T.C.
REPUBLIC OF TURKEY
HACETTEPE UNIVERSITY
GRADUATE SCHOOL OF HEALTH SCIENCES

CHARACTERIZATION OF ACUTE MYELOID LEUKEMIA STEM CELLS BY NICHE-LIKE COCULTURE SYSTEM

MSc. Berna ALKAN

Stem Cell Program

MASTER THESIS

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ETHICAL DECLARATION

In this thesis study, I declare that all the information and documents have been obtained in the base of the academic rules and all audio-visual and written information and results have been presented according to the rules of scientific ethics. I did not do any distortion in data set. In case of using other works, related studies have been fully cited in accordance with the scientific standards. I also declare that my thesis study is original except cited references. It was produced by myself in consultation with supervisor *Assoc. Prof. Fatma Visal OKUR* and written according to the rules of thesis writing of Hacettepe University Institute of Health Sciences.

12 / 09 / 2019

Berna ALKAN

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ABSTRACT

ALKAN B, Characterization of Acute Myeloid Leukemia Stem Cells by Niche Like Coculture System, Hacettepe University Graduate School of Health Sciences Stem Cell Program Master Thesis, Ankara, 2019. AML heterogeneity show evidence of hierarchical cellular organization and at the top of this hierarchical structure there is a rare group of LSCs. It was mentioned that there is an association between LSCs and disease prognosis. For better understanding of the leukemogenesis and therapy resistant mechanism, LSC *in vitro* culture is important and still challenging area. In this study, LSCs were maintained using niche like co-culture system in AML patient samples for long-term culture. Proliferation rate, blastic colony formation capacity, leukemia cobblestone formation capacity and ALDH activity was evaluated for determining the LSC frequency, and their self-renewal and leukemia formation capacity in leukemia population. For short-term culture, proliferation index was determined using CFSE analysis. Unlike healthy donor samples, blastic colony formation and cobblestone area formation were observed in AML samples. Majority of cell populations in the control samples show low ALDH activity whereas, AML samples show intermediate ALDH activity. Besides, in ALDH intermediate population LSC percentage was stable during long-term culture for AML samples. For remission samples the LSC percentage decreased. There was a dispersion among sample proliferation capacity because of the heterogeneity. Selected LSC surface markers (VEGFR-2, CD25, TIM3 ve CLL-1) can be used to determine LSC in leukemia samples for distinguishing HSCs and their expressions levels higher in AML diagnose and relapse samples than remission samples. In conclusion, using this niche like coculture system especially at the time of diagnose and ALDH assay, we can determine the LSC frequency and get information for early prognose. This *in vitro* assay can be used to assess investigating pathways of chemoresistance and screening of new LSC-targeted therapies.

Key words: Acute Myeloid Leukemia, Leukemia Stem Cells, Niche, Co-culture, Bone Marrow

ÖZET

ALKAN B, Niş Benzeri Kokültür Sistemi ile Akut Myeloid Lösem Kök Hücrelerin Karakterizasyonu. Hacettepe Üniversitesi Sağlık Bilimleri Enstitüsü Kök Hücre Programı Yüksek Lisans Tezi, Ankara, 2019. AML heterojenitesinin lösemi hücrelerinin hiyerarşik organizasyonundan kaynaklandığı ve bu hiyerarşik yapının en üstünde yer alan LKH'lerin hastalık prognozu ile ilişkisi gösterilmiştir. Hem lökomogenezin hemde ilaç direnç mekanizmalarının anlaşılabilmesi için çok önemli olan LKH'lerin çalışılabileceği *in vitro* kültür sistemlerine ihtiyaç duyulmaktadır. Bu çalışmada, niş benzeri kokültür sistemi ile AML örneklerinde LKH' lerin, uzun dönem idamesi sağlanmıştır. Lösemik popülasyondaki LKH' lerin sıklığının belirlenmesi yanında, LKH' lerin kendini yenileyebilme ve lösemi oluşturabilme potansiyellerinin incelenmesi için proliferasyon hızlarına, blastik koloni ve lösemik kaldırım taşı oluşturma kapasitelerine ve ALDH aktivitelerine bakılmıştır. Kısa dönem kültürde, CFSE analizi ile proliferasyon indeksi hesaplanmıştır. LKH' lerin tespit edilmesi ve HKH ayırımında kullanılabilecek faklı belirteçlerin (VEGFR-2, CD25, TIM3 ve CLL-1) etkinlikleri değerlendirilmiştir. AML örneklerinde lösemik koloni ve kaldırım taşı alanlarının oluşumu gözlenirken, sağlıklı kemik iliği örneklerinde bu oluşumlar gözlenmemiştir. Kontrollerde düşük ALDH aktivitesi gösteren ana hücre popülasyonun aksine, AML örneklerinde orta ALDH aktivitesinin olduğu ve bu popülasyondaki LKH oranının kültür boyunca stabil kaldığı, remisyon örneklerinde ise bu oranın düştüğü saptanmıştır. Heterojenite nedeniyle proliferasyon indekslerinin geniş bir dağılım aralığı görülmüştür. Seçilen LKH yüzey belirteçlerinin tanı ve relaps örneklerinde remisyon örneklerine göre daha fazla ifade edildiği ve HKH ayırımında kullanılabileceği gösterilmiştir. Sonuç olarak, tanı anında yapılacak niş-benzeri kokültür ile ve ALDH aktivitesine bakılarak LKH sıklığı ve fonksiyonları hakkında bilgi edinilebilir. Hastalık prognozu hakkında erken dönemde elde edinilen bu bilgiler ile tedavi planlaması yapılabilir.

Anahtar Kelimeler: Akut myeloid lösemi, lösemik kök hücre, niş, kokültür, kemik iliği

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ABBREVIATIONS

AML	Acute Myeloid Leukemia
ALDH	Aldehyde-Dehydrogenase
APL	Acute Promyelocytic Leukemia
ASXL1	Additional Sex Combs Like
Bcl-2	B-cell Lymphoma 2
BFU-E	Burst Forming Unit-Erythroid
BM	Bone Marrow
BM-MSCs	Bone Marrow Mesenchymal Stem Cells
CD	Cluster of Differentiation
CEBPA	CCAAT/Enhancer Binding Protein Alpha
CAFC	Cobblestone Area Forming Cell
CART	Chimeric Antigen Receptor Mediated T
CFSE	Carboxyfluorescein Succinimidyl Ester
CFU-C	Colony Forming Unit-Cells
CFU-E	Colony Forming Unit-Erythroid
CFU-G	Colony Forming Unit-Granulocyte
CFU-GM	Colony Forming Unit-Granulocyte/Monocyte
CFU-M	Colony Forming Unit-Monocyte
CFU-GEMM	Colony Forming Unit Granulocyte /Erythrocyte /Monocyte /Megakaryocyte
CFU-S	Spleen Colony Forming Units
CLL-1	C-Type Lectin-like Molecule 1
CNS	Central Nervous System
CR	Complete Remission
DMSO	Dimethyl Sulfoxide
DMEM-LG	Dulbecco's Modified Eagle Medium-Low Glucose
EDTA	Etilendiamine Tetraasetic Asid

FAB	French American British
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FITC	Fluoresceinisothiocyanate
FLT-3	FMS-like Tyrosine Kinase 3
G-CSF	Granulocyte-Colony Stimulating Factor
HSCs	Hematopoietic Stem Cells
HPCs	Hematopoietic Progenitor Cells
IL-3	Interleukin-3
IMDM	Iscove's Modified Dulbecco's Medium
L-CAFC	Leukemia Cobblestone Area Forming Cell
LDA	Limiting Dilution Assay
LICs	Leukemia Initiating Cells
LSCs	Leukemia Stem Cells
LTC-IC	Long-term Culture Initiating Cells
MDS	Myelodysplastic Syndromes
MFI	Median Fluorescence Intensity
MNCs	Mononuclear Cells
MRD	Minimal Residual Disease
MSC	Mesenchymal Stem Cells
NFκB	The Nuclear Factor Kb
NMP1	Nucleophosmin
NOD/SCID	Nonobese Diabetic/Severe Combined Immunodeficiency
PE	Phycoerythrin
PB	Peripheral Blood
PBS	Phosphate Buffered Saline
Rpm	Revolutions per minute
RUNX1	Runt-related Transcription Factor 1
Sca-1	Stem Cell Antigen-1
SCF	Stem Cell Factor

SP	Side Population
THD	The Turkish Society of Hematology
TGFβ	Transforming Growth Factor Beta
TPO	Thrombopoietin
VEGF-2	Vascular Endothelial Growth Factor 2
WHO	World Health Organization
7-AAD	7-Aminoactinomycin D

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1.INTRODUCTION

Acute myeloid leukemia (AML) is a rapidly progressive hematological malignancy, characterized by accumulation of epigenetic and genetic alterations in the hematopoietic stem (HSC)/progenitor cells (HPC), resulting in the expansion of clonal myeloid progenitors. Inability of the clonal myeloid progenitors i.e. leukemic blasts to differentiate into mature blood cells leads to inhibition of normal hematopoiesis [2]. The lack of normal thrombocytes, erythrocytes and white blood cells, which are also critical for a functional immune system, causes severe multilineage cytopenias and life-threatening systemic infections. Although it is possible to treat the majority of AML patients with risk-adapted, intensive, multidrug-chemotherapy treatment, followed by allogeneic hematopoietic stem cell transplantation in patients with high-risk of relapse or those with relapsed/refractory disease, long term survival ratio has not improved significantly for over 30 years and almost one third of AML patients who achieved complete remission with conventional chemotherapy suffers from relapsed disease [3]. Current risk-stratification relies mainly on the response to induction therapy, somatic mutations and cytogenetic alterations. However, molecular signatures of the AML cells, which contribute to myeloid leukemogenesis can evolve from diagnosis to relapse, making the identification a specific target and selection of the appropriate treatment strategy more difficult. Treatment failure in AML patients, who are stratified into an intermediate risk group due to the lack of specific molecular markers identifying the risk for relapse, create another obstacle to the improvement of survival. Although phenotypically similar and presenting with the same leukemia subgroups (M0-M7), adult and pediatric AML differs with respect to biology of this complex and genetically heterogeneous disease. Evidence suggests that the gradual acquisition of somatic mutations lead to leukemogenesis in adults, while chromosomal rearrangements are likely the initiating event in children [4, 5], and the paucity of potential therapeutic targets is more prominent in pediatric AML. Thus, early detection of imminent relapse or even better, prediction of relapse in AML patients who carry high risk at

the time of diagnosis, could guide the initial treatment stratification and it is great importance.

Evaluation of AML patient samples demonstrates that there is a hierarchical cellular organization, similar to normal hematopoiesis. A minor fraction of leukemic cells with the ability of self-renewal, quiescence and drug-resistance constitutes “leukemic stem cells” (LSCs) or “leukemia-initiating cells” (LICs). LSCs are found at the apex of this hierarchy, and they are highly similar to their healthy counterpart i.e. multipotent HSCs and are responsible for both disease maintenance and relapse [2, 6]. LSCs are defined by their self-renewal ability, and which give rise to leukemia in serial transplantations into immunocompromised mice. They can also partially differentiate into non-LSC leukemic blasts, which cannot self-renew, but cause the development of bulk disease. Even though targeting and elimination of LSCs seems to be the ideal strategy for disease eradication and cure, aberrant immunophenotypes, distinct genetic/epigenetic properties and clinical significance of LSCs still needs to be defined.

The bone marrow (BM) niche composed of various cell types with different functions, neuronal and vascular networks, is a dynamic microenvironment and modulates normal hematopoiesis through interactions between these cellular components. Extrinsic and intrinsic factors may cause disturbance of cellular homeostasis in the BM niche, and may contribute to the emergence of hematological malignancies [7]. Most of the studies investigating niche-induced leukemogenesis, molecular/cellular characteristics of LSCs, LSC-niche interactions and its impact on treatment resistance have been using mouse models [2]. Interspecies mismatches in microenvironment and immune interactions may have contributed to model-dependent engraftment potential, but also identification and frequency of LSCs. Thus, considering the molecular/functional heterogeneity of human AML LSCs, more reliable experimental models are needed for evaluation of clinical significance of LSCs and specific targeting of LSCs for disease eradication to serve as a real validation of cancer stem cell model.

The primary objective of this study was to establish a BM niche-like coculture system for maintenance and/or enrichment of AML LSCs as an alternative approach to study cellular and functional properties of LSCs and associations of LSCs with clinical outcome. Besides defining LSCs as an early predictive marker for disease prognosis, such an optimized coculture system may also be used to investigate patterns of chemotherapy resistance and efficiency/toxicity of new therapies targeting AML LSCs in future studies.

2.BACKGROUND

2.1. Acute Myeloid Leukemia

Leukemia is a type of blood cancer that is classified as acute or chronic according to the rate of growth and the leukemic cells i.e. blasts, are derived from either myeloid lineage or lymphoid lineage. Myeloid stem/progenitor cells in the bone marrow are involved in formation of different types of blood cells, including white blood cells (excluding lymphocytes), red blood cells and platelets. When there is a blockage in one of the differentiation steps of myeloid stem/ progenitor cells, a molecular defect leading to uncontrolled cell proliferation and/ or inhibition of apoptosis, malignant transformation occurs and acute myeloid leukemia (AML) develops. AML is the most common type of adult leukemia and has an unfavorable prognosis. It shows aggressive behavior and grows and spreads quickly. Acute leukemias which are characterized by interruption of differentiation at the hematopoietic stem/progenitor cell level, comprises 20- 25 % of childhood malignancies and is the second most common type of leukemia in both childhood and adolescence [8]. Uncontrolled proliferation and accumulation of leukemic cells in bone marrow results in severe cytopenias due to lack of normal hematopoiesis and is observed in more than 60% of patients, leading to typical leukemia-associated symptoms such as fatigue, arthralgia, fever, weight loss, exhaustion, paleness and bleeding.

The French American British (FAB) and World Health Organization (WHO) AML mainly classification systems have been used for defining AML subtypes. In the FAB classification established in 1976 French, American and British hematologists classified AML into subtypes (M0 to M7 at Table2.1) according to blast morphology i.e. cell shape/size, nucleocytoplasmic ratio, cytoplasmic vacuolization, the appearance of nucleolus and granularity. This classification shows at which stage interruption of hematopoiesis occurs [9].

Table 2. 1. AML Subtypes according to FAB Classification

FAB Subtype	**Name**
M0	Undifferentiated acute myeloblastic leukemia
M1	Acute myeloblastic leukemia with minimal maturation
M2	Acute myeloblastic leukemia with maturation
M3	Acute promyelocytic leukemia (APL)
M4	Acute myelomonocytic leukemia
M4 eos	Acute myelomonocytic leukemia with eosinophilia
M5	Acute monocytic leukemia
M6	Acute erythroid leukemia
M7	Acute megakaryoblastic leukemia

The WHO system classifies AML by looking not only at cell morphology but also to immunophenotype and genetic features of leukemic cells. In this system, there are four major groups of AML [10]. These groups are defined by certain genetic abnormalities, such as translocations or inversions including t(8;21), t(16;16), inv(16) or t(6:9), and myelodysplastic features. Some mutations in certain genes like *FLT3*, *RUNX1*, *TP53* and *ASXL1* are indicators of an unfavorable disease prognosis. So, being aware of AML subtypes and risk stratification at the time diagnosis is very important for prediction of disease course, prognosis and for selection of the best treatment strategy for a patient.

For the diagnosis of leukemia, the blast percentage should be higher than 20% in the bone marrow. Another indicator for AML is the identification of Auer rods which are needle-like elongated shapes and crystalline structures, seen in the cytoplasm of blasts. This structure gets formed from the fusion of granules and occasionally multiple rods may be present in blast cells. Auer rods are considered pathognomonic for AML, especially for AML M3 subtype. Auer rods can also be present in cases of AML- M1, and AML- M2 [11].

In comparison to other types of cancers, AML has a poor survival rate [12, 13]. The National Cancer Institute states that almost 30% of AML patients survive for more than 5 years. Multidrug chemotherapy is the mainstay of therapy for both pediatric and adult leukemia. Nevertheless, disease recurrence occurs in almost 50% of patients who achieved complete remission (CR) after chemotherapy. Moreover, more than half of the patients develop resistant disease [14].

AML is also classified into two categories depending on disease pathogenesis; primary or *de novo* AML and secondary or treatment-related AML. Secondary AML may develop after exposure to chemotherapy/radiotherapy applied for the treatment of other malignancies, such as malignant lymphomas, myelomas, epithelial cancers or exposure to environmental toxins/mutagenic agents and constitutes 5% to 15% of all AML cases. *De novo* AML is the type of AML which evolves without prior exposure [15].

AML patients may have an extensive number of chromosomal alterations. In pediatric AML, the alterations consist of distinct categories according to their chromosomal events, such as t(8;21) or Inv(16)/t(16;16) translocations, which have been found in 25% of pediatric patients, whereas *MLL* gene rearrangements and t(15;17) translocations have been found in 12% of pediatric patients [16]. These alterations have remarkable age-related variations with a high prevalence in pediatric patients. In more than 90% of pediatric patients at least one detectable genomic alternation is observed. 3 somatic mutations (*NPM1*, *FLT3* and *CEBPA*) have been implicated as markers in pediatric AML as important for prognosis and therapeutic response [16]. *FLT3* mutations are associated with a poor outcome and high relapse risks and are found in 15% of pediatric AML. In contrast, *CEBPA* mutations are associated with improved survival and is observed in 5% of pediatric AML patients [4, 16].

The genetic background of pediatric AML differs from adult AML, leading to a distinct disease biology in pediatric patients. According to recent data reported by The Turkish Society of Hematology (THD), pediatric and adolescent AML are responsible for almost 15 – 20% of all leukemia cases. Every year, about 1200 – 1500

new cases of leukemia were diagnosed in children under the age of 16. Although leukemia may occur at every age during childhood or adolescence, it was most frequently diagnosed in children between the ages of 2 to 5 years. There are some cancer predisposing syndromes such as Down syndrome (Trisomy 21), Fanconi Aplastic Anemia, Diamond Blackfan Anemia, Kostmann Syndrome, neurofibromatosis, and Klinefelter Syndrome, in which patients carry an increased risk for AML. Approximately 80% of AML patients have clonal chromosomal abnormalities. Environmental factors like smoking, being exposed to certain chemicals, drugs and radiation also increase the chance of developing the disease. However, these risk factors do not always give rise to AML and sometimes patients do not have any of these risk factors [17]. Although a remission rate of 80% is reported for AML patients, the actual survival rate is around 50%, due to the high risk of relapse [12, 14].

After treatment, there is a period called remission, in which all leukemia-associated signs and symptoms disappear, and leukemic cells are cleared from both the bone marrow and other infiltration sites. When the AML returns after a period of remission, it is called relapse. The risk of relapse is highly related to the aggressiveness of the disease. AML heterogeneity is a major challenge in leukemia treatment and disease monitoring, and treatment may vary according to the severity and subtype.

The evolution of the disease is not only dependent on genomic alternations, but also additional epigenetic and genomic changes occurring between diagnosis to relapse [18]. Specific mutations that have occurred in some AML patients with additional mutations may be observed as a major clone at diagnosis. Alternatively, minor/subclones may emerge after chemotherapy and contribute to relapse [19]. Craddock et al. demonstrated that patients who receive azacytidine and sodium valproate, show persistence of LSCs, as measured by flow cytometry, even in complete remission patients [2, 20]. Therefore, new alterations may arise and selection may be promoted and result in resistance to therapy. Moreover, at the time

of relapse, new clones may arise with a new genomic profile distinct from the original cases.

2.2. Leukemia Initiating Cells/Leukemia Stem Cells

The first definition of “cancer stem cell” was proposed by Lapidot and coworkers in 1994. They tried to detect cell surface markers to identify a rare population with stem like properties in AML samples [18]. According to the cancer stem cell hypothesis, leukemia originates from a rare population of stem cell-like leukemia initiating cells [19]. Leukemia stem cells are too heterogenic cells with distinct capabilities and functions and have the ability to initiate tumor growth upon transplantation into a receptive animal model [19]. Leukemia initiating cells are a rare subpopulation of leukemic cells. They have stem cell properties with which they differ from bulk leukemia cells [23]. Because of their ability of self-renewal and differentiation into other types of cells, AML is theorized to arise from a stem cell origin. Accordingly, cells that are causing aforementioned malignancies are called leukemia initiating cells (LICs) or leukemia stem cells (LSCs). Stem cells and leukemia initiating cells share many functions and properties that are crucial for their survival, growth and drug resistance, recurrence and remission [24]. LSCs have been shown to differentiate into leukemic blasts and possess self-renewal properties [25]. LSCs have also been suspected to be responsible for relapse after treatment and may survive and maintain malignancy after therapy [26]. Thus, LSCs may be the main reason of disease relapse. Therapy optimization requires a full understanding of the cells and their microenvironment. However, thus far it has proven hard to expand LSCs in vitro.

