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Differentiation plasticity of hematopoietic cells

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PhD tesis

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CONTENT

Abstrakt (CZ)

Klíčová slova: krvetvorba, regulace diferenciace, liniový přesmyk, akutní leukémie, myelodysplastický syndrom, rezistence na azacitidin

Hematopoéza byla mnoho let považována za nezvratný přímočarý proces, který postupným omezováním liniové plasticity vedl k vzniku zralých krevních elementů. V posledních letech se však objevily práce, které poukazují na nečekaně široký potenciál hematopoetických buněk, kdy i z lymfoidních prekurzorů můžou vzniknout zralé myeloidní buňky a naopak. Za fyziologických okolností je krvetvorba striktně regulována a definitivní osud buněk je ovlivněn mnoha faktory, které vedou ke změnám regulačních sítí zahrnujících transkripční faktory, epigenetické či post-transkripční modulátory. Jakékoli narušení této přísné kontroly, způsobené mutacemi nebo jinými událostmi, ovlivňuje proliferaci a liniovou plasticitu hematopoetických prekurzorů. To může vést ke klonálnímu růstu rozličné signifikance či k leukemogenezi. Změny v jednotlivých dráhách pak mohou ovlivnit i citlivost hematologických malignit na léčbu.

Pro lepší pochopení hematopoetické regulace jsme se rozhodli popsat změny genové exprese v průběhu fyziologického vývoje zdravých lymfoidních a myeloidních buněk, jakož i u buněk leukemických a to pomocí vlastní zjednodušené platformy založené na PCR v reálném čase. V sortovaných hematopoetických progenitorech a jejich maligních protějšcích jsme vyšetřili expresi 95 genů spojených s diferenciací vybraných leukocytárních populací nebo s leukemogenezí. Také jsme zkoumali změny exprese v jednotlivých subpopulacích nově popsané skupiny dětské ALL – leukémie s liniovým přesmykem. Tuto specifickou skupinu jsme dále popsali za použití průtokové cytometrie, detekce Ig/TCR přestaveb, cytogenetických a mutačních analýz a vyšetření metylačního statusu promotoru CEPBA. Nakonec jsme pomocí sekvenování nové generace vyhodnotili mutace v další jednotce ze širokého spektra hematologických malignit – MDS s vysokým rizikem. Opakované odběry vzorků před a během hypometylační léčby azacitidinem nám umožnily vyhodnotit dynamiku mutací v průběhu onemocnění.

V průběhu vývoje jednotlivých linií jsme pozorovali vyšší expresi různých genů. Například *PAX5*, *FOXO1*, *TCF3*, *BCL11A* a další byly více exprimovány v průběhu vývoje B-lymfocytů, zatímco *BCL11B*, *TCF7*, *HOXB4*, *NOTCH3* byly upregulovány v T-buněčném vývoji; během myeloidního vývoje jsme vedle jiných detekovali vyšší expresi genů z CEBP rodiny, *ID2*, *KLF4* nebo *MNDA*. Mezi zdravými a maligními populacemi bylo také pozorováno několik významných rozdílů. Vedle očekávané upregulace genů z rodiny HOX či genu *FLT3* byl nejvýraznější kontrast

zjištěn v úrovni exprese *CCDC26* a *PAWR*, u nichž se předpokládá vliv na léčebnou odpověď u pacientů s ALL. Dále jsme popsali fenomén liniového přesmyku u ALL, který se většinou vyskytuje ve dnech 1 až 33 indukční terapie. U všech pacientů s tímto typem leukémie byla v čase diagnózy detekována membránová exprese CD2, kdežto u pacientů bez liniového přesmyku byla CD2 pozitivita zachycena pouze vzácně. Monocytoidní populace vzniknuvší po liniovém přesmyku vykazovala stejný imunofenotyp i mRNA expresní profil jako normální monocyty od zdravých dárců. V této monocytoidní populaci však byly zjištěny přestavby imunoglobulinů specifické pro danou leukémii, což potvrzuje její leukemický původ. Expresní profilování poukázalo na *CEBPA* jako možný klíčový regulátor transdiferenciace, což dále podpořil průkaz hypometylace *CEBPA* promotoru. V naší kohortě nebyla zjištěna žádná další společná molekulární ani cytogenetická změna. Všechna uvedená expresní data ze zdravých i leukemických vzorků jsou dostupná ve veřejné databázi LeukoStage.org. Nakonec jsme popsali dynamiku mutací u pacientů s MDS během hypometylační léčby a identifikovali několik možností vývoje, které korelují s průběhem onemocnění. Objevili jsme mutace ovlivňující citlivost na azacitidin či přímo selektované terapií. Analýzou sekvenčně odebraných vzorků jsme byli schopni předvídat i blížící se klinický relaps onemocnění.

Abstract (EN)

Key words: hematopoiesis, cell fate regulation, lineage switch, acute leukemia, myelodysplastic syndrome, azacitidine resistance

Hematopoiesis has been for many years seen as a straightforward process based on sequential restriction of cell fate potential leading to production of mature blood cells. In the last decade, however, several works documented an unexpected plasticity of hematopoietic cells with expanded potential of myeloid development from lymphoid progenitors and vice versa. Under physiologic conditions hematopoiesis is tightly controlled and the definite cell fate is denominated by multiple factors that all lead to changes in regulatory networks that include transcription factors, epigenetic changes and post-transcriptional modulations. Any disruption of this strict regulation, caused by mutations or other events, affects the proliferation and lineage fidelity of hematopoietic precursors. This may lead to clonal growth of variable significance or leukemogenesis and may possibly affect the treatment sensitivity of the hematological malignancies.

For better understanding of hematopoietic regulation we described gene expression changes during physiological development of lymphoid and myeloid lineages and in leukemic specimens using our own simplified real-time PCR based platform. We investigated expression of 95 genes connected with lymphoid and myeloid differentiation or with leukemogenesis in sorted hematopoietic progenitors and their malignant counterparts. We also investigated the expression changes in separate subpopulations of the newly described subset of childhood ALL - lineage switching leukemia and confronted them with observations in nonmalignant populations. Further, using flow cytometry, Ig/TCR rearrangements detection, cytogenetics and mutation studies and methylation status of *CEPBA* promoter we described this specific subset within its molecular context including phenotypic and genotypic peculiarities or epigenetic changes. Finally, using massively parallel sequencing we evaluated mutations in another unit from the spectrum of hematological malignancies - high risk MDS. Using sequential sampling before and during hypomethylation treatment with azacitidine we were able to evaluate mutations dynamics within the disease course.

We identified several lineage associated genes being upregulated during particular population development. *PAX5*, *FOXO1*, *TCF3*, *BCL11A* and others were upregulated during B-lymphocytes evolution whereas *BCL11B*, *TCF7*, *HOXB4*, *NOTCH3* are examples of T-cell fate

regulators; besides others CEBP family, *ID2*, *KLF4* or *MNDA* were detected during myeloid maturation. Several significant differences were also observed between healthy and malignant populations. Besides the expected HOX family or *FLT3*, the most striking contrast was detected in expression levels of *CCDC26* and *PAWR*, which both correlate with therapy response in patients with ALL. We further described a rare phenomenon of lineage switch in ALL that mostly occurs within days 1 to 33 of induction regimen. All switching samples were $CD2^{pos}$ at time of diagnosis, which was not true for non-switching leukemias. After the switch, the formed monocytoid population presented with the same phenotype and mRNA expression profile as normal monocytes from healthy controls. However, leukemia specific immunoglobulin rearrangements were detected in this population proving its leukemic provenience. Our expression platform depicted CEBPA as a possible key regulator of the lineage switch, which was further supported by the finding of hypomethylation of *CEBPA* promoter. No other common molecular or cytogenetic markers were identified in our cohort. Using the above mentioned mRNA expression data from healthy and leukemic specimens we prepared publicly available LeukoStage.org database. Finally, we described mutation architecture dynamics in MDS patients during hypomethylation treatment and identified several dynamics patterns correlating with the disease course. We were able to depict mutations that preclude resistance to the demethylation therapy or those selected by the treatment. We were also able to predict ongoing clinical relapse.

List of abbreviations

AL	Acute leukemia
ALAL	Acute leukemia of ambiguous lineage
ALL	Acute lymphoblastic leukemia
AML	Acute myeloid leukemia
ANC	Absolute neutrophil count
AZA	5-Azacitidine
B-ly	B lymphocytes
BM	Bone marow
CFU	Colony forming unit
CLP	Common lymphoid progenitor
CMML	Chronic myelomonocytic leukemia
CMP	Common myeloid progenitor
CR	Complete remision
DEC	Decreasing
DNMT	DNA mehtyltransferase
EGIL	European Group for the Immunological Classification of Leukemia
EPO	Erythropoietin
Ery	Erythrocytes
ETP	Early thymic progenitor
G(M)-CSF	Granulocyte(macrophage) colony-stimulating factor
GMP	Granulocyte-monocyte progenitor
Gra	Granulocytes
HI	Hematological improvement
HSC	Hematopoietic stem cell
CH	Clonal hematopoieis
CHIP	Clonal hematopoieis of indetermined potential
Ig	Immunoglobulin
IL	Interleukin
INC	Increasing
InDel	Insertion or deletion

IWG	International Working Group
MBL	Monoclonal B-lymphocytosis
MDS	Myelodysplastic syndrome
MGUS	Monoclonal gamopathy of undetermined potential
MLPA	Multiplex ligation-dependent probe amplification
Mo	Monocytes
MPO	Myeloperoxidase
MPS	Massively parallel sequencing
MRD	Minimal residual disease
MSC	Mesenchymal stem cell
OS	Overall survival
PB	Peripheral blood
pB/My-AL	Acute leukemia of ambiguous lineage with B-precursor and myeloid phenotype
pB-ALL	B-precursor acute lymphoblastic leukemia
PCA	Principal component analysis
PFS	Progression free survival
PG	Progression
PR	Partial remision
SD	Stable disease
SNV	Single nucleotide variant
ST	Stable
SW-AL	Switching acute leukemia
T-ALL	T-lymphoblastic leukemia
TCR	T-cell receptor
T-ly	T lymphocytes
TPO	Thrombopoietin
VAF	Variant allele frequency
WGS	Whole genome sequencing
WHO	World Health Organization

1 Introduction

1.1 Hematopoiesis

Human blood system contains several different cell types (lineages) with various functions. These cell populations arise from hematopoietic stem cells (HSCs) in the process of hematopoiesis. Herein we will describe hematopoiesis with its basic steps; its regulation by exo- and endogenous signals together with resulting molecular changes that further lead to changes in blood cell progenitors' phenotype.

For a long time hematopoiesis has been seen as a one-directional process of continuous restriction of cell fate potential leading to sequential determination of particular lineages. The first models were thus represented as a hierarchical tree with individual branches containing lineage specific progenitors undergoing further commitment steps to develop particular populations (reviewed by Li et al. 2010). Traditionally, mature blood cells are divided into two main clusters: lymphoid, derived from common lymphoid progenitor (CLP), which is believed to maintain a weak myeloid-forming potential (Doulatov et al. 2010), and myeloid evolving from common myeloid progenitor (CMP). Some works rather suggest formation of common myeloid-lymphoid precursor (Adolfsson et al. 2005), but there is no consensus within the scientific community. Shortly HSCs (that can be subdivided into long-term HSCs with life-long selfrenewal activity and short-term HSCs that reconstitute hematopoiesis for about 6 weeks) give rise to population of multipotent progenitors (MPPs), that maintain their capacity to differentiate into various lineages, but do not retain the self-renewal potential. CMP and CLP arise from this cell population; CMP cells further differentiate into megakaryocytic-erythroid progenitor (MEP) or granulocyte-monocyte progenitor (GMP) giving rise, through different developmental stages, to platelets, and erythrocytes or granulocytes and monocytes respectively (Figure 1). CLPs give rise to B-lymphoid progenitors with further differentiation into B-lymphocytes (B-ly) or, after migration to thymus (together with MPPs), they found T-lymphoid (T-ly) lineage leading to different T-ly subpopulations (Fiedler and Cornelia 2012)

The commitment into one of the myeloid or lymphoid lineages was thought to be strict and irreversible. However, in last years several works documented an increased potential of myeloid lineage development from lymphoid progenitors or even from mature blood cells after specific molecular hits (Buske et al. 2001; Kondo et al. 2000), thus conferring unexpected plasticity to the hematopoietic cells. Whether the lineage switches or infidelity markers on hematopoietic cells are present in hematopoiesis under physiological or pathological non-malignant conditions remains

unknown, as well as is the possible regulation of this behavior, although several extrinsic and intrinsic factors are being discussed as will be discussed below.

Hematopoiesis under physiological conditions is believed to be precisely regulated on multiple levels in order to sequence adequately proliferation and maturation. The definite cell fate is denominated by the microenvironment, cell-cell interactions, hormones and cytokines – all leading to changes in expression profiles – via several regulatory levels (transcription factors and modulators, epigenetic changes, post-transcriptional regulations including non-coding RNA etc.). All these regulatory pathways are described below, since their understanding is important for comprehending of how particular hematopoietic lineages are formed; how the cell fate is orchestrated and what stays behind different physiological and pathological changes.

1.1.1 **(Micro) environmental regulation**

The evolution of particular lineages is regulated by variety of cyto- and chemokines in respond to current need of the organism under various physiological or pathological conditions. It is also affected by cell-cell contact in hematopoietic tissue or by paracrine regulation via primarily non-hematopoietic supportive cells.

One well known example of hematopoietic regulation depending on extrinsic conditions is induction of erythropoiesis during hypoxia. Hypoxia leads (through von Hippel-Lindau/Hypoxia Inducible Factor (HIF)/prolyl-4-hydroxylase domain oxygen-sensing pathway) to decreased degradation of HIF-α subunits, which then bind to hypoxia-sensitive enhancer of erythropoietin (EPO) gene resulting in upregulation of EPO expression. This increases serum EPO levels up to several hundred folds. EPO binds to its homodimeric receptor (EPO-R), which is also hypoxia inducible, and its main action lies in prevention of apoptosis of erythroid cells - after binding to its receptor, EPO triggers PI3K/Akt and MAPK activation that together with STAT5 and downstream Id1 and JAK2 upregulation ensure erythroid progenitors renewal and differentiation (reviewed in Haase 2010).

Similarly, thrombopoietin (TPO) maintains the platelets level. TPO binds to its receptor MPL (myeloproliferative leukemia protein, also known as CD110) on hematopoietic progenitor cells, megakaryocytes and platelets and is internalized and destroyed, which is one of the mechanisms of regulating TPO serum levels by platelet count. Its production is also increased by interleukin-6 (IL6), which explains increased platelet levels after inflammatory stimuli; BM stromal cells also increase their TPO production during thrombocytopenia, although this

regulatory mechanism is not clearly understood. Binding of TPO to MPL activates signal transduction that includes STAT3, STAT5 and JAK2 and PI3K/Akt or Ras activation. These pathways then regulate MEP proliferation, differentiation towards megakaryocytes and increase in lineage-specific markers expression, such as glycoprotein Ib or IIb/IIIa (reviewed in Hitchcock and Kaushansky 2014).

Myelomonocytic differentiation is regulated by G-CSF (granulocyte-colony-stimulating factor, M-CSF (monocyte-CSF) and GM-CSF (granulocyte-macrophage-CSF), synergistically with IL3 (historically also called multi-CSF). Production of all these CSFs is activated by various immune and inflammatory stimuli and some of them (GM-CSF) were also described to be expressed by leukemic cells in autocrine manner. They stimulate proliferation and maturation of myeloid progenitors giving rise to monocytes, neutrophils and eosinophils. GM-CSF also enhances functions of mature effector cells such as cytokine secretion, phagocytosis (reviewed in Gasson 1991; Leonard et al. 1988). G(M)-CSF stimulation leads to activation of MYC, STAT5 and STAT3 thus mediating myeloid progenitor proliferation as well as CEBPA upregulation that leads to further myeloid differentiation (Johansen et al. 2001; Zhang et al. 2010). The well known differentiation shift triggers are presented within the hematopoietic tree model on Figure 1.

The lymphoid commitment is mostly reported to be driven by IL7 after its binding to IL7 receptor (IL7-R) on non-committed progenitor cells (Dias et al. 2005), although some reports challenging this observation were published (Tsapogas et al. 2011). Further activation of IL7-R binding in CLP (and lymphoid-primed MPP) in BM enhances B-ly program initiation via activation of STAT5 signaling and increase of EBF-1 expression (Tsapogas et al. 2011).

The main site of T-ly formation is thymus, which is believed to provide specific microenvironment with particular chemokines' stimulation, although extrathymic T-ly differentiation was also described (Zepponi et al. 2015). T-ly progenitors in thymus show strong response to thymus-expressed chemokine, chemokine (C-X-C motif) ligand 12 (CXCL12) or IL7 (Bleul and Boehm 2000). Entry into the defined thymic microenvironment activates NOTCH pathway, which in turn triggers T-ly differentiation (Harman et al. 2003).

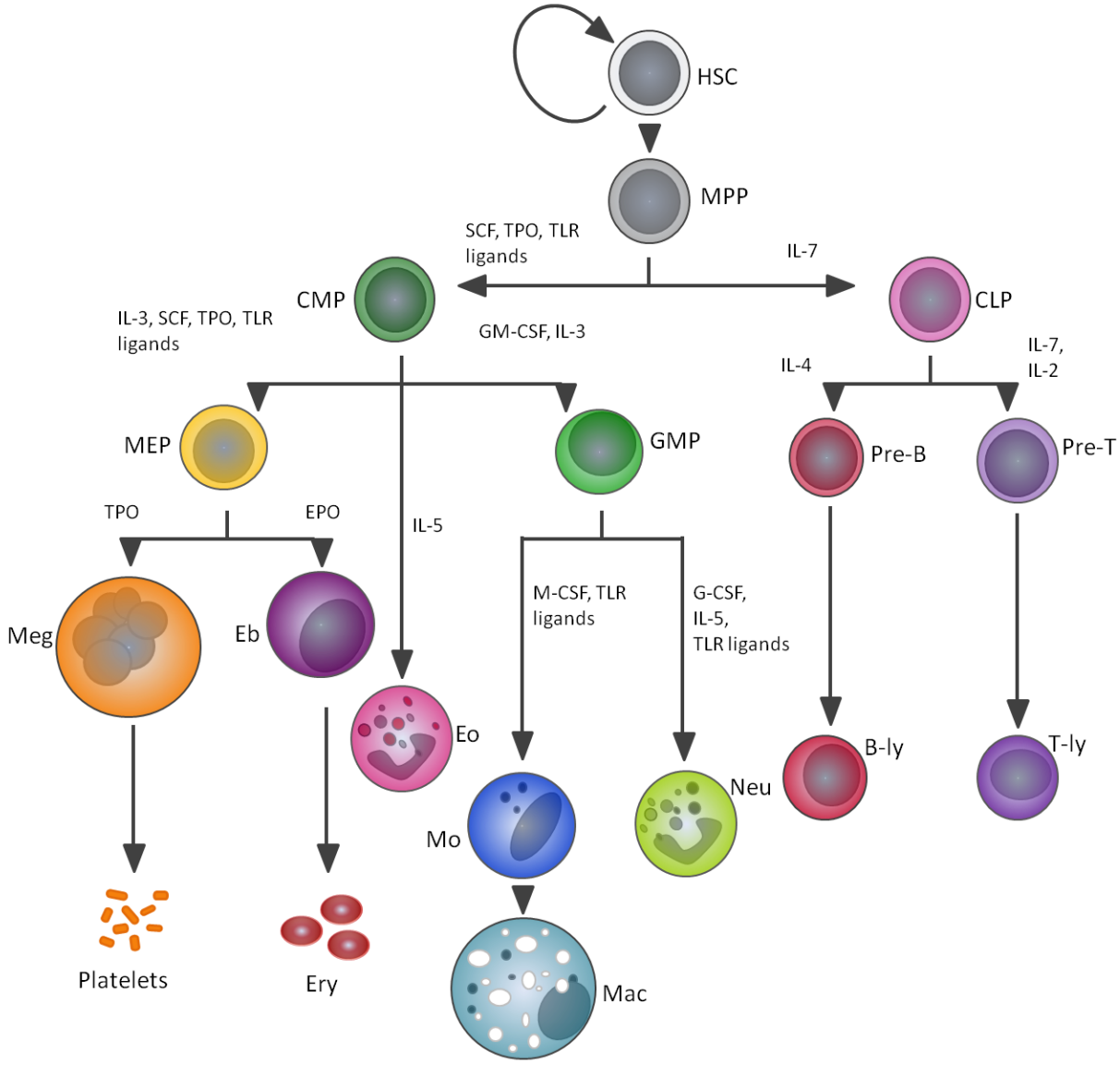


Figure 1. Hematopoiesis model outlining cyto- and chemokines that shift the precursors towards the differentiation of particular lineages. HSC – hematopoietic stem cell; MPP - multipotent progenitor; CLP - common lymphoid progenitor; CMP – common myeloid progenitor; GMP - granulocyte-monocyte progenitor; MEP - megakaryocyte-erythroid precursor; Meg - megakaryocyte; Eb - erythroblast; Ery - erythrocyte; Eo - eosinophil; Mo - monocyte; Mac - macrophage; Neu – neutrophil; B-ly – B lymphocyte; T-ly - T-lymphocyte; IL - interleukin; TPO - thrombopoietin; EPO - erythropoietin; TLR -Toll like receptor; SCF - stem cell factor; G(M)-CSF - granulocyte(-macrophage) colony stimulating factor (adopted from Graf 2002).

Bone marrow (BM) is the major site of hematopoiesis in humans. Besides HSCs that ensure durable blood cell production, it contains different other cells that are discussed to affect hematopoiesis, such as mesenchymal stem cells (MSCs), osteoblasts, adipocytes, endothelial cells,

neuronal and glial cells. These are thought to contribute to hematopoietic homeostasis via CXCL12, stem cell factor (SCF), angiopoietin, thrombopoietin (TPO), tumor growth factor β (TGF-β), IL-8, IL-3, colony forming factors (CSFs) and other chemo/cytokines which play crucial role in maintaining HSCs functions – retention in BM, quiescence and repopulating activity (Anthony and Link 2014; Mattiucci et al. 2018; Naka and Hirao 2017; Tzeng et al. 2011).

Changes in the BM niche were also described within different hematologic malignancies such as myelodysplastic syndrome (MDS) (Abbas et al. 2017; Medyouf et al. 2014), chronic myeloid leukemia (CML) (Aggoune et al. 2017; Anderson et al. 2013), acute myeloid leukemia (AML) (Huang et al. 2015; Konopleva et al. 2002) or acute lymphoblastic leukemia (ALL) (Johnson et al. 2016) suggesting that BM microenvironment support proliferation and survival of malignant cells and thus it may contribute to the pathogenesis or therapy resistance of particular diseases. Malignant cells may be protected from cell death induced by cytotoxic therapy by specific interaction with stromal cells, the C-X-C chemokine receptor type 4 (CXCR4) and CXCL12 interaction being considered to be most important (Vianello et al. 2010). Changes in BM microenvironment are also believed to mediate defects of normal hematopoiesis that are often seen in hematopoietic malignancies. This is in contrast with first explanation of cytopenias based on the concept of anatomic "crowding out" of normal hematopoietic progenitors by rapidly growing malignant cells, which, however, was not able to explain suppression of normal hematopoiesis occurring also in relatively low tumor burden (Colmone et al. 2008; Lagneaux et al. 1993).

Lagneaux et al. (1993) showed high TGF-β production and low IL-6 secretion of BM stromal cells in patients with chronic lymphocytic leukemia leading to insufficient support of colony forming unit (CFU) formation. Changes in TGF-β and subsequently in IL-6 secretion in BM were observed also in other B-lymphoid malignancies (Yasui, Hideshima, and Anderson 2008).

Malignant cells can also directly affect hematopoietic progenitors proliferation and commitment through soluble factors. It was proven that leukemic cells were able to produce SCF and thus causing atypical migration of $CD34^{pos}$ progenitors into the malignant niche (Colmone et al. 2008). $CD34^{pos}$ progenitors from this study showed also impaired mobilization from BM after the granulocyte colony stimulating factor (G-CSF) treatment and were also less responsive to their natural chemoatractant CXCL12. However, the exact reason of the decreased responsiveness of hematopoietic progenitors to particular cytokines or their decline after homing in atypical malignant microenvironment remains unclear.

Lastly, it was shown that malignant cells are able to change their non-malignant counterparts' phenotype inducing epigenetic changes similar to theirs (Werbowetski-Ogilvie et al. 2011). These changes are thought to be mediated by microparticular transport with protein, mRNA and non-coding RNA cargo and are possibly included in the biased malignant microenvironment. As seen above, all extracellular signals initiate a cascade of intracellular changes with activation or inhibition of different kinases' pathways that again lead to changes in expression profiles all together leading to changes in blood cell formation.

1.1.2 **Transcription factors**

The lineage determining programs are defined by regulated networks of different "master" transcription factors (TFs). TFs regulate other genes expression, coordinate cell division, apoptosis and differentiation and orchestrate the developmental changes accompanying particular lineages formation.

From the early differentiation regulators, the PU.1 transcription factor is the best described. It is a key differentiation regulator, which can interact with a variety of different molecules, such as GATA-1, GATA-2, RUNX1, IRF-4, IRF-8, CEBPA, CEBPB and others. Intensity of its expression delineates the alignment of the cell towards particular lineage (Iwasaki et al. 2005).

High PU.1 expression levels drive myeloid differentiation (Nerlov and Graf 1998). PU.1 interacts with GATA1 inhibiting its ability to activate erythroid genes. PU.1 coexpression with CEBPA (or CEBPB in stress hematopoiesis) leads to granulocyte-monocyte progenitor (GMP) formation. CEBPA as a key regulator, further targets downstream genes, such as *GFI1*, *CSF3R* or *CEBPE*, thus regulating proliferation and granulocytic differentiation. CEBPA together with PU.1 also upregulates downstream myeloid genes such as for lysosyme or CD13 and together with CBF and c-MYB, other key regulators, activates MPO expression. The expression of erythroid genes during myeloid maturation is also inhibited by MAFb by its interaction with Ets-1. During granulopoiesis GFI1 further restricts activity of monocytoid genes such as *CSF1* and *CSF1R* favoring granulopoiesis over monopoiesis (Friedman 2007; Wang et al. 2001).

In high levels PU.1 interacts with IRF8 and AP-1 family transcription factors and activates monocyte specific genes. Also, heterodimerization of AP-1 with CEBPA in high PU.1 levels leads to decreased CEBPA activity thus favoring monopoiesis (reviewed in Friedman 2007). The schematic overview of myeloid differentiation, including not only key TFs but also extrinsic signals and micro-RNAs (miRs) is delineated on Figure 2A.

Decreased *PU.1* expression together with high expression levels of *GATA-1*, *GATA-2* and their interaction with FOG1 lead to MEP formation via repression of *PU.1* expression (Mancini et al. 2012; Cobaleda et al. 2007). GATA-1 and GATA-2 are the key TFs that orchestrate erythroid and megakaryocytic differentiation. GATA-2 is expressed in both, erythroid and megakaryocytic progenitors, but its enforced expression favors differentiation towards megakaryocytic lineage. During erythroid maturation GATA-2 is downregulated by GATA-1 (which itself is a GATA-2 target). This downregulation is mediated by interaction between FOG1 and nucleosome remodeling and histone deacetylase (NuRD) complex. After that, GATA-1 replaces GATA-2 on the β-globin locus and induces up-regulation of hemoglobin genes expression. The GATA regulatory complex also includes LMO2, SCL, E2A and LDBM which primarily activate the GATA regulated genes (Doré and Crispino 2011). Erythroid maturation with the most important regulators including TFs, extrinsic/microenvironmental factors and miRs is outlined on Figure 2B.

Low level of PU.1 favors CLP development, where PU.1 is involved in regulation of components of IL-7 signaling pathway (DeKoter et al. 2002). After upregulation of Ikaros with further expression of GFI, the PU.1 expression is antagonized. These changes, together with upregulation of E2A and EBF1 lead to expression of further B-specific genes such as *POU2AF1* and *FOXO-1* (Zandi et al. 2008). With further PAX5 expression the B-lymphocyte fate is thought to be definitive and the progenitor loses it T-cell development potential, see Figure 3A. PAX5 also antagonizes development into other than B-ly lineage repressing non-B-ly specific genes and further activating B-ly developmental program. PAX5 remains expressed in mature B-ly and its deletion from mature cells lead to their dedifferentiation to immature progenitors with unrestricted developmental potential but also with risk of malignant transformation (Cobaleda et al. 2007).

