markers and often are well-studied drug transporters, cytokine receptors, and signalling pathway members. In CD34+ adult AML, the group of GJ Schuurhuis demonstrated that combining a six-marker PE-cocktail, CLL-1/TIM-3/CD7/CD11b/CD22/CD56, together with CD45RA, CD123, CD33 and CD44, performed excellently in identifying up to 87% of the LSC population[25](#_ENREF_25). CD45RA was shown to possess the highest discriminative power in adult AML to distinguish HSC from LSC[26](#_ENREF_26). However, several groups have advocated that LSC might co-exist in all CD34/CD38 defined subpopulations, which was recently validated in a large-scale xenograft study by Ng. et al.[27](#_ENREF_27). This observation has prompted to pursue a search for new membrane markers characterizing cells with LIC, irrespective of the CD34/CD38 phenotype.

Pabst et al.[28](#_ENREF_28) observed that G-Protein-Coupled Receptor 56 (GPR56) positivity identified a LSC compartment with high repopulating potential *in vivo*. Moreover, they observed that the presence of a GPR56+ population is associated with a detrimental outcome in adult AML. This feature was later on confirmed by expression profiling data[29-31](#_ENREF_29), showing a correlation between GPR56 expression and a LSC signature predicting worse survival.

Recently, the Junctional Adhesion Molecule-C (JAM-C), an adhesion molecule contributing to the homeostasis of HSC in the BM micro-environment[32](#_ENREF_32),[33](#_ENREF_33), was found higher expressed in leukemic cells than in hematopoietic stem and progenitor cells. The authors identified a high frequency of CD34+/CD38-/CD123+/JAM-C+ cells in *de novo* AML patients as an independent prognostic marker for disease outcome[34](#_ENREF_34).

Most recently, Paczulla et al.[35](#_ENREF_35) demonstrated that Natural Killer Group 2D Ligands (NKG2D-L) are generally expressed on bulk AML cells whilst absent on the surface of LSC. NKG2D-L negative LSC were able to serially engraft, initiate leukaemia and survive chemotherapy in patient-derived xenotransplant models, while NKG2D-L positive LSC are detected and eliminated by the immune system.

### Light scatter properties

Light scatter aberrancies have a two-tiered added value in LSC identification[36](#_ENREF_36). First, LSC events gated based on CD34/CD38 expression with/without LAIP markers can be further refined by inspecting homogeneous forward scatter (FSC) and sideward scatter (SSC) properties. Second, aberrant scatters, i.e. higher FSC and SSC, will allow the identification of LSC in the absence of LAIP markers. Kersten et al. illustrated that LSC with high CD45RA expression harbor significantly higher scatter properties compared to HSC[26](#_ENREF_26). Although LSC fractions with intermediate CD45RA expression also showed higher FSC and SSC, this difference was not significant. Hence, aberrant scatter properties may not replace the use of monoclonal antibodies directed against LAIP markers, but should be combined in order to maximally eliminate HSC contamination in the leukemic CD34+/CD38- compartment.

### Functional markers

The side population (SP) is characterized by high efflux of the fluorescent dye Hoechst 33342 via the ABCG2 multidrug resistance-mediating transporter[37](#_ENREF_37). Based on their chemoresistant capacities, and the fact that nearly all stem cells in normal tissues and solid tumours reside in this compartment, the SP was proposed as a surrogate compartment for LSC in AML. Integration of both immunophenotypic (CD34/CD38) and functional (SP) characteristics into a more restricted, single LSC definition tremendously (>500-fold) decreased the putative LIC frequency in both CD34+ and CD34- AML[38](#_ENREF_38).

Aldehyde dehydrogenase (ALDH) enzymes are known to protect HSC from the destructive properties of oxidative aldehydes and are involved in drug resistance and detoxification[39](#_ENREF_39). ALDH expression can be used as a surrogate marker to distinguish HSC from LSC and is applicable in CD34- AML. Gerber et al. showed that HSC harbour in >75% of the cases ALDHhigh expression, and readily generated serial engraftment[40](#_ENREF_40). By contrast, merely all LSC express ALDHlow or ALDHintermediate levels. In addition, ALDH levels may provide relevant information regarding the chemoresistance of the disease. In solid tumours, high ALDH expression is associated with therapy refractoriness[41](#_ENREF_41).

## Molecular detection and LSC signatures

A handful of LSC-specific gene expression signatures were recently generated in adult AML[27](#_ENREF_27),[30](#_ENREF_30),[42-46](#_ENREF_42), of which some hold significant prognostic value in independent cohorts. The signatures published by Eppert et al.[30](#_ENREF_30) and Ng et al.[27](#_ENREF_27) were established using bulk leukemia GEPs, correlated with outcome and high-risk disease. Hence, a high LSC-score theoretically functions as a surrogate marker for high LSC activity. In pediatric AML, the LSC17 score was demonstrated to hold prognostic significance. Recently, Lamba and colleagues[31](#_ENREF_31) developed a pLSC6-score with a highly significant prognostic value in poor-risk pedAML patients.

In addition, Yassin et al. recently identified the ERG gene enhancer+85 (ERG+85) region as a molecular biomarker representing stemness features[47](#_ENREF_47). Moreover, they developed a fluorescent lentiviral reporter construct that allows detection of the cellular stemness state in LSC and HSC, and furthermore, is able to predict disease outcome and drug sensitivity.

# Conclusion

As the role of LSC in AML biology, and more specifically in the disease outcome, has become clear, characterizing their properties and introducing methods for their detection has been high on several research agendas. Nevertheless, several unresolved issues and challenges persist. Specifically, it remains unclear whether leukemic transformation occurs in stem and/or progenitor cells at different maturation stages, or, whether distinct LSC with different phenotypes identified within one patient have evolved from one single clone through the acquisition of additional hits. Furthermore, xenografting patient material is not a feasible option in order to deliver results timely and influence treatment decisions. Therefore, much hope has been raised in establishing flow cytometric and molecular scores for LSC identification. The flow-based analysis is however hampered by the multiple compartments in which LSC reside. No marker has yet been identified that (i) provides a clear-cut discrimination between leukemic and normal stem cells, (ii) shows universal expression in all LSC, (iii) shows exclusive expression in LSC whilst absent in leukemic blasts, (iv) is able to identify LSCs regardless of the inter- and intra-patient intrinsic heterogeneity and (v) shows a stable expression over time. The design of smart combinatorial approaches will be most likely the way forward in LSC identification and development of LSC-targeted therapy.

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