Although many studies have tried to characterize LSCs, a single unique marker has not been detected yet. In 1997, according to Dick and Bonnet and in 1994, according to Lapidot et al. leukemia initiating cells were found to be CD34+CD38- as confirmed by their engraftment capacity in SCID mice [21, 27]. However, some types of AML are associated with lack of CD34 expression [28]. Therefore, LICs may show a heterogeneous phenotype within samples. In addition, the most accepted markers

for leukemia stem cells are CD34+CD38- which is also found on healthy hematopoietic stem cells. Therefore, LSC share certain characteristics with HSCs.

LSCs are defined as quiescent cells in the G0 phase of the cell cycle [29]. However, some other studies mentioned that actively cycling cell populations may contain LSCs as well [30-32]. Chemotherapy targets dividing cells and therefore LSCs cannot always escape the therapy. LSCs are rarely observed with values ranging from 1 in 10 thousand to 1 in 5 million in bone marrow cells. Ho and colleagues have proposed that, after relapse the LSCs frequency increases indicating that the frequency of LICs is related to prognosis and pathogenesis. Understanding the LSC dynamics and changeable nature are needed to develop therapeutic targeting for the disease [32].

Because of the heterogeneity, in certain cases, standard FDA approved therapy may not provide sufficient treatment for AML. Similar to other aggressive type of cancers, AML cells can often become resistant to standard treatment options. Therefore, slowing down the process has been shown to be difficult for this type of leukemia. Therefore, leukemia cell maintenance and proliferation need to be assessed in vitro [2, 33].

Personalized medicine or precision medicine is patient-specific treatment according to the characteristics of the disease and patient's unique profile. This offers not only the possibility of more appropriate treatment, but also the chance of early stage diagnosis and detection. Awareness of the leukemia cell profile and understanding of its unique characteristics are important for developing a patient-specific treatment plan. Finding the curative therapy for AML remains complex and challenging, and many factors may affect prognosis and applied therapy protocols. Understanding of the complexity and the heterogeneity of the disease may give a clue to identify novel the therapeutic targets.

2.2.1. Leukemia Stem Cell Biology

Data from several populations of LSCs within the same patient confirms that the LSC population can evolve to a complex phenotype by epigenetic and genetic

alterations. FLT-3 mutations are common in AML and it is thought considered as related to relapse ratethat there may be a relation between relapse rate and the mutation. Mutations in FLT-3 have been observed at diagnosis, but not at the time of relapse in the same patient and the visace versa [34]. Therefore, clonal evolution and mutations in preleukemic stem cells may not be stable and elimination of the dominant clone may not be sufficient for treatment but should also include eradication of clones and subclones to achieve complete remission.

2.2.2. Leukemia Stem Cell Identification and Characterization

Many researchers have confirmed LSCs in AML samples. LSC is defined by its capacity to initiate leukemia and they are considered responsible from relapse of disease. To understand and identify of the LSCs, ALDH assay, side population assay and multicolor flow cytometry can be used. Understanding of phenotype is important to enhance classification and can guide clinical approach for identifying new therapeutic targets. Elimination of LSCs can improve the AML outcome by preventing reoccurrence. LSC populations able to engraft and initiate leukemia in a recipient mouse, to give rise after retransplantation into secondary and tertiary (preferable) recipients to indicate self-renewal capacity as a Golden standard . For the presence of LICs, xeno-transplantation model has been declared as evidence by their engraftment capacity of samples. Sufficient number of cells are used to confirm presence of LSCs which is retrospectively assessed after engraftment of cells [27]. Like Hematopoietic Stem Cells, LSCs are characterized by their unlimited potential of repopulation and self-renewal ability. LSCs are responsible for blastic cell formation. Moreover, it has been mentioned that LSCs also express the stem cell markers and in animal models LSCs can survive upon serial transplantations. Similar to normal HSCs, LSCs are characterized as extremely rare population [35].

The ultimate proof for LSC is the engraftment of leukemia cells in a xenogeneic transplantation model in vivo. NOD/SCID mice model was found to be most efficient and transplanted cells show similar properties to the original disease [27]. Therefore, xenotransplantation model is used as the characterization assay.

Moreover, limiting dilution assay (LDA) is used to determine the frequency of LSCs. Sufficient number of leukemia cells is seeded for colony formation assay or transplantation assays and the differentiate property or engraftment capacity is assessed to estimate of the original LSC number. Because, in vivo transplantation assays are costly and time consuming, a number of in vitro bioassays are widely used for characterization of LSC in a short time [35].

CD34 is used as a starting point for selection of LSC. Normal CD34+cd38- progenitor cells have similar features with LSC, so targeted therapies require specific markers to eradicate and monitor the LSC [28]. CD45RA, CD11b, CD123, CLL-1, CD44 and CD47 are commonly used markers for LSCs [36-38]. However, each patient shows different expression level so development of a multicolor panel is crucial for therapy. LSCs have extreme molecular and phenotypic heterogeneity and can be found with different frequencies of CD34 and CD38 expressions. Accordingly, Taussing et. al. some AML samples have both CD34+ and CD34- LSC. Taken together, majority of CD34+ fractions and minority of CD34- fractions contain LSCs [36, 39, 40].

The frequency of LSC vary widely between patients (1/10.000 to 1/5 x 106). Side population (SP) was identified specific cell population by flow cytometry in which the specific Hoechst dye 33342 is released by ABC drug transporter pump. This population resist to AML therapies such as anthracyclines and initiate leukemia in NOD/SCID mouse models and contain both CD34+ and CD34- cells [41-43]. Thus, SP assay used for characterization of LSCs. But, SP can contain HSC so specific assays should be used to complement this assay [35, 43].

Various distinct assays are used to measure frequency and differentiation property of LSCs in vitro such as colony forming unit (CFU-C) assay, long-term culture initiating cell assay (LTC-IC) and cobblestone area forming cell assays (CAFC). CFU-C assays provide detection and quantification of myeloid progenitors in the population. The culture condition is the suitable to differentiation, maintenance and proliferation by growth factors, cytokines and nutrients. Thus, for characterization of LSC this assay is important. The CAFC and LTC-IC assays based on culture of stem cells with adherent

cells that mimic the microenvironment for LSC (and HSC) maintenance and survival. In vitro culture assays of LSC include the capability of the cells to generate cobblestone area for long-term cultures (L-CAFC). The L-CAFC assay and LTC-IC measures the frequency of LSC and their self-renewal capacity [3, 35, 44].

With the divisional kinetics LSC can be identified. LSC have slower divisional kinetics than HSC and extensive ability of self-renewal capacity [45, 46]. Thus, proliferation kinetics can be used to characterize LSC as a parameter. During proliferation dilution of cytosolic CFSE can be used for that purpose [35]. Another recent marker for stem cell characterization is ALDH. Stem cells protect themselves against DNA damage by ALDH (aldehyde dehydrogenase) so, high expression of ALDH indicates the stem/progenitor cells [39]. According to Cheung et al. ALDH activity can be used to isolate and characterize the LSC in AML samples [47]. Slow dividing and (ALDH+) LCS cells can repopulate in NOD/SCID mouse model [48]. And they show that ALDH+ cell isolation is more efficient than using other methods for functional experiments [35, 48]. Moreover, HSCs show high ALDH+ and LSCs show ALDH+ activity (both intermediate and high). In AML samples ALDH bright cells considered to contain only HSC and ALDH intermediate cells considered to contain LSCs [35, 45, 46]. Therefore, based on ALDH activity LSC can be identified and purified from HSCs and used for LSC targeted therapy.

2.3. Leukemia Stem Cell Targeted Therapies

Targeted therapies are categorized as selectively for LSC, “LSC specific” or for both the bulk and LSC populations, “LSC active”. In 1997 LSCs determined to be CD34+ CD38-, and using these surface markers LSC can be isolated and used for research. However, healthy HSCs also express the CD34+CD38- surface markers. Therefore, removal of LSCs based on these surface markers is impossible. Discovery of specific LSC markers may allow isolation of a pure population, tracking of the population during therapy and progression and be used to develop marker specific therapeutic-targeted strategy. However, the phenotype of LSC can differ among patients and

within patients [20, 21]. Moreover, LSCs can evolve and acquire a new phenotype between diagnosis and relapse [22].

Antibody-based therapies developed by binding toxins to antibodies using the latter as a “delivery vehicle”, is a novel approach for targeting LSCs and include Gemtuzumab ozogamicin (Mylotarg; Wyeth-Ayerst; New Jersey) a recombinant human - mouse monoclonal anti-CD33 antibody covalently conjugated to calicheamicin, an anti-tumor agent. Although, LSCs have variable expression of CD33, gemtuzumab ozogamicin in combination with chemotherapy has been shown to affectively eradicate LSCs in some patients [23]. Antibodies against CD123, CD33 and CD25 have also been used for antibody drug conjugate therapy in the treatment of AML [24-26].

Another strategy for antibody dependent therapy is triggering the innate immunity using CD33 and CD123 antibodies in combination with anti-CD3. This T cell, synthetic transmembrane structure, has a specific antibody target recognition side and an immune effector mechanism. The use of LSCs markers like CD123 as targets for CART “chimeric antigen receptor mediated T” cell based therapies offer alternative opportunities for AML specific therapy [27, 28].

LSC metabolic properties have also been investigated for targeted therapy. LSCs have distinct metabolisms and responses under stress condition. NF-κB activation during the stress response is specific for LSCs, not for quiescent HSCs [29]. On the basis of mitochondrial activity, B-cell lymphoma 2 (Bcl2) inhibition was also used to disrupt oxidative phosphorylation of LSC [30].

2.4. Leukemia Microenvironment “Leukemic Niche”

Hematopoietic cells are located in a special microenvironment called the bone marrow niche. Stromal cells, all blood cells, regulatory cytokines, extracellular matrix components are all part of this niche. In the BM HSCs stay quiescent for maintenance of the HSC pool and reactivate to assure blood production [19]. This stem cell specialized microenvironment was first proposed in the 1970s by Schofield where he suggested the presence of a local environment supportive of CFU-S “spleen colony

forming units" and hematopoietic stem cells [31]. Endothelial cells, stromal cells, osteoblasts and CXCL12 abundant reticular cells are found in this hematopoietic niche. The BM niche consist of at least two distinct areas known as the endosteal and perivascular niche. The endosteal niche is high in osteoblast content. In contrast, the perivascular niche contains mainly of extracellular matrix and stromal cells. Quiescence and differentiation of HSCs are regulated by communication with the microenvironment components. For instance, bone marrow endothelial cells play a crucial role for HSC self-renewal and production of growth factors, such as SCF and CXCL12 [32]. In addition, different mesenchymal stem cell (MSC) subtypes play a significant role as a scaffold for ECM components and a regulatory role by secretion of specific factors for regulating the localization and migration of HSCs. Megakaryocytes which do not circulate provide factors for HSC quiescence, such as CXCL4, TPO and TGFβ1 [33, 34].

Similarly, the leukemia stem cell microenvironment has a crucial role in the regulation of their behavior: Signals coming from the BM niche, are also crucial for LSC survival and maintenance. Alterations in the complex interactions between cells and their environment may lead to diseases and support instead of normal hematopoiesis, leukemogenesis [35]. Moreover, because of the nonspecific treatment, there is a considerable rate of recurrence and resistance of therapy in AML. Nowadays leukemic blasts and their microenvironment interactions are a hotspot area for researchers. Leukemia development may be related to a genetic mutation, epigenetic changes and immune dysregulation. And some researchers have claimed that changes in the niche may cause leukemia progression [36]. Because of the uncontrolled proliferation and cellular growth of the leukemia cells, the normal hematopoietic cells microenvironment is changed and normal red blood cell, neutrophil and platelet proliferation may be affected. As a consequence, it is common for a patient with leukemia to have anemia, weakness, thrombocytopenia, proneness to infections and a delay in wound healing.

Different mutations and DNA damage can accumulate in HSCs by intrinsic and extrinsic factors. These accumulations can cause malignant transformations and LSCs

may settle in the HSC niche and benefit from environmental signals such as expression of CXCL12 and E-selectin play a vital role in HSC homing [37]. Moreover, they provide homeostasis by supporting proliferation, self-renewal and quiescence of LSCs. Subsequently, LSCs can alter the HSC niche to support further leukemia growth and disturb hematopoiesis [38].

Many factors are secreted by the niche compartments to support LSC growth and maintenance. For instance, Interleukin-3 (IL-3) produced by T cells and mast cells, is responsible for growth, survival, proliferation and differentiation of primary HSCs. IL-3 is crucial for activation of SHP-2, which plays role in growth and proliferation of HSCs [33, 39]. AML patient samples overexpressing the IL3 R alpha chain on LSC are related to a negative outcome [40, 41]. Therefore, IL-3Rα is important for the maintenance of LSC and could be a target for therapy.

Thrombopoietin (TPO) plays a role in megakaryocyte production and platelet regulation. Matsumura and colleagues showed that TPO is important for AML cell proliferation. In addition, G-CSF (granulocyte-colony stimulating factor) stimulates survival, differentiation, the proliferation of granulocytes and neutrophils and supports development of AML.

Bone marrow mesenchymal stem cells (BM-MSCs) from the HSC niche have been shown to display the same chromosomal aberrations as seen in MDS and AML. Therefore, Blau *et. al.* have been implied that these aberrations in stromal cells may play an important role in the initiation of the disease [34]. On the other hand, AML cells themselves can change the niche through BMP signaling, which induces MSC osteoblastic differentiation instead of adipogenic differentiation [42]. This further provides support for AML development.

2.5. Microenvironment as A Potential Target for AML Therapy

Disruption of the supportive microenvironment of the LSCs can be used as a potential modality to leukemia treatment. In 2006, CD44 antigen antagonists were used for interruption of LSC growth [43]. However, more recent studies suggest that

LSC suppressive techniques may require strategies, specific to the stage of pathogenesis [44].

LSC metabolism may be altered by its microenvironment via cell to cell interaction and environmental factors. LSCs mostly reside in the endothelial region of the bone marrow and reside in adipose tissues. This alteration of metabolism may lead to chemotherapy resistance [45]. AML cells also borrow mitochondria from adherent stromal cells. This mitochondria transfer leads to an increase in oxidative phosphorylation and support recovery of DNA damage after chemotherapy. Moreover, the proinflammatory pattern can positively affect LSC survival and proliferation. Therefore, inhibition of these factors rendering the microenvironment more suitable for normal stem cells instead of LSCs may show potential as targeted therapy.

The most critical issue for targeting therapy is timing. More recently, It was shown that after chemotherapy, LSCs can gain heterogeneity compared to the primary samples [22]. Therefore, it is crucial to interfere at an early stage of pathogenesis. At diagnosis, according to critical trials, LSC-directed therapy alone is the most useful method for LSC eradication in comparison to conventional therapy and/or combination with conventional therapy. Thus, whereas combined therapy may result in significant toxicity, conventional therapy may lead to induction of heterogenous LSC populations [6]. It is possible to apply the LSC-directed therapy after consolidation chemotherapy or at the time of recurrence. This LSC-directed therapy can also be applied after allogenic stem cell transplantation to eliminate the risk of relapse or during the pre-transplantation conditioning regimen.

Gemtuzumab ozogamicin is a synthetic drug, covalently bound to calicheamicin (antitumor-antibiotic) [46]. Several studies showed that there were no significant response rates to gemtuzumab ozogamicin but, it can benefit patients with a significant survival rate. Therefore, LSC directed therapy is important for improving disease free and overall survival.

Therapy optimization requires a better understanding of leukemic cell behavior and the interaction of the leukemic cell with its microenvironment.

However, it is hard to expand leukemic cells *in vitro*. Primary leukemia cells derived from patients cannot survive *ex vivo* without support [42]. They need stromal cells, metabolites, chemokines, cytokines and nutrients. Thus, interactions through paracrine signals and adhesion molecules are crucial for maintenance and expansion of leukemic cells, the cellular compartment that contains LSCs. Therefore, stromal cell support and cytokine cocktails, providing a niche-like environment, may be crucial for successful cultures of LSCs.

2.6. Aim of the Thesis

Development and optimization of assays to study LSCs is pivotal in understanding of leukemia stem cell biology and mechanisms of leukemic transformation. This would assist in the discovery of new therapeutic targets of LSCs. Osteoblastic, endothelial and mesenchymal cells are used as feeder cells together with various cytokine cocktails to establish a “niche-like coculture systems” [47]. Data acquired from current *ex vivo* culture systems indicate that cell to cell contact has an impact on the expansion, maintenance and migratory potential of LSCs. Niche-like coculture systems seem to be ideal to understand the relationship between AML-LSCs and its stromal niche and could help identify mechanisms of leukemia relapse and disease persistence.

The main objectives of this thesis are; i- long term culturing of patient-derived primary AML samples on MS-5 stromal cells in order to expand and enrich LSCs, ii- evaluation of proliferation kinetics and frequency of LSCs in this optimized niche-like coculture system, iii- demonstration of self-renewal capacity of AML LSCs through observation of long term growth, cobblestone area forming cells and colony forming units, iv- assessment of proliferation kinetics and frequency of LSCs in short term culture and the association of LSCs with risk stratification and/or clinical outcome of AML patients.

3. MATERIALS AND METHODS

3.1. Patient and Donor Selection

Peripheral blood and/or bone marrow samples were obtained from three healthy donors and twelve AML patients (8 newly diagnosed, 4 relapse cases) prior to treatment, at remission (5 pairs of diagnosis-remission) and at the time of relapse after receiving informed consents. AML diagnosis was done for all patients enrolled in this study by conventional methods (morphology, immunophenotyping, cytogenetic/molecular analyses). Control subjects were selected among age and gender matched healthy bone marrow donors. AML sub-groups (M0-M7) were decided via examination of bone marrow aspirates and immunophenotyping of leukemic blasts. All AML patients were stratified into risk groups at the time of diagnosis according to the criteria defined in the BFM AML 2013 treatment protocol and received treatment according to their defined risk groups [48]. The patients who had disease relapse were treated with salvage chemotherapy and underwent allogeneic hematopoietic stem cell transplantation after achieving disease remission. Treatment response was evaluated at certain time points according to the protocol; morphological and cytogenetic analyses were performed to learn about disease status and/or minimal residual disease. The study was approved by Hacettepe University Ethical Committee (number: GO 16/824-27) (Supplement 1).

3.1.1. Isolation of Mononuclear Cells from Peripheral Blood and Bone Marrow Samples

Fresh peripheral blood and/or bone marrow samples were diluted 1:1 with 1x PBS (Phosphate Buffered Saline, pH 7,4). Lymphoprep density gradient medium (1.077 g/mL, Germany, Stem Cell Technology, cat no 07851) was used at the same ratio and transferred to a new tube. Diluted samples were added with a pasteur pipette. Centrifugation was done at 1600 rpm 40 min. without brake. After that, mononuclear cells (MNCs) were harvested by pasteur pipette in the new tube. PBS

was added at twice sample volume. The samples were centrifuged at 1500 rpm for 5 min. The supernatant was poured and the pellet which contains MNCs were resuspended with cell culture medium. Culture medium containing DMEM-low glucose (Gibco, Thermo Fisher, cat no 31885023) 10% FBS (Fetal Bovine Serum) (heat inactivated, Thermo Fisher cat no 10500064), 1% L-glutamine (200mM, Thermo Fisher, cat no 25030081) and 1% Penicillin Streptomycin (10.000 U/mL, Thermo Fisher, cat no 21980065) is used. Isolated cells were counted with Turk's solution. Samples were centrifuged at 1500 rpm 5 min. After that, the cell pellet was resuspended with freezing medium on ice. Freezing medium contained 20% FBS, 10% DMSO, 70% Basal medium (DMEM-LG). The cell concentration was between 10-20x 10^6/vial. Cells were frozen at a cooling rate of 1°C/ minute in freezing containers (Mr Frosty, Thermo Scientific, cat no 5100 0001) placed in a -80 °C freezer. The freezing container was stored for at least 4 hours. After which, the cells were transferred to the liquid nitrogen storage tank and stored until use.