CLP are also able to enter the thymus forming early thymic progenitors which still retain multilineage potential. However, thymic niche with increased IL-7, Kit-ligand and Notch activating ligands blocks alternative cell fates and induces T-ly development (Petrie and Zuniga-Pflucker 2007). The T-ly commitments is associated with upregulation of TCF-1 which then further induces GATA-3 and BCL11b expression and represses the NK cell development. The decision process of $CD4^{pos}CD8^{pos}$ T-ly stage is then driven by thPOK (regulated by GATA-3) which leads to $CD4^{pos}$ T-helpers and RUNX3 (regulated by IL-7 and STAT5) leading to $CD8^{pos}$ transcription factors activation (Park et al. 2010; Wang et al. 2008) (Figure 3B).

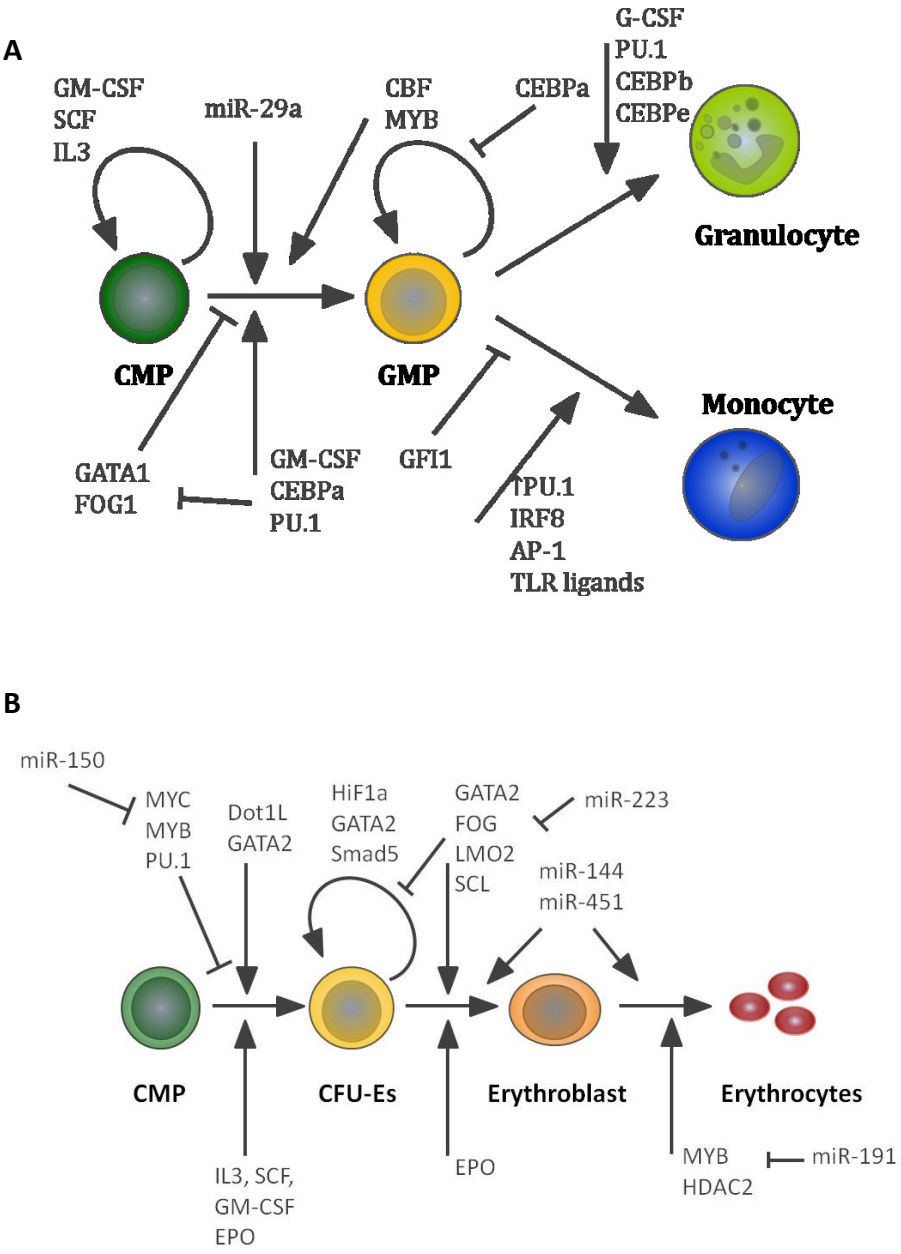


Figure 2. Scheme of maturation of granulocytes and monocytes (**A**) and erythrocytes (**B**) with basic regulators orchestrating the specific lineage fate including growth factors/cytokines, transcription factors or miRNAs. Arrows represent activation of particular lineage program, cut lines indicate repression, and cyclic arrows indicate maintenance and self-renewal.
CMP - common myeloid progenitor; GMP - granulocyte-monocyte progenitor; CFU-E - colony forming unit-erythrocyte; miR – micro RNA; IL3 - interleukin 3; SCF - stem cell factor; G(M)-CSF - granulocyte(-macrophage) colony stimulating factor; EPO -erythropoietin. Adopted from Navegantes et al. 2017 and Hattangadi et al. 2011.

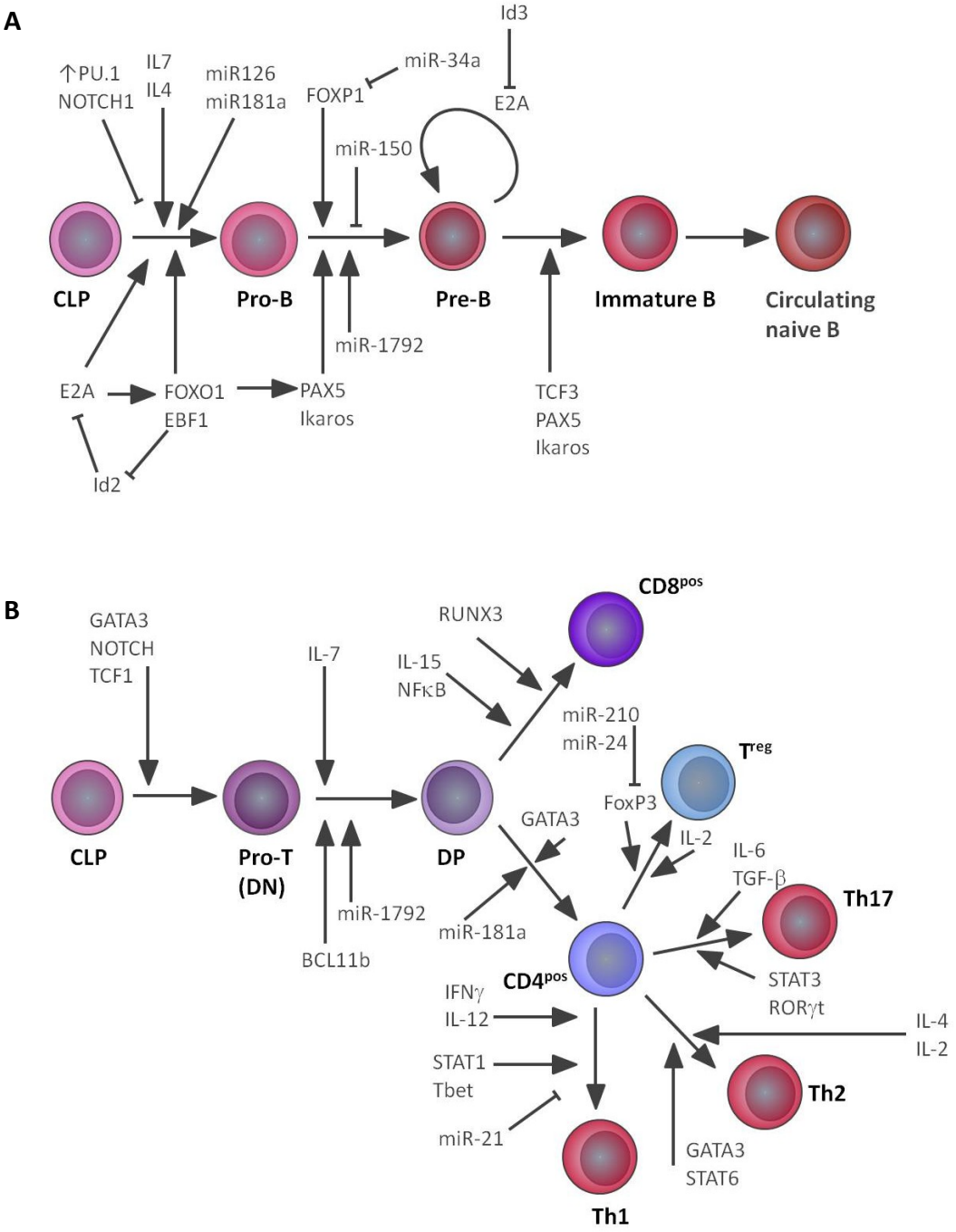


Figure 3. Maturation scheme of B-lymphocytes (**A**) and T-lymphocytes (**B**) including basic regulators orchestrating the specific lineage fate such as growth factors/cytokines, transcription factors or miRNAs. Arrows represent activation of particular lineage program, cut lines indicate repression, cyclic arrows indicate maintenance and self-renewal.

CLP - common lymphoid progenitor; DN - double negative ($CD4^{neg}CD8^{neg}$) T-lymphoid precursor, DP - double positive ($CD4^{pos}CD8^{pos}$) T-lymphoid precursor; Th - T-helper lymphocyte; T^{reg} – T-regulatory lymphocyte; IL – interleukin; miR - microRNA. Adopted from Somasundaram et al. 2015 and Rothenberg 2014.

1.1.3 **Epigenetic regulation**

Epigenetic modifications represent an important part of hematopoiesis regulation since different structural conformations of chromatin, depending on epigenetic modifications, may affect the accessibility of DNA for transcription factors. Epigenetic control mechanisms include DNA methylation, histone modification and chromatin remodeling and in specific context also microRNA regulation.

1.1.3.1 DNA methylation

DNA methylation of cytosine at CpG dinucleotides is essential for normal development and is maintained by DNA methyltransferases (DNMTs). DNMT1 is responsible for maintenance of existing methylation pattern during replication, whereas DNMT3a and DNMT3b are responsible for *de novo* methylation. Another regulatory level altering DNA methylation status is represented by oxidizing 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) by methylcytosine-dioxygenases from TET family (Fiedler and Cornelia 2012).

Several works showed that DNA methylation underwent significant changes during cell differentiation (Broske et al. 2009; Meissner et al. 2008). It was also shown that absence of normal methylation regulation by corrupting DNMT1 function led to defect in HSCs survival and absence of the differentiation potential. However, decreased but present DNMT1 activity led to hypomethylation in HSCs with decreased self-renewal ability and shift to myeloid-erythroid differentiation in oppose to lymphoid development (Broske et al. 2009). DNMT3a and DNMT3b have partially overlapping functions in hematopoiesis and both are most highly expressed in long term HSCs (Challen et al. 2014). Challen et al. (2011) also showed that DNMT3a and DNMT3b were crucial for HSCs self-renewal/differentiation decision with DNMT3a loss being more dramatic compared to DNMT3b loss. The DNMT3s loss of function led to changes in DNA methylation pattern resulting in interruption of normal DNMT3s mediated repression of the stem cell genetic network and blocked differentiation (Challen et al. 2011, 2014).

Changes in 5-hmC distribution that correlated with gene transcription were also described during myeloid and lymphoid development (Tekpli et al. 2016). Decrease of 5-hmC in loss of function of TET2 (which catalyzes oxidation of 5-mC to 5-hmC) led to an increased pool of HSCs due to their aberrant self-renewal and expansion and also skewed their differentiation towards the granulo-monocytoid lineage at the expense of lymphoid and erythroid ones (Ko et al. 2011). This was in line with the finding that disruption of TET2 function in mice induced chronic myelomonocytic leukemia (CMML)-like disease (Moran-Crusio et al. 2011).

Since TET2 function is α-ketoglutarate dependent it may also be disrupted by mutations R132 of *IDH1* or R140 and R172 of *IDH2* leading to neomorphic enzymatic activity of IDH (isocitrate dehydrogenase) aberrantly catalyzing the NADPH-dependent reduction of α-ketoglutarate to (D)-2-hydroxyglutarate. (D)-2-hydroxyglutarate could then potentially interfere with Fe(II) binding and destabilize the intermediate product of 5-mC to 5-hmC conversion. This results into inhibition of 5-mC hydroxylation and further demethylation and thus to hypermethylation status in *IDH* mutated cells. *IDH1* and *IDH2* mutations with resulting hypermethylation were observed in AML (Figueroa et al. 2010).

1.1.3.2 Histone modifications

Another important regulation mechanism is the posttranslational histone modification which includes methylation, acetylation, phosphorylation and sumoylation. These modifications change the nucleosome mobility and turnover and affect gene expression. Methylation of lysine 4 and 27 (K4 and K27) on histone 3 (H3) is of particular interest. Presence of both, activating (H3K4Me3) and repressing (H3K27Me3) histone modification marks a poised state for gene transcription (Zentner and Henikoff 2013). Thus enzymes and other proteins (such as polycomb and trithorax proteins) involved in histone de/methylation are believed to play significant role in hematopoiesis regulation. A histone demethylase KDM2B (which is in HSCs expressed preferentially compared to KDM2A) was proven to comprise to the regulation of stem cell maintenance and lineage commitment and its loss is connected to downregulation *Tcf7, PAX5, IKZF1* or *Gfi1b* and compromises lymphoid differentiation (Andricovich et al. 2016). Also, its overexpression was proven in ALL more than AML and silencing led to downregulation of MYC or EZH2 pathways (Andricovich et al. 2016; Kuang et al. 2017).

EZH2 is the catalytic subunit of Polycomb repressive complex 2 (PRC2) responsible for K27H3 methylation which mediates a higher repressive state of chromatin thus inhibiting gene expression (Margueron and Reinberg 2011). PRC2 (together with PRC1) plays important role in hematopoiesis since it promotes pluripotency maintenance and regulates cell fate decision (Herviou et al. 2016). In myeloid progenitors it inhibits definitive differentiation programs and its deletion may decrease leukemogenic activity in leukemic stem cells (Tanaka et al. 2012). EZH2 is also an important regulator of lymphopoiesis, since it interacts with STAT5. It also blocks DNA repair response pathway in activated B-cells after AID (activation-induced cytidine deaminase) activation and hypermutation of Ig genes (Caganova et al. 2013). Its overexpression is related to wide spectrum of hematologic malignancies, such as non-Hodgkin lymphomas, ALL, AML and high-risk MDS (McCabe et al. 2012; Tanaka et al. 2012; Xu et al. 2011).

ASXL1, a human homolog of Drosophila Additional sex combs (Asx), serves as important regulator of both polycomb and trithorax proteins thus playing role in activating and silencing of gene expression (Katoh 2013). Its loss-of-function mutations lead to loss of PRC2-mediated K27H3 methylation which is associated with upregulation of HOXA genes in HSCs and promotion of leukemogenesis (Omar Abdel-Wahab et al. 2012). Indeed, in AML and MDS patients, mutations of *ASXL1* are observed and are related to adverse prognosis (Chen et al. 2014).

1.1.3.3 Chromatin remodeling

Additional epigenetic regulation of hematopoiesis is based on chromatin remodeling complexes which are able to change nucleosome formation location and also may include proteins with histone de/acetylase activity (Bowen et al. 2004). These complexes include Mi-2/nucleosomal remodeling and deacetylase (NuRD), the imitation switch (ISWI) and SWI/SNF (SWItch/Sucrose Non-Fermentable)-family (Vignali et al. 2000).

SWI/SNF proteins form complexes that both repress and activate different gene subsets on ATPase-dependent manner. These multi-protein complexes contain one of the two catalytic ATPase subunits, that are mutually exclusive – BRM (brahma, also known as SMARCA2) and BRG1 (brahma-related gene, also known as SMARCA4). The SWI/SNF complexes are capable of altering nucleosome position along DNA and can promote binding of transcriptional activators and also play role in formation of pre-initiation and elongation complexes (reviewed in Wilson and Roberts 2011); besides transcription they also affect DNA replication, recombination or repair (Wu et al. 2017). The role of SWI/SNF complexes in mammalian development was shown by $Brg1^{-/-}$ mice. $Brg1^{-/-}$ embryos die at peri-implantation stage of the development and conditional knock-outs have defects in erythropoiesis, T-lymphopoiesis, angiogenesis or limb-development (Gebuhr et al. 2003; Griffin, Brennan, and Magnuson 2008). It was proven, that BRG1 is necessary for maintaining the self-renewal ability of embryonic stem cells but participates also on their lineage commitment (X. Zhang et al. 2014). Conditional deletion of other subunit of this complex (BAF53a) lead to BM failure and aplastic anemia mediated due to changes in SWI/SNF target regulating cell-cycle and apoptosis thus disrupting the HSCs pool maintenance (Krasteva et al. 2012). Bakshi et al. (2010) proved that SWI/SNF complex interacts with RUNX1 and by association with its target genes' (such as *CSF2*, *CSF1-R*, *IL3* or *p21*) promoters regulates their transcription. Also, SWI/SNF complex enables CD79a expression in presence of EBF1 and PAX5 (Ramirez and Hagman 2009).

Mi-2/NuRD is a family of complexes that are assembled around catalytic core proteins consisting of helicase or ATPase subunits of the chromodomain helicase DNA-binding domain

(CHD), which beside of mobilizing nucleosomes may participate in scaffolding of other components of the complex, again mostly by ATP-dependent manner (Dege and Hagman 2014). CHD4 is highly expressed in HSCs and lymphoid as well as myeloid early progenitors (Kim et al. 1999). Other described subunits are metastasis-associated factors (MTA1-3) that are present in mutually exclusive fashion within Mi-2/NuRD complexes and contribute to its specialized functions. MTA1 and MTA2 interact with variety of TFs including FOG1 and FOG2, thus recruiting NuRD complexes to GATA TF family member and repressing gene transcription in erythroid cells. Mi-2/NuRD complexes are also associated with DNA methylation via their association with methyl-binding-domain (MBD) protein family, namely MBD2 and -3 (Dege and Hagman 2014). Mi-2/NuRD mediated regulation is required for HSCs self-renewal, thus maintaining the HSCs pool and regulation of multilineage differentiation (Yoshida et al. 2008). It was shown to regulate B-ly and T-ly differentiation, for example, by its association with Ikaros, which leads to repression specific genes expression and promotes lymphoid differentiation (Kim et al. 1999) or together with SWI/SNF (in opposite manners) it mediates the effect of EBF1 and PAX5 activation during early B-cell development (Gao et al. 2009).

The ISWI family was shown to regulate nucleosome spacing, DNA damage repair, transcription and replication. ISWI complexes are composed of 2-4 subunits that mediate ATPase activity or interactions with DNA, DNA binding proteins, nucleosomes or other structures within nuclei (Corona et al. 1999). It was shown that the ISWI nuclear ATPase SMARCA5 was required for proliferation of HSCs and for erythroid commitment (Kokavec et al. 2017) and it was upregulated in AML (Stopka et al. 2000). SMARCA5 together with CCCTC-binding factor and members of the Cohesin complex also associates with PU.1 thus regulating its expression and myeloid differentiation program (Dluhosova et al. 2014).

1.1.3.4 Micro RNA

MicroRNAs (miRs) are small (~22 nucleotides (nt)) non-coding single-stranded RNA molecules which are able to repress translation of mRNA by binding to 3' untranslated regions of their specific targets leading to their degradation or just translation repression, thus serving as negative post-transriptional regulators of gene expression. They add further layer of epigenetic regulation of lineage restriction process. It is estimated that miRs constitute at least 1 % of all predicted genes in mammals (Lim et al. 2003), and they were identified to play role in development, cell differentiation, proliferation, apoptosis or malignant transformation (Cai et al. 2009). The miRs' biogenesis starts with transcription of long primary transcript that may contain one or more hairpins since miRs may be clustered together giving rise to polycistronic

transcriptional units; then the hairpin is excised from this primary transcript by Microprocessor complex resulting in pre-microRNA (60-110 nt) that is actively transported from nucleus to cytoplasm. In the cytoplasm pre-miR is further processed by RNA endonuclease enzyme Dicer to mature miRNA which after losing one complementary (also called passenger) strand is loaded onto the RNA-induced-silencing complex (RISC) (Lee et al. 2002). There are currently 1917 human miR sequences assigned to the microRNA database (mirnabase.org) and the number is still rising. Several works proved the importance of miRs as ubiquitinous regulators of cell cycle, apoptosis, proliferation or differentiation (reviewed in Carleton, Cleary, and Linsley 2007 and Mens and Ghanbari 2018). This is true also for the process of hematopoiesis, where conditional knockout of Dicer leads to increased apoptosis of HSCs which however can be rescued by single miR – the miR-125a that downregulates proapoptotic protein Bcl2-antagonist/killer1 (Bak1) (Guo et al. 2010). Other molecule, miR-196b is responsible for maintaining HSCs in undifferentiated state by regulating *HOXA8* and *HOXB8* genes (Lazare et al. 2014). MiR-196b is regulated by MLL and its overexpression leads to increased proliferation capacity and survival with blocked differentiation, suggesting its potential role in leukemogenesis (Popovic et al. 2009).

MiR-196b and also miR-21, which are the key molecules that orchestrate myelopoiesis are downregulated by GFI1. The downregulation of miR-196b is necessary to direct progenitor cells towards myeloid differentiation; miR-21 is believed to regulate monocytic differentiation (Velu, Baktula, and Grimes 2009). Myeloid differentiation is further regulated by miR-223 (Figure 2A). This molecule was described as lineage determining as it is upregulated by CEBPA and its levels are increased during granulopoiesis, but not monopoiesis (Fazi et al. 2005). It is believed to target MEF2 and to regulate not only myeloid progenitor proliferation and differentiation, but also the function of mature granulocytes (Johnnidis et al. 2008). Another crucial molecule in deciding granulocyte versus monocyte cell fate is miR-34a also regulated by CEPBA and favoring granulopoiesis (Pulikkan et al. 2010). Monocytic differentiation is stimulated by miR-424 that is regulated by PU.1 and inhibits NFI-A whose decrease is necessary for activation of CSF1 expression (Rosa et al. 2007); and by miR-17-5p and miR-106a that suppress RUNX1 and thus also stimulate CSF1 expression (Fontana et al. 2007).

During erythropoiesis, miR-223 was described to inhibit LMO2, a key factor in erythrocytic differentiation (Felli et al. 2009), see Figure 2B. Other crucial molecules are miR-144 and miR-451 which increase during erythroid differentiation. Their effect is mediated by inhibition of RAB14, a member of Ras family which negatively regulates human erythropoiesis (Kim et al. 2015). On the contrary, miR-150 by targeting MYB, shifts the development of

megakaryocytic-erythrocyte progenitors towards megakaryocytes on expend of CFU-e and also induces apoptosis in erythroid progenitors (Sun et al. 2015).

In lymphopoiesis miR-17-92 cluster is of particular importance since it targets pro-apoptotic factors Bim and Pten thus inhibiting apoptosis of pro-B cells and allowing their transition towards pre-B cells (Lai et al. 2016). Its upregulation is connected to lymphoproliferative diseases and autoimmunity (Xiao et al. 2008). Similar effect is observed in T-lymphoid development where miR-17-92 is involved in regulation of double negative stage (DN, i.e. $CD4^{neg}CD8^{neg}$) to double positive (DP, i.e. $CD4^{pos}CD8^{pos}$) (Saki et al. 2015). In B-lymphoid development premature expression of miR-150 and miR-34a (leading to deregulation of MYB and FOXP1 respectively) blocks transition from pro-B to pre-B cells (Rao et al. 2010; Zhou et al. 2007). Later stages of B-ly development are affected by changes in miR-155 expression. B cells lacking miR-155 generated reduced responses upon antigen stimulation which correlated with reduced IgG rearrangements (Figure 3A). This effect is mediated by PU.1 targeting (Vigorito 2007). In T-lymphocytes, latter developmental stages leading to different (sub)populations of $CD4^{pos}$ and $CD8^{pos}$ T-ly include miR-125 that regulates expression of IL2 receptor β or IFNγ, miR-181a that is involved in positive as well as negative selection in thymus, miR-155 which by targeting MAF enhances Th1 development on extend of Th2 or MiR-146a that by affecting IRAK1, STAT1 or TRAF6 regulates Th1 and T^{reg} responses (reviewed in Saki et al. 2015).

As seen above, the continuously increasing family of miRs together with their targets that include TFs, growth factors, their receptors and other molecules form a delicate regulatory net that orchestrates self renewal and differentiation of hematopoietic precursors leading to formation of all blood cells according to actual need. Interruption of this regulation leads to various defects in terms of apoptosis that may cause cytopenias, but also increased survival, proliferation or differentiation blocks that may result to leukemias; lastly malfunction of this regulation may cause defective immune responses leading to immune compromise and/or autoimmunity.

1.1.4 **Transdifferentiation**

In last three decades several papers describing switching of differentiated cells into various hematopoietic lineages were published (Graf 2002), see Figure 4, which also includes known molecular hits. These switches were mostly induced by different molecular or genetic hits. It was proved that pre-B lymphoma cell line may be transformed into macrophages by treatment with the demethylation agent 5-azacitidine (AZA) or by induction of concomitant *c-myc* and *v-raf*

overexpression. These lymphoma-derived macrophage-like cells were able to phagocyte particles, expressed different myelomonocytic markers but also retained immunoglobulin rearrangements characteristic of the original cells ruling out the possibility of contamination of the culture with variable cell lineages (Boyd and Schrader 1982; Klinken et al.1988). *MYC* is also involved of another model of lineage infidelity, where the authors created transgenic mouse line containing *MAX* gene (*MYC* associated factor X) which after crossing with Eμ-*myc* mice (Harris et al. 1988) resulted in Eμ-*myc*/*max* 41 animals that besides developing pre-B cell lymphomas contained elevated levels of granulocytes. Obtained lymphomas were transplantable and again gave origin to higher numbers of granulocytes. These again included the original cells immunoglobulin rearrangements (Lindeman et al. 1994).

Common lymphoid progenitors (CLP) were also proven to be highly plastic. Ectopically expressed human IL-2 receptor β-chain (IL-2Rβ) in mice CLP coupled with IL-2 common γ chain forming functional IL-2 receptor. The treatment of these modified CLP with IL-2 then induced granulocytic and macrophage rather than B-cell differentiation (Kondo et al. 2000). Similar results were observed after IL-2 treatment of pro-T cells from thymus, but not in case of IL-2Rβ-pro-B cells from BM from the same mice (Kondo et al. 2000).

Most of above mentioned examples of observed lineage switches were performed in transgenic animals, thus raising the question, whether similar transdifferentiation could be observed also without such molecular hits. However, several works suggested that this phenomenon was not only restricted to cells with abnormal growth properties and that subset of B-ly residing mainly peritoneal and pleural cavities and, in lesser extent, spleen (so called B-1 cells) indeed was able to transform into macrophage-like cells (Popi 2015). It is believed that in this B-ly subset Ikaros, a zinc-finger transcription factor, plays an important role regulating their lineage infidelity (Oliveira et al. 2018; Popi 2015).

There were several works reporting fetal B-macrophage progenitors (Mebius et al. 2001). Montecino-Rodriguez et al. (2001) proved such cells also in adult mice BM. In their experiments they show a subset of $CD45R^{neg}$ $CD19^{pos}$ BM cells expressing early lymphoid antigens (CD127, AA4.1) and B-ly progenitor potential but lacking T –ly, NK cell or multipotent myeloid potential. Under specific cytokine conditions, these cells were, besides B-ly, also able to differentiate into macrophages *in vitro*.

Similarly, enforced transcription of particular genes may change the predicted lineage of megakaryocytic/erythroid progenitors (MEP) into myeloid and vice versa. For example, enforced expression of *PU.1* in MEP induced macrophage/granulocyte precursors, whereas upregulation of *CEBPA* induced eosinophils formation (Nerlov et al. 1998; Nerlov and Graf 1998). The opposite

direction of lineage switch was observed in myeloid cells with enforced expression of *GATA1* turning into MEPs (Kulessa et al. 1995). The MEP phenotype can be induced also by increased expression of *FOG1* in eosinophils (Querfurth et al. 2000).