3.2. Murine Stromal Cell line (MS-5) Expansion

Gelatin was prepared as 0,1% in PBS for coating the plate prior to MS-5 cell culture. In a glass bottle, 0.5 g gelatin was weighed and 500 ml dH_2O was added. Then, the glass bottle was autoclaved. 12-well plates were covered with 0.1% gelatin and incubated for 4 hours at 37°C in 5% CO_2 in order to tightly attach the MS-5 to the well plate.

MS-5 is a murine stromal cell line, was used as a feeder layer for LSCs enrichment culture (DSMZ, Germany, cat no ACC 441). Irradiation of the stroma was not performed.

The gelatin was aspirated, 40.000 MS-5 cells / well were seeded, and cultured in IMDM media (Thermo Fisher, cat no 21980065) containing 10 % heat inactivated FBS, 1% Penicillin Streptomycin (10.000 U/mL, Thermo Fisher, cat no 21980065), 1% L-glutamine (200mM, Thermo Fisher, cat no 25030081) for one day. When MS-5 cell confluency reached approximately 80%, culture medium was changed with basal medium consisting of Myelocult H5100 (Stem Cell Technologies, cat no 05150) and

10^4 M hydrocortisone (Stem Cell Technologies, cat no 07904), and cells were cultured with this coculture media for 24 hours before co-culture. (Required confluency was reached for co-culture within 2 days.)

3.3. Thawing AML Patient Samples and Coculture with MS-5

At day 0, Cryopreserved cells were thawed rapidly in the water bath (37 °C) until there was a little ice cube left it. (The recovery rate is approximately in the range of 70-95% for our samples.) Before the vials were transferred into a laminar hood, the outside of the cryovials were cleaned with ethanol (70%). Then, the cell suspension was transferred into the tubes containing the same pre-warmed basal medium (DMEM - LG). These steps were done quickly and gently; otherwise, cryopreservation may damage the cells. The samples were centrifuged at 1500 rpm 5 min. Thereafter, the supernatant was decanted, and the cell pellet was resuspended with Myelocult H5100 media (Stem Cell Technologies, cat no 05150) with 10^4 M hydrocortisone , TPO (Stem Cell Technologies, cat no 2922), G-CSF (Stem Cell Technologies, cat no 78012) and IL-3 (Stem Cell Technologies, cat no 78040) each at 2 ng/mL concentration. Thawed AML cells were seeded on confluent MS-5 cells in 12 well plate with this medium (200.000 MNCs/well).

Fresh medium was changed twice a week with half of the medium changed. One a week, cultures were fed by demi-population of the non-adherent cells and 500 µL fresh medium replacement. Half of the non-adherent cells were removed due to demi-population by gently shaking the plate and collecting the half of the cells and medium. Myelocult H5100 media (Stem Cell Technologies, cat no 05150) with 10^4 M hydrocortisone , TPO (Stem Cell Technologies, cat no 2922), G-CSF (Stem Cell Technologies, cat no 78012) and IL-3 (Stem Cell Technologies, cat no 78040) each at 2 ng/mL concentration used as co-culture medium.

Feeder layers were renewed by passaging, around week 3 to week 5. At day 35 cocultures were replated, but in case MS-5 stoma cells incapable of sustaining the cells we had replated earlier than day 35, at day 21 or day 28.

3.4. Assessment of LSC Maintenance and Enrichment by Short-term and Long-term Cultures

3.4.1. Evaluation of Cell Proliferation

Every week, one well was harvested by 0.25% Trypsin/EDTA (Trypsin-EDTA, 0.25%, Thermo Fisher, cat no 25200072) for evaluation of proliferation. Firstly, suspension cells were collected in a conical tube and counted with Turk's dye. Then, the MS-5 layer was washed gently with PBS. To remove remaining nonadherent cells. All cells were trypsinized and passed through a 40 μm Cell Strainer (Corning, Falcon, cat no 352340). Then, the harvested cells were incubated for 1 hour. These incubation and filtration steps were performed with aim to get rid of MS-5 feeder cells. Cell counting was done to evaluate proliferation kinetics and at day 7 and passage day, cells were reserved for assays.

To determine the phenotype of the cells, CD34 and CD38 surface markers were measured via flow cytometry analysis at day 0, day 7 and passage day. Gating strategy was shown in Figure 4.7. Onto FSC vs SSC plot viable cell population was selected, then leukemic cell population was evaluated according to CD34 – CD38 expression patterns (CD34+CD38-, CD34+CD38+, CD34-CD38+).

All analyses were done using the same gating strategy except Patient 11 whom leukemic cells were CD45 negative. Thus, CD45 negative fraction was used in all analyses of this patient.

3.4.2. Cobble-stone Area Forming Cells as Indicators of Self-renewal Ability of AML Stem Cells

During co-culture cells showed distinct localizations. Most of the cells were attached and some resided in suspension. A small fraction of cells were located beneath the feeder cells and these cells formed "leukemic cobblestone areas" (≥5 cells observed under phase-contrast microscopy). Presence of "leukemia-cobblestone area forming cells" (L-CAFC) in culture and their reformation after serial plating indicate the self-renewal ability of LSCs.

Cells were cultured at 37°C in 5% CO_2 5 to 8 weeks. Every week, to observe cobblestone areas, inverted microscopy was performed. Pictures were taken at 4x – 20x magnification range (Olympus, IX73).

At day 35 cocultures were replated, but in case MS-5 stoma cells uncapable of sustaining the cells we had replated earlier than day 35, at day 21 or day 28. After passage, to observe reformation of cobblestone areas, cells were cultured for 3 more weeks.

3.4.3. Colony Forming Assay and Wright's Staining of Samples

The quantification of hematopoietic stem/progenitor cells and the evaluation of their differentiation potential are done using CFU assay. Leukemic colonies are morphologically distinct from normal hematopoietic colonies.

3 ml Methocult H4435 medium aliquot (Stem Cell Technologies, cat no 04445), which is commercially available methylcellulose based medium with enriched human recombinant cytokines, was thawed at room temperature before use. For long term culture at the first passage 20.000 - 40.000 cells per sample were reserved for this colony forming unit assay. The cells were washed with IMDM medium supplemented with 2% FBS and centrifuged at 300 *xg* for 10 min. The supernatant was discarded and the pellet was resuspended with 0,3 mL 2%FBS IMDM medium and transferred to the Methylcellulose H4435 media for a duplicate assay. The tube was vortexed vigorously to mix the cells and medium. Thereafter, the tube was stood to allow bubbles to dissipate. 1.1 ml mixture was transferred to 35 mm petri dish by using a blunt end 16-gauge needle (Stem Cell Technologies, cat no 28110). For each sample 2 x 35 mm petri dishes were used, and 1 uncovered dish was filled with 3 mL of sterile water for humidity. All petri dishes were placed into a 100 mm petri dish with a lid. The samples were incubated for 14 days at 37°C in 5% CO_2. After incubation time, colonies were observed and counted by microscope (Olymupus, IX73).

At the end of 14 days the colonies were collected and pooled, cytospins were prepared for cytological examination. Cells were harvested and washed with PBS and with IMDM medium supplemented with 2% FBS. The washing step was repeated at

least 4 times until the media was removed and single cells were obtained. Cells were counted and 40.000 cells per slides were resuspended by 0.3 mL IMDM medium. The slides were mounted with the paper pad which has the same opening area with the plastic funnel. Prepared slides were placed in the metal holder. The samples were put into plastic funnel and cytospin preperation was done at 700 rpm for 3 min. Then, slides were stained with Wright's stain and washed with tap water. The cells were under microscope (Leica, DMI6B).

3.4.4. ALDH Assay as a Functional Analysis of LSCs.

ALDH assay was performed to identify AML-LSCs and discriminate them from normal hematopoietic stem/progenitor cells which are also located in bone marrow. By distinguishing AML LSCs from normally functioning HSCs, it may be a valuable tool for early identification of AML cases with high risk of relapse or refractory disease.

According to manufacturer's directions (ALDEFLUOR kit, Stem Cell Technologies, cat no 1700) 25 µl DMSO was added to the vial of Aldefluor Reagent and 1 min. incubation was done at room temperature (15-25°C). Then, 25 µl 2N HCl was added and 15 min. incubation was done at room temperature (15-25°C). 360 µl Aldefluor assay buffer was added and mixed well. Activated Aldefluor Reagents was aliquated and stored at -20°C.

At day 0, day 7 and passage day cells were harvested by 0.25% Trypsin EDTA and same protocol was applied as in part 3.4.1. Samples were counted. 400.000 cells were used for control and test tube. 400.000 cells were washed and resuspended with 400 µl assay buffer. 2,5 µl Aldefluor DEAB Reagent was added to control tube without cells. 2,5 µL of the activated ALDEFLUOR Reagent were added onto total cells and cells were mixed. 200 µl of cell suspension was transferred to the control tube immediately. 30 min incubation was done at 37°C in 5% CO_2. Samples were washed with assay buffer and stained with CD45, CD34 and CD38 antibodies. The washing step was repeated after 15 min incubation at room temperature at dark. Cells were analyzed by flow cytometry (BD Accuri C6, using BD Samples software).

Gating strategy was shown in Figure 4.16. For every sample 200.000 cells/tube were used with selected antibodies and a minimum of 100.000 events in gated sample were acquired. (Gated sample selected as P1 on FCS vs SSC plot for live-cell population (Fig.4.16.). CD45 antibody was used for selection of leukocyte population and CD34 – CD38 antibodies were used to identify different leukemic cell populations within each ALDH subgroup defined according to the ALDH expression level as dim, intermediate and bright.

Samples were analyzed using 4-color antibody panel. Antibodies used to analyses in this part were as follows: ALDH, CD45-PerCP (Biolegend, clone: 2D1, cat no 368506), CD34-PE (Biolegend, clone: 561, cat no 343606), CD38-APC (Biolegend, clone: HB-7, cat no 356606).

3.5. Early Identification of AML LSCs during Short-Term Culture

3.5.1. CFSE Cell Proliferation Assay

CFSE (Carboxyfluorescein succinimidyl ester) is a dye dilution assay used to track frequency of cell division *in vivo* and *in vitro*. Using CFSE assay in short term culture of AML cells we aimed to investigate the frequency and proliferation kinetics of LSCs. This may be an important prognostic tool which may be used in early risk stratification and prediction of outcome.

At day 0, 20 µL FBS (2% FBS) was added in 1.0 ml PBS. 2 µL 5 mM CFSE (Cell Trace CFSE Cell Proliferation Kit, Thermo Fisher, cat no C34570) stock solution was added to prepared 10 mM working solution. Thawing cells were washed with PBS at 1500 rpm 5 min. The supernatant was discarded and resuspend the cell with prepared coculture enrichment medium. After that, the cells were incubated for 4 hours. After the incubation, samples were washed and prepared working solution was added to cells (100 µL for 1 x 10^6 cells). Cells were incubated for 15 min at 37 °C with 5% CO_2. 5 ml cold PBS was added, and cells were incubated at 4°C for 5 min. Samples were washed twice at 1500 rpm 5 min. Then, 200.000 cells/well (52.631 MNCs / cm^2) were cultured on pre-established confluent MS-5 cells with co-culture medium in 12 well

plate. Plate was incubated at 37°C in 5% CO_2 humidified incubator. 50.000 cells were run in a flow cytometry to confirm CFSE labeling.

After 18 hours and 7 days 1 CFSE stained well and 1 unstained well were harvested by 0.25% Trypsin EDTA and same protocol was applied as in part 3.4.1. Cells were counted and washed with PBS. Pellet was resuspended and approximately 300.000 - 200.000 cells were resuspended with 100 µL FACS buffer, stained with rat anti-mouse Sca-1 as MS-5 marker, 7-AAD for selection of viable cells and mouse anti-human CD45 as a common leucocyte marker. Samples were analyzed using 4-color antibody panel. Antibodies used to analyses in this part were as follows: CFSE, CD45-APC (Biolegend, clone:2D1, cat no 368512), Sca-1-PE (Biolegend, clone: D7, cat no 108108), 7-AAD (Biolegend, cat no 420403).

Incubation was performed for 15 min at room temperature at dark. Prepared samples were washed with PBS at 1500 rpm for 5 min. Then, stained and unstained samples were analyzed by flow cytometry BD Accuri C6 using BD Samples software. For every sample 300.000 - 200.000 cells/tube were used with selected antibodies and a minimum of 150.000 events in gated sample were acquired. (Gated sample selected as P1 on FCS vs SSC plot for live-cell population (Fig.4.23.)).

Debris were eliminated by FSC vs SSC plot and doublets were eliminated by SSC-A vs SSC-H plot. CD45+Sca-1- cells selected to define the R1 gate of bulk AML population. 7-AAD + cells were excluded to discriminate viable population. Gating strategy was shown in Figure 4.23.

Proliferation index for each sample was calculated by dividing the 18-hour CFSE MFI (median fluorescence intensity) with one-week CFSE MFI as an indicator of leukemia stem cell expansion that demonstrated a high variance because of AML cell heterogeneity and used to assess the presence and frequency of AML-LSCs [49]. We preferred to use bulk leukemic population instead of selecting a group of leukemic cells at a certain state of the cell cycle through sorting since this might not represent the heterogeneity of AML cells.

3.5.2. AML LSC Characterization by Flow-cytometry Using LSC-Specific Markers

At day 0, day 7 and at the day of passaging, cells were harvested by 0.25% Trypsin/EDTA and the same protocol was applied as in part 3.4.1. Samples were counted. Approximately 300.000 - 200.000 cells per tube were stained with selected antibodies and incubated for 15 min. at room temperature in the dark. Unlabeled antibodies were removed by washing cells with PBS at 1500 rpm for 5 min. The negative fraction was determined by using unstained samples and analyzed by flow cytometry BD-Accuri-C6. Gating was performed using BD Samples software. Expression levels were shown as percentages at Table 4.3. and 4.4.

For every sample 300.000 - 200.000 cells/tube were used with selected antibodies and a minimum of 150.000 events were acquired in the final selected gate. (Gated sample selected as P1 on FCS vs SSC plot for live-cell population (Fig.4.24.).) Samples were analyzed using 4-color antibody panel (Table 3.1). VEGFR-2, CD25, TIM-3 and CLL-1 were selected as LSC- specific markers to determine and quantify the LSCs within AML samples and the negative fraction indicates the healthy hematopoietic stem/progenitor cells within AML samples. Moreover, in order to determine the change in expression levels after treatment we compared diagnose samples with their follow up samples (n=5) and assessed the association of LSCs with risk stratification, treatment response and disease outcome. CD-45 antibody was selected for leukocyte count and CD34 – CD38 antibodies were selected to identify and classify the cells within AML samples. Gating strategy was shown in Figure 4.24. Within the blast population presumed progenitor cells were defined as CD34+CD38-/FSClow/SSClow.

Antibodies used to analyses in this part were as follows: CD45-PerCP (Biolegend, clone: 2D1, cat no 368506), CD34-FITC (Biolegend, clone: 561, cat no 343604), CD38-APC (Biolegend, clone: HB-7, cat no 356606), VEGFR-2 (Biolegend, clone: 7d4-6, cat no 359904), CD38-PE (Biolegend, clone: HB-7, cat no 356604), CD25-APC (Biolegend, clone: M-A251, cat no 356110), TIM-3-PE (Biolegend, clone: F38-2E2, cat no 345006), CLL-1-PE (Biolegend, clone: 50C1, cat no 353604).

Table 3. 1. Flow cytometry panel used for analysis of AML LSCs.

1.	CD45	CD34	CD38	VEGFR-2
2.	CD45	CD34	CD38	CD25
3.	CD45	CD34	CD38	TIM-3
4.	CD45	CD34	CD38	CLL-1

3.6. Statistics

Data were analyzed using Paired Student's-test for statistical significance and represented as the mean ± standard error of the mean (SEM). P value <0.05 was considered as significant. Statistical analyses were performed using PRISM (Graphpad Software, CA, USA). (**** p< 0.0001, ***: p=0.0001- 0.001, **: p=0.001- 0.01, *: p=0.01 - 0.05)

4. RESULTS

For this study, twelve pediatric AML patients' bone marrow and/or peripheral blood samples and three healthy donors' bone marrow samples were collected and MNCs were isolated. Twelve AML samples included samples from eight newly diagnosed AML cases and samples from four relapse cases, obtained prior to therapy. Also, samples of five patients who had diagnosis and remission pairs were studied as follow-up samples. Patient and disease characteristics are presented in Table 4.1.

Short-term cultures were established to investigate the presence and enrichment of AML stem cells in AML samples, and to evaluate whether there is a correlation between frequency of LSCs and risk stratification and/or early outcome. Long-term cultures were established to assess maintenance and self-renewal capacity of leukemic stem cells (Table 4.1).

4.2. Expansion of the AML Cells on MS-5 Stromal Cells and Maintenance/Enrichment of AML-LSCs.

4.2.1. Expansion of AML Cells on MS-5 Stromal Cells with Long-term Culture

Long-term cultures on MS-5 stromal cells were performed using 12 AML samples (8 newly diagnosed AML and 4 relapsed AML), 5 follow-up AML samples (diagnosis-remission pairs) and two healthy donor BM samples. Total cell counting was done weekly to evaluate the expansion and viability of bulk leukemic cell population.

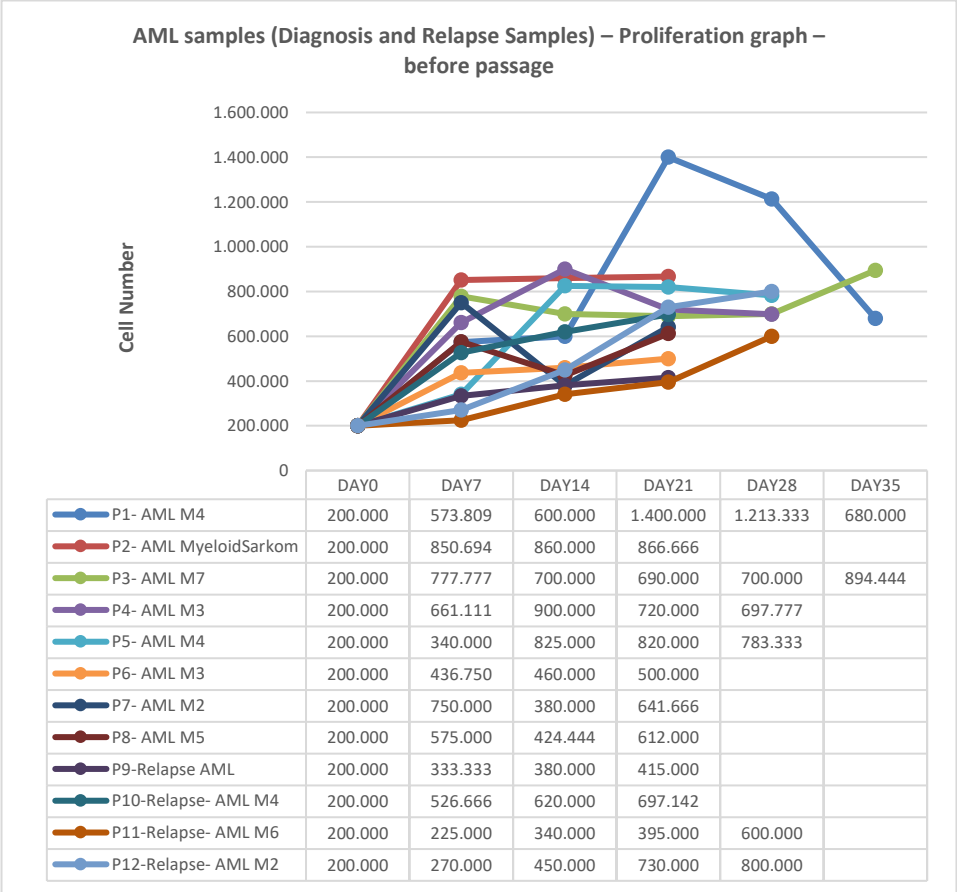
Table 4. 1 Patient and disease characteristics.