Several other works investigating transdifferentiation potential of particular hematopoietic progenitors or mature cells under variable conditions were published (Kee and Murre 1998; Lee et al. 2001; Rolink et al. 1999; Yamaguchi et al. 1998).

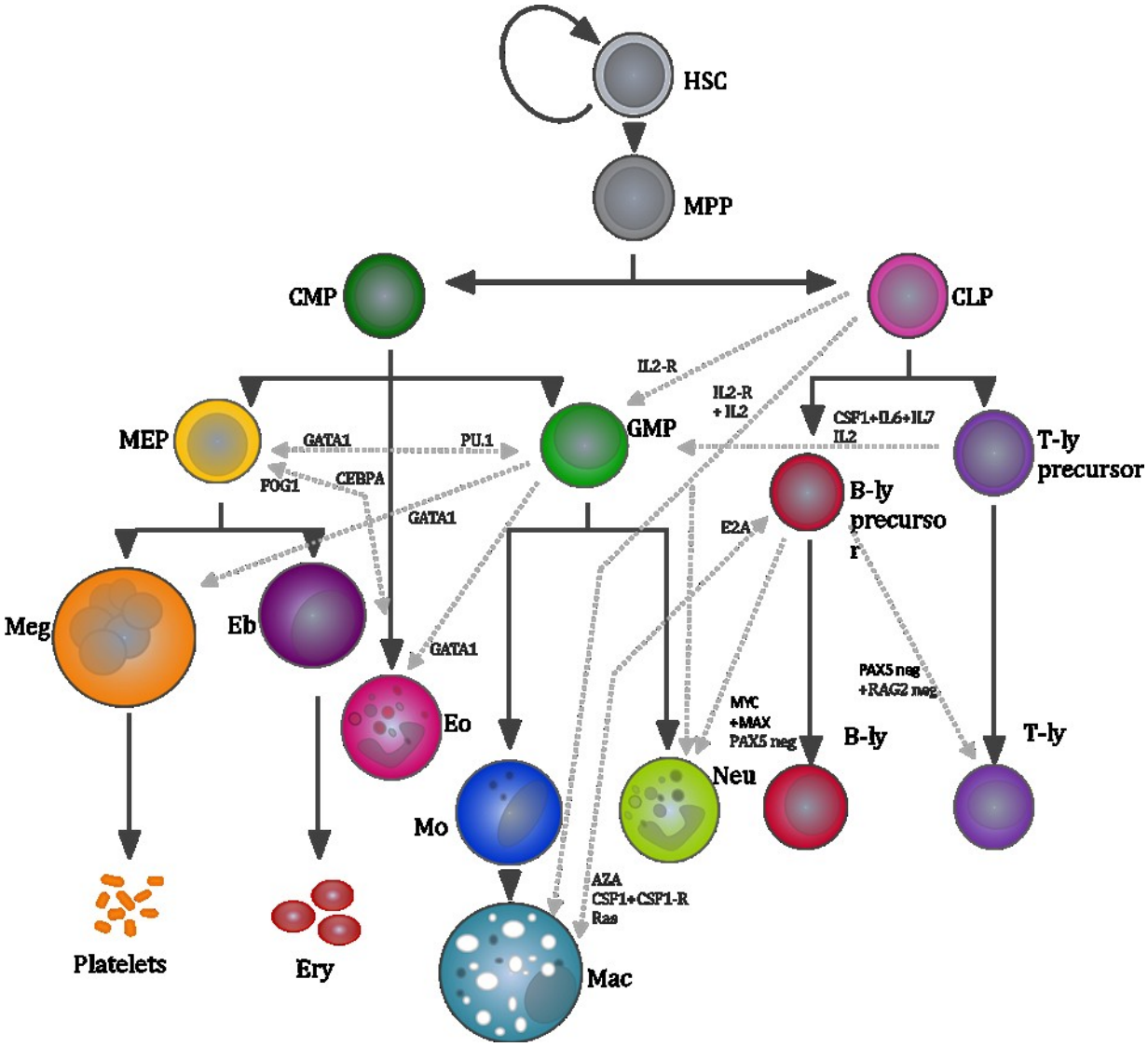


Figure 4. Model of hematopoiesis with transdifferentiation and switching phenomena described in literature marked by grey dotted arrows. HSC – hematopoietic stem cell; MMP – multipotent progenitor; CLP – common lymphoid progenitor; CMP – common myeloid progenitor; GMP - granulocyte-monocyte progenitor; MEP - megakaryocyte-erythroid precursor; Meg - megakaryocyte; Eb - erythroblast; Ery - erythrocyte; Eo - eosinophil; Mo - monocyte; Mac - macrophage; Neu - neutrophil; B-ly - B lymphocyte; T-ly - T-lymphocyte; IL - interleukin; (adopted from Graf 2002).

1.2 Hematopoietic disorders

Hematopoietic disorders form a broad spectrum of several clinical units with specific behavior. They include different non-malignant conditions, such as clonal hematopoiesis of indetermined origin (CHIP), idiopathic cytopenias of undetermined significance (ICUS), clonal cytopenias of undetermined significance (CCUS) which are believed to be benign but may increase the risk of hematopoietic malignancy development (Bejar 2017; Genovese et al. 2014). Hematologic cancers lie on the other side of the spectrum. They again include different subtypes of indolent or aggressive diseases. Since the description of the features of all units that can be classified as a hematopoietic disorder or deregulation is beyond the extent of this thesis, only units relevant for the thesis will be described.

1.2.1 Clonal hematopoiesis of indetermined potential (CHIP)

Clonal hematopoiesis (CH) with somatic mutations is a condition where single HSC disproportionately contributes to formation of mature blood cells. In first DNA-sequencing based studies CH was relentlessly connected to presumed driver mutations in hematological malignancies (Genovese et al. 2014). It was however observed that HSCs accumulated mutations during their life history without an apparent effect on their phenotype (Holstege et al. 2014; Welch et al. 2012). These mutations were then passed on HSCs descendants and could be detected using DNA sequencing, thus revealing contribution of particular HSCs to hematopoiesis. Indeed, Zink et al. (2017) using whole genome sequencing (WGS) technology observed CH in otherwise healthy individuals without any cytopenia or dysplastic changes and lacking known hematologic malignancy (which was then defined by Steensma et al. (2015) as CH of indetermined potential – CHIP) and showed its increased incidence in elderly. Mutations in genes such as *DNMT3A*, *TET2*, *ASXL1* or *PPM1D* that are often observed in different myeloid neoplasia, were also detected in the cohort, however their prevalence was low and strongly depending on age. Another study, on the contrary, showed high prevalence of *DNMT3A* and *TET2* mutations in healthy individuals (Young et al. 2016). Busque et al. (2012) also observed presence of *TET2* mutation in otherwise healthy individuals; moreover they proved also changes in *TET2* function resulting in changed methylation pattern. On the other hand, even if detected in healthy individuals, CHIP may predispose them to higher risk of hematologic malignancy and all-cause death suggesting that some of the mutation may present early events in the development of hematologic cancers mostly

of myeloid origin (Genovese et al. 2014; Jaiswal et al. 2014; Zink et al. 2017). Non-hematologic mortality was mostly connected to cardiovascular and metabolic disease and is thought to be connected to changes in inflammasome and accelerated atherosclerosis (Jaiswal et al. 2014; Sano et al. 2018).

Beside CHIP there is also monoclonal B-lymphocytosis (MBL) and monoclonal gammopathy of undetermined significance (MGUS), both similarly to CHIP being believed to precede lymphoid neoplasms in low proportion of patients (Rawstron et al. 2008; Zingone and Kuehl 2011).

1.2.2 **Myelodysplastic syndrome (MDS)**

Myelodysplastic syndrome (MDS) is a heterogenic group of clonal BM neoplasms characterized by ineffective hematopoiesis (due to exaggerated apoptosis of late hematopoietic precursors) leading to peripheral cytopenia(s), BM morphologic dysplasia (more than 10 % of dysplastic cells in erythroid, myeloid and/or megakaryocyte lineages) and is connected to higher risk of AML development (Lichtman et al. 2017).

The disease spectrum ranges from indolent form with mild to moderate anemia through more troublesome multicytopenias without presence of increased blast number to oligoblastic forms with blasts up to 19% of cells in BM. If BM blasts count 20 % and more the disease is already considered as AML. It is rather rare disease with increasing incidence from age 40 years (0.2/100 000 persons) to age 85 years (incidence 45/100 000 persons). It is very rare in childhood (incidence rate 0.1/100 000 children to 15 years per year) when it more often evolves from inherited syndromes, such as Fanconi anemia. Clinical features arise mostly from presence of cytopenia– pallor, dyspnea, palpitations in anemia (hemoglobin < 110 g/l), easy bruising, petechiae, hemorrhage in thrombocytopenia (platelet count < 100 000/μl), and recurrent infections in neutropenia (absolute neutrophil count (ANC) < 1500/μl) (Lichtman et al. 2017).

Peripheral cytopenias together with blast number in BM and particular cytogenetic changes present fundamental criteria for MDS diagnosis, classification (Table 1) and prognostic scoring (Greenberg et al. 1997; Greenberg et al. 2012) (Table 2 and Table 3). Cytogenetic changes may be categorized as (very) good, such as del(11q), -Y or del(5q), del(20q), intermediate, such as +8, and (very) poor, e.g. -7 or complex abnormalities (Lichtman et al. 2017).

The treatment decision is based on risk category, accompanying cytopenias and also on the frailty of the patient, since the disease is often present in elderly. Current treatment options include EPO analogues to relieve anemia, G-CSF, lenalidomide, particularly for del(5q) MDS, hypomethylating agents (5-azacitidine (AZA) or decitabine) and allogenic HSC transplantation for

high risk or non-responding disease. Ongoing therapeutic trials include new epigenetic targets (IDH1 and IDH2 inhibitors), check-point inhibitors and different signal transduction inhibitors (Lichtman et al. 2017).

Table 1. WHO 2016 classification of myelodysplastic syndrome subtypes including bone marrow and peripheral blood findings.

Name	Dysplastic lineages	Cytopenias	Ring sideroblasts as % of marrow erythroid elements	BM and PB blasts
MDS with single lineage dysplasia (MDS-SLD)	1	1 or 2	<15%/<5%	BM <5%, PB<,1%, no Auer rods
MDS with multilineage dysplasia (MDS-MLD)	2 or 3	1-3	<15%/<5%	BM <5%, PB <1%, no Auer rods
MDS with ring sideroblasts (MDS-RS)				
MDS-RS with single lineage dysplasia (MDS-RS-SLD)	1	1 or 2	≥15%/≥5%	BM <5%, PB <1%, no Auer rods
MDS-RS with multilineage dysplasia (MDS-RS-MLD)	2 or 3	1-3	≥15%/≥5%	BM <5%, PB <1%, no Auer rods
MDS with isolated del(5q)	1-3	1-2	None or any	BM <5%, PB <1%, no Auer rods
MDS with excess blasts (MDS-EB)				
MDS-EB-1	0-3	1-3	None or any	BM 5%-9% or PB 2%-4%, no Auer rods
MDS-EB-2	0-3	1-3	None or any	BM 10%-19% or PB 5%-19% or Auer rods
MDS, unclassifiable (MDS-U)				
with 1% blood blasts	1-3	1-3	None or any	BM <5%, PB = 1%, no Auer rods
with single lineage dysplasia and pancytopenia	1	3	None or any	BM <5%, PB <1%, no Auer rods
based on defining cytogenetic abnormality	0	1-3	<15%	BM <5%, PB <1%, no Auer rods
Refractory cytopenia of childhood	1-3	1-3	None	BM <5%, PB <2%

Table 2. International prognostic scoring system (IPSS) to estimate prognosis of MDS patients; 0 points represents low risk category with median survival of 5.7 year; 0-1 points represent Intermediate 1 risk category with median survival of 3.5 year; 1.5-2 represents Intermediate 2 and 2.5-3 point high risk categories with median survival of 1.2 and 0.4 years respectively. Adopted from Greenberg et al. 1997.

prognostic variable	**score value**				
	0	**0.5**	**1**	**1.5**	**2**
BM blasts	<5%	5-10%	x	11-20%	21-30%
Cytogenetic category	good	intermediate	poor		
No. of cytopenias	0-1	2.3			

Table 3. Revised international prognostic scoring system (IPSS-R) to estimate prognosis of MDS patient; The prognostic risk groups are very low (≤1.5 points), low (>1.5-3), intermediate (>3-4.5), high (>4.5-6) and very high (>6 points) with median survival (and median time to 25% AML progression) of 8.8 years (not reached), 5.7 years (10.8 years), 3 years (3.2 years), 1.6 years (1.4 years) and 0.7 (0.7 years) respectively. Adopted from Greenberg et al. 2012.

prognostic variable	**score value**						
	0	**0.5**	**1**	**1.5**	**2**	**3**	**4**
BM blasts	≤2%	x	2-5%	x	5-10%	>10%	x
Cytogenetic category	very good	x	good	x	intermediate	poor	very poor
Hemoglobin	≥100 g/l	x	80-100 g/l	≤80 g/l			
Platelets	≥100 $x10^9$/l	50-100 $x10^9$/l	<50 $x10^9$/l				
ANC	≥0.8 $x10^9$/l	<0.8 $x10^9$/l					

1.2.2.1 Mutations in MDS

Pathogenesis of this disorder remains obscure, but as the massively parallel sequencing (MPS) studies revealed, half of the patients carried at least one somatic mutation in 18 most often

mutated genes (Bejar et al. 2011) and ~90% of them carried at least one somatic mutation in 40 most often mutated genes observed in myeloid neoplasia. These mutations include known apoptotic regulators such as *TP53*, *CDKN2A*, epigenetic modulators such as *TET2*, *ASXL1*, *DNMT3A* or *KDM6A*, transcription factors such as *RUNX1*, *GATA2*, *CEBPA*, members of tyrosine kinase and MAP kinase signaling such as *FLT3*, *KRAS*, *NRAS* or members of spliceosome (e.g. *SF3B1*, *ZRSR2*, *SRSF2*, *UA2F1*) and cohesin complexes (*RAD21*, *STAG2*, *SMC3*) (Bejar 2017). Some of these mutations are associated with specific cytogenetic changes (*TP53* mutations with complex karyotype (Bejar et al. 2011)), or specific disease phenotype (*SF3B1* with presence of ring sideroblasts (Patnaik et al. 2012), some predict poor (*TP53*, *ASXL1*, *RUNX1*, *EZH2*) or better (mutations of spliceososme) overall survival (Bejar et al. 2011). Distinct mutational profiles are observed in de novo versus therapy related MDS suggesting different pathogenesis of these two subtypes of the disease (Ok et al. 2015).

Mutations in specific genes are also believed to predict therapy response; *TET2* in absence of *ASXL1* mutation predicts good response to treatment with hypomethylating agents (i.e. AZA or decitabin) (Bejar et al. 2014; Itzykson et al. 2011); however, the response duration and overall survival do not seem to be affected by *TET2* mutational status (Itzykson et al. 2011); in some genes, such as *DNMT3A* or *SF3B1* the studies on predictive value of response rate in mutated status are conflicting with no consensus on their effect (Martín et al. 2017; Walter et al. 2011).

1.2.2.1.1 Mutations of spliceosome

Components of spliceosome are within the most often mutated genes in MDS, observed in up to 60 % of patients. They are believed to be mutually exclusive to each other and usually occur as heterozygous missense mutations at highly restricted residues. The first member of this group observed was *SF3B1* which is mutated in ~80 % of patients with MDS with ring sideroblasts. Despite the high frequency, the mechanistic consequences of *SF3B1* mutations are not very clear. It is a member of the U2 snRNP complex responsible for 3'SS recognition. Mutations of *SF3B1* are usually associated with alterations in splicing as a consequence of aberrant 3'SS (splice site) binding. Many of the cryptic 3'SS used by mutant SF3B1 proteins were shown to be located upstream of the canonical 3'SS causing frameshifts that could potentially lead to degradation of higher fraction of resulting transcripts. In erythroid lineage, mutations of *SF3B1* lead, besides their changes, in aberrant splicing of Mitoferrin-1 and /or ABCB7, both genes being connected to congenital sideroblastic anemia (reviewed in Inoue et al. 2016).

SRSF2 encodes protein that facilitates spliceosome assembly by binding to exonic splicing enhancer sequences within pre-mRNA which are recognized through its consensus motif of SSNG. Mutated SRSF2 proteins exhibit altered to exonic splicing enhancer sequences recognition and may also change SRSF2 interactions with other spliceosomal proteins.

U2AF1 is a subunit of heterodimer U2AF which recognizes the consensus motif yAG at the intron/exon boundary. Most of the observed mutations lie within one of the zinc finger domains and rather than loss of function cause its alteration and lead to aberrant 3'SS recognition.

Mutation of *U2AF1* and *SRSF2* seem to be associated with altered splicing of various epigenetic modulators including EZH2, BCOR or ASXL1 and are connected to more aggressive MDS subtypes (reviewed in Inoue et al. 2016).

Mutations of *ZRSR2*, a component of U12 snRNP that recognizes 3'SS of U12-type introns (comprising ~0.5 % of human introns), are usually loss of function. Analysis of genes that were misspliced in ZRSR2 mutated cells revealed enrichment for genes involved in MAPK and ErdB signaling. (reviewed in Inoue et al. 2016)

1.2.2.1.2 Mutations of epigenetic regulators

Second common class of mutation comprises of epigenetic regulators responsible for DNA methylation and histone modification. This group includes *TET2*, *EZH2*, *ASXL1*, *IDH1*, *IDH2*, *DNMT3A* or *KDM6.* Since a significant part of patients with MDS is treated with hypomethylating agents mutations of this group members are extensively studied in respect of affecting the response to the treatment (Bejar et al. 2014; Jung et al. 2016).

DNMT3A is an enzyme that adds a methyl group to cytosine in CpG dinucleotides and is responsible of de novo DNA methylation. Its mutations are present in 5-20 % of MDS and similar rate of de novo AML. In MDS they are usually associated with higher age, worse prognostic group and higher risk of AML transformation. About 60 % of *DNMT3A* mutations occur at R882 resulting in decreased catalytic activity of the enzyme. In majority of cases, mutations are heterozygous, suggesting that haploinsufficiency is sufficient to lead to malignant transformation; alternatively, since DNMT3A functions as an oligomer, mutations can lead to dominant-negative form inhibiting the wild type protein. Although overall methylation of the genome is not significantly changed in mutated cells, analysis of methylome revealed specific genomic loci with decreased 5-mC levels including key metabolic, apoptotic and self-renewal regulators, as well as genes from HOXB group leading to expansion of long term HSCs pool in BM (Jeong et al. 2018).

Conversion of 5-mC to 5-hmC is catalyzed by TET enzymes in Fe(II) and α-ketoglutarate manner. Mutations in *TET2* are observed in 10-20 % of MDS and ~50 % of CMML and are believed to be an early event in disease initiation. 5-hmC blocks the binding of methyl-DNA proteins that silence transcription. Thus, increase 5-hmC of CpG near transcriptional starting site lead to increase expression of particular genes. Moreover, 5-hmC may lead to passive demethylation during DNA replication and also to active demethylation via activation-induced deaminase DNA repair pathway. Similarly to *DNMT3A* mutations, studies of global 5-hmC changes in *TET2* mutated cells led to conflicting results. Nevertheless, disruption of TET2 function leads to aberrant hematopoietic differentiation with expansion of HSCs which in mice resembles CMML like disease (Moran-Crusio et al. 2011). Another TET2-KO mice model revealed development of different myeloid MDS-like disease with erythroid expansion (Li et al. 2011).

IDH1 and *IDH2* mutations are present in ~5 % of MDS patients (comparing to ~20 % of *de novo* AML cases). Their incidence is higher in patients with trisomy of chromosome 8 or in absence of cytogenetic changes. *IDH* mutated status is related to high risk disease with lower ANC and higher BM blast count. However, no clear independent prognostic impact was observed in patients bearing IDH1 or IDH2 mutations (DiNardo et al. 2016). As mentioned above, IDH1 and IDH2 catalyze conversion of isocitrate to α-ketoglutarate in cytoplasm and peroxisomes (IDH1) or mitochondria (IDH2). Cancer associated mutations occur mostly in highly conserved arginine residues IDH1-R132, IDH2-R172 and IDH2-R140 and lead to aberrant gain-of-function with conversion of α-ketoglutarate to 2-hydroxyglutarate. This activity leads to inhibition of TET proteins for which α-ketoglutarate is an essential cofactor (Reitman et al. 2010). Indeed, experimental data show, that *IDH1* and *IDH2* mutants in mouse BM cells exhibit similar phenotype to those with *TET2* knock-down. Besides TET proteins, other α-ketoglutarate dependent enzymes may be inhibited by 2-hydroxyglutarate as well. This includes family of jumonji-domain-containing family of histone demethylases, which are responsible for H3K9 and H3K36 demethylation (Hoffmann et al. 2012).

EZH2, a H3K27 methyltransferase, is the enzymatic component of PRC2 complex and from polycomb proteins it is one of the most frequently implicated in human cancer. Broad spectrum of different missense, nonsense and frameshift mutations was observed in myeloid malignancies and most of them are believed to be loss of function (in contrast to particular lymphoid neoplasms, where rather activating mutations were observed). Its role in MDS pathogenesis is not fully understood, since animal studies suggest that its haploinsufficiency as

well as its overexpression may lead to myeloid transformation; studies shown differential expression of plethora of the genes possibly involved in transformation in *EZH2* mutated cells, including NOTCH, JAK-STAT pathways or *MYC*. *EZH2* mutations are present in ~11 % of MDS patients and often co-occur with *TET2*, *RUNX1*, *NRAS* and *ASXL1* mutations. They are also related to chromosome 7 abnormalities. Correlation studies connect *EZH2* mutations with shortened survival but not AML transformation (Mcgraw et al. 2016).

ASXL1 is an important regulator of repressive polycomb and activating trithorax complexes including the polycomb-repressive deubiquitynase which deubiquitylate H2AK119. Its mutations are believed to be loss-of function and surprisingly do not lead to significant changes in H2AK119 ubiquitylation in myeloid hematopoietic cells but result in loss of H3K27 repressive trimethylation (Katoh 2013). This is probably due to its interaction with PRC2 complex resulting in its recruitment and stability. It was proven, that in absence of ASXL1 EZH2 recruitment was significantly decreased in myeloid cells. Conditional knock-out in hematopoietic compartment in mice led to MDS-like disease (Omar et al. 2013). *ASXL1* mutations are observed in ~20 % of MDS and ~50 % of CMML patients often appearing with chromosome 8 aberrancies and with mutations in *RUNX1*, *EZH2*, *IDH*, *NRAS*, *JAK2*, and *SRSF2*; interestingly it is negatively associated with *SF3B1*mutation. Studies evaluating prognostic impact of *ASXL1* mutated status are conflicting, however it is mostly thought that *ASXL1* mutation reflect poorer disease prognosis in MDS and MPN comparing to wild-type status (Chen et al. 2014).

1.2.2.1.3 Cohesin complex mutations

Cohesin is a large ring-shaped multiprotein complex composed of RAD21, SMC3, SMC1A, STAG1 and STAG2 proteins which is best known for mediating sister chromatid aligning during mitosis. It also plays crucial roles in gene expression regulation, probably via long-range communications events mediated by DNA-loop forming, and DNA damage repair. It also plays an important role in organizing global genomic architecture. Cohesin is involved in expression regulation of plethora of hematopoietic transcription factors including RUNX1 which is enhanced in hematopoietic cells in cohesin depletion (Viny et al. 2015).

Mutations of cohesin complex members are observed in 10-20 % of MDS patients (with higher prevalence in high-risk patients), lead usually to loss-of-function and are believed to be mutually exclusive. They are significantly associated with *RUNX1*, *ASXL1* and *BCOR* mutations and are associated with adverse prognostic impact and higher risk of AML transformation (Thota et al. 2014).

1.2.2.1.4 Transcription factors mutations

Most frequently mutated transcription factor in myeloid neoplasms is *RUNX1*. Other examples include *GATA2*, *NPM1* or *WT1*. Heritable mutations of some of these factors such as *RUNX1, CEBPA* or *GATA2* are known to cause familial MDS or AML (Nickels et al. 2013).

RUNX1 is a sequence-specific DNA binding protein that plays an important role in early hematopoietic development, self-renewal activity of early hematopoietic progenitors and its conditional knockout in HSCs leads to defects in thrombopoiesis and lymphopoiesis. It may function in conjunction with its coactivators p300 and CBP which posses histone-acetyl transferase activity leading to modification of chromatin-associated histones with transcriptional activation. RUNX1 can also repress the transcription in cooperation with other proteins such as EAR2, Sin3 or other partners with histone deacetylase or histone methyltransferase activity. In hematopoietic genes' transcription regulation, factors such as CEBPA, PU.1 or c-MYB were described to cooperate with RUNX1 to organize the lineage restricted transcriptional programs (reviewed by Mikhail et al. 2006). Mutations of *RUNX1* are mostly loss-of function and are, together with *NRAS* and *TP53* mutations, strongly related to higher blast count and thrombocytopenia as well as to poor prognosis; no connection was observed in terms of anemia or neutropenia grade (Bejar et al. 2011). Interestingly, correlation of loss-of-function *RUNX1* mutations with radiation, both accidental and therapeutic, was observed, suggesting that *RUNX1* may be particularly sensitive to radiation induced DNA damage. This observation, together with frequent development of *RUNX1* mutations after alkylating agents exposition explain its higher incidence in therapy related MDS compared to *de novo* disease.

GATA2 is a zinc-finger transcription factor with broad distribution among hematopoietic cells, particularly in early progenitors and crucial role in regulation of maintenance and proliferation of HSCs preserving a pool of immature cells. It interacts with variety of other transcription factors including CEBPA, PU.1 or c-MYB. High levels of GATA2 inhibit erythroid differentiation and its changes, together with increasing GATA1 levels orchestrate the megakaryocyte-erythroid cell fate, since higher *GATA2* expression is needed for terminal thrombopoiesis. GATA2 (together with GATA1) also regulates myeloid differentiation via variable repression of PU.1 (Vicente et al. 2012). Germline *GATA2* mutations are well known for their connection with cytopenias in childhood and adolescence with high propensity to myeloid disease. Several familial syndromes such as monoMac syndrome (monocytopenia and mycobaterial infections) or DCML (syndrome associated with defects in dendritic cells,

monocytes, and B and NK lymphoid cells) with predisposition to MDS and AML development were described to be caused by *GATA2* mutations (Hyde and Liu 2011).

1.2.2.1.5 TP53 mutations

TP53, encoding p53 sometimes dubbed as the "guardian of the genome", is a tumor suppressor gene that transmits different stress-inducing signals into antiproliferative responses. After its activation by DNA damage, oncogene activation with excessive mitogenic signaling, hypoxia or other conditions it initiates and directs cell cycle arrest, senescence, apoptosis or autophagy. Majority of cancer associated mutations affect its DNA binding domain.

TP53 mutations occur in about 10 % of MDS patients probably as an early event. They are associated with higher BM blast count, higher risk category group with higher risk of AML transformation and shorter survival (Kulasekararaj et al. 2013). They often co-occurre with *TET2*, *ASXL1*, *DNMT3A* or *IDH2* mutations and is also associated with chromosomes 5 and 7 aberrations and complex karyotype. Several works reported adverse outcome of *TP53*-mutated patients even after adjustment for other risk factors but *TP53*-mutational status impact on response to different treatment modalities including hypomethylating agents remains conflicting (Al-Issa et al. 2016; Kulasekararaj et al. 2013).