Patient ID	Age	Sex	FAB Classification	Blast % at diagnosis	Cytogenetics	Treatment	Risk Group	Relapse Status	Disease Status
PATIENT1	3 mo	M	M5	100%	Normal	AML BFM 2013	high		remission
PATIENT2	3 mo	F	Myeloid Sarkom	6-7%	Normal	AML BFM 2013	high		remission
PATIENT3	11y	F	M7	68%	Normal	AML BFM 2013	intermediate		exitus
PATIENT4	5y	F	M3	100%	t(15:17)	AML BFM 2013	standart		remission
PATIENT5	14y	F	M4	100%	FLT3 ITD, trizomy 8, WT-1 expression	AML BFM 2013	high	+	exitus
PATIENT6	14y	F	M3	62%	t(15:17)	AML BFM 2013	standart		remission
PATIENT7	3y	F	M2	89%	t(8:21), WT-1 expression	AML BFM 2013	standart		remission
PATIENT8	8y	F	M5	47%	PTPN11, WT-1 expression	AML BFM 2013, *Allogeneic HSCT*	high		remission
PATIENT9	8y	M	M5	100%	Trizomi 10, WT-1 expression	AML BFM 2013 *Allogeneic HSCT*	intermediate at diagnosis	+	exitus
PATIENT10	6y	F	M4	100%	WT-1 expression	AML BFM 2013, *Allogeneic HSCT*	intermediate at diagnosis	+	remission
PATIENT11	16y	F	M6	28%, 60% normoblast	WT-1 expression	AML BFM 2013, *Allogeneic HSCT*	intermediate at diagnosis	+	remission
PATIENT12	6y	F	M2	80%	t(8:21), WT-1 expression	AML BFM 2013 *Allogeneic HSCT*	intermediate at diagnosis	+	remission

When we compared cell counts during long-term culture, we observed that total cell numbers were found to be increased in all AML samples using the niche-like coculture system. However, when we looked further into the proliferation pattern; cell numbers remarkably increased in diagnosis and relapse samples while cell numbers fluctuations were observed in remission samples, similar to healthy donor BM samples (Figs. 4.1., 4.3. and 4.5.). During short-term culture, cell numbers significantly increased in diagnosis and relapse samples (n=12, 200.000 for day 0 vs. [527.000 (±60.2)] for day 7, p= 0.0002]. The cell number change in remission samples were not statistically significant for short-term cultures (n=5, 200.000 for day 0 vs. [496.307 (±114.9)] for day 7, p= 0.06,] similar to healthy donor BM samples (200.000 for day 0 vs. [502.592 (±132.7)] for day 7, p=0.15) even though there was an increasing trend, possibly due to small sample size. There was also no significant change in the cell numbers when the follow up samples (diagnosis-remission pairs, n=5) were evaluated (p=0,8). The proliferation potential of AML samples showed variations among patients. After passaging, the cell numbers continued to increase (Figs. 4.2. and 4.4.).

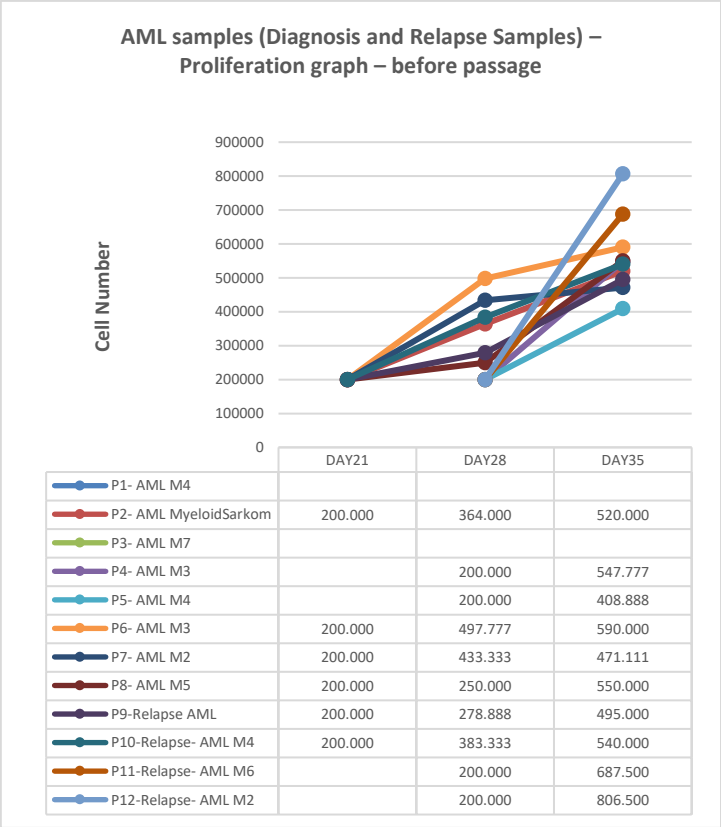
There was a difference between AML samples and donor BM samples regarding the increase in cell numbers during long-term culture [599.268 (±41.1)] vs. [357.222 (±56.2)], p=0.016). Likewise, a similar difference was observed when the increase in cell numbers of AML samples at diagnosis and remission were compared. [599.268 (±41.1)] for AML samples vs [435.333 (±60.3)] for remission samples, p=0,04).

The cell number increment in AML samples was also shown as fold change by comparing with healthy donor BM samples (Fig. 4.6.). The most significant change was observed at day 14 of long-term culture. Similarly, when we compared the cell numbers in all AML samples (diagnosis and relapse) with those in the healthy donor BM samples, no significant change was found (n=12, AML samples, n=3 donor samples, p=0,8).



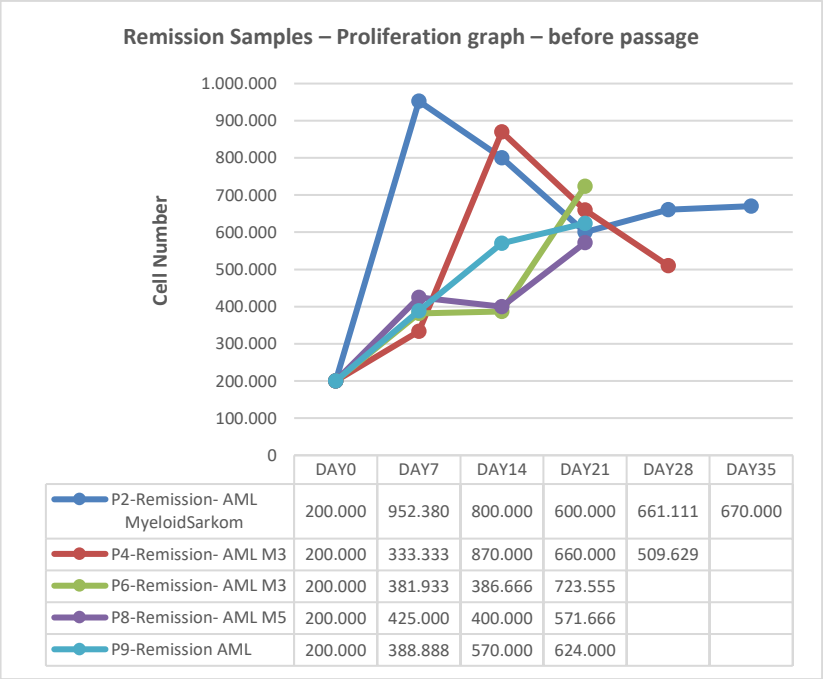
	DAY0	DAY7	DAY14	DAY21	DAY28	DAY35
P1- AML M4	200.000	573.809	600.000	1.400.000	1.213.333	680.000
P2- AML MyeloidSarkom	200.000	850.694	860.000	866.666		
P3- AML M7	200.000	777.777	700.000	690.000	700.000	894.444
P4- AML M3	200.000	661.111	900.000	720.000	697.777	
P5- AML M4	200.000	340.000	825.000	820.000	783.333	
P6- AML M3	200.000	436.750	460.000	500.000		
P7- AML M2	200.000	750.000	380.000	641.666		
P8- AML M5	200.000	575.000	424.444	612.000		
P9-Relapse AML	200.000	333.333	380.000	415.000		
P10-Relapse- AML M4	200.000	526.666	620.000	697.142		
P11-Relapse- AML M6	200.000	225.000	340.000	395.000	600.000	
P12-Relapse- AML M2	200.000	270.000	450.000	730.000	800.000	

Figure 4.1. Proliferation kinetics of AML samples (diagnosis and relapse) before passaging. Cell counts represents the cumulative cell numbers indicating all AML cells which are calculated weekly during long-term culture.



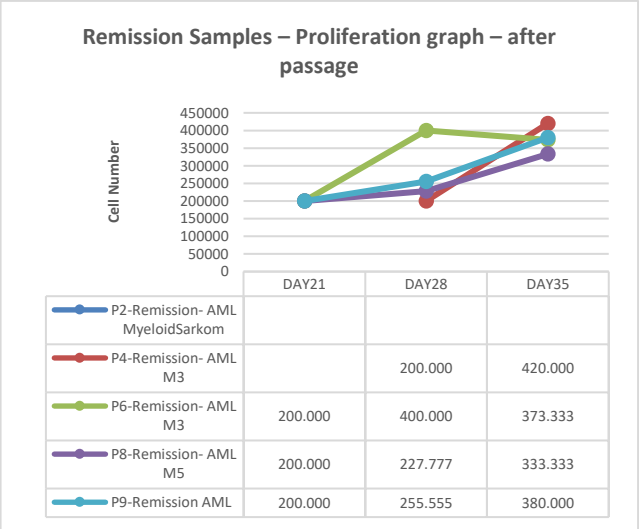
	DAY21	DAY28	DAY35
P1- AML M4			
P2- AML MyeloidSarkom	200.000	364.000	520.000
P3- AML M7			
P4- AML M3		200.000	547.777
P5- AML M4		200.000	408.888
P6- AML M3	200.000	497.777	590.000
P7- AML M2	200.000	433.333	471.111
P8- AML M5	200.000	250.000	550.000
P9-Relapse AML	200.000	278.888	495.000
P10-Relapse- AML M4	200.000	383.333	540.000
P11-Relapse- AML M6		200.000	687.500
P12-Relapse- AML M2		200.000	806.500

Figure 4.2. Proliferation kinetics of AML samples (diagnosis and relapse) after passaging. Cell counts represents the cumulative cell numbers indicating all AML cells calculated after passaging the samples, around days 21-35 (weeks 3-5) according to the time of passaging.



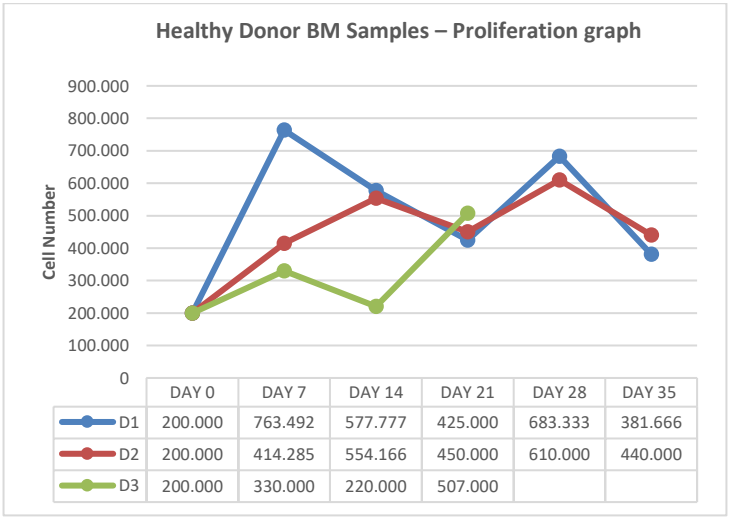
	DAY0	DAY7	DAY14	DAY21	DAY28	DAY35
P2-Remission- AML MyeloidSarkom	200.000	952.380	800.000	600.000	661.111	670.000
P4-Remission- AML M3	200.000	333.333	870.000	660.000	509.629	
P6-Remission- AML M3	200.000	381.933	386.666	723.555		
P8-Remission- AML M5	200.000	425.000	400.000	571.666		
P9-Remission AML	200.000	388.888	570.000	624.000		

Figure 4. 3. Proliferation kinetics of follow-up samples (remission) before passaging. Cell counts represents the cumulative cell numbers indicating all cells in suspension, adherent cells which are calculated weekly during long-term culture.



	DAY21	DAY28	DAY35
P2-Remission- AML MyeloidSarkom			
P4-Remission- AML M3		200.000	420.000
P6-Remission- AML M3	200.000	400.000	373.333
P8-Remission- AML M5	200.000	227.777	333.333
P9-Remission AML	200.000	255.555	380.000

Figure 4. 4. Proliferation kinetics of follow-up samples (remission) after passaging. Cell counts represents the cumulative cell numbers indicating all cells in suspension, adherent calculated after passaging the samples, around days 21-35 (weeks 3-5) according to the time of passaging.



	DAY 0	DAY 7	DAY 14	DAY 21	DAY 28	DAY 35
D1	200.000	763.492	577.777	425.000	683.333	381.666
D2	200.000	414.285	554.166	450.000	610.000	440.000
D3	200.000	330.000	220.000	507.000		

Figure 4. 5. Proliferation kinetics of healthy donor BM samples. Cell counts represents the cumulative cell numbers indicating all cells in suspension, adherent calculated weekly during long-term culture (n=3).

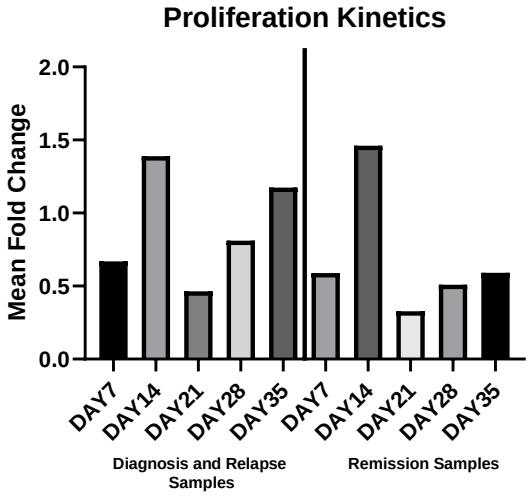


Figure 4. 6. Expansion of AML cells during long-term cultures. The data show the mean fold expansion of AML samples (n=12, for primary and relapse samples) and follow-up samples (n=5, remission samples) during long-term culture stimulated with TPO, G-CSF and IL-3. Normalization was done according to the expansion of donor BM cells.

4.2.2. Maintenance and Enrichment of AML LSCs during Long-term Culture.

4.2.3. Evaluation of AML LSC expansion on the basis-of CD34 expression during long-term culture

Immunophenotyping is used to identify and classify the cells. Heterogenous cell populations like AML patient samples can be analyzed by flow cytometry to determine presence and proportion of the LSCs and characterize remaining cell population. In order to evaluate the phenotype of leukemic cell population and specifically, retention of the CD34+ CD38- phenotype which indicates LSCs during long-term culture, multi-parameter staining was applied to AML samples, follow-up samples and healthy donor BM samples. Long-term cultures on MS-5 stromal cells stimulated with TPO, G-CSF and IL-3 were performed using 12 AML samples (8 newly diagnosed AML and 4 relapsed AML), 5 follow-up AML samples (remission) and 3 healthy donor samples.

The cell surface phenotypes of AML and healthy donor samples were evaluated at day 0, day 7 and passage day (weeks 3-5). Changes in CD34+CD38-,

CD34+CD38+ and CD34-CD38+ cell populations over time were presented in Table 4.2. Gating strategy was shown in Figure 4.7.

Following one-week culture, frequency of LSCs defined as the percentage of CD34+ CD38- cell population was 5.5% (±2.3) for 12 AML samples, 3.4% (±1.9) for 4 follow-up samples and 2.2% (±0.9) for 3 control samples. In 8 patients (6 newly diagnosed and 2 relapse samples), there was an upgrade trend in CD34+ CD38- population at the first week (0.4% ±1.3 at day 0 and 6.6% ±3.2 at day 7).

Different expansion patterns were observed when the results of long-term cultures were evaluated. CD34+ CD38- populations were found increased in all AML samples, except Patient 4 and Patient 7 and in 4 out of 5 remission samples during long-term culture. AML LSC frequency decreased in the other two patients (Patient 4 and Patient 7).

When the frequency of CD34+CD38- LSCs for all AML samples (n=12) was evaluated it remained stable as 1.5 % (±0.7) at day 0 and 1.7% (±0,4) at the passage day during long-term culture. There was no significant change in LSC frequency for neither short-term ($p>0.05$) nor long-term cultures ($p>0.05$). There was a significant decrease in CD34+CD38+ population ($p=0.033$) for both short-term and for long-term cultures ($p=0.026$). Although, a decrease was observed for CD34-CD38+ population, it was found significant for only long-term culture ($p=0.034$) (Fig 4.8).

In order to see whether there is any significant change in the frequency of AML LSCs after treatment, we compared the CD34+CD38- cell frequencies in 5 AML samples with their follow-up samples at day 0, at day 7 and at the passage day. Although it seemed that the frequency of CD34+CD38- cells were higher for follow-up samples at day 0 (n=5) [9.44% (±9.2)] this probably resulted from the high frequency of CD34+CD38- cells in the remission sample of one patient (Patient 9 remission sample, day 0; 46.1%, for day 7; 2.2%, for passage day; 3.4%) who presented with relapsed disease and achieved morphological remission after therapy. However, since Patient 9 did not have any molecular disease markers, minimal residual disease and molecular remission status could not be evaluated. The patient 9 did not survived after allogeneic stem cell transplantation due to early

transplant-related complications. A similar trend in CD34+CD38- cell frequency was present for all time points for follow-up samples excluding the results of this patient (n=4) and control samples (n=3). CD34+CD38- population was maintained for all samples (Figs 4.9. and 4.10.).

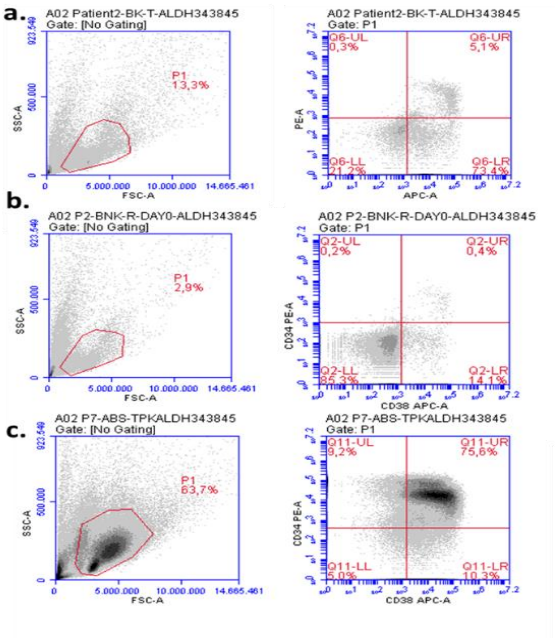
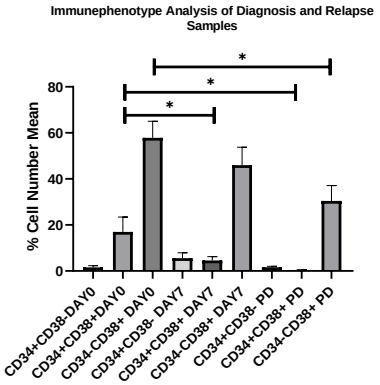


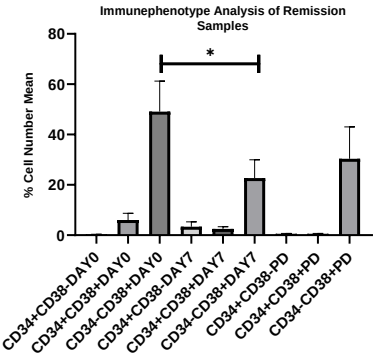
Figure 4. 7. Representative flow cytometry analysis of CD34 expression during long-term culture. Onto FSC vs SSC plot our population was selected and percentages of CD34+CD38-, CD34+CD38+ and CD34-CD38+ leukemic cell populations were calculated.



AML PATIENT SAMPLES	CD34+CD38- % (mean ±SEM)	CD34+CD38+ % (mean ±SEM)	CD34-CD38+% (mean ±SEM)
Day 0	1.5 (±0.7)	16,9 (±6,5)	57,8 (±7,2)
Day 7	5,5 (±2,3)	4,5 (±1,7)	46 (±7,8)
PD	1,7 (±0,4)	0,45 (±0,14)	30,4 (±6,7)

Figure 4. 8. Immunophenotype of AML samples (diagnosis and relapse) during long-term culture. At day 0, day 7 and PD, samples were harvested and stained with CD34 and CD38 surface markers to determine any change in immunophenotype of AML

cells (n=12). CD34+CD38- population (LSCs) were maintained the phenotype for 5 weeks (PD: passage day of AML cells i.e. around days 21-35). Data given were the average ± SEM of population percentages. (p<0.05 accepted as significant.)



AML PATIENT SAMPLES	CD34+CD38- % (mean ±SEM)	CD34+CD38+ % (mean ±SEM)	CD34-CD38+% (mean ±SEM)
DAY0	0,27 (±0,1)	6,025 (±2,6)	49,1 (±12,1)
DAY7	3,4(±1,9)	2,4 (±0,8)	22,6 (±7,2)
PD	0,5 (±0,1)	0,57 (±0,1)	30,3 (±12,6)

Figure 4. 9. Immunophenotype of AML follow-up samples during long-term culture. At day 0, day 7 and passage day samples were harvested and stained with CD34 and CD38 surface markers to determine the immunophenotype of AML cells (n=4) (PD: passage day of AML cells i.e. around days 21-35). Data given were the mean ± SEM of population percentages. (p<0.05 accepted as significant.)

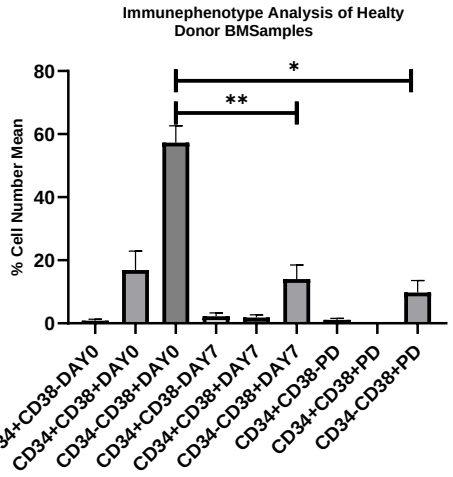
CD34+CD38- population frequency in remission samples was not observed to be changing from day 0 to day 7 (p>0.05) similarly from day 0 to passage day (p>0.05) Likewise, CD34+38+ population frequency remained stable from day 0 to day 7 (p>0.05) and to passage day (p>0.05). The frequency of CD34-CD38+ cells decreased from day 0 to day 7 (p=0.016) but from day 0 to passage day it was not significant (p>0.05).

In diagnosis and remission sample pairs (n= 4) the change was not significant at day 0 in CD34+CD38- population (0.6±0.2% vs. 0.27±0.1% , p>0.05), for CD34+CD38+ population (7.7±3% vs. 6.0±2.6%, p>0.05) and for CD34-CD38+ population (69.3±3,5% vs. 49.10±12,1%, p>0.05). Patient 9 was excluded due to unexpectedly high frequency of CD34+CD38- percentage.

At day 7 similarly, the change was not significant for CD34+CD38- population (5%±2.8 vs. 3.4±2%, p>0.05), for CD34+CD38+ population (2.4% ±0.9, diagnose vs. 2.5 % ±0.9, p=0.05) and for CD34-CD38+ population (31.3 %±7.4 vs. 22.7 %±7,3%, p>0.05).

Table 4. 2. Phenotype change in AML patient samples during long-term culture.

	CD34+ CD38-% DAY0	CD34+CD38+% DAY0	CD34-CD38+% DAY0	CD34+CD38-% DAY7	CD34+CD38+% DAY7	CD34-CD38+% DAY7	CD34+ CD38-% PD	CD34+ CD38+% PD	CD34-CD38+% PD
P1- AML M4	0,1	0,7	92,2	19,8	1,6	9,2			
P2-AML MyeloidSarkom	0,3	5,1	73,4	2,2	2,5	33	0,8	0,5	40,2
P2-Remission MyeloidSarkom	0,2	0,4	14,1	1	0,2	2,9	0,3	0,7	12,1
P3- AML M7	0,3	7,9	63,8	1,2	0,2	84	1	0,5	29
P4-AML M3	1,3	1	62,3	13,2	4,8	23,9	0,5	0	8
P4-Remission- AML M3	0,2	7	55,9	9,2	4,1	22,9	0,8	0,3	5,2
P5-AML M4	1,3	10,6	65,4	0,2	0,1	70,5	2	0,1	33,3
P6-AML M3	0,1	9,1	76,8	1,2	0,8	51,3	2,7	1,7	46,7
P6-Remission- AML M3	0,3	3,8	67	2,1	3,6	37,7	0,5	0,8	49,6
P7-AML M2	9,2	75,6	10,3	1,6	20,9	56	4,3	0,5	7,3
P8-AML M5	0,5	15,2	64,5	3,3	1,4	16,9	1,5	0,4	5,6
P8-Remission- AML M5	0,5	12,9	60,1	1,2	2	27,2	0,8	0,5	54,6
P9-Relapse- AML M5	0,4	14,9	72,1	22,6	9,5	8,3	3,1	0,3	18,7
P9-Remission-AML M5	46,1	13,7	23,9	2,2	1,1	35,1	3,4	0,5	54,6
P10-Relapse- AML M4	0,2	48,9	28,7	1,7	7,2	49,9	0,6	0,3	82,5
P11-Relapse- AML M6	3,1	4,2	19	0,7	4,1	68,2	3,8	0,6	25,8
P12-Relapse- AML M2	1,4	9,6	65	0,3	1,5	80,2	1,6	0,1	37,6
D1	0,3	27,3	66,1	4,2	2,1	19,6	0,4	0,1	2
D2	1,8	16,9	47,7	0,4	0,2	5,2	2	0,2	13,6
D3	0,3	6,5	58,1	1,6	3,2	17,3	0,8	0,1	13,6



HEALTHY DONOR BM SAMPLES	CD34+CD38- % (mean ±SEM)	CD34+CD38+ % (mean ±SEM)	CD34-CD38+% (mean ±SEM)
DAY0	0,8 (±0,5)	16,9 (±6,004)	57,300 (±5,327)
DAY7	2,2 (±0,9)	1,833 (±0,876)	14,033 (±4,466)
PD	1,067 (±0,481)	0,133 (±0,033)	9,733 (±3,867)

Figure 4. 10. Immunophenotype of healthy donor BM samples during long-term culture. At day 0, day 7 and passage day, samples were harvested and stained with CD34 and CD38 surface markers for determining the immunophenotype of AML cells (n=3) (PD: passage day of AML cells i.e. around days 21-35). Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

Healthy donor BM samples CD34+CD38- percentage change was not significant from day 0 to day 7 ($p>0.05$) similarly from day 0 to passage day ($p>0.05$). CD34+38+ percentage change was not significant from day 0 to day 7 and to passage day ($p>0.05$). CD34-CD38+ cell population decreased from day 0 to day 7 ($p=0.0015$) and from day 0 to passage day ($p=0,032$).

When we compared AML patient and donor samples there was no significant change in the CD34+CD38-, CD34+CD38+ and CD34-CD38+ percentages between day 0 and day7 ($p>0.05$).

4.2.4. Monitorization of self-renewing AML-LSCs through demonstration of cobblestone area forming cells and CFU assay

Cobblestone Area Forming Cells Assay

Cobblestone area forming cells show the self-renewal capacity of stem cells and are more a rapid screening assay than *in vivo* studies [50]. It has been mentioned that more immature and dormant cells migrate under the adherent feeder cells [51].

During co-culture with MS-5 cells, most of the LSCs were attached and some resided in suspension. After the first week, with this niche-like coculture system, the cell clusters forming under the feeder layer started to appear and their numbers increased during long term-culture. These clusters are called “leukemia- cobblestone area forming cells” (L-CAFCs) (Fig. 4.11) [52]. Those beneath the MS-5 stromal cells (Phase-dim) have a more immature phenotype compared to the non-adherent AML cells and these cells are crucial for long-term maintenance because of their stemness property [51]. We observed L-CAFCs for all of the AML samples around day 7 to day 21. In contrast CAFC could not be observed in follow-up samples and healthy donor BM samples. While all of the intermediate- and high-risk group AML samples formed L-CAFCs after passaging, L-CAFCs were not seen in two standard-risk AML samples (Patient 4 and Patient 7). Only in one follow-up sample, L-CAFCs were observed before passaging, which was Patient 9 whom molecular remission status was in question but after passaging L-CAFCs were not observed in this patient’s sample.

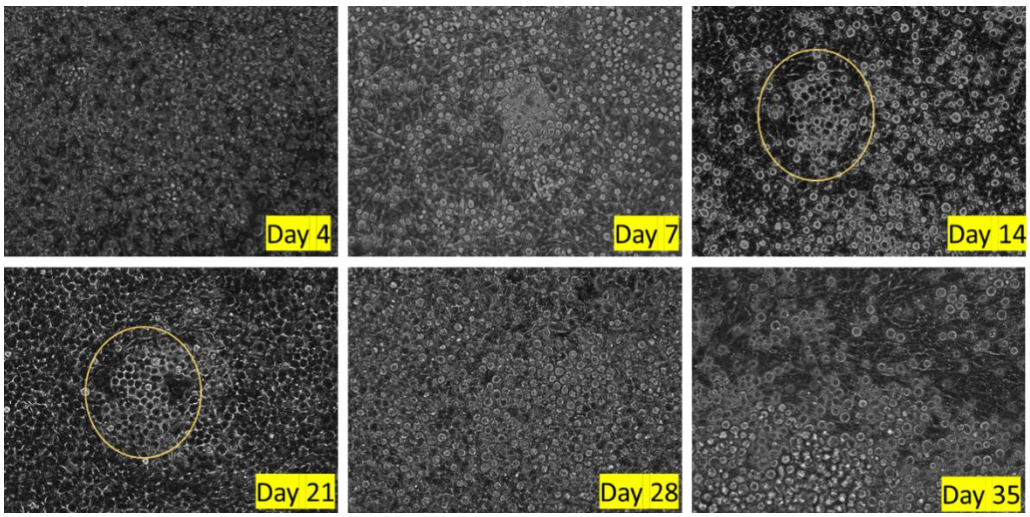


Figure 4. 11. Cobblestone area forming cells derived from AML samples. Representative image of localization and morphology of a primary AML sample (Patient 5) and L-CAFCs formation during long-term coculture. This sample was passaged at day 28, second L-CAFCs were observed at day 49 which indicate their self-renewal capacity. To observe cobblestone areas, we performed an inverted microscopy (Olympus, IX73, 20X magnification). In coculture, we assessed 3 distinct localization. Few cells were located beneath the MS-5 feeder cells, some of them were found on the surface of the MS-5 layer and the others (nonadherent cells) resided in suspension. Around day 7 to day 14 cobblestone areas were observed for all AML samples.

Leukemia Stem Cell Colony Forming Assays and Wright's Staining

Colony forming assays are used to demonstrate (CFC- colony forming cells) self-renewal and differentiation patterns of hematopoietic stem/progenitor cells. It has been used widely for quantifying and evaluating of the progenitor/stem content of a cell population.

In this study, all colony assays were performed at the passage day after enrichment of LSCs in culture. Firstly, we collected suspension cells and then, adherent and underneath cells respectively for CFU assay. Colony forming capacity of these three cell populations was found different. Suspension cells formed mostly normal CFU-GM-like spread colonies and rare small blastic colonies. While all adherent and underneath cells formed blastic colonies (Fig. 4.12). Therefore, we decided to use a mixed cell population for CFU assay. Clonogenic difference was observed between AML patient and donor samples and also among the AML patients with different AML subtypes (Figs. 4.13.and 4.14). For some AML patient samples, predominance of leukemic-CFUs (L-CFUs) were more obvious.

These blastic colonies dominated the whole plate, making the assessment of other types of colonies impossible. For remission samples, blastic colonies observed only in the samples of Patient 9 and Patient 8. For healthy donor BM samples, all type of hematopoietic colonies were observed.

Morphological analysis of cells obtained by pooling different types of colonies in the plate provides to understand the cell lineage and to determine the degree of maturation of the cell populations. For cytological confirmation, cytospin samples were evaluated and AML-specific blasts were observed in AML samples (Fig. 4.15.).

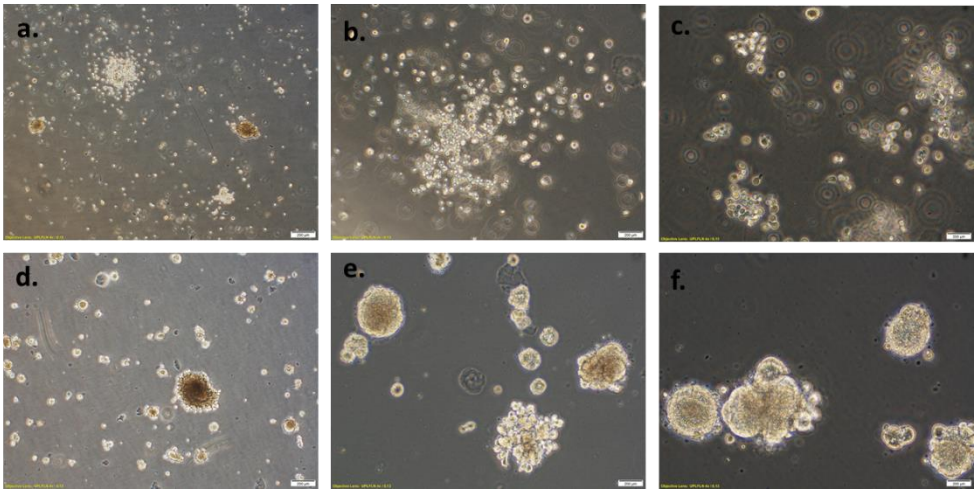


Figure 4. 12. Colony forming capacity of AML samples from distinct localizations after long-term culture. a. Patient 3 AML samples, CFU-GM-like colonies and blastic colonies from suspension cells (4X magnification), **b.** Patient 3 AML samples, CFU-GM like colonies from suspension cells (10X magnification), **c.** Patient 3 AML samples, CFU-GM like colonies from suspension cells (20X magnification), **d.** Patient 3 AML samples, blastic colonies from adherent and underneath cells (4X magnification), **e.** Patient 3 AML samples, blastic colonies from adherent and underneath cells (10X magnification), **f.** Patient 3 AML samples, blastic colonies from adherent and underneath cells (20X magnification) (Olympus, IX73).

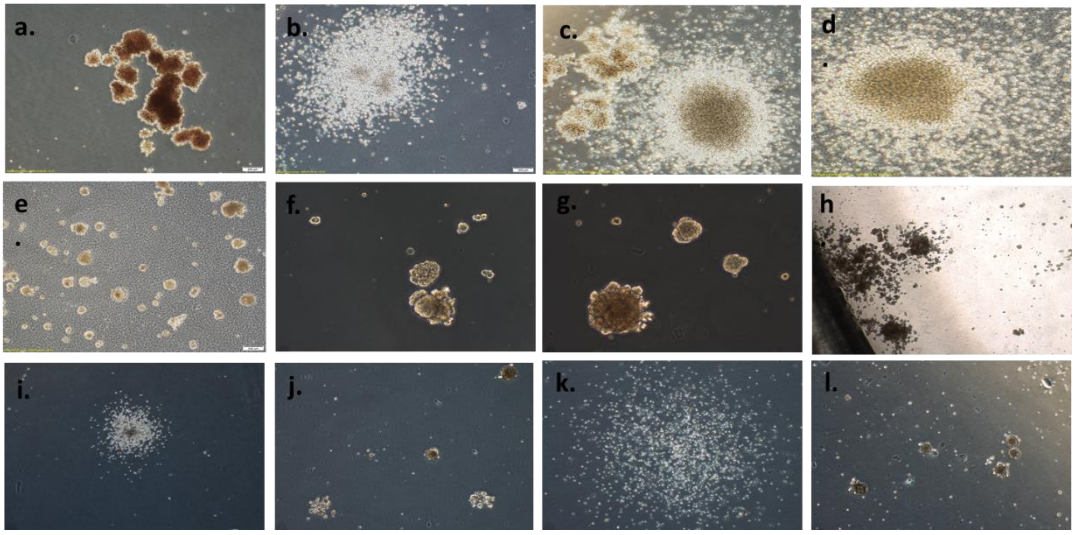


Figure 4. 13. Colony forming capacity of AML samples and healthy donor BM samples after long-term culture. Colonies in culture were scored with an inverted microscope. Colony of forming units were identified as clusters consisting of more than 40 cells [1]. Representative examples of colonies from AML samples and healthy donor BM samples; **a.** BFU-E colonies of healthy donor BM sample (10x magnification), **b.** CFU-GM colonies of healthy donor BM sample (10x magnification), **c.** CFU- GEMM colonies of healthy donor BM sample (10x magnification), **d.** CFU- GM colonies of healthy donor BM sample (10x magnification), **e.** Patient 7; primary AML sample blastic colonies (4x magnification), **f.** Patient 7; primary AML sample blastic colonies (10x magnification), **g.** Patient 3; primary AML sample blastic colonies (20x magnification)**, h.** Patient 8; primary AML sample, BFU-E-like colony (4x magnification), **i.** Patient 8; Remission sample, CFU-GM (4x magnification), **j.** Patient 8; Remission sample, blastic colonies (4x magnification), **k.** Patient 8; remission sample, CFU- GM (4x magnification), **l.** Patient 8; remission sample, blastic colonies (4x magnification) (Olympus, IX73).

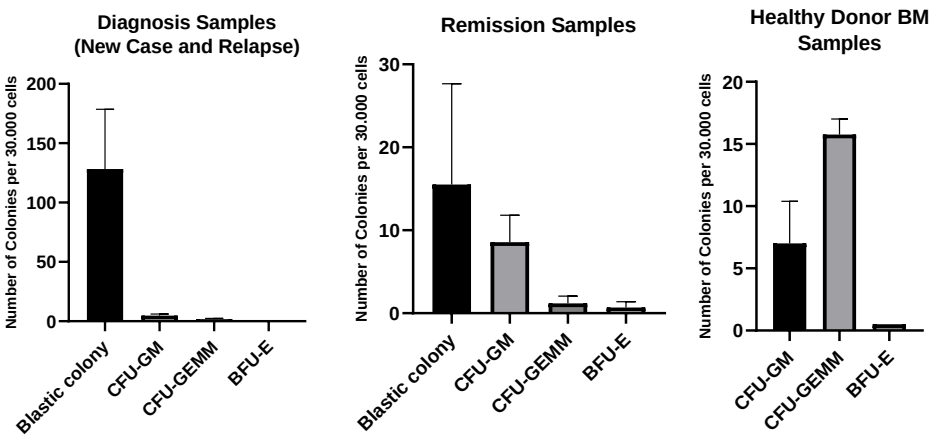


Figure 4. 14. The results of CFU-assays performed prior to passaging AML samples. Colonogenic potentials of AML samples at diagnosis and remission compared to donor samples. CFU-GM represents GFU-M, CFU-G and CFU-GM colonies (CFU-E: colony forming unit- erythroid, CFU-G: colonyformingunit- granulocyte. CFU-M: colony forming unit-macrophage, CFU-GM: colony forming unit-granulocyte/macrophage, CFU-GEMM: colony forming unit-granulocyte/erythrocyte/macrophage/megakaryocyte, BFU-E: burst-forming unit-erythroid) (AML samples n=12, remissions amples n=5, healthy donor BM samples n=2). Data given were the mean ± SEM of colony numbers.