1.2.3 **Acute myeloid leukemia (AML)**

Acute myeloid leukemia (AML) is a heterogeneous neoplastic disease originating from HSCs leading to uncontrolled clonal proliferation of abnormal blasts in BM and impaired normal hematopoiesis that results in different cytopenias with variable leukocyte count in peripheral blood depending on blast number in PB. There are different AML subsets depending on the blast phenotype, cytogenetic and molecular changes. Classification is thus based on morphological findings (French-American-British classification, (Bennett et al. 1976)) or more complex criteria including certain genetic abnormalities, myelodysplastic features, occurrence after chemotherapy and/or radiotherapy for another disease, etc. (WHO2016 classification; for more details see Arber et al. 2016). All these factors, together with age and disease status (primary versus secondary AML) are determinants of prognosis prediction (Liersch et al. 2014, Bejar 2014). In last decades the genomics of AML was, similarly to MDS, subject of intensive studies with aims to explain the complex pathogenesis of the disease, to find novel therapeutic targets and to predict the prognosis more precisely. With the integration of MPS high number of molecular changes undetectable by

standard cytogenetic testing in AML has been detected. These are shared with those observed in MDS and include mutations in epigenetic modulators, nucleophosmin (NPM1), transcription factors, tumor suppressors, different signaling and kinase pathways, or spliceosome and cohesion complexes. Several works show distinct molecular subgroups of AML in terms of their morphological/cytogenetic phenotype, the disease ontogeny or their prognosis (Eisfeld et al. 2018; Herold et al. 2018; Ok et al. 2015; Rose et al. 2017). For example, mutations in genes *SF3B1*, *ZRSR2*, *U2AF1*, *SRF2*, *EZH2*, *BCOR*, *STAG2* and ASXL1 were with 95% specific for secondary AML (i.e. developing after antecedent myeloid malignancy), whereas *de novo* cases more often presented with *NPM1* mutations (which are rather rare in MDS patients) or *MLL* and *CBF* rearrangements (Lindsley et al. 2015).

Papaemmanuil et al. (2016) in large cohort of 1540 patients identified driver mutations in 76 genes or genomic regions (out of 111 that were sequenced) with 2 or more driver mutations observed in 86 % patients. The study identified 11 disease classes based on the co-mutation pattern, each class having distinct clinical features and prognostic outcome, and proposed new genomic classification of AML.

Mutational profiling also allows tracking clonal evolution of the disease, evaluation of the response and early detection of relapse (Graubert and Mardis 2011).

1.2.4 **Acute lymphoblastic leukemia (ALL)**

Lymphoid malignancies comprise a wide spectrum of clinically and morphologically distinct syndromes. For purpose of this thesis, only acute lymphoblastic leukemia (ALL) will be described in this work. ALL is a malignant disease of immature lymphocytes or lymphocytic progenitor cells of B- or T-lineage, the latter being less frequent. Accumulation of lymphoblasts usually leads to impaired formation of normal blood cells and the leukemic cells may reside in various extramedullary sites. Each ALL subtype is phenotypically defined and often is associated with particular molecular hits (Table 4). ~80 % of patients carry a cytogenetic or molecular abnormality with different distribution within different age groups (Iacobucci and Mullighan 2017). These abnormalities include hypo- or hyperdiploidy, *BCR/ABL1* translocation (t(9;22)), *ETV6/RUNX1* translocation (t(12;21)), *KMT2A/AFF1* (t(4;11)), *E2A/PBX1* (t(1;19)), del(6q), intrachromosomal amplification of chromosome 21, and others and may define clinical phenotype of the disease or its prognosis. For example, *ETV6/RUNX1* positive ALL with ~25 % prevalence in childhood B-precursor ALL (pB-ALL) which generally implies a good prognosis is often present with more severe anemia compared to hyperdiploid or *BCR/ABL* positive ALL (Lichtman

et al. 2017). Hyperdiploidy (> 50 chromosomes) which occurs in ~33 % of childhood and ~6 % of adult ALL cases is also associated with favorable prognosis; on the contrary, hypodiploidy (<45 chromosomes) is connected to poor outcome (Lichtman et al. 2017).

Table 4. Immunophenotypic and clinical features of different ALL subtypes. cCD3 – cytoplasmic CD3, cIG – cytoplasmic immunoglobulin, mIG – membrane immunoglobulin, TdT – terminal deoxynucleotidyl transferase. Adopted from Lichtman et al. 2017.

	subtype	typical markers	associated features
B cell precursor		$CD19^{pos}$, $CD22^{pos}$, $CD79a^{pos}$, HLA-DR^{pos}, $cIg^{pos/neg}$, $mIg\mu^{neg}$	
	Pro-B	$CD10^{neg}$	infants and adult age group, high leukocyte count, initial CNS leukemia, MLL-rearrangements, unfavorable prognosis
	Early pre-B	$CD10^{pos}$	favorable age group (1-9 years), low leukocyte count, hyperdiploidy
	Pre-B	$CD10^{pos/neg}$, cIg^{pos}	high leukocyte count
	Mature B cell	$CD19^{pos}$, $CD22^{pos}$, $CD79a^{pos}$, cIg^{pos}, mIg^{pos}	male predominance, initial CNS involvement, abdominal masses, often renal involvement
T lineage		$CD7^{pos}$, $cCD3^{pos}$	
	T cell	$CD2^{pos}$, $CD1^{pos/neg}$, $CD4^{pos/neg}$, $CD8^{pos/neg}$, HLA-DR^{neg}, $TdT^{pos/neg}$	male predominance, hyperleukocytosis, extramedullary disease
	Pre-T	$CD2^{neg}$, $CD1^{neg}$, $CD4^{neg}$, $CD8^{neg}$, HLA-$DR^{pos/neg}$, TdT^{pos}	male predominance, hyperleukocytosis, extramedullary disease, unfavorable prognosis
	Early T-cell precursor	$CD1^{neg}$, $CD8^{neg}$, $CD5^{weak}$, $CD13^{pos}$, HLA-$DR^{pos/neg}$, $CD33^{pos}$, $CD65^{pos}$, $CD117^{pos}$, $CD11b^{pos}$	male predominance, age>10 years, poor prognosis

Approximately 40% of pB-ALL carry mutations in key transcription factors that promote early B-lymphoid differentiation, such as *IKZF1* (encoding a zinc finger transcription factor Ikaros that is indispensable for lymphoid differentiation), *IKZF3*, *PAX5* (being the most frequently mutated), *EBF*, *BLNK* and others (Mullighan et al. 2007). Some of them, such as *IKZF1* mutations, which in most cases are dominant-negative, have negative impact on prognosis (Mullighan et al. 2009), which, however, could be alleviated by MRD-directed treatment of ALL (Volejnikova et al. 2013).

T-ALL as well as pB-ALL are often describe to aberrantly express various myeloid or cross-lineage antigens (and not reaching the EGIL criteria for ambiguous lineage acute leukemia, see further), such as CD79a in T-ALL or CD66c on pB-ALL, or CD13 and CD33 in both, T- and pB-ALL (Suggs et al. 2007). The aberrant expression often accompanies particular cytogenetic changes, e.g. CD66c expression in pB-ALL is connected to *BCR/ABL* translocation and to hyperdiploid blasts (Guillaume et al. 2011; Tang et al. 2015), CD13 and CD33 are more often present in MLL rearranged as well as ETV6/RUNX1 pB-ALL (Chiaretti et al. 2014, Hrusak et al. 2002).

1.2.5 **Acute leukemia of ambiguous lineage (ALAL)**

Acute leukemias of ambiguous lineage (ALAL), in which assigning a single lineage of origin is more complicated, comprise ~5 % of all diagnosed AL. According to European Group for the Immunological Classification of Leukemia (EGIL) criteria it is defined by reaching score >2 for the myeloid and one of the lymphoid lineages phenotypes (Table 5) (Bene et al. 1995). WHO criteria are believed to underestimate the incidence of this unit (Manola 2013). Rare cases of B-T mixed lineage or trilineal phenotypes were also described (Matutes et al. 2011; Yan et al. 2012). The pathogenesis of this unit remains obscure; till now, no correlation between immunophenotype and specific cytogenetic aberration has been discovered, although it is believed that mixed phenotype is more common in MLL rearranged AL, del(1) or t(2;5) (Manola 2013). WGS data showed *DNMT3a* mutations (common in AML but rare in ALL) in 33 % of ALAL (Eckstein et al. 2016). Other often mutated genes include *TP53*, *NOTCH1*, *NRAS*, EZH2, *IKZF1* or *TET2* (Eckstein et al. 2016; Yan et al. 2012).

1.2.6 **Switching acute leukemia (SW-AL)**

The lineage switch in acute leukemia, i.e. conversion of leukemic lineage (lymphoid or myeloid) during the disease course, is a rare phenomenon with reported incidence from 0.6 % to 5 % firstly described in late 80's (Ihle et al.1989). It is observed more frequently in children than

in adults and interestingly higher incidence of lineage switch is present in patients with rare congenital acute leukemia. Most cases involve switch from ALL to AML and the opposite phenomenon is very rare with only few cases being reported (Dorantes-Acosta and Pelayo 2012). Several hypotheses were proposed to explain this phenomenon including aberrant effect of specific fusion genes, particularly abnormalities involving *MLL* (*KMT2A*) gene were blamed (Rossi et al. 2012). This is in line with observation of higher frequency of switch in congenital AL which in 40 % contain translocation involving region 11q23 (that includes *KMT2A*), reprogramming the malignant pluripotent cells, transdifferentiation (Tirtakusuma et al. 2013) of already committed B-ly progenitor or selection of pre-existing chemotherapy resistant minor clone of different lineage that the predominant leukemic population (Dorantes-Acosta and Pelayo 2012). It is possible that different mechanisms play role in particular cases; precise molecular mechanisms however have not been elucidated yet.

Table 5. According to European Group for the Immunological Classification of Leukemia EGIL criteria, ALAL is diagnosed when score >2 is reached for the myeloid and on of the lymphoid lineages. Each lineage is defined by cytoplasmic (c) or membrane (m or without preffix) expression of particular proteins; MPO – myeloperoxidase, TdT – intranuclear terminal deoxynucleotidyl transferase.

Points	B-lineage	T-lineage	Myeloid lineage
2	CD79a	(c/m) CD3	(c) MPO
	cIgM	$TCR^{\alpha/\beta}$	
	cCD22	$TCR^{\gamma/\delta}$	
1	CD19	CD2	CD13
	CD10	CD5	CD33
	CD20	CD8	CDw65
		CD10	CD117
0.5	TdT	TdT	CD14
	CD24	CD7	CD15
		CD1a	CD64

2 Introduction to the thesis

Hematopoiesis is a process regulated on multiple levels that leads to formation of many specific cell types with variable functions. During their differentiation expression of plethora of lineage specific regulatory molecules such as PAX5, PU.1, CEBP family, HOX family, GATA, IKZF, or growth factors and their receptors is up- or downregulated as needed to direct the cells towards their destiny. This process can be corrupted at various stages, thus leading to aberrant maturation or even malignant transformation, which can be reflected on molecular level. To account for any kind of alternative behavior, changes occurring during physiological differentiation must be described first. Although the key differentiation regulatory steps have already been described, a concise collection addressing particular stages of different lineages' maturation has been missing for a long time since the available datasets were evaluated by microarray technology, which besides being robust, brings several troubles possibly leading to biased results, thus needing to be further confirmed by gold standard quantitative real-time PCR (Draghici et al. 2006; Steger et al. 2011), or they were focused mostly on already differentiated cells or limited number of populations (Tuomela et al. 2012). Precise delineation of what occurs during early stages of normal hematopoietic maturation may help to understand the background of promiscuous behavior that was described in some acute leukemias. Specific cases are presented by AL of ambiguous lineage or switching acute leukemias that change their lineage from one to another. Several hypotheses were made on how and why the switch may occur (Dorantes-Acosta and Pelayo 2012; Rossi et al. 2012), however valid molecular description of the switching process has been missing for a long time.

The last decade and expansion of massively parallel sequencing brought new insight into the etiopathology of various clonal disorders. It was shown that mutations of different hematopoietic regulators are present in healthy individuals without apparent hematologic disease. This enhanced the effort to categorize different conditions into precisely defined units such as CHIP or CCUS (clonal cytopenia of undetermined significance). On the other hand, mutations of huge variety of different genes were described to contribute to development of clonal disorders; they were proven to affect the disease phenotype, its behavior and also its response to the treatment and patients' prognosis (Bejar et al. 2011). MPS also allows tracking the clonal evolution, which then may help to identify driver hits leading to the disease progression (Ding et al. 2012). In MDS, a clonal disorder leading to defective hematopoiesis with increased risk of AML development, mostly mutations of epigenetic regulators and cohesin and spliceosome complexes members are pointed out. This is of particular interest, since the disease is often treated

with demethylation agents implying direct relationship between presence of some of these mutations and efficacy of the treatment. The positive or negative impact of particular mutations was indeed proven; however, most of the published studies focused on the mutational profile before the treatment and did not evaluate impact of its changes during the disease course on the therapy effect (Bejar 2014).

2.1 Hypothesis

Cell fate during hematopoietic differentiation is dependent on complex transcriptional networks that are changing during lineage restriction. Deeper knowledge of these expression profiles' changes could improve the understanding of molecular background of the lineage infidelity in malignant populations. Using current knowledge and our own profiling data we hypothesized which factors and regulatory pathways may contribute to aberrant expression of particular molecules and to the lineage switch in specific subgroup of childhood AL.

Mutations hitting specific regulatory levels that participate on orchestrating hematopoietic cell fate may not only lead to defective hematopoiesis or affect the definitive phenotype of clonal population but also impact on therapy response and thus on disease prognosis. Thus, we though, that pursuing dynamics of these mutations (using MPS) and clonal evolution during disease course in MDS patients treated with AZA could disclose the effect of particular mutations on the efficacy of this treatment. Revealing mutations that provide AZA resistance could further help to classify the progression/relapse risk and to predict further outcome of the patient.

2.2 Aims of the Thesis

The aims of this thesis were:

- To describe changes of regulatory networks determining cell fate during hematopoietic differentiation on mRNA expression level. This includes changes during physiological development of lymphoid (B- and T-lymphocytes) and myeloid (granulocytes and monocytes) lineages, as well as depiction of variations in malignant lymphoid an myeloid specimens. Comparing our own observations and confronting them with current knowledge we also aimed to reveal potential markers of lineage infidelity.

- To connect mRNA expression changes with transdifferentiation potential of switching leukemias thus finding key regulatory molecules and processes responsible for the phenotypic changes. Describing this new subgroup of hematological malignancies within its molecular context we also aimed to find common characteristics allowing prediction of possible lineage switch and thus improving clinical evaluation during the treatment.

- To describe clonal development in myelodysplastic syndrome during demethylation treatment with azacitidine and to identify mutations that are selected by this therapy or those that preclude resistance to the treatment.

3 Materials and Methods

3.1 Material

3.1.1 Biological material

3.1.1.1 Patient samples and healthy controls

For expression profiling study (enclosed article Polgarova et al. 2016), cell type subsets from healthy donors without signs of infection, immune system activation, systemic disease, hematological malignancies or other possibly considerable diseases were separated from PB (mature B-lymphocytes, monocytes and granulocytes) or from BM (earlier developmental stages of B-lymphoid and myeloid lineage). T-lymphocytes and their immature precursors were obtained from thymi removed during cardiosurgery for congenital heart disease from otherwise healthy patients. Three specimens for every cell-type subset from physiological samples were included.
Malignant specimens for expression profiling were separated from diagnostic BM samples of pediatric B-cell precursor acute lymphoblastic leukemia (pB-ALL, N=14), acute T-lymphoblastic leukemia (T-ALL), acute myeloid leukemia (AML), acute leukemia of ambiguous phenotype (pB/My-AL) and switching leukemia (SW-AL) with their particular subpopulations – B-lymphoblasts (SW-B), intermediate or transition population (SW-I) and monocytoid population (SW-Mo); (N=32) that were sent to laboratory center of Department of Pediatric Hematology and Oncology (CLIP, 2nd Faculty of Medicine, Charles University), see Table 6.

Table 6. Exact numbers of AL samples and their particular subtypes included in the gene expression study.

AL lineage	AL subtype/subpopulation	Number of samples
pB-ALL	Hyperdiploid	2
	ETV6/RUNX1	3
	MLL+	2
	Other	7
T-ALL		4
AML		6
pB/MY-AL		5
SW-AL	sw-B(Dg)	10
	sw-B(D8)	4
	sw-I	9
	sw-Mo	9

Switching leukemia study (enclosed article Slamova et al. 2014) also included patients with pB-ALL (N=708) treated according protocols ALL-BFM95 (N=269), ALL-BFM2000 (N=154), ALL-BF2002 (N=257), POG9407 (N=5), Interfant1999 (N= 12) and Interfant 2006 (N=11); 3 patients died at day of diagnosis, and were not included in SW-AL incidence analysis. Additional 3 SW-AL cases were included for molecular studies. The study included a validation cohort of 44 patients diagnosed with pB-ALL from Austria. The samples were or used fresh or the Ficoll-Paque premium gradient was used to separate the mononuclear fraction which was then stored in liquid nitrogen till use.

Malignant and control specimens for sequencing project (enclosed article Polgarova et al.2017 – Supplementary 3) were obtained from diagnostic and restaging samples of patients with myelodysplastic syndrome (MDS, N=38) that were sent to tissue biobank of 1st Internal Clinic - Clinic of Hematology (1st Faculty of Medicine, Charles University in Prague) within the years 2011-2015, and they were stored in liquid nitrogen till use. The study included MDS patients with Int-2/high IPSS risk or MDS/AML with less than 30 % myeloblasts in BM cytology that were not eligible for allogenic SCT and were treated by AZA in 1-month cycles until PG with AZA 75 mg/m2 (Vidaza, Celgene) in 5+2+2 regimen with median of 14 applied cycles (range 4-34 cycles). Disease classification was performed according to WHO 2008 criteria (since samples and clinical data were collected before the novel classification was released) but equivalent clinical category according to WHO2016 classification criteria (table 7) is stated when appropriate.

Marrow and cytogenetic responses were monitored every 4 months or in suspected progression. In total 97 BM samples were obtained. Response criteria for CR, HI, or SD were used according to International Working Group criteria (Cheson et al. 2006).

Treatment responses were as follows: complete remission (CR; N=11, 29 %), stable disease with hematological improvement (SD with HI; N=11, 29 %), stable disease (SD; N=12, 32 %), and finally no response with progression on AZA (PG; N=4, 10 %).

Overall survival (OS) on AZA for particular response groups was as follows: CR >12 months (29.6 months), CR<12 months (17.6 months), SD w HI (31 months), SD (32.4 months) and PG (6.1 months). 25 (66 %) patients progressed to AML. Median OS range was 4-48 months; 5 patients are currently alive with the follow-up exceeding four years. Further details describing the cohort and sampling strategy are available in table 7 and fig. 5.

Both, malignant and non-malignant specimens were separated after written informed consent was obtained. All procedures were performed in accordance with the ethical standards of the Institutional Review Board and the Helsinki declaration.

Table 7: MDS cohort clinical characteristics. Diagnosis (Dg) is stated according to WHO2008 classification (in regard to time of Dg for patients); particular WHO2016 category is mentioned in parenthesis.

No. of patients				**38**				
No. of samples	Total				Per patient (median, range)			
	97				2 (1-5)			
Age at Dg Median (range)	70 (57-82)							
Gender (Female/Male)	20/18							
Dg before AZA	**RA** (MDS-SLD)	**RARS** (MDS-RS-SLD)	**RCMD** (MDS-MLD)	**RAEB-1** (MDS-EB 1)	**RAEB-2** (MDS-EB 2)	**MDS/ AML**	**CMML 1**	**CMML 2**
	0	0	2	12	18	5	1	1
IPSS-R risk group:	Very low	Low		Intermediate		High		Very high
	0	1		15		18		5
Cytogenetic risk group:	Very good	Good		Intermediate		Poor		Very poor
	0	28		7		2		2
Clinical characteristics	BM blast %		Hb g/L		ANC x 10^9/L		PLT x 10^9/L	
Median (range)	10 (2-26)		91 (62-128)		1.24 (0.01-11.6)		80 (2-576)	
OS on AZA Median (range)	25 (4-49) months							
PFS (months) Median (range)	17 (4-37) months							
Primary response	CR>12 Mo		CR<12 Mo		SD w HI	SD w/o HI		PG
	4		7		10	12		4

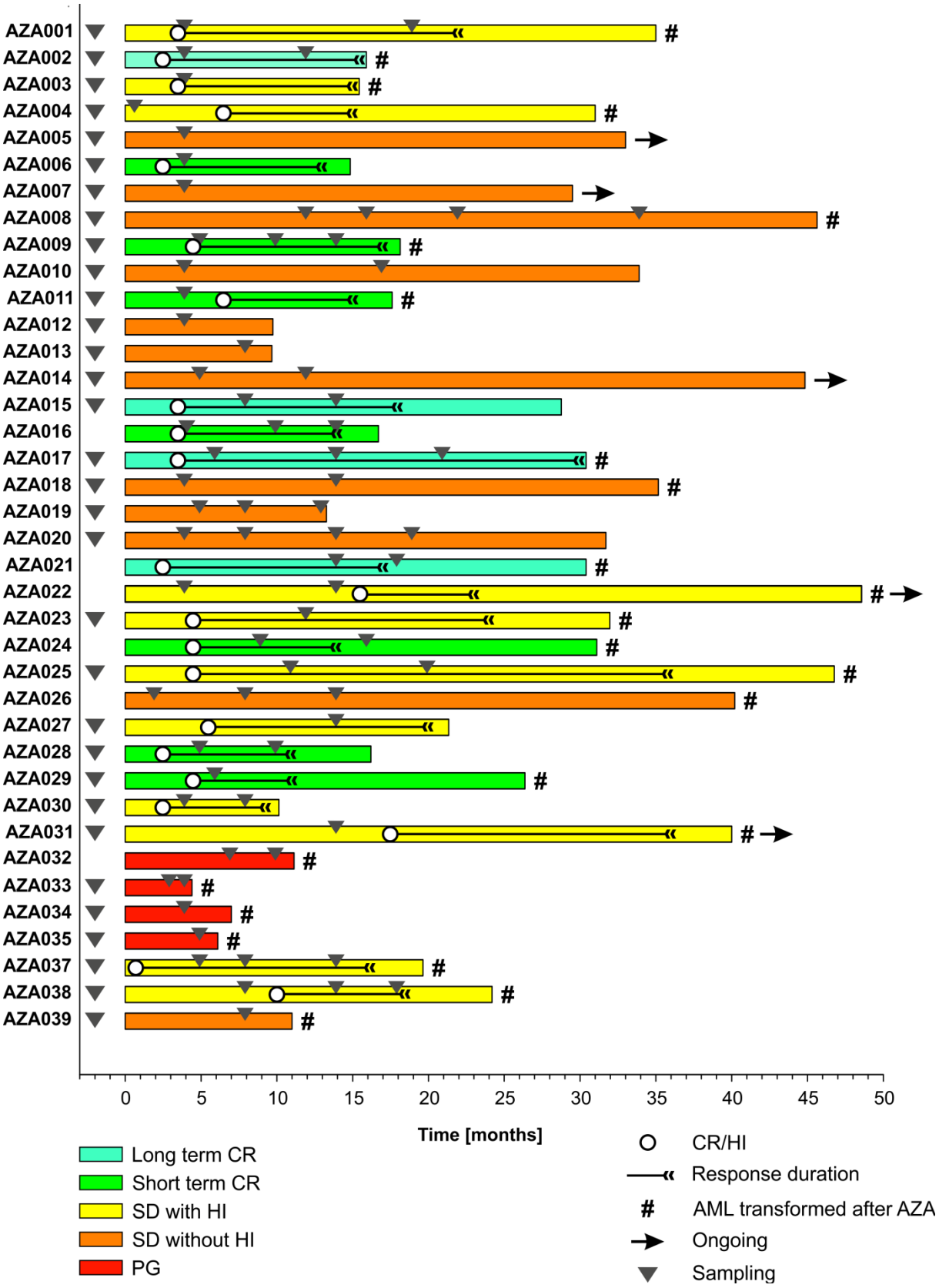


Figure 5: Swimmers' plot of 38 patients treated with AZA. Responses (in different colors: CR, SD, HI, or PG), their duration and patients' survival (in months), sampling (triangles), AML transformation (#) and ongoing follow-up (→) are indicated.

3.1.1.2 Cell lines

pB-ALL (REH, SP-B15, RS4;11), T-ALL (Jurkat) and AML (NB4, Molm13, K562, Kasumi) cell lines were used for expression profiling; they were obtained from the German Tissue Culture Collection (DSMZ; Jurkat, REH, SUP-B15, RS4;11, NB4, and Kasumi) or the American Tissue Culture Collection (ATCC; Molm13, respectively). Cell lines were cultivated at 37 °C in 5 % CO_2 in RPMI1640 supplemented with fetal bovine serum, streptomycin and penicillin. For gene expression analysis, cells between 4 and 10 passages after defrosting were used.

3.1.1.3 Antibodies

Monoclonal antibodies targeting following antigens were used:

Target	Fluorochrome	Manufacturer
CD1a	APC	BD Biosciences, San Jose, CA, USA
CD3	APC	Beckman Coulter, Miami, FL, USA
CD3	ECD	Beckman Coulter, Miami, FL, USA
CD3	FITC	Exbio Praha a.s., Vestec, Czech Republic
CD3	PerCpCy5.5	Exbio Praha a.s., Vestec, Czech Republic
CD4	APC	Exbio Praha a.s., Vestec, Czech Republic
CD4	ECD	Beckman Coulter, Miami, FL, USA
CD5	PC7	Beckman Coulter, Miami, FL, USA
CD7	ECD	Beckman Coulter, Miami, FL, USA
CD7	PE	Beckman Coulter, Miami, FL, USA
CD8	A700	Exbio Praha a.s., Vestec, Czech Republic
CD10	ECD	Beckman Coulter, Miami, FL, USA
CD117	PE	Beckman Coulter, Miami, FL, USA
CD13	PC5	Beckman Coulter, Miami, FL, USA
CD14	APCH7	BD Biosciences, San Jose, CA, USA
CD14	PE	Exbio Praha a.s., Vestec, Czech Republic
CD15	APCH7	BD Biosciences, San Jose, CA, USA
CD16	PB	Exbio Praha a.s., Vestec, Czech Republic
CD19	FITC	Beckman Coulter, Miami, FL, USA
CD19	PC7	BD Biosciences, San Jose, CA, USA
CD20	PB	Exbio Praha a.s., Vestec, Czech Republic
CD22	APC	BD Biosciences, San Jose, CA, USA
CD33	APC	Beckman Coulter, Miami, FL, USA
CD33	PE	BD Biosciences, San Jose, CA, USA
CD34	PC7	Beckman Coulter, Miami, FL, USA
CD34	PerCpCy5.5	BD Biosciences, San Jose, CA, USA
CD38	A700	Exbio Praha a.s., Vestec, Czech Republic
CD45	PerCP	Exbio Praha a.s., Vestec, Czech Republic
CD45	PO/OC515	Exbio Praha a.s., Vestec, Czech Republic
CD66c	PE	Beckman Coulter, Miami, FL, USA
CD99	FITC	BD Biosciences, San Jose, CA, USA

3.1.1.4 Real-time PCR assays

Following real-time PCR assays based on commercial fluorogenic probe Taqman® platform (Applied Biosystems, ThermoFisher Scientific, MA, USA) were used for expression profiling of healthy and leukemic (incl. SW-AL) specimens.

gene ID	Taqman® assay commercial numbers
ABL1	Hs01104728_m1
AIF1	Hs00610419_g1
IKZF3	Hs00232635_m1
RUNX	Hs00231079_m1
B2M	Hs00984230_m1
BCL11A	Hs00256254_m1
BCL11B	Hs00256257_m1
BCR	Hs00244716_m1
BLNK	Hs00179459_m1
CCDC26	Hs01886265_s1
KIT	Hs00174029_m1
CD14	Hs00169122_g1
CD19	Hs00174333_m1
CD2	Hs00233515_m1
CD22	Hs00233533_m1
CD24	Hs00273561_s1
CD25	Hs00907779_m1
CD2AP	Hs00961451_m1
CD3E	Hs01062241_m1
CD4	Hs00181217_m1
CD79A	Hs00233566_m1
CD8A	Hs00233520_m1
CEBPA	Hs00269972_s1
CEBPB	Hs00270923_s1
CEBPD	Hs00270931_s1
CEBPE	Hs00357657_m1
CRLF2	Hs00845692_m1
CSF1	Hs00174164_m1
CSF3R	Hs00167918_m1
CUL1	Hs01118950_m1
CDKN1A	Hs00355782_m1
TCF3	Hs01012685_m1
EBF1	Hs00395513_m1
IKZF4	Hs00223842_m1
EPOR	Hs00959427_m1

FCGR3A	Hs01569121_m1
FCGR2A	Hs01017702_g1
FLT3	Hs00174690_m1
FOXC1	Hs00559473_s1
FOXO1	Hs01054576_m1
FOXO3	Hs00818121_m1
FOXP3	Hs01085834_m1
GAPDH	Hs99999905_m1
GATA1	Hs01085823_m1
GATA3	Hs00231122_m1
GLS	Hs00248163_m1
GUSB	Hs00939627_m1
HOXA10	Hs00172012_m1
HOXA9	Hs00365956_m1
HOXB3	Hs00231127_m1
HOXB4	Hs00256884_m1
HPRT	Hs01003267_m1
ID2	Hs00747379_m1
IKZF1	Hs00172991_m1
IKZF2	Hs00212361_m1
IL6R	Hs01075667_m1
IRF4	Hs01056533_m1
IRF8	Hs01128710_m1
ITGA6	Hs01041011_m1
LTK	Hs00950634_m1
KLF4	Hs00358836_m1
CAECAM6	Hs00366002_m1
MAFB	Hs00534343_s1
LAT	Hs01065378_g1
LCK	Hs00178427_m1
LGALS1	Hs00355202_m1
LILRA2	Hs00429044_m1
LMO2	Hs00153473_m1
LTF	Hs00914334_m1
MLL	Hs00610538_m1
MNDA	Hs00159210_m1
MSH6	Hs00264721_m1
MYB	Hs00920554_m1
MYC	Hs00905030_m1
NDN	Hs00267349_s1
NFIL3	Hs00705412_s1
NOTCH1	Hs01062014_m1
NOTCH3	Hs01128541_m1
PAWR	Hs01088574_m1
PAX5	Hs00172003_m1
PBX1	Hs00231228_m1

POU2AF1	Hs01573371_m1
SPI1	Hs02786711_m1
RAG1	Hs00822415_m1
RBM47	Hs00219308_m1
RNF130	Hs00218335_m1
S100A10	Hs00741221_m1
STAT5A	Hs00234181_m1
TCF7	Hs00175273_m1
TNFSF10	Hs00921974_m1
TOP3A	Hs00172806_m1
RNF125	Hs00215201_m1
TRAP1	Hs00212474_m1
TSC2	Hs01020387_m1
UBASH3A	Hs00957643_m1

3.2 Methods

3.2.1 Cell-type subset separation

3.2.1.1 Magnetic cell sorting

Mature B-ly and mature monocytes from PB of healthy donors for expression profiling were separated using magnetic beads (the Human Naive B-cell Enrichment Kit based on negative selection, the CD14 Positive Selection Kit from EasySep^{TM} (STEMCELL Technologies, Canada)) after mononuclear fraction was obtained from full PB by Ficoll density gradient centrifugation.