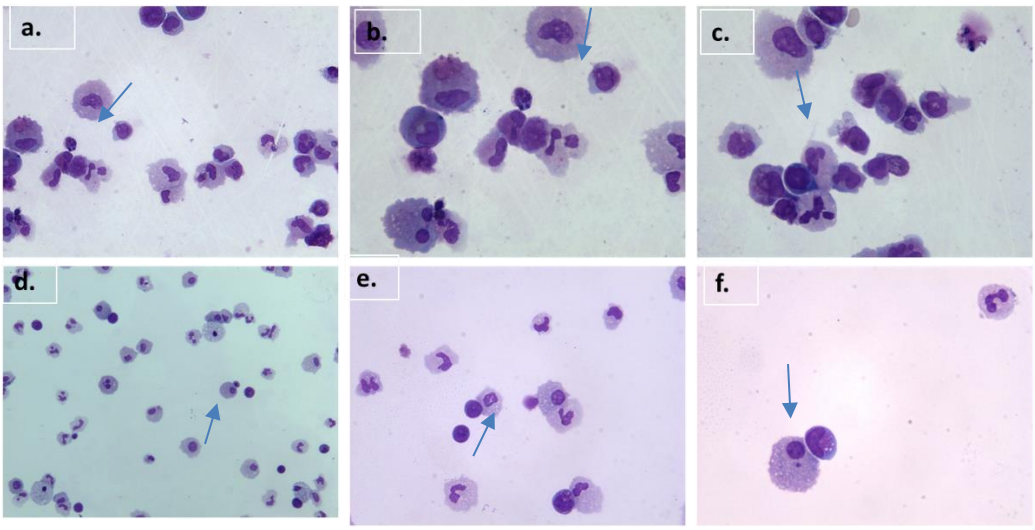


Figure 4. 15. Wright's staining of cytospin preparations from AML patient samples after CFU assay. After 14 days of incubation, cells were harvested, washed and stained with Wright's dye. Cytological representative images of 1 AML patient's blasts **a.** Patient 9, relapse AML sample at 40x magnification, **b.** Patient 9 relapse, AML sample at 63x magnification, **c.** Patient 9, relapse AML sample, at 63x magnification **d.** Patient 9, remission AML sample at 20x magnification **e.** Patient 9 remission AML

sample at 40x magnification **f.** Patient 9, remission AML sample at 63x magnification (Leica, DMI6B). (blue arrows indicate the blastic cells).

4.2.5. Separation of AML LSCs from Normal HSCs/ HSPCs by ALDH Activity

ALDH (aldehyde-dehydrogenase) activity is used as a putative stemness marker in hematopoietic system. Although leukemia initiating potential of cells with high ALDH activity is still controversial, recent studies confirm that LSCs might be enriched in the ALDH intermediate subsets [53]. Increased ALDH activity in primary AML samples have been demonstrated to be associated with adverse disease outcome. ALDH activity might also play a crucial role in chemotherapy resistance since ALDH is a cytosolic enzyme involved in detoxification of alkylating agents [54]. Thus, ALDH activity together with CD34 expression, can be used as a prognostic marker for AML patients. We evaluated ALDH-dim, ALDH-intermediate and ALDH-bright leukemic cell subsets in AML samples and healthy donor samples by flow cytometry. Moreover, using CD34 and CD38 surface markers, we determined the phenotype of these subsets. And using day 0, day 7 and passage day data, we characterized the maintenance of leukemic cells and LSCs. At day 0, day 7 and passage day, cells were labeled with ALDH and CD34, CD38 and CD45 antibodies. Gating strategy was represented in Fig. 4.16. In this part of the study, ALDH activity was used for identification of LSCs and separation of LSCs from normal HSCs/HPCs. Three cell subsets were determined according to brightness. CD34 and CD38 expressions were used to confirm presence of LSCs within the bulk leukemic cell population and identification of leukemic cell maintenance and proliferation as an indirect indication of self-renewing and differentiating LSC presence within the bulk population.

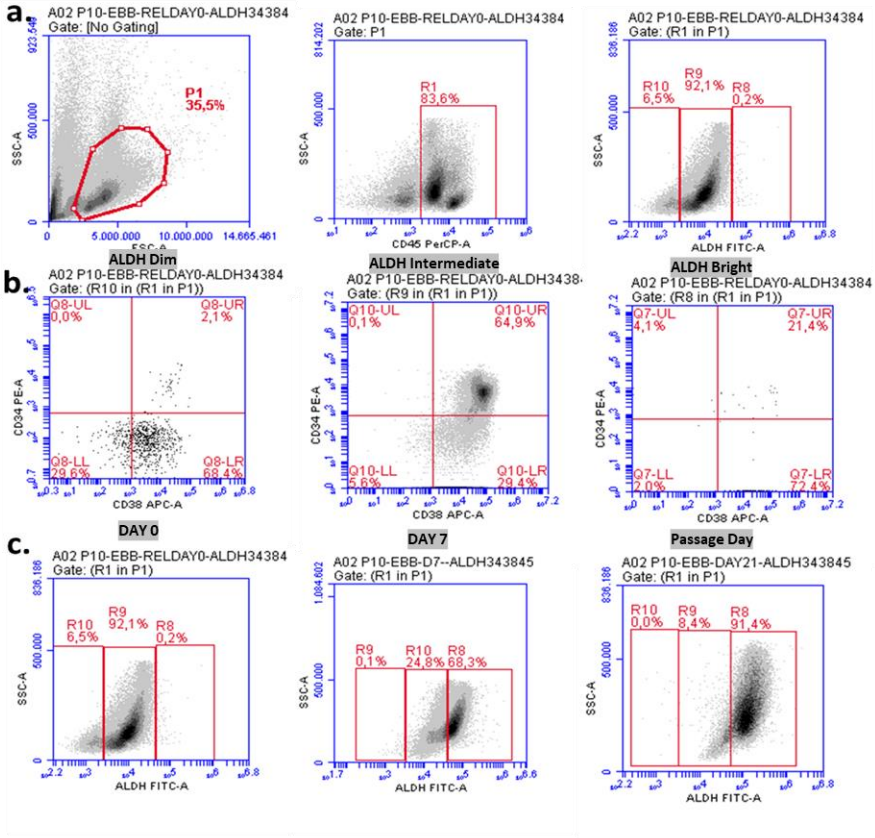


Figure 4. 16. Representative flow cytometry analysis of ALDH activity during long-term culture. a. Represantative gating strategy for ALDH assay. ALDH-dim, ALDH-intermediate and ALDH-bright leukemic cell subsets in CD45+ populations. **b.** ALDHdim, ALDHintermediate and ALDHbrightsubset populations phenotype. **c.** ALDH subset population percentages at Day0, Day7 and passage day respectively. For some AML (diagnosis and relapse) samples shifted from dim to bright.

Gating strategy; onto FSC versus SSC plot based on the size of the cells and by back-gating strategy, our leukemic population was selected, which was located in P1. Then on CD45 plot, CD45 positive cells were gated as R1. In R1 gate, ALDH positive cells were separated into 3 areas according to the brightness as R2, R3 and R4 [55]. Their mean percentages were presented in figure 4.15 for newly diagnosed and relapse AML samples, in figure 4.17 for remission samples, and in figure 4.19 for donor samples. After that, for each area three different cell subset were evaluated according to their CD34 - CD38 expression levels

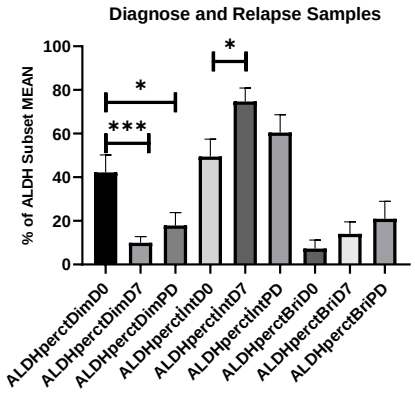


Figure 4. 17. ALDH activity of AML samples (diagnosis and relapse) according to fluorescence intensity (n=12). ALDHdim, ALDHintermediate and ALDHbright subset populations percentages for day 0, day 7 and passage day. Enrichment of LSCs and/or HSCs was assessed by looking at ALDHintermediate and ALDHbright side populations. Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

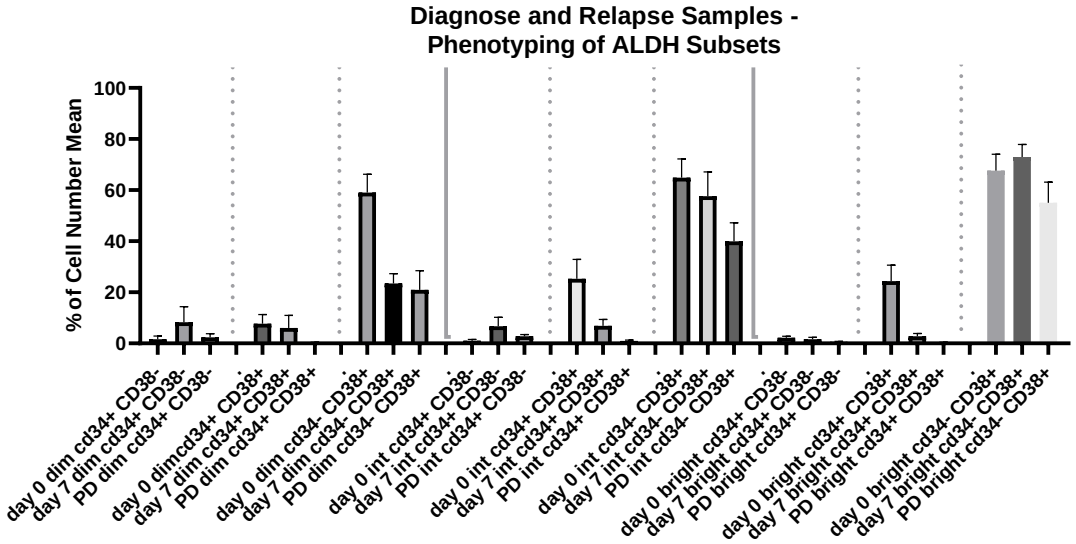


Figure 4. 18. Separation of different cell subsets according to ALDH activity and CD34 expression in AML samples (diagnosis and relapse) (n=12). Enrichment of LSCs and/or HSCs was assessed by looking at the percentage of CD34+ CD38- cells in the ALDHintermediate and ALDH bright side populations. Data given were the average ± SEM of population percentages. ($p<0.05$ accepted as significant.)

For all AML samples; CD45 positive cells were mostly found in the ALDH intermediate area at day 0, ALDH-int 49.46% (±8). Following 7-day culture and at the passage day (week 3 to week 5) the majority of these CD45 positive cells remained on the ALDH intermediate area (day 7; 75% (±6) and for passage day 60.4% (±8)) (Fig 4.17). The increase in ALDH-int cell population within the first week of culture was statistically significant (p=0.029). but following long-term culture this significance was lost (p>0.05). ALDH-intermediate cells maintained overall during long-term culture.

Within ALDH-intermediate population, enrichment of LSCs was assessed by looking at the percentage of CD34+ CD38- cells. This population slightly increased from 1.03% (±0.5) to 6.7 % (±3.5) following short-term culture but statistically it was not significant (p>0.05) and remained unchanged (2.75% (±0.7)) for long-term culture (p>0.05). (Fig 4.18). CD34+CD38+ cells within the ALDH intermediate population rapidly decreased following short-term culture (p=0.0083) and this decrease continued during long-term culture (p=0,0071) (25.28% (±7), 6.8% (±2.5) and 0.92% (±0.4), respectively). CD34-CD38+ cells within the ALDH intermediate population did not demonstrated any significant change following short-term culture but during long-term culture this population significantly decreased (p=0.0426).

ALDH-bright cell population increased during long-term culture from 7.3% (±4) to 14% (±5.6) at day 7 and then to 21% (±8) at passage day but statistically this increase was found to be not significant (p>0.05).

Within ALDH-bright population, CD34+ CD38- cells showed a significant decrease during long-term culture (p=0.0384) from 2.14% (±0.6) to 0.6% (±0.2) but the change was not significant for short-term culture (p>0.05) (Fig 4.18.).

ALDH bright CD34+CD38+ population decrease was significant from day 0 to passage day (p=0.0036) and similarly the for short-term culture (p=0.0027).

ALDH bright CD34-CD38+ population change was not significant from day 0 to passage day and similarly the for short-term culture (p>0.05), (Fig 4.18.).

ALDH dim subset decreased rapidly from day 0 to day 7 (p=0.0002) and this decrease continued during long-term culture ([42.2% (±8)]; [9.9% (±2,8)]; [17.9% (±5.9)], p=0.01).

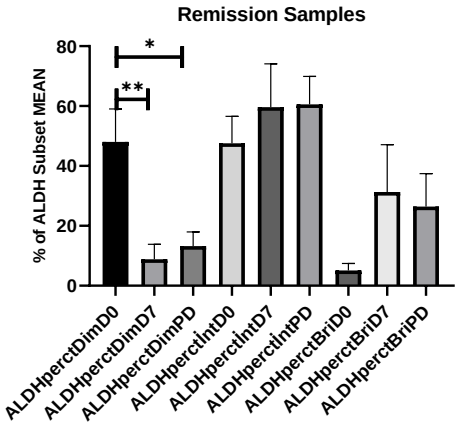


Figure 4. 19. ALDH activity of AML remission samples according to fluorescence intensity (n=5). The mean percentages of ALDH-dim, ALDH-intermediate and ALDH-bright subset populations for day 0, day 7 and passage day. Data given were the mean ± SEM of population percentages Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

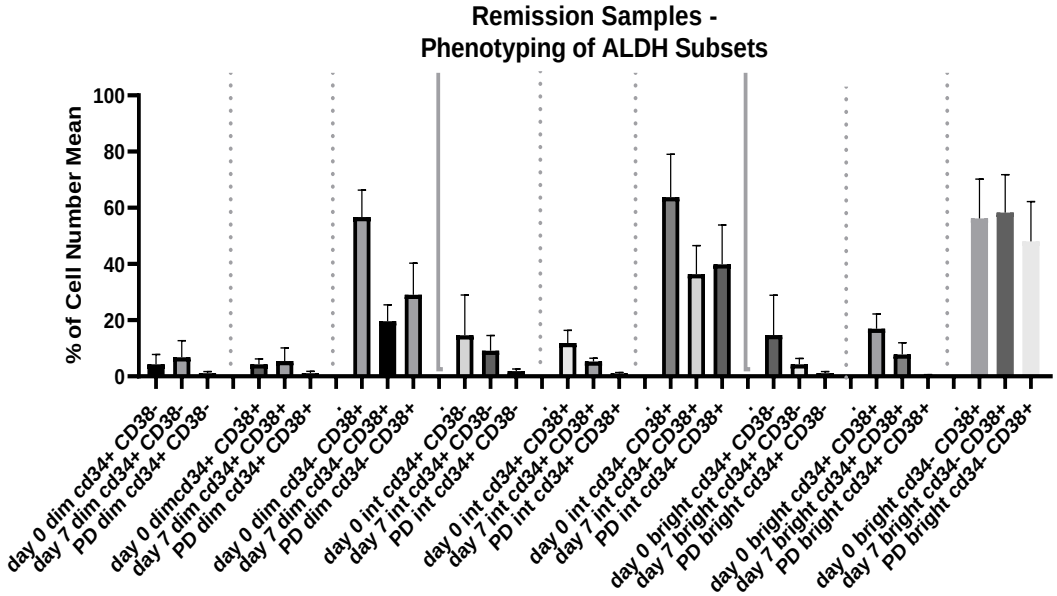


Figure 4. 20. Separation of different cell subsets according to ALDH activity and CD34 expression in AML remission samples (n=5). Enrichment of hematopoietic stem cells was assessed by looking at the percentage of CD34+ CD38- cells in the

ALDH bright side population. Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

For AML follow up (remission) samples, CD45 positive cells were mostly located in the ALDH intermediate area during long-term culture (day 0 [48% (±11)], day 7 [59.6% (±14.5)], passage day [60.5% (±9.3)]) (Fig 4.19). The change was not significant for all time points ($p>0.05$). CD34+CD38- cells within the ALDH-intermediate population decreased from [14.6% (±14.3)] to [9.14% (±5,4)] at day 7, and to [1.9% (±0.7)] at passage day) but, the change was not found significant for neither short-term nor long-term culture. CD34+CD38+ cells in the ALDH intermediate population remained stable during short-term and long-term culture ($p>0.05$) similar to CD34-CD38+ cells in the ALDH intermediate population ($p>0.05$) (Fig 4.20).

Although, ALDH bright cells increased from [5.0% (±2)] to [31.3% (±15.8)] at day 7 and remained almost unchanged [26.42% (±11)] at passage day, this was not significant ($p>0.05$).

Within the ALDH-bright population, CD34+CD38- cells decreased from [14.7% (±14)]to [4.3% (±2)] at day 7 and [1.14% (±0.6)] at passage day. But this change did not reach any statistical significance ($p>0.05$). CD34+CD38+ cells in the ALDH bright population decreased significantly from [16% (±5)] to [7.8% (±4)] following short-term culture and [0.5% (±0.1)] during long-term culture ($p=0.03$). CD34-CD38+ cells in the ALDH bright population did not show any significant change during culture (day 0 [56.2% (±14)], day 7 [58% (±13)] and passage day [48% (±14)] (Fig 4.20).

ALDH dim subset decreased significantly following both short-term ($p=0.005$) and long-term cultures ($p=0.027$).

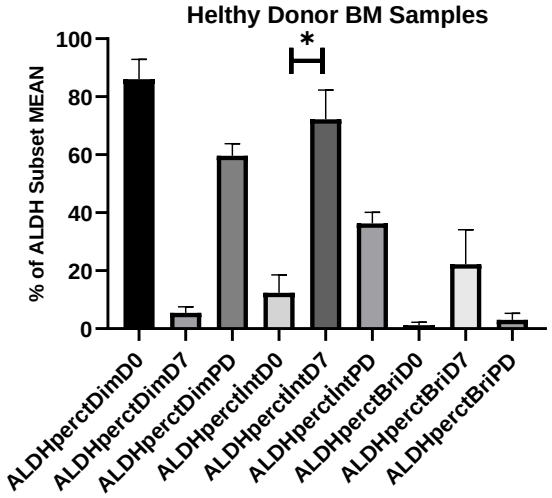


Figure 4. 21. ALDH activity of healthy donor BM samples according to fluorescence intensity (n=2). The mean percentages of ALDH-dim, ALDH-intermediate and ALDH-bright subset populations for day 0, day 7 and passage day. Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

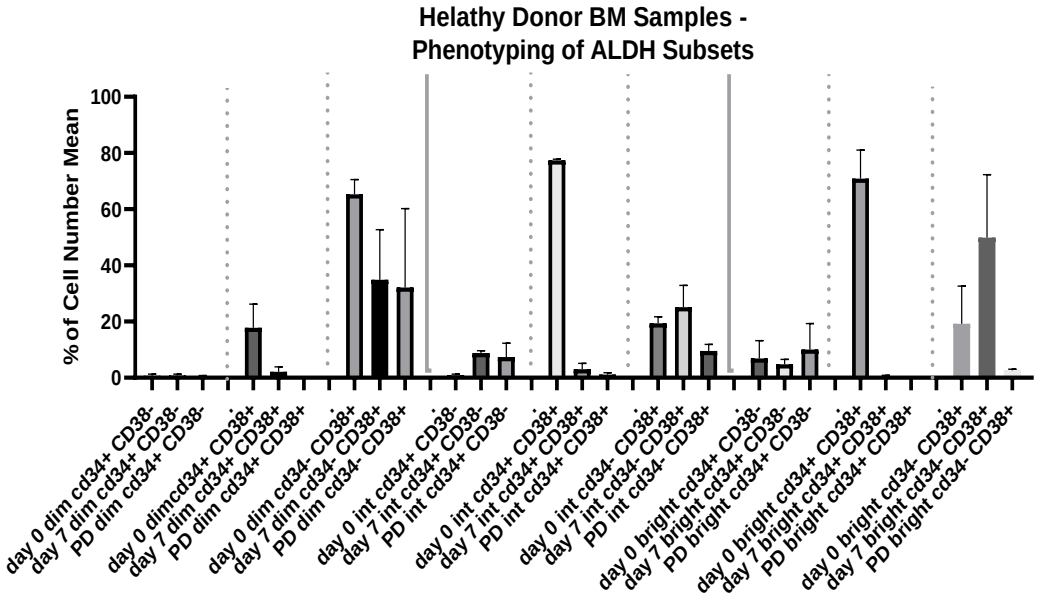


Figure 4. 22. Separation of different cell subsets according to ALDH activity and CD34 expression in healthy donor BM samples (n=2). Enrichment of hematopoietic stem cells was assessed by looking at the percentage of CD34+ CD38- cells in the

ALDH bright side population. Data given were the mean ± SEM of population percentages. ($p<0.05$ accepted as significant.)