Similarly, T-ly ($CD3^{pos}$) and enriched myeloid ($CD3^{neg}$) fraction from BM of MDS patients for MPS (article 3) were separated using and the CD3 MicroBeads (MACS, Miltenyi) following the manufacturer's instructions.

Efficacy of the separation process and purity of the obtained fractions was then evaluated by flow cytometry using anti-CD3, -CD14, -CD33 and -CD19 antibodies (BD FACS LSR II, BD Bioscience, CA, USA).

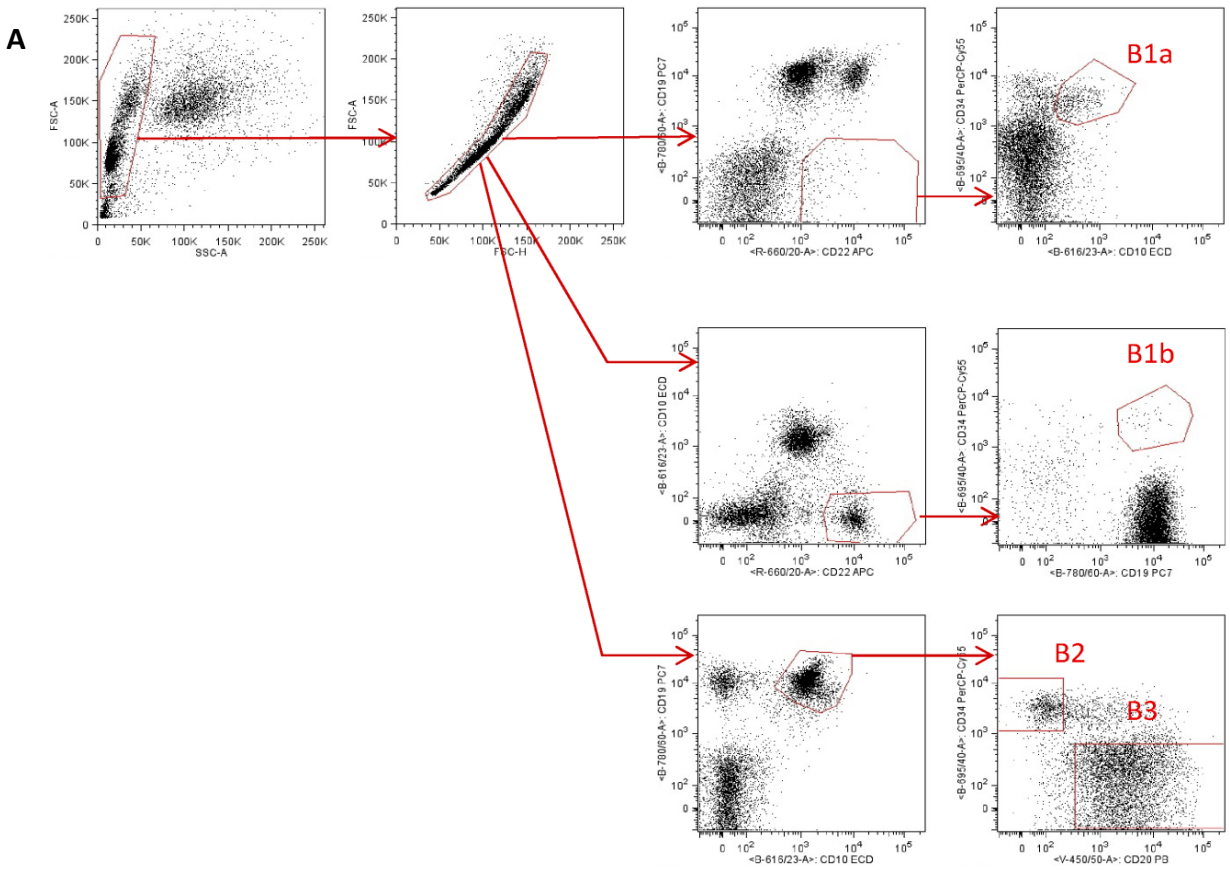
3.2.1.2 Fluorescence-activated cell sorting (FACS) of AL and non-malignant specimens

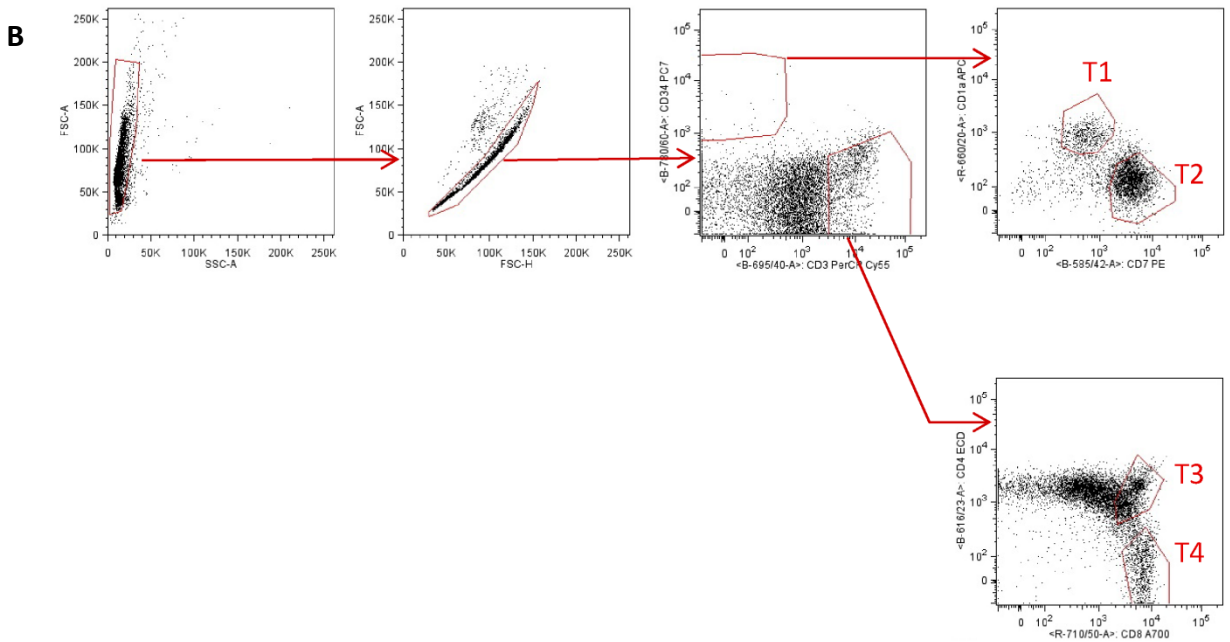
PB granulocytes, BM monocytes and B-ly and thymic T-ly for expression profiling were separated using FACS (BD FACS Aria III, BD Bioscience, USA). Immunophenotypic definitions of all non-malignant populations are described in Table 8 and the sorting strategy is presented in the Figure 6.

Sorting of non-malignant specimens from PB and BM for expression profiling was performed after red cell lysis by ammonium chloride (NH_4Cl_2) for 15 min at room temperature and subsequent PBS wash. Malignant specimens were sorted according to their individual phenotypes using thawed mononuclear cells separated from diagnostic BM sample by Ficoll-Paque premium gradient or fresh BM samples residues from diagnostic procedure, again, after red cell lysis.

Table 8. Immunophenotypic definition of sorted non-malignant specimens of various lineages used for mRNA expression profiling

Stage	Immunophenotype	Tissue
B1	$CD22^{pos}CD34^{pos}CD19^{neg}CD10^{pos}$ (B1a) $CD22^{pos}CD34^{pos}CD19^{pos}CD10^{neg}$ (B1b)	bone marrow
B2	$CD34^{pos}CD19^{pos}CD10^{pos}CD20^{neg}$	bone marrow
B3	$CD34^{neg}CD19^{pos}CD10^{pos}$	bone marrow
B4	$CD19^{pos}CD10^{neg}CD20^{pos}CD27^{neg}$	peripheral blood
T1	$CD34^{pos}CD1a^{neg}CD7^{pos}$	thymus
T2	$CD34^{pos}CD1a^{pos}CD7^{pos}$	thymus
T3	$CD3^{pos}CD4^{pos}CD8^{pos}$	thymus
T4	$CD3^{pos}CD4^{neg}CD8^{pos}$	thymus
Mo1	$CD33^{high}CD4^{pos}CD14^{neg}$	bone marrow
Mo2	$CD33^{high}CD4^{pos}CD14^{pos}$	bone marrow
Mo3	$CD33^{high}CD4^{pos}CD14^{pos}$	peripheral blood
Gr1	$CD13^{pos}CD16^{neg}CD117^{pos}$	bone marrow
Gr2a	$CD13^{high}CD16^{neg}CD117^{neg}$	bone marrow
Gr2b	$CD13^{neg}CD15^{high}CD16^{neg}$ (Gr2a) $CD13^{low}CD15^{high}CD16^{low}$ (Gr2b)	bone marrow
Gr3	$CD13^{high}CD16^{high}CD117^{neg}$	bone marrow
Gr4	$CD13^{high}CD15^{high}$ $CD16^{pos}$	peripheral blood


A
FSC-A
SSC-A
FSC-H
<R-660/20-A>: CD22 APC
<B-780/60-A>: CD19 PC7
<B-695/40-A>: CD34 PerCP-Cy55
<B-616/23-A>: CD10 ECD
B1a
B1b
B2
B3
<V-450/50-A>: CD20 PB


B
FSC-A
SSC-A
FSC-H
<B-695/40-A>: CD3 PerCP Cy55
<B-585/42-A>: CD7 PE
<R-660/20-A>: CD1a APC
<R-710/50-A>: CD8 A700
<B-616/23-A>: CD4 ECD
T1
T2
T3
T4

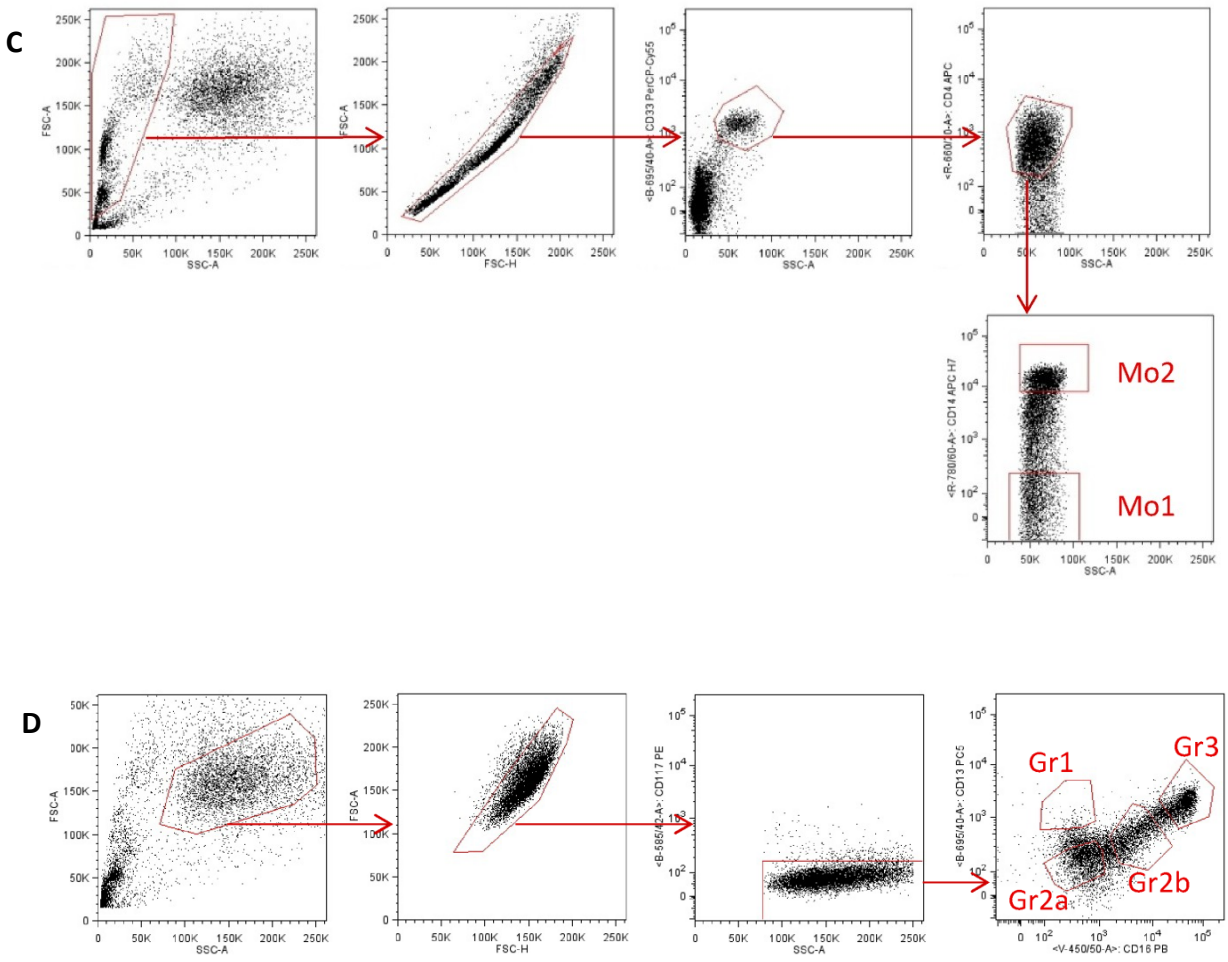


Figure 6: Gating strategies for fluorescence activated cell sorting of B-ly (A), T-ly (B), Mo (C) and Gr (D) populations. Sorting of each cell population was based first on lympho-mono gate (A, B, C) or granulocytes gating (D) according to their forward (FSC-A) and side scatter (SSC-A) characterization with further selection of singlets (first two gating images from the left of each subfigure). After that, particular antigens' expression was evaluated as defined in table 8.

3.2.1.3 Immunophenotyping and cell-sorting of SW-AL subpopulations

A prospective 8-color SW-AL combination including anti-CD45, CD14, CD10, CD20, CD19, CD34, CD33 and anti-CD3 antibodies (i.e. 8CSAC) was designed to assess the incidence of transdifferentiation during the first weeks of treatment. This panel was used to analyze samples from all pB-ALL patients from the Czech cohort used for SW-AL study between 09/2007 and 05/2010 (N=179) at diagnosis, day 8, day 15 and 33. CD2 expression was analyzed in all available patients at time of diagnosis (N=704). Data were acquired on BD FACS LSR II (BD, Bioscience, San Jose, USA). Data were analyzed using FlowJo software (Treestar, Ashland, USA).

The 8CSAC panel was also used for sorting B-lymphoblasts, intermediate and monocytoid populations (Table 9 and Figure 7).

Table 9. Immunophenotypic definition of sorted SW-AL subpopulations

Subpopulation	Immunophenotype
sw-I	$CD14^{pos}CD33^{pos}CD34^{pos}CD19^{pos}$
sw-Mo	$CD14^{pos}CD33^{pos}CD34^{neg}CD19^{neg}$

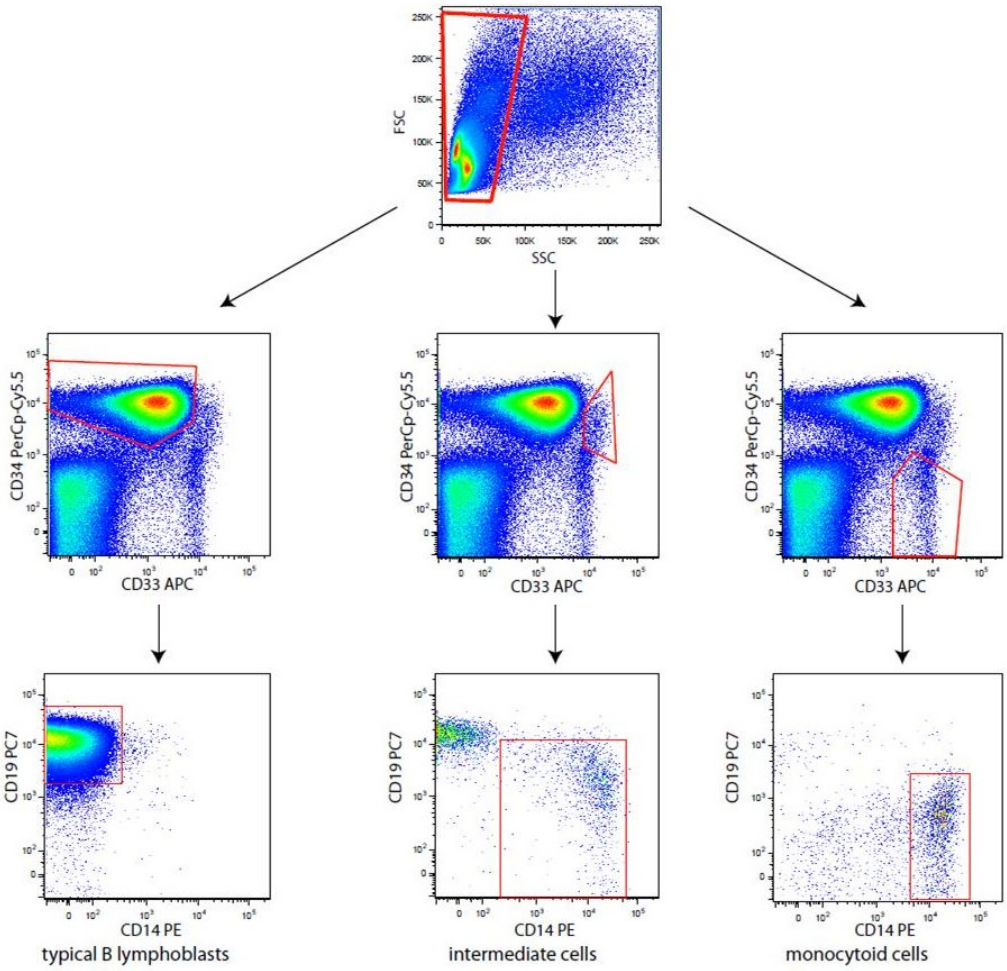


Figure 7. Defining criteria and cell sorting strategy to obtain lymphoblasts with typical pB-ALL phenotype, intermediate population and monocytoid population. The gating strategy starts with defining lympho-mono gate (Forward scatter and side scatter (i.e. FSC and SSC) with further evaluation of selected antigens expression as defined in table 9.

3.2.2 Gene expression profiling

3.2.2.1 Quantitative real-time PCR expression array assessment

For gene expression profiling we selected 95 genes using our own preliminary and already published data (Slamova et al. 2014) as well as publicly available microarray gene expression data (A. R. Abbas et al. 2005; van Zelm 2012). The gene set included several subgroups of cell fate regulators: known hematopoietic differentiation regulators, such as *SPI1*, *IKZF1*, *PAX5*, the CEBP family of genes, etc.; genes involved in leukemogenesis, e.g., genes from the HOX family; genes that encode cell surface markers such as *CD19* or *CD3e* to confirm appropriate cell sorting; genes with uncertain functions that have a possible regulatory role in hematopoiesis, such as *CCDC26*; and finally internal control or reference genes (RGs: *GAPDH*, *HPRT1*, *ABL1*, *GUSB*, and *B2M*). The complete list including assay numbers is available in section 3.1.2.2.

3.2.2.2 RNA extraction, reverse transcription

Harvested cells were submitted to RNA extraction (RNeasy Micro Kit, Qiagen). DNase treated RNA was eluted in 14 µl H_2O and then transcribed into cDNA (iSCRIPT, BioRad). Reverse transcription reaction was prepared according to manufacturer's intructions:

- 4 µl 5x iSCRIPT master mix
- 1 µl reverse transtricptase
- 11 µl RNA
- 5 µl H_2O

The whole mix was then incubated for 5 min at 25 °C followed by 30 min at 42 °C, 5 min at 85 °C and copled to 4 °C. Transcribed cDNA was then directly used for further analyses or stored at -20 °C until use.

3.2.2.3 Preamplification

Equal volumes of 20x TaqMan assays for each of 95 genes were pooled together and diluted in TE to achieve final concentration of 0.2x for each assay. The preamplification mix was prepared as follows:

- 25 µl of TaqMan preAmp Master Mix
- 12.5 µl of the TaqMan assay pool

- cDNA up to 100 ng (according to photometrical measurements of concentrations purity (Nanodrop, ThermoFisher Scientific, MA, USA))
- H_2O to final volume of 50 µl.

The preAmp program was run on the Thermal Cycler (Bio-Rad Laboratories, CA, USA):

10 min	95 °C
14x	
15 sec	95 °C
4 min	60 °C

After the final run, samples were diluted 1:20 in water and stored at -20 °C. Preamplified and diluted cDNA was then used as a template for qPCR. The possibility of bias caused by the pre-amplification step was ruled out by comparing expression data from the preamplified and non-amplified samples using Pearson correlation coefficient and Bland-Altman comparison (see Figure 8)

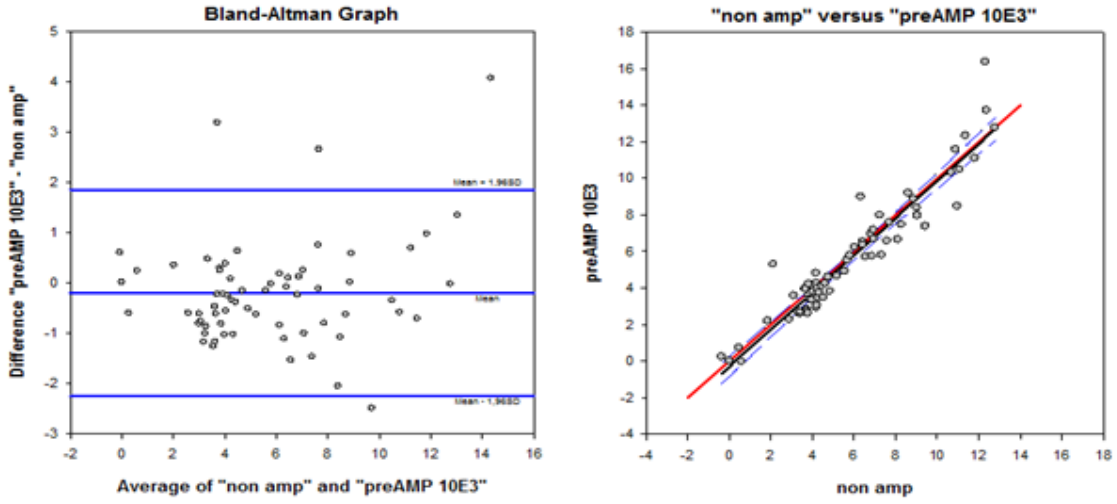


Figure 8: Bland-Altman comparison and Pearson correlation coefficient comparing non-amplified and premplified samples showing good agreement between the two ways of samples processing.

3.2.2.4 Quantitative real-time PCR

Real-time qPCR experiments were performed on 7500 Fast Real-Time PCR System (Life Technologies, ThermoFisher Scientific, MA, USA) using commercially available hydrolytic

probes and Taqman Universal Master Mix II with UNG (both Life Technologies, ThermoFisher Scientific, MA, USA). Preamplified and diluted cDNA was used as a template in real time PCR mix:

- 1 µl of TaqMan assay
- 10 µl of master mix
- 5 µl of pre-amplified (or non-amplified) sample
- 4 µl H_2O

The real time qPCR run:

2 min	50 °C
10 min	95 °C
45x	
15 sec	95 °C
1 min	60 °C

3.2.2.5 Data analysis

For analysis, linear regression method was used to assess Cq values, which were obtained by LinReg software to avoid the bias possibly introduced by the subject of evaluator (Ruijter et al. 2009). Normalized gene expression was then calculated using the ΔCq method. An appropriate combination of internal control genes was obtained by performing intra- and intergroup variation analysis using the NormFinder tool (Andersen, Jensen, and Ørntoft 2004). Expression data were analyzed using the ΔCq and $2^{\Delta Cq}$ methods. Based on the NormFinder results, a combination of *HPRT1* and *GUSB* was used as the internal control. Hierarchical clustering using complete linkage method and Pearson correlation as recommended by D'haeseleer (2005) and principal component analysis (PCA) were performed using the MultiExperiment Viewer (MeV) and R software (Lucent Technologies). We also aimed to identify individual differences between different lineages and their developmental stages performing Kruskal-Wallis non-parametric test. Significance of differences in particular genes expression between malignant and non-malignant specimens was evaluated using Mann-Whitney test. The data were then uploaded to a publicly available database – leukostage.org using R-project for the database assessment.

3.2.3 **Switching leukemia characterization**

Flow cytometry analyses, cell sorting and gene expression analyses for SW-AL study (enclosed article Slamova et al. 2014 – Supp. 2) were performed as described above (part 3.2.1.3).

3.2.3.1 Ig/TCR rearrangements evaluation

Patients' specific immunoglobulins (Igs) and TCR rearrangements were detected using quantitative real-time PCR which was performed for whole BM samples as well as sorted populations as previously described (van der Velden et al. 2007)and was used for MRD detection during standard clinical reevaluation.

3.2.3.2 Cytogenetics and fusion genes determination

Cytogenetic and FISH analyses were included in clinical evaluation and risk stratification prior to therapy according to standard protocols and were performed in the laboratory of Centre of oncocytogenetics of General Faculty Hospital, Prague. All patients were screened for recurrent, non-random genetic aberrations specific for ALL, including *MLL* rearrangements, *ETV6/RUNX1* fusion gene (also called *TEL/AML1*) and hyperdiploidy, using interphase fluorescence in-situ hybridization (I-FISH) with locus specific and/or centromeric Vysis probes (Abbott Molecular). The presence of *ETV6/RUNX1*, *BCR/ABL* and *E2A/PBX1* was also examined as a part of routine ALL diagnostics. The presence of the *MLL/AF4*, *MLL/AF6*, *MLL/AF9*, *MLL/AF10*, *MLL/ENL*, *MLL/ELL* gene fusions was evaluated using multiplex real-time PCR, as already published (Andersson et al. 2001).

3.2.3.3 Single nucleotide polymorphism (SNP) Array

Genomic DNA was extracted from mononuclear cells (obtained by Ficoll-Paque density centrifugation) from diagnostic and remission (i.e. control) BM samples by QIAamp® DNA Blood Mini Kit or the EZ1 DNA Blood Kit (both Qiagen GmbH, Hilden, Germany).
Genotyping was then performed by the Affymetrix platform as previously described (van Delft et al. 2011) or the Illumina HumanOmniExpress BeadChip according to the Infinium HD assay Ultra protocol (Illumina Inc., CA, USA). Illumina GenomeStudio software was used for genotype analysis. CNV analysis was performed using the cnvPartition 2.4.4 software tool within GenomeStudio.

3.2.3.4 Detection of IKZF1 alterations and ERG deletions

IKZF1 deletions were analyzed following the instructions of multiplex ligation-dependent probe amplification based SALSA MLPA P335 kit (multiplex ligation-dependent probe amplification; MRC Holland, Amsterdam, Netherlands) with probes covering exons 1-8. The results were evaluated using the Coffalyser v9.4 software. The gene expression of various *IKZF1* isoforms was evaluated by real-time qPCR as published previously (Volejnikova et al. 2013).
To detect intragenic *ERG* deletions multiplex PCR was performed using 1 forward and 3 reverse primers (forward: 5'-AGGAGAGAAAGGAACCACTGCTTTG-3',
reverse 1: 5'-CCCTATGTTGAAATCTTAACCCGCAG -3',
reverse 2: 5'-CATCTCAGATGTCTTGCTAGGGGACTC-3',
reverse 3: 5'-GTCTAACTCAGAAGCATCTCACGGTAAGG-3').
PCR reaction based on HotStarTaq Master Mix Kit (Qiagen GmbH, Hilden, Germany) contained 0.4 mmol/l of each primer and 200 ng genomic DNA. PCR products were analyzed using agarose gel electropohoresis.

PCR program was as follows:

15 min	95 °C
50x	
20 sec	95 °C
20 sec	62 °C
40 sec	72 °C
10 min	72 °C

3.2.3.5 Methylation of CEBPA promoter

Methylation status of *CEBPA* promoter was analyzed in diagnostic samples from 16 SW-AL, 30 pB-ALL, 4 T-ALL and 14 AML using bisulfite conversion method with further sequencing analysis. Shortly, genomic DNA was treated by EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation, Irvine, USA) and amplified using *CEBPA* promoter specific primers. The PCR product was then cloned into a CR®2.1 vector using TOPO® TA Cloning® Kit (Invitrogen Corporation, Carlsbad, CA, USA) that included also competent E. coli bacteria that were then transformed with the plasmid. After bacteria growth, plasmids were isolated using

QIAprep Spin Miniprep kit (Qiagen GmbH, Hilden, Germany) and subjected for sequencing. CpG sites were then identified as methylated CpG or demethylated TpG.

3.2.3.6 In vitro simulation of the first week of the therapy

Lymphoblasts obtained by Ficoll-Paque gradient centrifugation from diagnostic samples from pB-ALL (N=8) and SW-ALL (N=4) were cultured in RPMI supplemented with 10% fetal bovine serum and antibiotics and subjected to prednisolone treatment (4-8 days culture in presence of 0, 5, 10 or 100 μg/ml prednisolone). Immunophenotyping was performed using 8CSAC panel every 24 hours.

3.2.4 **Sequencing**

3.2.4.1 DNA extraction, quality control

Genomic DNA was extracted from control $CD3^{pos}$ and malignant $CD3^{neg}$ fraction, as well as whole BM, using DNeasy blood and tissue extraction kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer recommendation. DNA was diluted in water and stored at -20 °C till use. The amount of extracted DNA was measured using Qbit fluorometric system (ThermoFisher Scientific) and its quality was confirmed using agarose gel electrophoresis.

3.2.4.2 Library preparation, quality control, sequencing

Sequencing libraries were prepared from DNA (50 ng) in duplicates using TruSight Myeloid Panel Kit (Illumina Inc., CA, USA) following manufacturer's instruction. The quality of prepared libraries was then evaluated by agarose gel electrophoresis and also by KAPA library quantification Kit (Illumina) according to manufacturer's instructions. Only libraries reaching required quality were then used for sequencing. Sequence data were obtained on Illumina Mi-Seq and Hi-Seq platform (Illumina Inc., CA, USA)

The panel focuses on ~141 kb of genomic content, consisting of 568 amplicons of ~250 bp length designed against the human NCBI37/hg19 reference genome. The oligo pool targets 15 genes (exons only) plus exonic hotspots of an additional 39 genes, providing nearly 100 % coverage of all targeted regions. The uniform coverage of the target regions enables > 500x coverage for > 95 % of amplicons at > 5,000x mean coverage. Average amplicon coverage was > 1000. We have sequenced at least one sample per patient twice including two separate sequencing library preparations. Moreover, we use age-matched controls as internal controls.

3.2.4.3 Data analysis

Somatic variants were primarily detected using $CD3^{neg}$ fraction, while duplicate samples and $CD3^{pos}$ fraction data were used for control and filtering. FASTQ files produced by the sequencer were processed by custom pipeline. Initial quality control was performed by means of FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc). After sequencing adaptor trimming and low quality regions removal by cutadapt, reads were aligned to the human genome HG19 using bwa mem (bwa version: 0.7.15-r1140). Subsequently, bam files were processed by GATK IndelRealigner and primer sequences were removed from bam files.

We used two variant calling tools (FreeBayes and samtools mpileup; R-project) (H. Li et al. 2009) to detect single nucleotide polymorphisms (SNVs) and insertions/deletions (InDels). We then annotated detected variants using dbSNP and COSMIC databases. Criteria for selecting reported variants were as follows. We use data from the union of variant detections performed using FreeBayes and samtools mpileup with default set of parameters to these programs. For each sequencing unit from MDS patient, we computed VAF and reported only the variants with VAF value that was greater by 20 percentage points than the highest VAF for the variant in an internal control. ALL reported mutations were then reviewed using Integrative Genomics Viewer software (Robinson et al. 2011). Mutation was considered somatic and reported if not detected in the $CD3^{pos}$ fraction and if successfully detected in a duplicate library sample. Mutations that markedly affected protein structure also excluding polymorphisms were further filtered by experienced biologist. The arbitrary VAF was set at 5 % as inspired by other studies (de Leng et al. 2016).

4 Results

4.1 Gene expression profiling

4.1.1 Lineage-associated gene expression differences

Using our optimized gene expression profiling platform we evaluated expression of 95 genes in different developmental stages from BM, thymus and PB (including rare early progenitors) of B-ly, T-ly, Mo and Gra lineages aiming to define each lineage developmental expression changes.

We represented the expression data in heat maps using hierarchical clustering. The analysis showed strong clustering of particular lymphoid and myeloid lineages (Figure 9) which remained unbiased even after excluding the defining molecules (CD3, CD4, CD8, CD14, CD19, CD22, CD79a) (not shown, see enclosed article Polgarova et al. 2016). Similar clustering in repeated analysis after defining markers exclusion suggests that chosen genes appropriately describe evolution of expression changes during differentiation in all tested hematopoietic lineages confirming accurate gene selection algorithm used in real-time qPCR array assembly. As summarized in table 10, several genes were denominated as lineage specific, i.e. showed statistically significant difference between lineages (Kruskal-Wallis test, $p<0.05$).

Hierarchical clustering analysis was also performed using data from malignant specimens (Figure 10) revealing clustering of AL with their non-malignant counterparts. Interestingly, samples of biphenotypic pB/My AL cases clustered together with B-ly and pB-ALL. This observation further supports the idea of ALL-regimen based treatment for patients with ALAL achieving better results that was recently published (Hrusak et al. 2018).

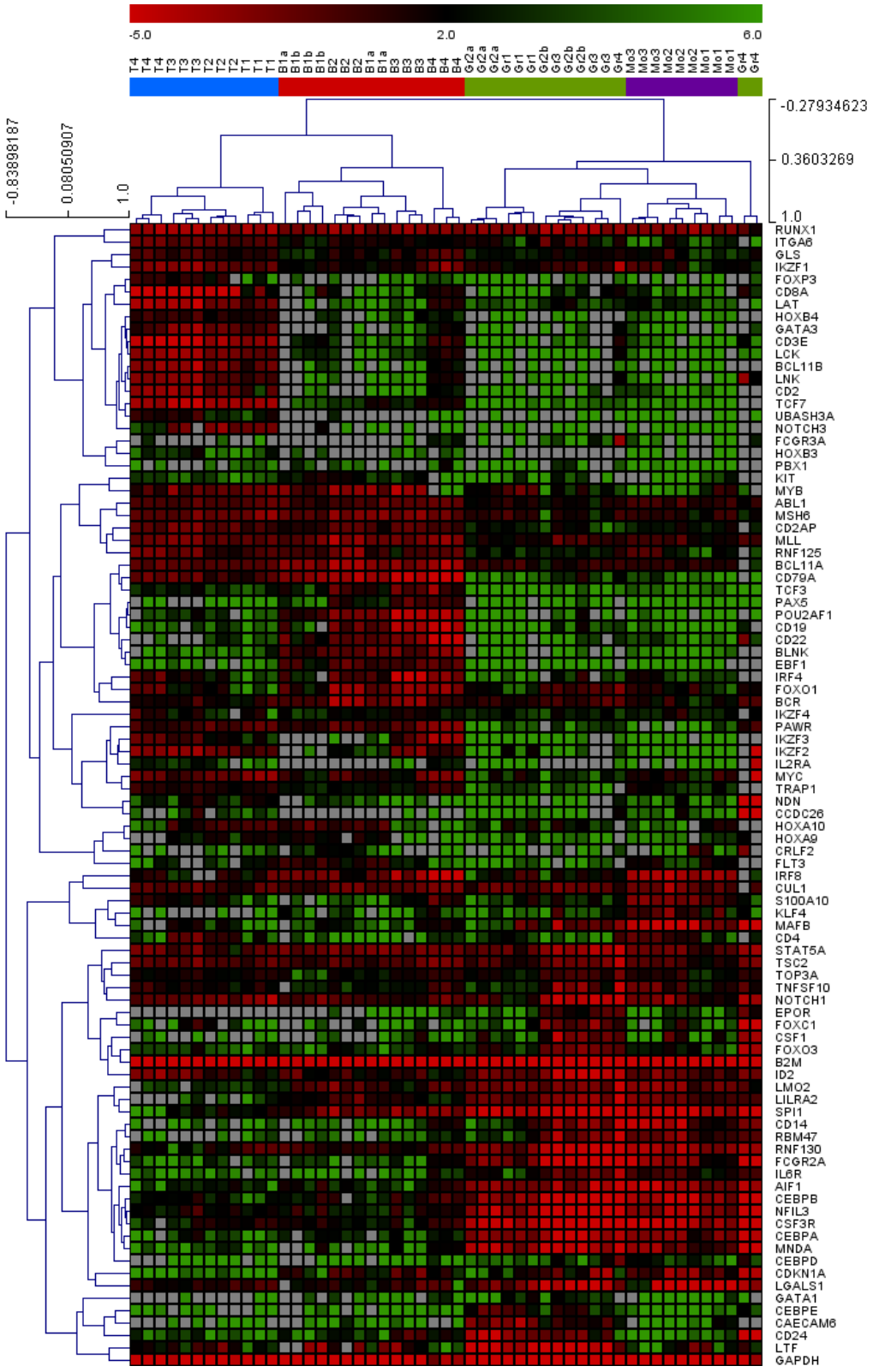


Figure 9: Heatmap showing B-ly, T-ly, Mo and Gra developmental stages clustering together (complete lineage hierarchical clustering, Pearson correlation); data are presented as delta CT; high expression is shown in red, low expression is marked by green; grey is reserved for undetectable expression.

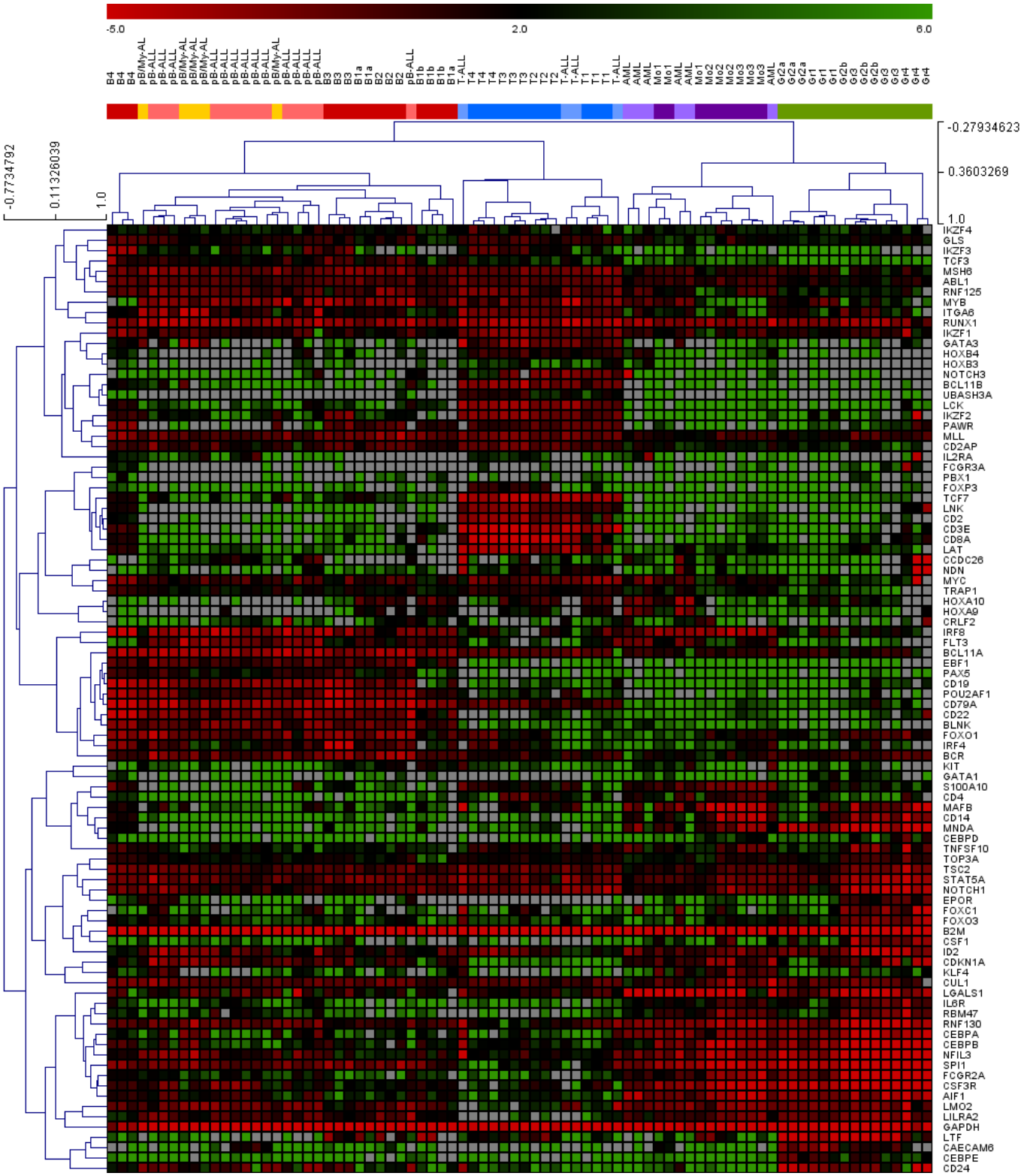


Figure 10: Heatmap showing malignant specimens clustering together with their non-malignant B-ly, T-ly, Mo and Gra counterparts (complete lineage hierarchical clustering, Pearson correlation); pB/MY-AL cluster together with pB-AL; data are presented as delta CT; high expression is shown in red, low expression is marked by green; grey is reserved for undetectable expression.

Table 10. Genes with lineage association

Myeloid lineage associated genes		**Lymphoid lineage associated genes**	
AIF1		MSH6	
CEBPD		MYB	
CEBPB		IKZF3	
RNF130		PAWR	
PU.1		***B-cell associated genes***	***T-cell associated genes***
FCGR2a			
CEBPA		CD19	CD8a
CSF3R		POU2AF1	HOXB4
IL6R		PAX5	CD3e
MAFB		CD22	ITK
MNDA		FOXO1	LCK
NFIL3		BLNK	TCF7
RBM47		EBF1	CD2
CD14		BCL11A	BCL11B
		CD79A	GATA3
Monocytes associated genes	***Granulocytes associated genes***	TCF3	UBASH3A
KLF4	CEBPE		NOTCH3
S100A	LTF		LAT
MAFB	CEACAM6		ITG6A

4.1.1.1 Gene expression changes during development of B- and T-lymphocytes

To investigate changes in transcriptional networks during lymphoid differentiation we compared gene expressions between particular lineages and also tracked genes' up- and downregulation during T and B-ly development. Our data show that the mRNA level of known B-cell fate regulators, such as *PAX5*, *IKZF1* or *FOXO1* (Medvedovic et al. 2011; Reynaud et al. 2008; Zandi et al. 2008) expectedly increased along with B-ly maturation; this was also true for *IKZF2*, *IKZF3*, *PAWR*, *POU2AF1*, and *S100A10* and B-ly markers, such as of *CD19*, *CD22*, *CD79A;* see Figure 11A for *POU2AF1* expression as an illustrative example. Other genes (e. g. *FLT3*, *HOXA9* and *HOXA10*), on the contrary, tended to decrease their expression during development. Most of these genes have already been described to regulate early differentiation steps, such as lymphoid commitment or (pre-)pro-B cell development (Somasundaram et al. 2015). Finally, some genes (*IRF4*, *MYB*) presented with more complicated expression dynamics with increasing levels during BM development (B1-B3) with further fall of expression in PB stages.

In T-ly differentiation genes for CD8a, LAT, ITK, ID2, IKZF3, IRF4 and S100A10 exhibited increasing expression levels. Similarly to the B-ly development, we also observed genes with lower expression in more differentiated stages, including, again, *HOXA9* and *HOXA10* or *NOTCH3*.

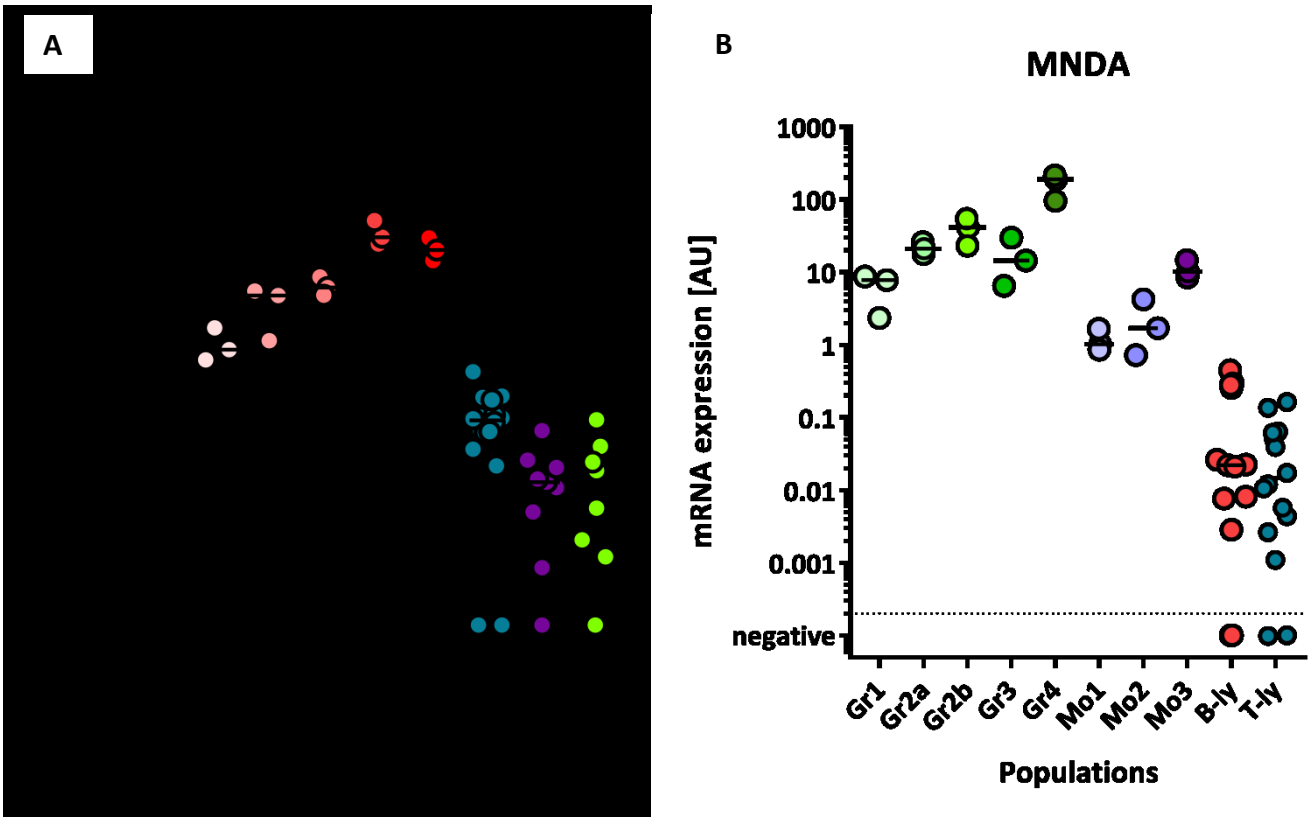


Figure 11. Expression of (A) *POU2AF1* in B-lymphocytes (B-ly) during B-cell development and its comparison with other lineages; and (B) MNDA during granulocyte (Gra) and monocyte (Mo) development in comparison with B- and T-lymphocytes (B- and T-ly); data are presented as $2^{-\Delta CT}$; samples with undetectable expression are marked as negative; horizontal lines represent medians.

4.1.1.2 Gene expression changes during development of myeloid lineages

We also determined myeloid (both, monocytic and granulocytic) lineage specific expression changes. During myeloid cells' development an expression increase was observed in genes *AIF1*, *CEBP* family (except for *CEBPE*), *CSF3R*, *FCGR2A*, *FOXO3*, *ID2*, *KLF4*, *LILRA2*, *MAFB*, *MNDA*, *NFIL3*, *NOTCH1*, *RNF130* and *S100A10*. In general, more intensive developmental changes were observed in granulocytes comparing to monocytes. This was true particularly for *FOXO3* and *LTF*. On the other hand, *S100A10* or *MAFB* were more dramatically changed in monocytes.

Several of these genes are, again, already known to regulate myeloid cell fate in different developmental stages; others' rising expression is connected to the function of mature monocytes and/or granulocytes (e. g. *FCGR2a, LTF*). The decrease of expression during differentiation was observed in *MYB* that is known to block granulocyte differentiation when being overexpressed (Patel et al. 1993).

4.1.2 Cross-lineage aberrant expression detected in healthy populations

Several works state aberrant expression of different lineage-specific molecules during various pathological conditions. B-ly lineage markers, such as CD79a or CD20 were repeatedly observed on the cell surface of T-cell lymphoma (Matnani et al. 2013) and in rare cases also in AML with t(8;21) (Kozlov et al. 2005); several myeloid markers were detected in T-ALL as well as pB-ALL (Chiaretti, Zini, and Bassan 2014), in some cases (such as CD66c) relation to specific molecular changes (e.g. *BCR/ABL* rearrangement) was observed (Tang et al. 2015). We thus searched for aberrant cross-lineage expression also in our dataset.

Our expression profiling revealed lineage-aberrant mRNA expression of several genes in non-malignant specimens. For example, *CD79A* was, on mRNA level, detected in healthy T-ly, although with lower expression level comparing to B-ly (Figure 12). This phenomenon was not explainable only by contamination, as concordant expression of the rest of (defining) B-ly molecules (*CD19* or *CD22*) was lacking. Whether this aberrant expression on normal T-ly founds the aberrant expression of membrane CD79A described in T-ly malignancies remains unknown. In above mentioned AML cases the aberrant CD79a expression was related to particular cytogenetic changes and we did not confer similar infidelity in myeloid lineages within our data. Similarly, expression of CD66c is connected to particular cytogenetic changes and we did not observed any parallel in healthy populations in our samples.

Surprisingly, the *FCGR3A* (*CD16a*) transcript was detected in mature B cells, which, although capable of phagocytosis, are by definition characterized as $CD16^{neg}$ at the protein level. Again, the contamination by myeloid cells should be considered, as the B4-ly stage was separated from PB; however, increased expression of the rest of myeloid genes, such as *CD14* or *CSF3R* was not observed.

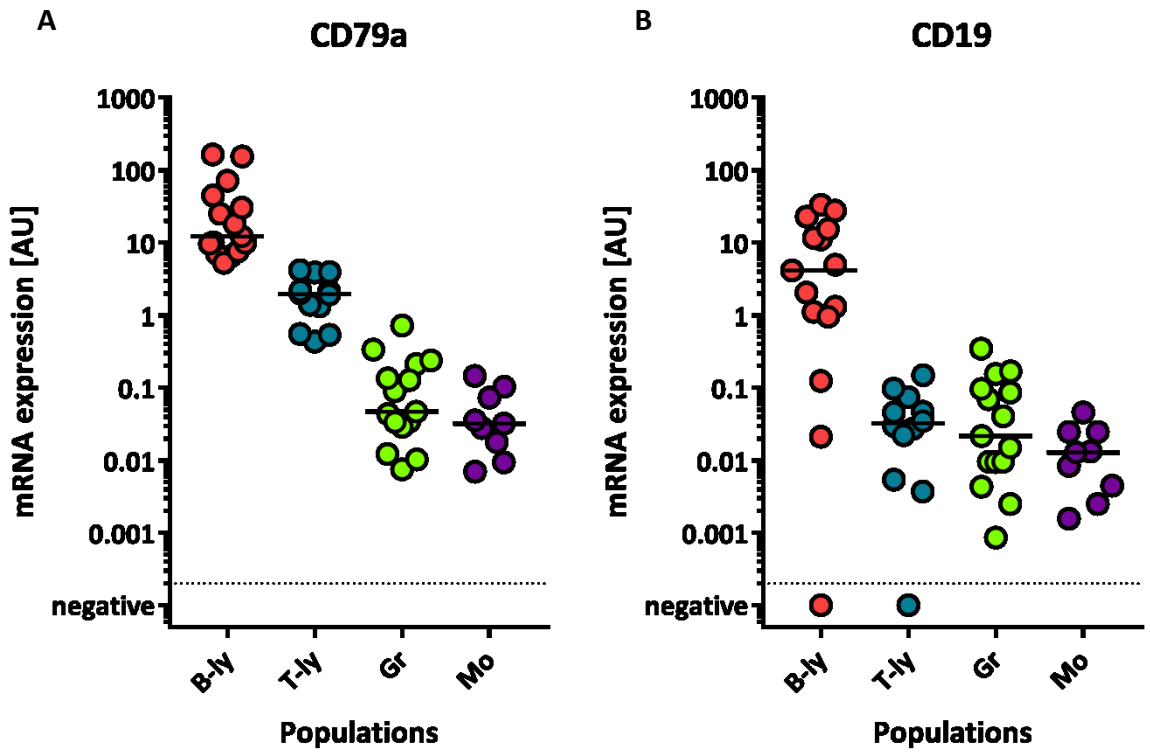


Figure 12. Expression of CD79a (A) in B-lymphocytes (B-ly), T-lymphocytes (T-ly), granulocytes (Gr) and monocytes (Mo) and its comparison with other highly expressed B-lymphoid genes, CD19 (B) excludes the possibility of falsely positive results due to B-ly contamination. Data are presented as $2^{-\Delta CT}$; samples with undetectable expression are marked as negative; horizontal lines represent medians.