For healthy donor BM samples, CD45 positive cells were mostly located in the ALDH dim population during long-term culture; at day 0 [86% (±6.9)], and at passage day [59.6% (±2.05)]. They showed a transient shift towards ALDH-intermediate area at day 7 [72.2% (±10.1)]. But the change was not significant for any time points ($p>0.05$).

ALDH-intermediate subset increased during long-term culture from 12.4 % (±6.1) to 72.2% (±10.1) at day7 ($p=0.04$), and then to 36.3% (±3.8) at passage day ($p>0.05$) (Fig 4.21).

Within the ALDH intermediate population, the increment in CD34+CD38- population was significant [day 0, 0.8% (±0.4) vs. day 7, 8.7% (±0.8)] during short-term culture ($p=0.03$) but the change was not significant for long-term culture [day 0, 0.8% (±0.4) vs. day 7, 7.4% (±5)], ($p>0.05$) (Fig 4.22). CD34+CD38+ population decreased rapidly from [77.4% (±0.45)] to [3.05% (±2)] at day 7 ($p=0,02$), and to [1.15% (±0.6)] at passage day ($p=0.001$). CD34-CD38+ population remained unchanged [19,35% (±2.3)] vs. [25.15% (±8)] vs. [9.5% (±2)].

ALDH bright subset was not increased significantly from day 0 to day 7 [1.2% (±1)] vs. [22.2% (±11.9)] and to passage day [3.05% (±2.2)] besides an increasing trend was seen ($p>0.05$). Within the ALDH-bright population, CD34+CD38- cells did not change significantly during short-term [6.8% (±6.3)] vs. [4.7% (±1.7)], and long-term culture [10.1% (±9)] ($p>0.05$) (Fig 4.22). Even though there seems to be a rapid decrease in CD34+CD38+ population from day 0 to day 7 [70.9% (±10) vs. [0.5% (±0.4)] and to passage day [0.15% (±,1)], it was not accepted reliable due to small sample size ($p>0.05$). The increase in CD34-CD38+ population [19.2% (±13.4)] vs. [49.85% (±22)] was not significant at day 7, and it remained unchanged during the long-term culture [2.65% (±0.35)], but this was not accepted reliable due to small sample size.

4.3. Early Identification of AML LSCs During Short-term Cultures and Its Contribution to Risk Stratification and Outcome

4.3.1. CFSE Assay as an Evaluation Tool for Demonstration of AML LSC Proliferation Pattern

CFSE assay performed following short-term culture using AML samples at diagnosis might be a practical and easy way to evaluate the proliferation pattern of heterogenous LSCs population and it might be used to predict the risk for refractory and/or relapse disease and outcome.

CFSE assay was performed on 12 AML samples (8 newly diagnosed and 4 relapsed AML) and 5 follow-up AML samples (remission). Proliferation of AML cells was assessed using proliferation index which was defined as the ratio MFI at 18hr CFSE divided by the CFSE at one week. It was used as an indicator of cell proliferation to assess the relationship between leukemic cell proliferation and LSC frequency. Gating strategy was shown in Figure 4.23. On FSC and SSC, we gated both of the two different cell populations considering the heterogeneity of AML cells. The mean of proliferation index was [20 (±5,93)] for AML samples (newly diagnosed and relapse, n=12), and [47 (8±32)] for remission samples, n=5 (Fig. 4.23).

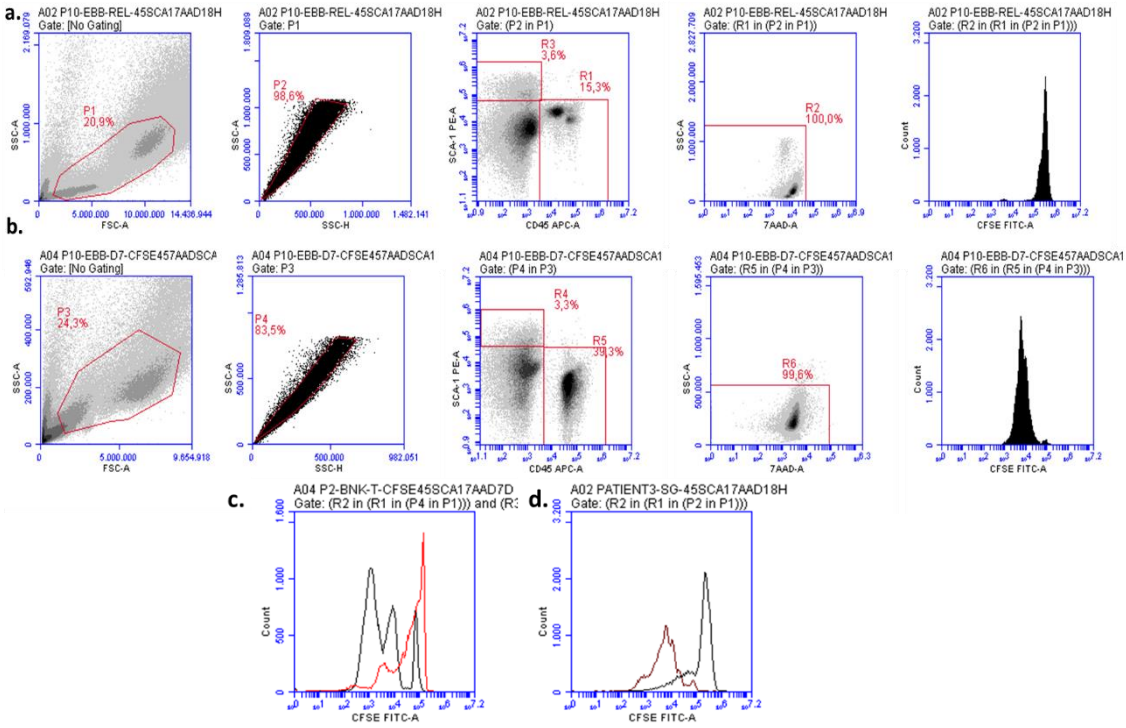


Figure 4. 23. Analysis of AML samples by CFSE proliferation assay. CFSE median fluorescence intensity was calculated on gated viable CD45+ viable positive cells at 18hrs and day 7 to characterize the short-term culture. **a.** Representation of an AML patient sample analysis after 18-hour incubation of CFSE (Patient 10). **b.** Representation of AML patient sample analysis after 7-day incubation of CFSE (Patient 10). **c.** Patient 2; diagnosis AML sample with low proliferation index (2.28) overlayed 18hr (black) vs one week (red) CFSE expression histograms. **d.** Patient 3; diagnosis AML sample with high proliferation index: 32.90, overlayed 18hr (red) vs. one week (black) CFSE expression histograms.

Gating strategy; onto FSC versus SSC plot based on size of the cells and by back-gating strategy our leukemic population was selected which was located in P1. Then, singlets were gated as P2. Then, in P2 onto Sca-1 negative and CD445 positive cells were gated as R1. The viable cells in R1 were selected by 7AAD staining (R2). The median fluorescence intensity for CFSE was used for further analyses. The proliferation index were calculated by the ratio of the 18-hour CFSE median fluorescence intensity divided by 7-day CFSE median fluorescence intensity [49].

4.3.2. Identification of AML LSCs at Diagnosis Using LSC- Specific Surface Markers

Identification and discrimination of LSCs from HSCs based on their immunophenotype, requires LSC-specific surface markers which were expressed on LSCs but not on HSCs. Besides the prediction of disease prognosis and treatment follow up, these markers may be used as valuable therapeutic targets for AML. Four different AML LSC-surface markers (VEGFR-2, CD25, TIM-3 and CLL-1) were selected in order to identify and quantify the LSCs within 12 AML samples (Table 4.4.) and 5 of their remission pairs (Table 4.5). Gating strategy was shown in Figure 4.24. Patient specific blast populations were gated and the expression of each marker was analyzed within CD34+CD38- cell population. CD38 negative and selected marker positive population was considered as LSCs. Double negative population was considered as HSCs. CD38 was used to separate the mature blast populations.

Expression levels were found to be different among patients. All patients (6 diagnosis and 4 relapse) except Patient 1 and 3, expressed VEGFR-2, TIM3 and CLL-1. CD25 expression levels varied among patients (>10%). 6 out of 12 patients (3 diagnosis and 3 relapse) expressed CD25 marker (>10%). The mean VEGFR-2 expression was 27% (±5.8) (max-min; 21.2 – 32.8) for AML samples (n= 12) and 16.9% (±10.2) (min-max 6.72 – 27.12) for remission samples. The mean CD25 expression was 15.4 % (±4.1) (min-max 11,3 – 19,5) for AML samples and 10.4% (±4.5) (max-min; 5.9 – 17.7) for remission samples. The mean TIM-3 expression was 39.3% (±7.2) (max-min; 32.1 – 46.5) for AML samples and 34.5% (±9.5) (max-min; 25 – 43) for remission samples. The mean CLL-1 expression was 44.8% (±7.9) (max-min; 36.9 – 52.8) for AML samples and 31.2% (±12.7) (max-min; 18.5 – 43.9) for remission samples (Tables 4.3. and 4.4.).

When we analyzed the data in order to see whether there was any change in expression levels after treatment, we found a decline in the expression levels of all 4 markers for patients following treatment (Tables 4.4.) (n=5). For diagnosis-remission pairs (n=5) although the decrease in the VEGFR-2 percentages was observed, statistically this decrease was not significant [29,7% (±6,8) for diagnosis and 16,9% (±10,3) for remission samples] (p=0.2) and similarly, for CD25 [22,9% (±7,7) for

diagnosis and 10,4% (±4,5) for remission samples] (p=0.07), for [53,4% (±10,5) for diagnosis and 34,5% (±9,5) for remission samples], (p=0.2) and for CLL-1 51,9% (±10,4) for diagnose and 31,2% (±12,8) for remission samples] (p=0.07). For HSCs population in AML cells, double positive areas were compared between diagnosis and remission sample pairs. Although the remission samples percentages were high for HSCs the change was not significant, for VEGFR-2 [65.3% (±5) for diagnosis and 78% (±10) for remission samples] (p=0.3) and similarly, for CD25 [60% (±14) for diagnosis and 90% (±4,5) for remission samples] (p=0.08), for [44% (±9) for diagnosis and 60% (±9) for remission samples], (p=0.3) and for CLL-1 42% (±8) for diagnose and 57% (±11) for remission samples] (p=0.2).

For selected CD34+CD38- stem cell population, on the marker positive and CD38 negative population considered as LSCs and double negative population was considered as HSCs. Thus, VEGFR-2 expression level was 57.7% (±7.03) for HSCs and 27% (±5.8) for LSCs (n=12). CD25 expressed 63,0% (±8,6) on HSCs and 15,8% (±4,16) on LSCs. TIM-3 percentage was 47,6% (±6,9) for HSCs and 39,7% (±7,3) for LSCs. CLL-1 percentage was 42,0% (±7,5) for HSCs and 45,3% (±8) for LSCs.

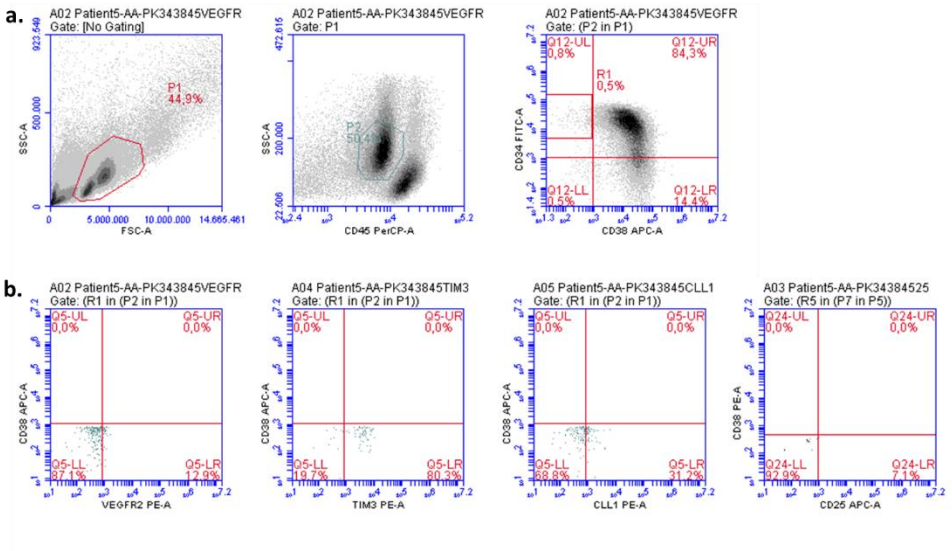


Figure 4. 24. Representative flow cytometry analysis of LSC-specific markers' expressions at diagnosis. CD34+CD38- populations were gated for detection of LSC/ progenitor cells. Marker positive cells indicate LSCs and negative cells indicate healthy progenitor cells. **a.** Representative analysis of population selection for LSCs characterization (Patient 5 diagnose sample at day 0) **b.** 4 selected marker percentages for Patient 5 in gated population at part a.

Gating strategy; Onto FSC versus SSC plot based on size of the cells and using back-gating strategy our population was selected which is P1. In P1, onto CD45 plot according to the type of AML, blast cells were gated as P2. Maturation curve was differed from patient to patient according to their subtype and their CD34 expression level is different among patients. Thus, we used same gate strategy for all samples and assessed the LSC by looking at the percentage of CD34+ CD38- percentage, gated as R1. Onto CD38 versus selected marker plot positive and negative populations were evaluated as AML LSCs and HSCs.

Table 4. 3. LSC-specific marker expression profiles of AML samples

	VEGFR-2- %	VEGFR-2+ %	CD25- %	CD25+ %	TIM-3- %	TIM-3+ %	CLL-1- %	CLL-1+ %
P1-AML M4	43,9	0	32,7	1	54,2	0	45,1	1,1
P2-AML MyeloidSarkom	63,2	11,8	48,6	45,9	62,5	25	43,9	26,3
P3- AML M7	90,5	6,3	92,4	7,6	91,9	5,4	88,9	8,1
P4- AML M3	78,7	21,3	89,4	10,6	10,5	89,5	50,9	49,1
P5- AML M4	87,6	12,4	92,9	7,1	32,1	67,9	73,1	26,9
P6- AML M3	50	50	20	2	50	50	30,8	69,2
P7- AML M2	27,9	40,2	87,6	12,4	24,9	43,4	1,1	65,8
P8- AML M5	74,6	25,4	91,1	8,9	55,6	44,4	66,7	33,3
P9-Relapse AML M5	60	40	48,1	37	41,7	58,3	18,5	81,5
P10- Relapse AML M4	30	70	69,2	30,8	70,2	29,8	46,6	53,4
P11- Relapse AML M6	16,1	19,4	12	8	14,8	25,9	26,2	40,5
P12- Relapse AML M2	70	30	71,9	18	62,9	37,1	12,1	87,9

Table 4. 4. Change in LSC-specific marker expression profiles of AML samples after treatment

	VEGFR-2- %	VEGFR-2 + %	CD25- %	CD25+ %	TIM-3- %	TIM-3 + %	CLL-1- %	CLL-1+ %
P2- AML MyeloidSarkom	63,2	11,8	48,6	45,9	62,5	25	43,9	26,3
P2-Remission- AML Myeloid Sarkom	68,4	5,3	72,7	27,3	52,6	21,1	40	0
P4- AML M3	78,7	21,3	89,4	10,6	10,5	89,5	50,9	49,1
P4-Remission- AML M3	93,8	6,2	90	10	70	30	71,4	28,6
P6- AML M3	50	50	20	12	50	50	30,8	69,2
P6-Remission- AML M3	96,9	3,1	93,2	6,8	84,9	15,1	81,4	18,6
P8- AML M5	74,6	25,4	91,1	8,9	55,6	44,4	66,7	33,3
P8-Remission- AML M5	87,5	12,5	100	0	63	37	68,3	31,7
P9-Relapse-AML M5	60	40	48,1	37	41,7	58,3	18,5	81,5
P9-Remission-AML M5	42,5	57,5	92	8	30,5	69,5	22,8	77,2

4.3.3. The Association of LSCs with Risk Stratification and Disease Outcome.

Quantification of LSCs can be used to obtain information about the refractory/relapse disease and outcome. Disease relapse can occur even in patients

with low or negative minimal residual disease (MRD) levels accepted as in molecular remission and also in the patients who are stratified into intermediate or standard risk groups at diagnosis and are treated accordingly [56, 57]. Thus, especially for AML patients in the intermediate group with no defined molecular marker for disease follow up, there is a great need for new methods that take shorter time and are performed easily in order to identify these patients. When LSCs in AML samples are quantified properly at diagnosis, this can help to stratify patient risk groups more accurately.

In this study, we used a short-term niche-like coculture system to analyze LSCs. After one-week culture, LSCs proliferation pattern was assessed using the CFSE assay and stem cell functionality was evaluated using ALDH activity. We also tried to demonstrate LSCs within bulk leukemic cell population by using LSC-specific surface markers. When we looked at these LSC-specific markers, we found that 6 out of 12 AML patients had a CD34+CD38- population expressing all four of these markers (>10%). When we looked at the expression of each marker, 3 out of 4 high risk group and 3 out of 4 intermediate and standard risk group patients expressed VEGFR-2, TIM-3 and CLL-1 for newly diagnose patient and all relapse samples expressed these markers. 2 out of 4 high risk group and 2 out of 4 intermediate and standard risk group patients expressed CD25 for newly diagnose patient and 3 out of 4 relapse samples expressed these markers. CD25 (>10%). Moreover, when we analyzed the expression of all markers, a similar trend was observed for AML patient samples and their follow-ups.

For patient 9 remission sample, all surface marker expression levels, CD34+CD38- population within ALDH-bright and ALDH-int subsets and the percentage of CD34+CD38- cells were considerably high although he was in clinical/morphological remission.

Table 4. 5.Distribution of AML patients according to AML LSC defining parameters and risk groups

Patient ID	Risk Group	VEGFR-2 + %	CD25+ %	TIM-3 + %	CLL-1 + %	Proliferation Index	ALDH bright % Day 0	ALDH intermediate % Day 0	ALDH bright % Day 7	ALDH intermediate % Day 7	CD34+CD38- DAY0	CD34+CD38- DAY7	Relapse Status	Disease Status
P1- AML M4	high	0	1	0	1,1	5,11	1,3	81,7	6	92,3	0,1	19,8		remission
P2-AML MyeloidSarkom	high	11,8	45,9	25	26,3	2,28	1,2	9,9	2,9	76,6	0,5	2,2		remission
P3- AML M7	intermediate	6,3	7,6	5,4	8,1	32,9	45,5	39,6	1,4	95,1	0,3	1,2		exitus
P4- AML M3	standart	21,3	10,6	89,5	49,1	17,35	9,1	85,1	7,3	92,6	1,3	13,2		remission
P5- AML M4	high	12,4	7,1	67,9	26,9	4,37	22,7	31,6	33,8	52,6	1,3	0,2	+	exitus
P6- AML M3	standart	50	2	50	69,2	12,72	0,5	50,6	2,3	88,4	0,1	1,2		remission
P7- AML M2	standart	40,2	12,4	43,4	65,8	21,31	0,2	71,5	7,9	87,9	9,2	1,6		remission
P8- AML M5	high	25,4	8,9	44,4	33,3	5,22	1,5	45,4	17,6	68,4	0,5	3,3		remisyon
P9-Relapse AML M5	İntermediate at diagnosis	40	37	58,3	81,5	1,96	1,2	45,1	5,5	91,4	0,4	22,6	+	exitus
P10- Relapse AML M4	İntermediate at diagnosis	70	30,8	29,8	53,4	49,81	0,2	92,1	68,3	24,8	0,2	1,7	+	remission
P11- Relapse AML M6	İntermediate at diagnosis	19,4	8	25,9	40,5	12,42	3	12,4	4,5	67,9	3,1	0,7	+	remission
P12- Relapse AML M2	İntermediate at diagnosis	30	18	37,1	87,9	13,82	1	28,5	10,5	58,5	1,4	0,3	+	exitus
P2-Remission- AML Myeloid Sarkom	high at diagnosis	5,3	27,3	21,1	0	39,86	12,5	64	5,3	91,5	0,1	1		remission
P4-Remission- AML M3	standart at diagnosis	6,2	10	30	28,6	82,61	8,6	66	18	80,9	0,2	9,2		remission
P6-Remission- AML M3	standart at diagnosis	3,1	6,8	15,1	18,6	27,43	3	21,6	4,2	69,8	0,3	2,1		remission
P8-Remission- AML M5	high at diagnosis	12,5	0	37	31,7	75,97	0,4	31	39,8	45,4	0,5	1,2		remission
P9-Remission-AML M5	İntermediate at diagnosis	57,5	8	69,5	77,2	7,99	0,9	55,3	89,1	10,5	46,1	2,2		exitus

5.DISCUSSION

In this study, we identified and characterized LSCs in AML patient samples using niche-like coculture system. Cell numbers were increased considerably during long-term culture for all AML samples. This increase indicates that the niche-like co-culture system supports the proliferation and maintenance of AML cells and LSCs. When cell proliferation kinetics was considered the proliferation capacity of AML samples changed between patients. We did not use CD34+ selected leukemic cell population for the analyses. Instead we preferred to unselect mononuclear cells presenting the heterogeneity of leukemic cells in order to avoid the loss of any specific leukemic cell subset. Therefore, differences observed between patients' samples is probably related to the cell population's impurity and to AML subtype. Depending on the AML subtype and/or risk group, LSCs frequency may change and long-term proliferation may not be achieved for some AML subtypes [49, 52]. But our all samples proliferated during long-term cultures. The difference in proliferative responses between AML samples indicates that our coculture conditions are not sufficient to support growth of all AML subtypes in concordance with the previous studies. But it can still be used to maintain AML cells. After passaging, cell numbers continued to increase in all AML samples at different rates. However, increase in cell number was minimal possibly due to the loss of some leukemic cells during culture period, in spite of self-renewing and differentiating LSCs. However, the variation in the cell numbers didn't reach any significance, possibly due to small sample size for any of the studied groups. Although, cumulative cell numbers seemed to increase for all samples during short-term culture, AML samples retained significantly higher absolute counts compared to remission and donor samples following short-term culture. Thus, this co-culture system is suitable to maintain and enrich the LSCs even though, the change in the cell numbers of diagnosis-remission pairs was not significant following short-term culture. On the other hand, the cell numbers were found higher in AML samples when compared to those in for remission and donor samples after long-term culture. Thus, this coculture system seems to support the

proliferation of LSCs better than that of normal hematopoietic stem/progenitor cells. The fluctuations observed in the cell numbers of remission and donor samples probably was resulted from the proliferation of normal hematopoietic stem/progenitor cells and the residual LSCs which could be present, even in the bone marrow of AML patients who are in remission [60].