4.1.3 **Healthy versus malignant populations**

Searching for the leukemia specific abnormalities we next compared malignant specimens with their non-malignant counterparts. We observed several differentially expressed genes. Some of them are known as cell commitment regulators, some regulate proliferation or apoptosis and some are connected to the physiological function of healthy mature cells and are thus expectedly deregulated in leukemic cells.

In B-lineage, one of the most significant differences was observed in expression of *CCDC26* in normal and malignant samples. Our analysis showed that this gene is variably expressed in all non-malignant specimens, except for B-ly, where its expression was completely abolished. In malignant samples the *CCDC26* expression was mostly present in similar or higher levels comparing to non-malignant counterparts. In pB-ALL the *CCDC26* expression was also detected with levels comparable to the rest of leukemic samples thus contrasting with mostly undetectable expression in healthy B-ly (pB-ALL vs. B-ly, $p<0.001$, Mann-Whitney test), Figure

13A. Similarly, *FLT3* tended to be upregulated in malignant, comparing to non-malignant B-ly samples, particularly comparing to more mature stages of B-cell development (Figure 14A).

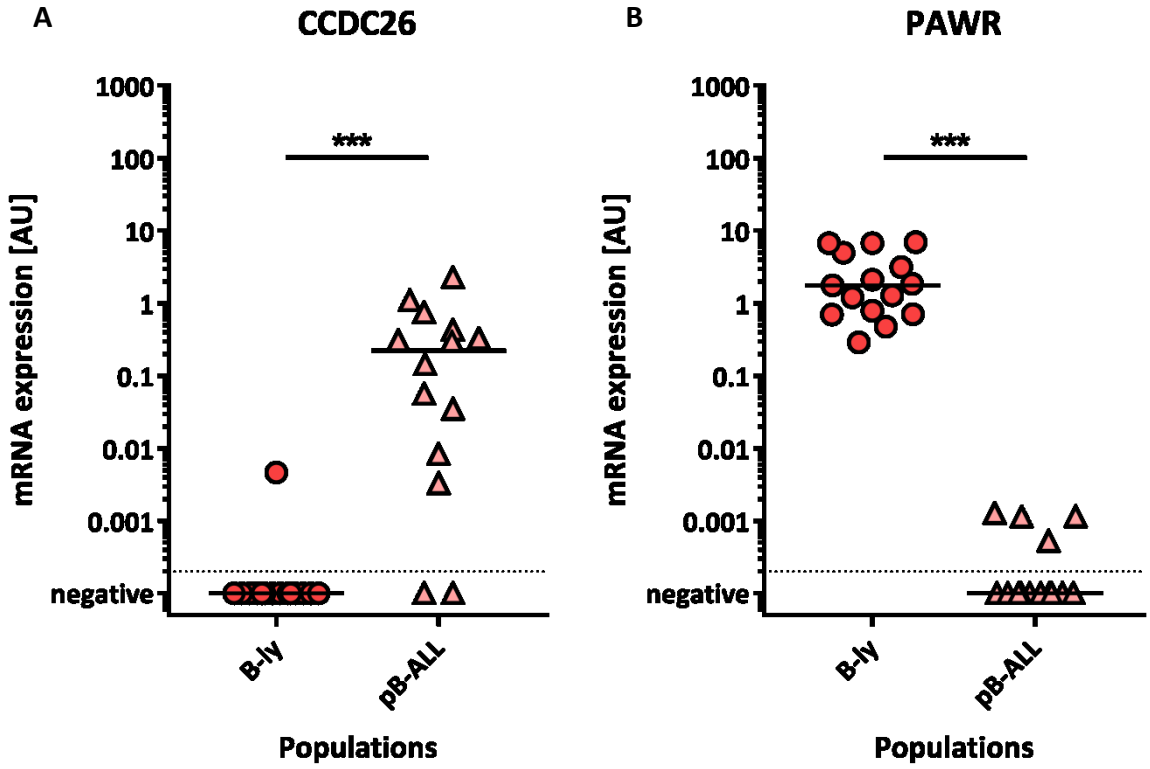


Figure 13. Expression of CCDC26 (A) and PAWR (B) in B-lymphocytes (B-ly; N=15) and B-precursor ALL (pB-ALL; N=14). Data are presented as $2^{-\Delta CT}$; samples with undetectable expression are marked as negative; horizontal lines represent medians. Statistical value of observed differences was calculated by Mann-Whitney non-parametric test with $p<0.001$ (***).

Other genes, on the contrary, were downregulated in leukemic specimens – *PAWR* (capable to induce apoptosis via Fas-pathway activation (Hebbar et al. 2012)) expression was mostly undetected or significantly lower in pB-ALL (and pB/MY-AL) compared to normal B-ly, where its expression increased along with more mature developmental stages (pB-ALL vs. B-ly, $p<0.001$, Mann-Whitney test; Figure 13B). This trend was partially present in AML vs. My-lineage but interestingly not in T-ALL vs. T-ly.

Interestingly, expression of both *HOXA9* and *HOXA10* was higher in early (B1 and B2) differentiation stages but was underexpressed (or undetectable) in pB-ALL. This was also true for T-ALL vs. T-ly (Figure 14B), but opposite trend was observed in myeloid lineage, where AML tended to express *HOXA9* and *HOXA10* at higher levels compared to healthy Gra or Mo confirming the described role of these genes in myeloid transformation (Thorsteinsdottir et al.

2001). Similar differences between healthy and malignant specimens were observed also in T-lymphoid lineage, where a decrease of expression levels was observed in leukemic samples in genes *CSF3R*, *FOXP3*, *HOXA9*, *HOXA10*, *IRF4* and *MNDA*. Some genes, such as *ID2* or *ITK*, exhibited expression comparable to early developmental stages (i.e. lower than more mature (T3-4) stages).

In myeloid lineage, we observed increased levels of *FLT3*, *HOXA9* and HOXA10 in malignant samples. On the contrary, genes from CEBP group, *MYB* or *NOTCH1* were downregulated in AML, resembling immature stages of physiological development. Expression of *PAWR* was, similarly to pB-ALL, mostly undetectable in AML. Generally, differences observed between AML and normal myeloid populations were less striking comparing to those observed in lymphoid lineages. Also, since our AML samples were mostly M4 or M5 subtypes according to FAB classification, the expression levels of myeloid genes mirrored more normal Mo than Gra.

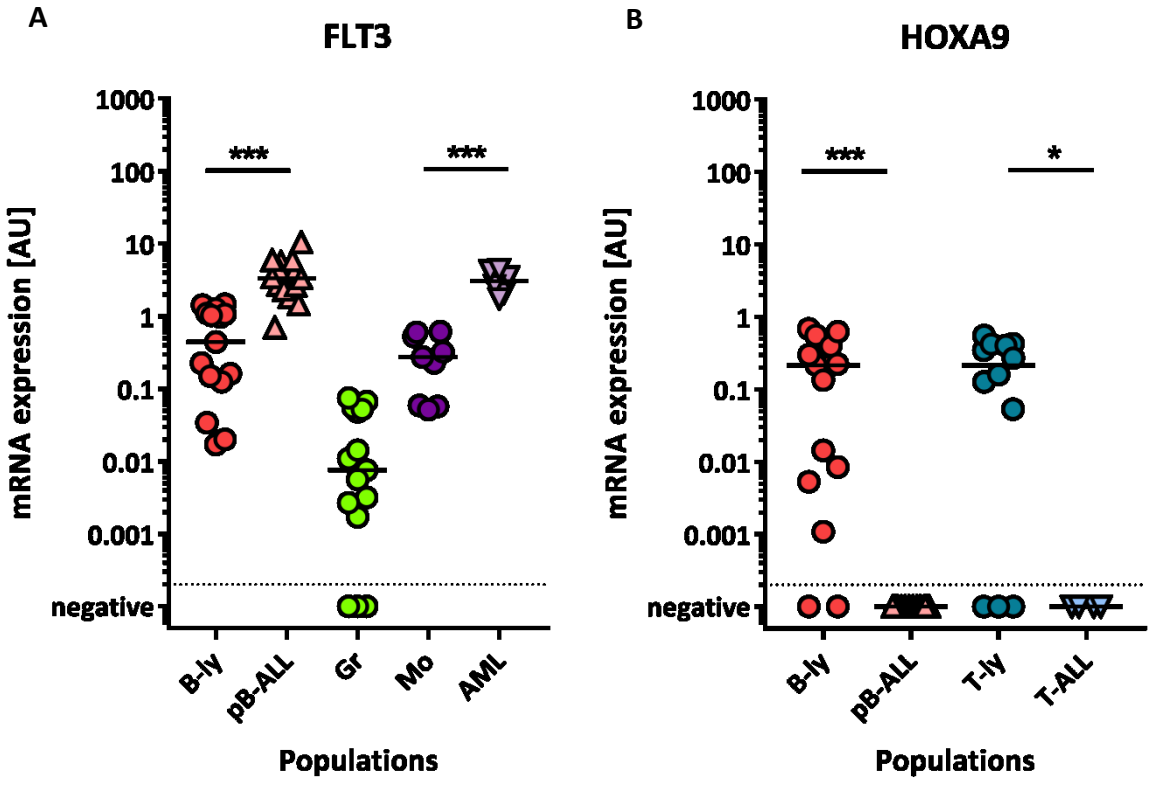


Figure 14. Expression of FLT3 (A) in B-lymphocytes (B-ly; N=15) versus B-precursor ALL (pB-ALL; N=14), granulocytes (Gr; N=15) and monocytes (Mo; N=9) versus AML (N=6); and HOXA9 (B) in B-ly versus pB-ALL and T-lymphocytes (T-ly; N=12) versus T-ALL (N=4). Data are presented as $2^{-\Delta CT}$; samples with undetectable expression are marked as negative; horizontal lines represent medians. Statistical value of observed differences was calculated by Mann-Whitney test with $p<0.05$ (*) or $p<0.001$ (***).

4.1.4 **Comparison of different leukemia subtypes**

Several cytogenetics related peculiarities in expression profiles have already been described in ALs (Guillaume et al. 2011; Moya et al. 2010). Therefore, we searched for such changes also in our dataset. Since number of specimens per particular AL subtype was low, we did not observe statistically significant differences between them. However, several genes exhibited subtype specific up or downregulation of its expression. E.g. *ITGA6* was expressed in higher levels in *ETV6/RUNX1* pB-ALL and also in most pB/MY-AL, whereas the rest of pB-ALL expressed this gene mostly on the level observed in normal B-ly. Likewise, the expression of *EPOR*, *KLF4* and *NFIL3* was higher in *ETV6/RUNX1* pB-ALL. We also detected higher expression of *S100A10* in *MLL* rearranged leukemias, which was previously described to mediate glucocorticoid resistance in this leukemia subtype (Spijkers-Hagelstein et al. 2013). *CEACAM6* (i.e. CD66c) presented higher expression in hyperdiploid pB-ALL, again with line with known observation of CD66c membrane positivity of this subgroup (Guillaume et al. 2011).

Interestingly, from the plethora of myeloid genes, *CEPBA* was the one with clearly upregulated expression in pB/MY-AL comparing to pB-ALL. The rest of genes from CEBP family, HOX family or other genes that were upregulated in myeloid lineage (healthy or malignant) were not observed to be particularly changed in our ALAL specimens.

4.1.5 **LeukoStage Database**

All expression data are available at web based database - http://www.leukostage.org, where it is possible to choose and compare different lineages throughout their differentiation or malignant with healthy samples. The data may be visualized by dot plots (expression is represented as $2^{\Delta CT}$ with combination of *GUSB* and *HPRT1* as default reference genes) with option of choosing other internal control gene. It is also possible to search for differentially expressed genes between two selected groups. A unique group of switching leukemias with different transdifferentiation stages was also presented in this database. Finally, several examples of commercially available cell lines (REH, SUP-B15, Jurkat, K562 and others as mentioned in part 3.1.1.2) were included.

4.1.6 **Summary**

In summary, we optimized expression profiling platform based on quantitative real-time PCR that includes 95 chosen genes with possible role during differentiation; we tested different developmental stages of four hematopoietic lineages – B- and T-lymphocytes, monocytes and

granulocytes from bone marrow, thymi and peripheral blood and identified various lineage-specific patterns. These observations were further confronted with those detected in malignant specimens relieving differences in genes involved in differentiation or apoptosis, such as *CCDC26* or *PAWR*. We also included peculiar observation from AL of ambiguous lineage as well as a specific AL group with switching phenotype. All these data are available for the scientific community on our web based database.

4.2 Lineage switching leukemias (SW-AL)

Acute leukemia switching its phenotype is a rare phenomenon previously described in literature, however without proper characterization or convincing explanation of its molecular nature. Herein we describe group of SW-ALs, that were diagnosed within a cohort of pediatric pB-ALL treated according different protocols that in all cases included glucocorticoid based induction phase.

4.2.1 SW-AL immunophenotype

We classified as SW-AL those pB-ALL cases that did not fulfill WHO or EGIL criteria for bilineal AL but exhibited leukemia-derived monocytoid cells in BM or PB within days 1 to 33 of ALL treatment (always including BFM corticosteroid-containing induction). Thus, 18 SW-AL patients were identified in total. The relation between pB-ALL and monocytoid population was confirmed by the presence of B-to-monocytoid transdifferentiation with an intermediate B/monocytoid stage with decreasing CD19 and CD34 and increasing CD33 and CD14 expression (Figure 7). The median peak of monocytoid cell frequency was 9.7 % (comparing to 2.2 % for pB-ALL) with the most pronounced differences within days 8 and 15 after treatment initiation. The analysis of retrospective cohort pointed out the superficial expression of CD2 that was present in SW-AL cases. The CD2 (partial)positivity was then confirmed also in the cohort of 179 pB-ALL prospectively screened for SW-AL using the optimized 8-color panel for flow cytometry – 8CSAC. In this cohort 7 patients (3.9 %) were identified as SW-AL, all being $CD2^{(partly)pos}$. Nine patients from the cohort were $CD2^{(partly)pos}$ but not classified as SW-AL and no SW-AL case was identified in $CD2^{neg}$ pB-ALL cases. The $CD2^{pos}$ phenotype was further confirmed also in Austrian pB-ALL cohort (2 SW-ALL from 44 patients) and one Swiss patient.

4.2.2 Ig/TCR rearrangements

To prove the relatedness of the monocytoid population with B-lymphoblasts we evaluated patient specific Ig/TCR rearrangements in different sorted subpopulations using quantitative real-time PCR. Patient specific Ig/TCR rearrangements were detected in 84 % (26/31 specimens) of monocytoid populations sorted from SW-AL samples from diagnosis and days 8, 15 or 33 after therapy initiation. In 7 cases also the intermediate stage was sorted and presence of specific Ig/TCR rearrangements was confirmed in all cases. On the contrary, only 1 out of 14 pB-ALL cases (7.1 %) revealed presence of Ig/TCR rearrangements in sorted monocytoid population.

4.2.3 Genetic changes

Aiming to find specific molecular background possibly explaining phenotypic changes occurring in SW-AL we evaluated different molecular hits and cytogenetic changes previously described in ALs using different methods, including cytogenetics and FISH, SNP arrays, MLPA, multiplex or quantitative real-time PCR.

No aneuploidy was detected in SW-AL cohort according to the DNA index; none of the patients exhibited any of the following: *MLL* gene rearrangements, *BCR/ABL*, *ETV6/RUNX1*, *E2A/PBX*, *BCR/ABL*, Flt3-ITD (not tested in 2 cases). Somatic copy-number alterations in SW-AL patients were less frequent comparing to already published pB-ALL or AML (Mullighan et al. 2007; Radtke et al. 2009). In our cohort 4 patients were detected to have deletions in *CDKN2A*, 2 patients had deletions that affected *CCDC26* gene, one patient had additional copy of this gene (due to chromosome 8 trisomy); 5 (out of 15 examined) patients exhibited *IKZF1* alteration – the frequency was higher than observed in previously published pB-ALL cohort from our laboratory (17 patients with *IKZF1* alteration out of 150); deletion of *ERG* was observed in 4 patients. Neither SNP array nor cytogenetic analysis revealed any common genetic aberration.

4.2.4 mRNA expression profiling

Using our gene expression profiling platform we evaluated expression of set of 95 genes in 32 sorted SW-AL specimens including B-lymphoblasts, intermediate population and monocytoid cells. We compared these populations with pB-ALL and AML samples that were described above. Clustering analysis revealed, that B-lymphoblasts resemble pB-ALLs, monocytoid cells mirrored normal Mo maturation and to lesser extend AML and the intermediate stages lied in between (Figure 15).

Comparison of particular genes' expression revealed downregulation of B-ly genes (or lymphoid), such as B-ly markers (*CD19*, *CD22*, *CD79A*), *POU2AF1*, *PAX5*, *EBF1*, *FOXO1*, *BCL11A*, or *MYB*) during transdifferentiation. Genes related to myeloid development, such as *CEBPD*, *CSF3R*, *FCGR2A*, *IL6R*, *MNDA*, *MAFB*, were, on the contrary, upregulated.

HOXA9 and *HOXA10* usually expressed in AML and downregulated in other AL from our cohort presented with very variable, but generally low expression throughout different SW-AL specimens. That was true also for some Mo related genes, such as *KLF4*, which has variable detection among all leukemic samples. Expression of *PAWR*, which was usually undetectable or very low in AML and pB-ALL, was also variable, but clearly detectable in more 20 cases (63 %).

Interestingly, *IKZF1*, whose low levels in early B-cell development were previously described by others to lead to transdifferentiation of B-ly to Mo (Reynaud et al. 2008) did not exhibit significant changes in expression during the evolution of monocytoid population.

Strikingly, *CEPBA* was upregulated already in B-lymphoblasts, when comparing to pB-ALL and showed stable pattern without further significant upregulation during monocytoid differentiation (Figure 16). *CEBPA* mRNA levels were significantly higher (Mann-Whitney test, $p<0.001$) in all SW-AL subpopulations (including B-lymphoblasts) comparing to pB-ALL samples or healthy B-ly. The expression was comparable to the expression observed in pB/MY-AL, AML or normal monocytes.

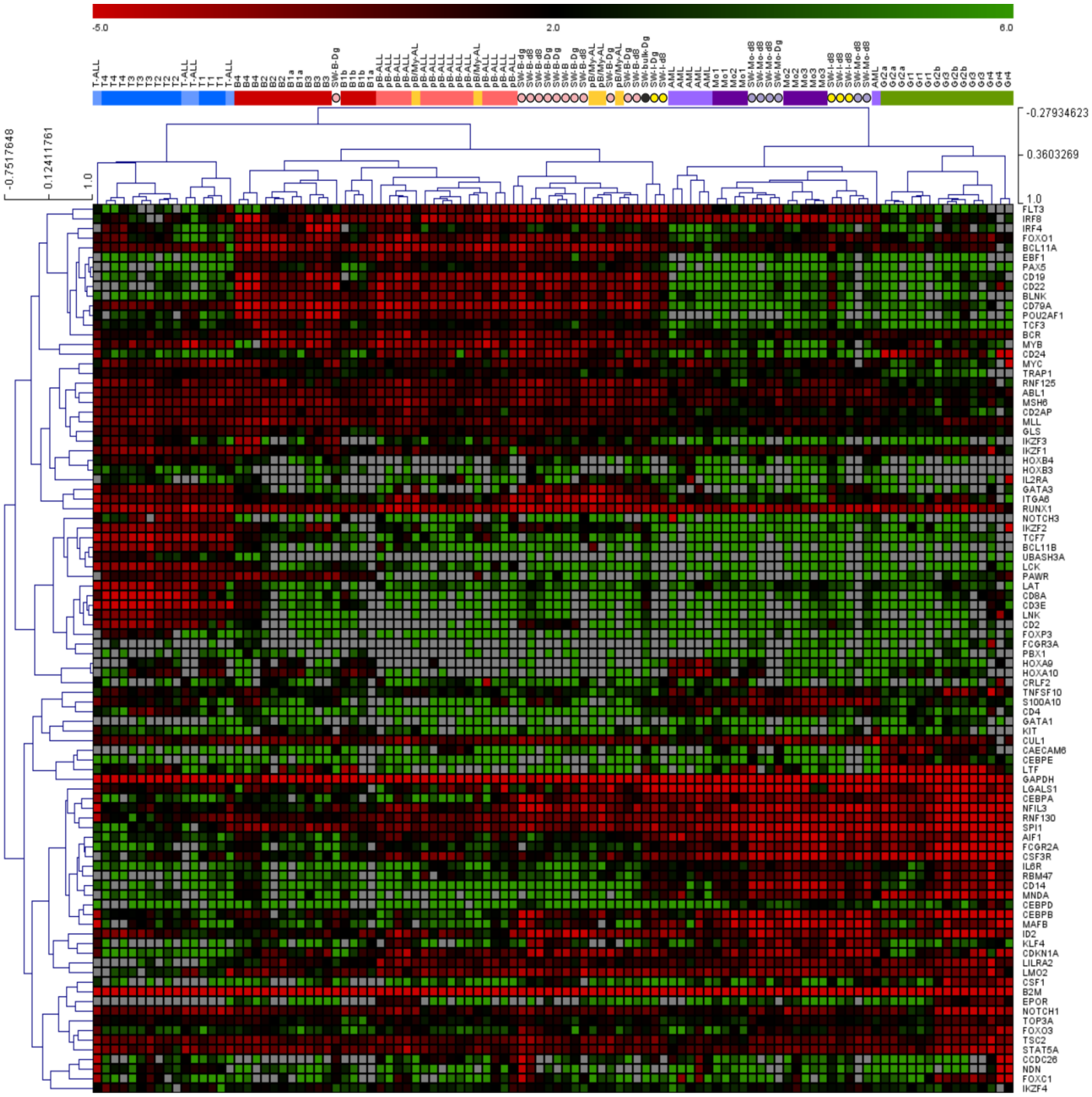


Figure 15. Heatmap showing clustering of particular SW-AL subpopulations within malignant and non-malignant specimens (complete lineage hierarchical clustering, Pearson correlation); SW-B – B-lymphoblasts (light red circles); different lineages are marked by different colors: B-ly red (pB-ALL light red), T-ly blue (T-ALL light blue), Mo violet, Gr green (AML light violet); SW-I – intermediate subpopulation (yellow circles); SW-Mo – monocytoid population (violet circles); Dg – sampling at diagnosis; d8 – sampling at day 8 from treatment initiation) within malignant and non-malignant specimens; data are presented as ΔCT; high expression is shown in red, low expression is marked by green; grey is reserved for undetectable expression.

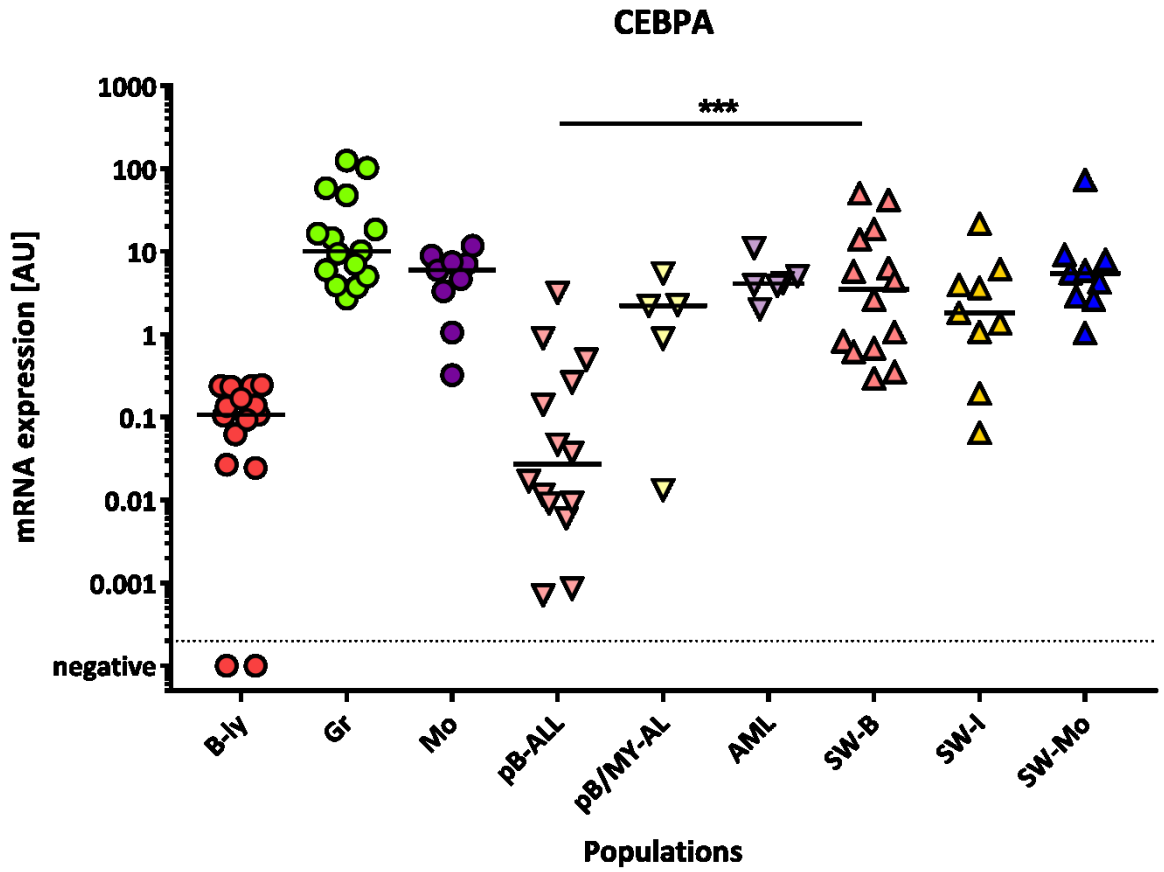


Figure 16. Comparison of expression of CEPBA in B-ly, non-malignant myeloid populations, pB-ALL, pB/My-AL, AML and B-lymphoblastic (SW-B), intermediate (SW-I) and monocytoid (SW-Mo) subpopulations. Note high CEBPA expression in SW-B subpopulation which is similar to expression observed in pB/MY-AL and AML and significantly higher than expression in pB-ALL (as calculated by Mann-Whitney test with $p<0.001$ (***)). Data are presented as $2^{\Delta CT}$; samples with undetectable expression are marked as negative; horizontal lines represent medians.

4.2.5 **Methylation of *CEBPA* promoter**

As mentioned above, we observed increased *CEBPA* expression in all subpopulations of SW-AL including B-lymphoblasts. *CEBPA* is one of the key transcription factors regulating differentiation towards the myeloid lineage. Thus, we concluded that this upregulation may be of the particular importance in the molecular background of SW-ALs. Since it was shown that *CEBPA* expression may be regulated epigenetically (Tada et al. 2006) we examined methylation status of CpG islands of *CEBPA* promoter by bisulfite sequencing.

Decreased promoter methylation in Dg (when compared to other pB-ALL), regulating *CEBPA* expression was observed in 14 out of 16 SW-AL cases (Fig. 4 of enclosed article Slamova

et al. 2014). Except for BCR-ABL and MLL-rearranged pB-ALL, hypomethylation of *CEBPA* promoter was related with increased *CEBPA* expression. The methylation level observed in SW-AL was similar to that found in AML.

4.2.6 Immunophenotypic changes after corticosteroid treatment in vitro

In order to recapitulate the transdifferentiation of B-lymphoblast to monocytes, cells from 4 diagnostic SW-ALL samples were maintained *in vitro* and cultured with prednisolone which lead to similar changes, that were observed in clinical SW-ALL settings (i.e. increased CD33 and CD14 expression and decreased CD19 and CD34 membrane expression observed by flow cytometry). These changes were not observed in pB-ALL samples without lineage switch during induction therapy with identical *in vitro* treatment.

4.2.7 Summary

Herein we described specific group of acute leukemias, which present at time of diagnosis as pB-ALL, but during induction treatment the B-lymphoblasts transdifferentiate into monocytoid population that is undistinguishable from normal monocytes. We performed immunophenotyping and molecular-genetic analyses to further characterize these SW-ALs. We also proved the relatedness of B-lymphoblasts with this monocytoid population and recapitulated the lineage-switch upon corticosteroids stimulation in vitro. Using our expression data we identified *CEBPA* as potential driver of the lineage-switch and further determined its promoter methylation as it is recognized way of *CEBPA* expression regulation.