The results of recent studies indicates that LSCs are mostly enriched within the CD34+CD38- cell fraction of AML samples, beside the presence of LSCs within the CD34+CD38+ and CD34CD38+ cell fractions in certain AML subtypes [19]. In long-term cultures, CD34 and CD38 expression levels were used to evaluate maintenance/enrichment of LSCs and separate immature and mature leukemic cells from LSCs. Following one-week culture, although an increasing trend was observed in the CD34+CD38- population in AML samples, this increase was not significant possibly because of the small sample size and presence of different AML subtypes in our study group. The proliferative capacity of AML cells may change considerably depending on AML subtype [58]. Reikvam and et al. reported that proliferative capacity of leukemic blasts may be related to their gene expression profile. Another study demonstrated that during the early days of *in vitro* culture, most of the AML cells go through apoptosis [59]. In agreement with these data, CD34+CD38- cells in long-term cultures of Patient 5, Patient 7, Patient 11 and Patient 12 increased despite a transient decrease in short-term culture. For AML samples CD34+CD38+ and CD34-CD38+ cell populations decreased during long-term culture. This indicates that CD34+CD38- population maintained in this coculture system, the blast population did not. The frequency of CD34+CD38- LSCs remained almost stable throughout the culture period. Since LSCs are rare ranging from 1 in 10 thousand to 1 in 5 million, even a difference of 0.2 percent in CD34+CD38- expression may be clinically relevant [60]. A similar trend in CD34+CD38- cell frequency was present for all time points for all remission samples except patient 9. The results of this patient were quite intriguing since he presented with relapsed disease, stratified into intermediate risk group at diagnosis with no unfavorable molecular marker and clinical/morphological remission was achieved after treatment. But the LSCs frequency of this patient did

not show any significant change following culture. Thus, one should always remember that there can be quiescence residual LSCs cells after treatment and they may cause disease relapse, as in our patient.

Distinct compartmentalization of LSCs was observed when cultured on MS-5 stromal cell during long-term cultures. LSCs can migrate underneath the MS-5 feeder cells or locate above the MS-5 feeder or in suspension. The CAFC assay is based on morphological criteria and used to identify progenitor/stem cells with self-renewal capacity [50, 51]. After the first week, L-CAFCs were observed at dim and dark phase by phase contrast microscopy, implying that these cells are probably LSCs [52]. Because more primitive/progenitor cells can attach to the stromal cell and some of them can pass through to under the feeder cells [50]. According to the subtype of AML and the LSCs frequency, the time of formation and number of L-CAFCs varied among samples (between 7 to 21 day), even though L-CAFC were observed in all AML samples. In contrast, CAFC could not be detected in the remission samples and healthy donor BM samples. Moreover, after replating the cells, we observed again L-CAFCs, which indicates that these cells maintained their self-renewal potential *in vitro* [52]. Only 2 patients (Patient 7 and Patient 4) did not form L-CAFC after replating. This data is in concordance with the decline in CD34+CD38- cell population for these two patients. Among the remission samples only one sample (Patient 9) contained cobblestone areas.

For morphologic analysis of the proliferated AML cells, CFU assay was performed. The CFU assay is a functional assay to identify hematopoietic stem/progenitor cells and their differentiation capacity. It shows the long-term maintenance/enrichment of LSCs that have the ability of self-renewal and blastic colony formation [21, 61]. We observed a difference between colony formation capacity of suspension cells, adherent and underneath cells. This results is compatible with the results of another study reporting that immature and dormant stem cells (LSCs) locate under the adherent feeder cells and they can generate blastic colonies [51]. Morphologically distinct blastic colonies were observed only in AML samples and it was the dominant type of the colony covering all plates. However, blastic

colony formation was not seen in Patient 4 and Patient 6, both diagnosed with AML M2 subtype and standard-risk patients. Therefore, there appears to be an association between the risk group and/or subtype of AML and the blastic colony formation ability which is closely related to LSC frequency. Leukemia-CFUs were observed specifically in AML samples at diagnosis, in contrary to the heterogeneous distribution of different types of hematopoietic CFUs in follow-up and control samples. Moreover, blastic colonies were observed in both diagnosis and remission samples of Patient 8 and Patient 9. They were both treated as high-risk patients.

ALDH is an enzyme which is crucial for intra-cellular aldehyde oxidation [62]. High ALDH activity has been determined as a stem/progenitor marker for both HSCs and LSCs [54, 55]. The ALDH assay was used to characterize and confirm the presence of LSCs and to understand whether the relation between ALDH activity and CD34 expression can be used as a prognostic marker. It was previously found that ALDH expression can be used to separate LSCs from HSCs in AML patients [55]. Therefore, we assessed enrichment of LSCs by looking at the percentage of CD34+CD38- in ALDH intermediate and bright populations [55, 62, 63]. According to the literature, LSCs show a different pattern for ALDH activity compared to normal HSCs [54]. Because HSCs show high ALDH activity, majority of ALDH bright cells considered as healthy HSCs in AML samples. Recent studies confirmed that LSCs can be discriminated by intermediate ALDH activity and their ability of engraftment [64]. Increase in cells with intermediate ALDH activity confirmed that the presence of LSCs in our samples. Moreover, ALDH-int CD34+CD38+ and CD34-CD38+ cells decreased by time. These populations mostly consist of immature/mature blast population. Thus, decrease was expected.

It was previously shown that; in remission samples ALDH activity and LSCs frequency are related to clinical outcome [65]. Therefore, in remission samples, we expected or observed ALDH bright and intermediate population increment for some patients (Patient 9). For remission samples, ALDH bright population increment was related to CD34-CD38+ progenitor cells. This cell population can survive in this co-culture system.

Unlike AML samples, cells were mostly located in the ALDH dim area during long-term culture in donor samples and ALDH bright population increment was related to CD34+CD38- HSCs. It supports the data above that HSCs can be maintained in this co-culture system.

LSCs can be found in both ALDH intermediate and ALDH bright fractions [55, 66]. ALDH activity may differ among patients and varies between AML samples [63]. In general, ALDH expression by intermediated risk groups intensified during-long term culture. According to Hoang *et al.* ALDH bright and intermediate group are related to a poor prognosis [55]. Moreover, cobblestone area formation and CFU assays support these data which may be related to a poor prognosis. Because AML clones are heterogeneous at diagnosis and may change after relapse, results varied among patient samples.

Overall, our data obtained through a combination of functional and phenotypical assays demonstrates that LSCs were enriched in ALDH intermediate area and HSCs in ALDH bright area [67].

CFSE staining is an easy and practical method to determine proliferation pattern of LSCs in short-term cultures. Because of the heterogeneity of the leukemic cells and variations in LSC frequency, our results varied widely among the samples. Division peaks were not observed for most of the samples because cell division was not synchronized which depends on morphological heterogeneity [49]. For diagnosis and relapse samples the proliferation index mean was 20 (±5.9), whereas in remission samples the mean was twice as much 47.8 (±32.0). Comparing the results of the AML cells proliferation in AML samples (diagnosis and relapse) and remission samples, our data demonstrate that there was considerable dispersion among samples. Moreover, the rapid dividing small cells and slowly dividing large cells can affect the results since these groups are not distinguishable. The blast percentages of the patients at diagnosis may affect the LSCs frequency which changes the proliferation rate of AML cells.

To identify a more specific immunophenotype of LSCs, we used 4 different markers to stain CD34+CD38- population. VEGF activation is an important pathway

for malignancies. Previously VEGF related pathways have been targeted in clinical trials for hematolymphoid malignancies [68]. In addition, VEGF can modulate leukemic cells by paracrine and autocrine signaling and VEGF stimulation can lead to proliferation, survival and migration of leukemias [68]. VEGFR-2 expression level differed depending on the LSC frequency. When we focus on the diagnose and their remission pairs, we can see that VEGFR-2 expression was lower at remission state than at diagnosis and relapse state, but it is not significant because of the sample size (p=0,2, NS). CD25 expression level fluctuated among patients and has been shown to be related with poor prognosis [69, 70]. Similarly, we found that CD25 expression levels varied in the diagnose samples. Also, for diagnose and remission pairs CD25 level was lower during remission. Therefore, CD25 level may be related with LSCs frequency. According to Haubner and colleagues, CLL-1 and TIM-3 are highly positive in AML cells compared with normal hematopoietic cells [71]. TIM-3 directly promotes LSC maintenance by affecting their proliferative capacity [72]. In addition, TIM-3 promotes self-renewal of LSCs and therefore is crucial for progression of the disease [73]. According to our results TIM-3 was expressed in all but two patients (Patient 1 and Patient 3). Hence, targeting TIM-3 might be used to eliminate LSCs. Similarly, CLL-1 expression differed in diagnosis and relapse samples and was not expressed by Patient 1 and Patient 3. Therefore, we concluded that CLL-1 is an important marker for some AML subgroups and may be a favored target. Patient 1 and Patient 3 did not express all selected markers. This indicates that selected 4 markers can be used as individually for combinational target therapy and characterization. Statistically most of our results were not significant. For validity appropriate sample size is important and by using suitable sample size we can represent the target population clearly. Thus, we should expand the sample size.

For prediction of prognosis and disease progression, we focus on short-term culture data which may contribute to risk group stratification and outcome at an early stage.

VEGFR-2, TIM-3 and CLL-1 marker were expressed on 3 out of 4 intermediate and standard risk group patient diagnosis and all relapse samples (≥10%). 3 out of 4 high risk group samples had marker expression for VEGFR-2, TIM-3 and CLL-1 markers

and when compared with their clinical progression 50% of the patients had passed away after their remission and relapse state. These levels are considerable when considered that LSCs are rare [6]. Therefore, these markers may be used as multicolor detection to reduce the false negative outcomes, predict the risk of relapse and specify remission quality. In addition, when we compared diagnose and their remission pairs expression levels mostly high at diagnose state. Because of the sample size it was not statistically significant but clinically it might have considerable value. CD34+CD38- population was found to be in ALDH-bright or ALDH-intermediate areas [54]. Therefore, a functional analysis combined with a panel of selected antibodies may be the best way to detect LSCs for early identification of high-risk patients.

In summary, in this study LSCs phenotype and self-renewal potential were maintained for long-term using a niche-like coculture system. We demonstrated that LSCs had intermediate ALDH activity as well as ability of cobblestone area and blastic colony formation, and increased expression of selected LSC-specific markers. This niche like co-culture system may also be useful for drug screening and better understanding of LSC biology besides the early prediction of disease prognosis.

6. CONCLUSION

Identification of specific markers for AML-LSCs may enhance prognosis and provide of early detection of relapse. According to the subtype of AML and risk groups, therapy is applied. Prediction of disease course and clinical outcome are challenging especially for intermediate-risk AML patients. Therefore, determination of LSCs frequency/functionality using *in vitro* culture systems at diagnosis might be useful for prediction of disease prognosis.

Some of the studies have reported that LSCs can be maintained by coculture systems but, some of these systems may induce differentiation and loss of stemness property. In this study AML-LSCs were maintained using a niche-like coculture more than 5 weeks. Proliferation kinetics, blastic colony forming ability and cobblestone area formation were assessed. Furthermore, detailed ALDH functional analysis has been used to understand the *ex vivo* maintenance of LSC and their characteristics.

We found that, the ability of long-term maintenance *in vitro* for leukemia stem cells differ between patients likely related to their specific AML subtypes. AML diagnosis and relapse samples express VEGFR-2, CD25, TIM-3 and CLL-1 but during remission the expression level of VEGFR-2, CD25, TIM-3 and CLL-1 decrease. In addition, blastic colonies were observed only in AML samples and it was the dominant type of the colony covering all plates and there was a relation between the risk group and/or subtype of AML and the blastic colony forming ability Moreover, LSCs in AML samples showed intermediate ALDH activity and high ALDH activity can be related to remaining HSCs populations in AML samples.

Understanding the relation between AML-LSCs and leukemic niche is crucial to better understand the mechanism of their survival after remission and relapse. Finding specific markers to detect LSC accurately may help to determine prediction of relapse/refractory disease especially for intermediate risk group with no defined molecular markers. The role of LSCs in relapse needs further investigation.

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8.APPENDICES

Appendix 1. Ethical Approval

T.C.
HACETTEPE ÜNİVERSİTESİ
Girişimsel Olmayan Klinik Araştırmalar Etik Kurulu

Sayı : 16969557-A20 ARAŞTIRMA PROJESİ DEĞERLENDİRME RAPORU

Konu :

Toplantı Tarihi	: 21 KASIM 2017 SALI
Toplantı No	: 2017/25
Proje No	: GO 16/824 (Onay Tarihi: 20.12.2016)
Karar No	: GO 16/824- 01

Kurulumuzun 20.12.2016 tarihli toplantısında Etik Kurul onayı almış olan Üniversitemiz Tıp Fakültesi Çocuk Sağlığı Enstitüsü Pediatrik Temel Bilimler Metabolizma Bilim Dalı öğretim üyelerinden Doç. Dr. Basri GÜLBAKAN' ın sorumlu araştırmacı olduğu, Doç. Dr. Fatma Visal OKUR, Prof. Dr. Bekir SALİH, Doç. Dr. Ş. Selin Aytaç EYÜPOĞLU, İnci Cevher ZEYTİN, Hemş. Hacer CÜZDANCI, Prof. Dr. Duygu Uçkan ÇETİNKAYA ve Arş. Gör. Ecz. Yağız ANASIZ ile birlikte çalışacakları GO 16/824 kayıt numaralı ve ***"Akut Myeloblastik Lösemi (AML) Hastalığında Aptamer Temelli Yeni Yüzey Belirteçlerinin Keşfi"*** başlıklı proje için vermiş olduğunuz 15.11.2017 tarihli dilekçeniz Kurulumuzun 21.11.2017 tarihli toplantısında değerlendirilmiş olup, projeye yüksek tez öğrencisi Berna ALKAN' ın eklenmesi ve hücre kültürlerinde lösemi başlatıcı hücrelerin zenginleştirilmesi, akım sitometri ile karakterizasyon ve aptamer profillemesi alt iş paketinin Berna ALKAN'ın yüksek lisans tezi olarak tamamlanması **uygun bulunmuştur**.

1.Prof. Dr. Nurten AKARSU	(Başkan)	10 Prof. Dr. Oya Nuran EMİROĞLU	(Üye)
2. Prof. Dr. Sevda F. MÜFTÜOĞLU	(Üye)	11 Yrd. Doç. Dr. Özay GÖKÖZ	(Üye)
İZİNLİ			
3. Prof. Dr. M. Yıldırım SARA	(Üye)	12. Doç. Dr. Gözde GİRGİN	(Üye)
		KATILMADI	
4. Prof. Dr. Necdet SAĞLAM	(Üye)	13. Doç. Dr. Fatma Visal OKUR	(Üye)
5. Prof. Dr. Hatice Doğan BUZOĞLU	(Üye)	14. Doç. Dr. Can Ebru KURT	(Üye)
İZİNLİ		İZİNLİ	
6. Prof. Dr. R. Köksal ÖZGÜL	(Üye)	15. Yrd. Doç. Dr. H. Hüsrev TURNAGÖL	(Üye)
7. Prof. Dr. Ayşe Lale DOĞAN	(Üye)	16. Öğr. Gör. Dr. Müge DEMİR	(Üye)
8. Prof. Dr. Elmas Ebru YALÇIN	(Üye)	17. Öğr.Gör.Dr. Meltem ŞENGELEN	(Üye)
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Appendix 3. Originality Report

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BENZERLIK ENDEKSI	İNTERNET KAYNAKLARI	YAYINLAR	ÖĞRENCI ÖDEVLERI

BIRINCIL KAYNAKLAR

1	"Expression of CXC Chemokines and Their Receptors Is Modulated during Chondrogenic Differentiation of Human Mesenchymal Stem Cells Grown in Three-Dimensional Scaffold: Evidence in Native Cartilage", Tissue Engineering Part A, 01/2008 Yayın	%1
2	Submitted to University of Sydney Öğrenci Ödevi	<%1
3	"Regenerative Medicine", Springer Nature, 2011 Yayın	<%1
4	Submitted to The Robert Gordon University Öğrenci Ödevi	<%1
5	Diana Hanekamp, Jacqueline Cloos, Gerrit Jan Schuurhuis. "Leukemic stem cells: identification and clinical application", International Journal of Hematology, 2017 Yayın	<%1
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9. CURRICULUM VITAE

I. Personal Details

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III. Skills and Capabilities

- Languagues:

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Second Languagues: English

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Cell culture techniques, cell isolation, basic biochemical research techniques, basic clinical biochemistry applications and methods, media preparation and sterilization techniques, basic staining techniques, isolation techniques, flow cytometry, cytogenetic techniques, Western blot techniques.

- Computer Skills

MS Office Applications

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IV. Academic and Professional Activities

-Conference Abstracts

- İ.Cevher, B. Alkan, D. Uckan Cetinkaya, F.V. Okur, HEMATOPOIETIC DIFFERENTIATION POTENTIAL OF INDUCED PLURIPOTENT STEM CELLS DERIVED FROM OSTEOPETROSIS PATIENTS' PERIPHERAL BLOOD MONONUCLEAR CELLS, Amsterdam, Nerherlands, ISSCR, (2019)

- S. Aygar, İ. Cevher Zeytin, B. Alkan, E. Kılıç, F.V. Okur, D.Uçkan Çetinkaya, Uyarılmış Pluripotent Kök Hücre (uPKH) Bankacılığında Kullanıma Yönelik, Besiyeri Tabanlı Karşılaştırma Çalışması, Kök hücre ve Hücresel Tedaviler Kongresi, İstanbul (2019)

- S. Aygar – İ. Cevher Zeytin – B. Alkan – E. Kılıç – F. V. Okur – D. Uçkan Çetinkaya, Investigating The Metabolıc Remodelling of Early and Late Passaged Induced Pluripotent Stem Cells (IPSCs), VI. Uluslararası Katılımlı Deneysel Hematoloji Kongresi, Gaziantep (2019)

-Relevant Experience:

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