4.3 Changes in mutational architecture in patients with myelodysplastic syndrome

4.3.1 Mutations dynamics in AZA treated patients

Using sequential tracking of mutational pattern in BM samples of MDS patients by TruSight Myeloid panel Kit by Illumina we identified a set of 116 somatic variants in 33 out of 54 evaluated genes. 35 patients (92 %) from our cohort bore at least 1 mutation with median of 3 mutations per patient (range 1-8 mutations per patient). Single nucleotide variants (SNVs, N=82) as well as in/dels (N=28) and complex mutations (N=6) were detected. The highest number of mutations was detected in genes *CUX1* (N=10, 8.6 %), *TP53* (N=9, 7.8 %), *BCORL1* (N=9,

7.8 %), *ASXL1* (N=7, 6 %), *RUNX1* (N=7, 6%), *BCOR* (N=6, 5.2 %), *TET2* (N=5, 4.3 %), *SF3B1* (N=5, 4.3 %), *SRSF2* (N=5, 4.3 %) and *STAG2* (N=5, 4.3 %) and 50 (43 %) of detected variants matched to the COSMIC database. Most variants were already present in samples from diagnosis, only 4 (3 %) were detected *de novo* during the disease course (in genes *SMC3*, *PTPN11*, *ASXL1*, *BCORL1*). We also noted, that approximately half of the mutations retained their allelic frequency stable during the disease course; other variants tended to decrease or increase their abundance substantially (i.e. two folds) during the treatment, and finally, some mutations presented with more complex dynamics. Changes in allelic burden often reflected the clinical course of the disease. The summary of detected variants and their dynamics during the AZA treatment is presented in Figures 17A and 17B; all detected variants together with their dynamics, amino acid change and COSMIC annotation are available in supplementary table 2 of the enclosed article Polgarova et al. 2017.

4.3.2 **Relation of variant dynamics with MDS clinical course**

As pointed above, we observed different types of mutation dynamics in our cohort: increasing (INC), decreasing (DEC), complex (DEC-INC) and stable (ST). We then assessed how variant dynamic mirrored the disease course. We hypothesized that presence of ST and INC dynamics would reflect the AZA resistance, thus the clones bearing these variants would exhibit stable (or increasing) allelic burden. Indeed, ST and INC dynamics contained mutations in genes that were previously associated with adverse outcome, such as *ASXL1*, *EZH2*, *RUNX1*, *TET2* or *TP53* (Bejar et al. 2014; Steensma et al. 2015) whereas DEC dynamics contained only few of them. In our cohort patients with ST dynamics showed a trend towards shorter OS on AZA treatment in comparison to those displaying DEC dynamic profile (OS median 19.7 months and 31 months respectively). Genes most frequently affected by mutations with ST/INC dynamics were *TP53* (median OS on AZA 14.8 months) and *BCORL1* (median OS 18 months). Other above mentioned examples for ST/INC dynamics included *EZH2* with OS 11.1 months, *TET2* (OS 16.7 months) or *CDKN2A* (OS 10 months). On the other hand, several patients with these (generally adverse outcome predicting) genes presented with longer OS indicating, that eventual pathogenicity of particular variants should be considered individually.

We also assessed relationship between mutation status and dynamics with clinical course of patients from our MDS cohort. The outcome of the cohort was following (Table 11): complete remission (CR) (according IWG criteria (Cheson et al. 2006)) was reached in 11 cases (29 %) with short duration (less than 12 months) in 7 patients and long duration (more than 12 months) in 4

patients, 11 (29 %) patients achieved stable disease (SD) with hematologic improvement (HI), 12 (32 %) SD without HI and 4 patients (10 %) progressed (PG) early on AZA therapy.

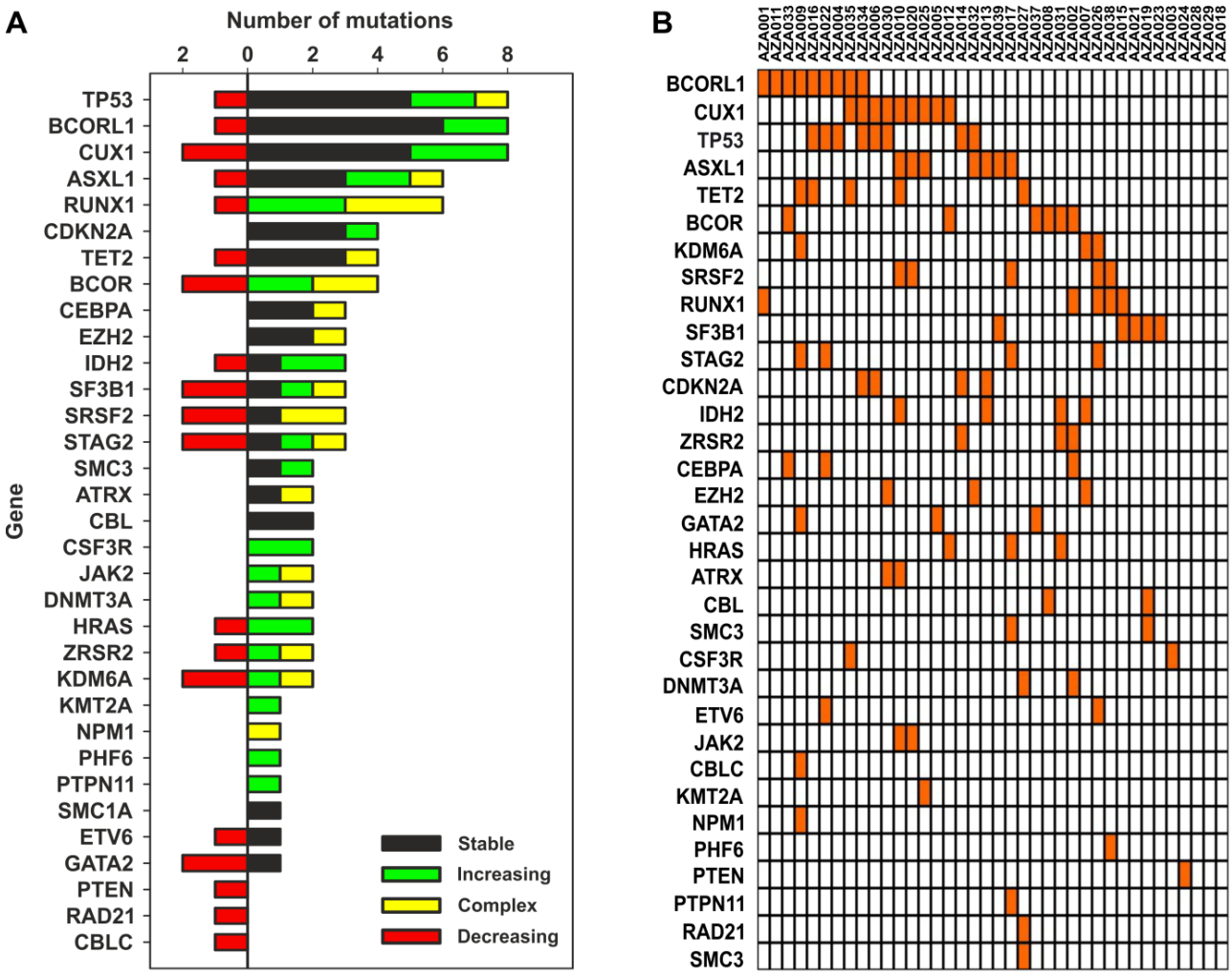


Figure 17. A) Number of mutations (X-axis) detected in particular genes (Y-axis) with color demonstration of their dynamics. B) Mutation heatmap that includes only genes with at least 1 detected mutation (rows: genes, column: patients), unmutated genes are not listed.

Table 11. Summary of the response achieved in MDS cohort of 38 patients including their median overall survival (in months). CR – complete remission (long – more than 12 months; or short – less than 12 months); SD – stable disease; w HI – with hematological improvement; PG - progression

Response	CR>12Mo	CR <12Mo	SD w HI	SD	PG
Number of patients	4	7	11	12	4
OS on AZA [Months]	29.6	17.6	31.0	32.4	6.1

Expectedly, PG patients expressed mostly ST variant dynamics, which indicates, that these variants indeed reflect disease refractoriness to AZA treatment. Samples from these patients were also enriched with mutations in genes *TP53* or *BCORL1*. Additionally, almost half of the patients achieving only short term CR contained ST variants suggesting possible role of these variants in early progression after response achievement. In patients achieving long term CR AZA therapy led often to DEC-INC dynamics, i.e. decrease of the variants' allelic burden at point of reaching clinical response with further reappearance of initially presented variants during ongoing treatment usually preceding clinical relapse of MDS. This confirms what is known from clinical experience - that AZA treatment hardly leads to lasting cure and all patients will finally relapse. For example, patient AZA17, who was initially diagnosed with RAEB1 (or MDS-EB1 according to WHO 2016) and bore somatic mutations in genes *ASXL1*, *HRAS*, *STAG2* and *SRSF2* at time of diagnosis reached CR after 6 cycles of AZA. In this time point all previously detected mutations decreased their allelic frequency below 5 % and thus were barely detectable. However, after 14 cycles of AZA, when still in clinical remission, 3 out of 4 mutations from diagnosis reappeared again. This was followed by further increase in allelic burden (almost reaching the original VAF of all variants) after 21 AZA cycles when disease progression was confirmed also clinically. Besides of restoration of initially detected variants, new mutations (in *PTPN11* and *SMC3*) were detected in progression sample. Similar pattern was observed in patient AZA009. The rest of patients achieving CR revealed less complexity in their mutations' dynamics. Interestingly, none of our patients reached mutation free status during AZA treatment regardless of achieving clinical CR - see Figure 18.

Similarly, we looked to changes in variants' allelic burden in patient with SD with or without HI. We did not observe differences in OS (so is true when comparing OS of patients with SD with those reaching long CR). We observed that some patients responding with HI showed decrease of VAF of some of the initial variants during response duration but again, restitution of the original VAF accompanied disease progression. Two patients with SD with HI had relatively stable mutational pattern (AZA001 and AZA031) and one patient (AZA038) displayed continuous increase of VAF of some of the initial variants till disease progression (Figure 19A), suggesting that presence of these variants does not affect eventual HI achievement.

Patients not achieving HI displayed broadly stable mutation patterns during the AZA treatment (Figure 19B). Patients with therapy failure, that progressed early on AZA showed stable mutation pattern with minimal changes in VAFs suggesting, that indeed AZA did not substantially affect the disease course and thus the clonal pattern (Figure 19C).

In conclusion, patients responding with HI or CR demonstrated dynamic changes in mutation pattern indicating possible clonal selection influenced by AZA. On the other hand, patients with SD or early PG retained their mutation pattern without substantial changes or even displayed increasing VAF of some mutations suggesting possible role of these variants in AZA resistance.

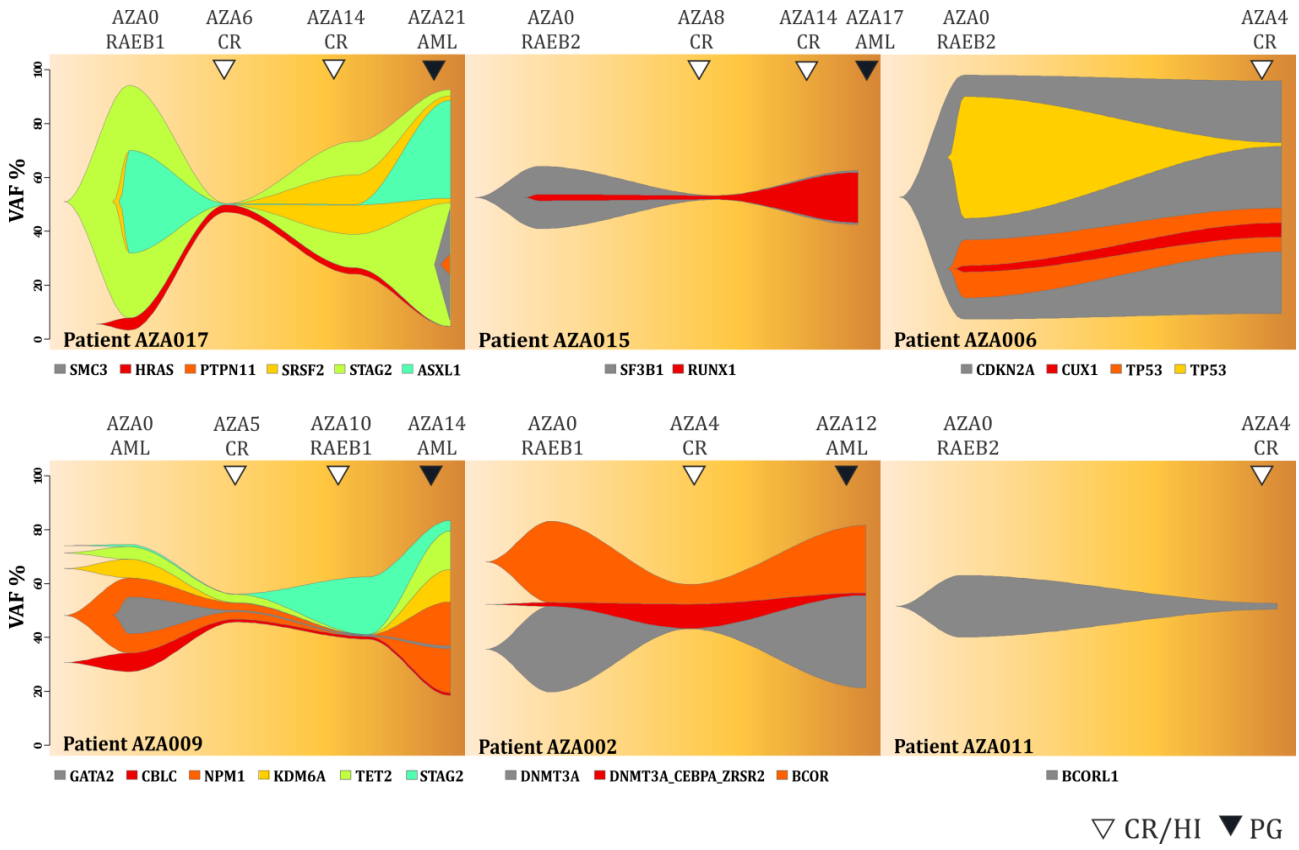


Figure 18. Fish plots of somatic variants detected in sequential BM sampling of MDS patients achieving complete remission (CR). Empty arrows indicate CR, black arrows indicate disease progression (PG), Y-axis represents VAF in %, X axis represents time course (in months). AZA0 indicates sample before starting the treatment, AZA(number) indicates number of AZA cycles before particular restaging.

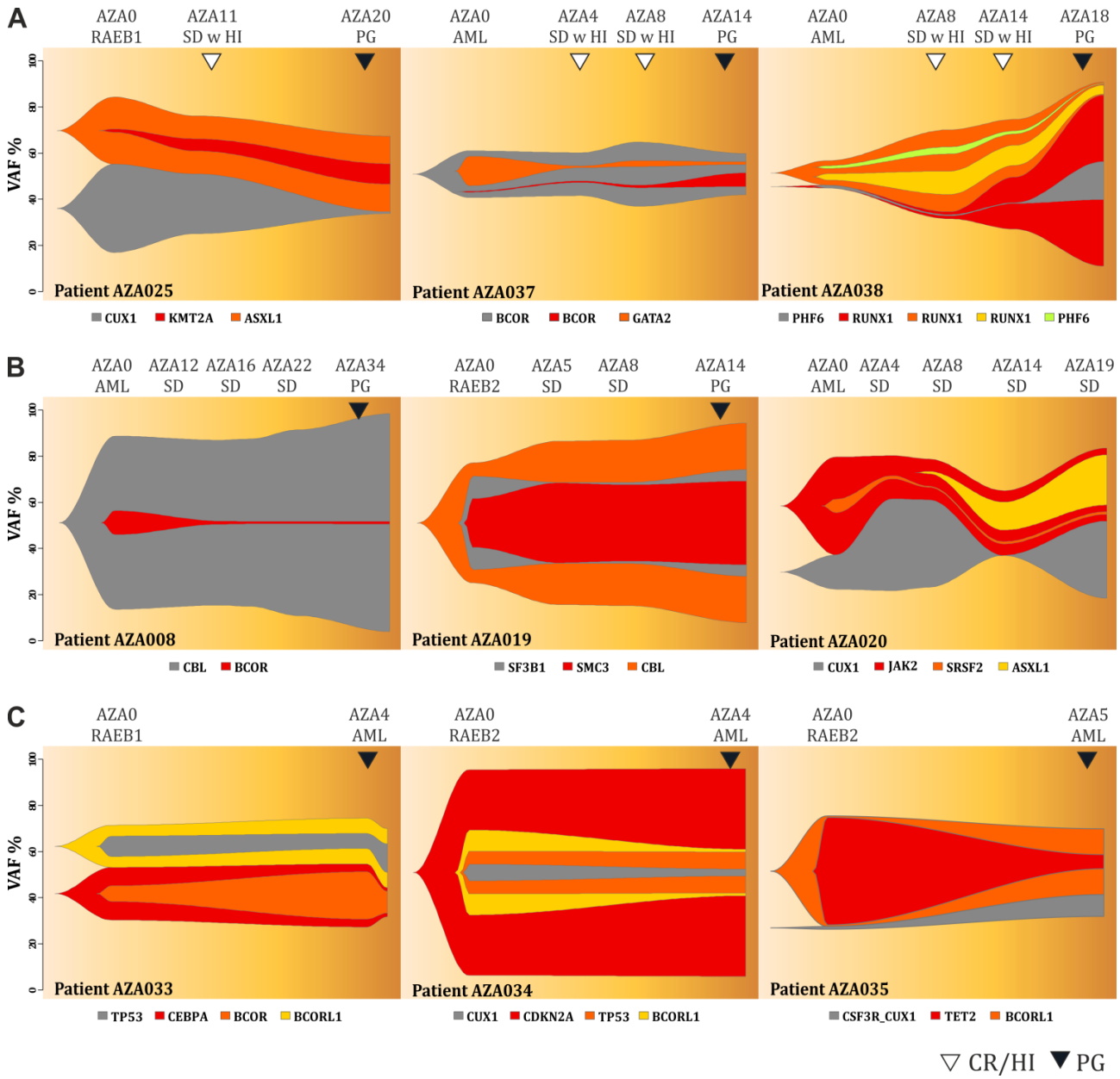


Figure 19. Fish plots of somatic variants detected in sequential BM sampling of MDS patients achieving stable disease with hematologic improvement (SD w HI) (A); achieving stable disease but without hematologic improvement (SD) (B); and primary progressing patients (PG) (C). Empty arrows indicate CR, black arrows indicate disease progression, Y-axis represents VAF in %, X axis represents time course (in months).

4.3.3 **Statistical evaluation of the mutations' dynamics**

We next evaluated further the impact of mutated genes and their dynamics during AZA treatment on the clinical response, its duration and OS. To decrease the bias resulting from relatively distinct time points in which VAFs were measured we took into account regression slopes coming from a regression model on VAFs. We then utilize a fitted joint model (providing a multivariate approach) to analyze response duration and OS on AZA which included time varying (e.g. VAFs) and constant (e.g. sex or globally increasing or decreasing mutation status) covariates. Table 12 shows estimated parameters (with corresponding p-values and confidence intervals) obtained by the Cox proportional hazards (PH) model together with the Poisson count model.

We observed significant effect on the overall survival caused by the following mutated genes: *TP53* (p-value 0.023), *CDKN2A* (p-value 0.023) and *KDM6A* (p-value 0.011). We also noted significant prognostic effect of previous 5q syndrome which retains its favorable impact even in presence of other mutations. Male patients from our cohort also tended to survive longer. The second part of the joint model represents the response duration on AZA. We observed significant effects of mutated or dynamically changing (increasing or decreasing pattern of allelic burden of) *ASXL1*, *BCOR*, *BCORL1*, *CDKN2A*, *CUX1*, *EZH2*, *KDM6A*, *RUNX1*, and *TP53*. In our cohort *BCOR*, *CDKN2A* and *EZH2* mutations significantly shortened the response duration suggesting that clones bearing these mutations were AZA resistant. Mutations in *ASXL1*, *CUX1*, *RUNX1*, and *TP53* were, on the contrary, found in patients with longer response duration, indicating that clones bearing these mutations could be controlled by AZA. However, globally increasing VAF for mutations in *ASXL1*, *BCORL1*, *CUX* and *TP53* led in our model to shorter response suggesting that not only presence/absence of the mutation is important, but also the VAFs' dynamics could change the patients' clinical outcome.

Table 12. Fitted joint model for overall survival time and response duration on AZA. (A) Cox PH model for OS time; Score (logrank overall) test.0012. (B) Poisson count model for Response duration; Likelihood ratio (overall) test <.0001. SE indicates standard error; CI confidence interval; HR hazard ratio; IR incidence rate.

* A positive coefficient estimate in the Cox PH model corresponds to a higher risk of death and thus on average a shorter OS time.

** A positive coefficient estimate in the Poisson count model corresponds to higher number of months of response duration.

Table: Fitted Joint Model for Overal Survival Time on AZA and Response Duration of AZA						
	Coefficient	SE	95% CI	Hazard ratio* / Incidence rate**	95% CI	P-value
A Cox PH model for OS time*				Hazard ratio		Score (logrank overall) test 0.0095
Male vs Female	-0.906	0.453	-1.795 , -0.018	0.404	0.166 , 0.982	0.046
Age	-0.006	0.030	-0.065 , 0.052	0.994	0.937 , 1.054	0.833
CDKN2A mutated>5%	1.698	0.749	0.229 , 3.166	5.461	1.258 , 23.704	0.023
KDM6A mutated>5%	-1.598	0.632	-2.836 , -0.360	0.202	0.059 , 0.698	0.011
TP53 increasing	-1.489	0.673	-2.808 , -0.169	0.226	0.060 , 0.845	0.027
5q-	-1.496	0.495	-2.466 , -0.525	0.224	0.085 , 0.592	0.003
B Poisson count model for Response duration**				Incidence rate		Likelihood ratio (overall) test <.0001
Sex (Female as intercept)	0.324	0.887	-1.413 , 2.062	1.383	0.243 , 7.869	0.715
Male vs Female	1.141	0.179	0.791 , 1.491	3.130	2.205 , 4.442	<.0001
Age	0.006	0.012	-0.018 , 0.029	1.006	0.982 , 1.030	0.637
ASXL1 mutated>5%	1.137	0.249	0.650 , 1.624	3.118	1.915 , 5.075	<.0001
ASXL1 increasing	-1.665	0.452	-2.550 , -0.779	0.189	0.078 , 0.459	0.0002
BCOR mutated>5%	-1.079	0.337	-1.739 , -0.420	0.340	0.176 , 0.657	0.001
BCOR increasing	1.699	0.384	0.947 , 2.451	5.467	2.577 , 11.597	<.0001
BCORL1 increasing	-0.576	0.224	-1.015 , -0.137	0.562	0.362 , 0.872	0.010
CDKN2A mutated>5%	-2.750	0.481	-3.693 , -1.808	0.064	0.025 , 0.164	<.0001
CUX1 mutated>5%	1.981	0.361	1.274 , 2.687	7.248	3.576 , 14.691	<.0001
CUX1 increasing	-3.105	0.435	-3.957 , -2.254	0.045	0.019 , 0.105	<.0001
EZH2 mutated>5%	-3.304	0.529	-4.340 , -2.267	0.037	0.013 , 0.104	<.0001
KDM6A increasing	1.209	0.398	0.429 , 1.989	3.350	1.536 , 7.308	0.002
RUNX1 mutated>5%	0.863	0.192	0.486 , 1.240	2.370	1.625 , 3.454	<.0001
TP53 mutated>5%	1.733	0.537	0.680 , 2.786	5.658	1.974 , 16.211	0.001
TP53 increasing	-1.022	0.501	-2.004 , -0.040	0.360	0.135 , 0.960	0.041

4.3.4 **Summary**

Using massive parallel sequencing technology based on targeted sequencing of 54 genes recurrently mutated in myeloid neoplasms we evaluated occurence of mutations, including rare variants, and their evolution in patients with MDS treated with hypomethylating agent AZA. We observed similar spectrum of mutations as described by others. We tracked the observed mutations, defined their dynamics and connected these data with the disease course. We then utilized a fitted joint model to analyze possible impact of detected mutations (and their dynamics) on response duration and overall survival. We also find out, that not only presence/absence of mutations in different genes is important, but also particular variant may play role in mediating possible AZA tolerance.

5 Discussion

This work aimed to describe mRNA expression changes of regulatory and lineage defining molecules during hematopoietic differentiation. Ninety five genes were evaluated in different developmental stages of particular leukocyte lineages including early progenitors as well as in their malignant counterparts and also newly described switching AL. Observed expressional changes were then used to define lineage infidelity markers of this specific AL group. We also described other characteristics and components of the molecular background of SW-AL.

Since somatic mutations were also described to affect development, survival, phenotype, therapy resistance and other features of hematologic malignancies, we pursued mutations and their dynamic in MDS patients treated with demethylating agent 5-azacitidine and connected changes in mutational profile to the treatment sensitivity and course of the disease.

5.1 Gene expression profiling

In our work we prepared a set of 95 genes that are known or suspected to play role in hematopoietic differentiation or determining the phenotype or function of particular lineages and we measured their mRNA expression by gold-standard quantitative real-time PCR. We optimized the cell sorting and RNA extraction process and we included and tested the preamplification step to ensure valid results for rare progenitor populations. We then prepared publicly available database (leukostage.org), where all data are to be shown according to the user preferences. Our data show expression changes of different molecules during particular cell population development as well as in malignant transformation. In some genes malignant expression profiles copied those of their healthy counterparts; however, also specific situations in terms of differentiation and proliferation regulation were observed.

5.1.1 **Developmental changes and lineage associated differences**

Various genes were observed to change their expression during B-ly, T-ly, Gra and Mo development. Changes in expression of *PAX5*, *FOXO1*, *LAT*, *BCL11B*, or *NOTCH*, *HOX* and *CEBP* family were mostly described previously and thus our results confirmed the current knowledge of mastering hematopoietic differentiation. For example, diverse cell-fate regulator ID2 is known to block E2A activation which controls the initiation of B-lymphopoiesis (Ji et al. 2008). In line with this knowledge, our data show lower expression levels in B-ly and

upregulation of ID2 during development of other lineages. The highest expression was observed in mature granulocytes, which is in line with an experimental observation of accelerated maturation of neutrophils and eosinophils in ID2 overexpression mice model (Buitenhuis et al. 2005). In T-ly, ID2 represses E2A mediated activation of IL-10 locus, thus playing a regulatory role in T-cell proliferation and also modifying T-cell effector functions (Masson et al. 2014).

Another observed B-ly specific gene (besides chronically known regulators such as PAX5, EBF1, FOXO1 etc., as mentioned in Table 10) was *POU2AF1* that has long been considered as strictly B-cell restricted (Luo et al. 1992). This gene encodes activation factor OCA-B that together with OCT2 (a POU family member) regulate immunoglobulins (Ig) expression (Luo et al. 1992) and germinal B-cells Ig hypermutation and proliferation. OCA-B and OCT2 regulate a broad spectrum of other B-lineage specific transcription factors, such as IRF4, TCF3 or STAT3 as well as oncogenic signaling pathways that include MYD88 or JAK. They are also essential for survival of diffuse large B-cell lymphoma (both, germinal center like and activated B-cell like) (Hodson et al. 2016). Interestingly, OCA-B is also believed to regulate the differentiation of $CD4^{pos}$ T-ly into follicular T-helper lymphocytes via BCL6 activation (Stauss et al. 2016). We could not confirm its expression in T-ly since we did not examine this particular subpopulation of mature T-ly.

Several molecules showing expression changes during differentiation were related to the functions of maturated cells; e.g. immunoglobulin Fcγ receptors (i.e. FCGR2A and FCGR3A from our dataset) are necessary for phagocytosis mediated by antibody opsonization and are upregulated in mature mono- and granulocytes. Similarly to FCGR2A and FCGR3A, expression of LILRA2 (Leukocyte immunoglobulin-like receptor subfamily A member 2) that allows sensing of microbially cleaved immunoglobulins is strongly expressed in the most differentiated developmental stages of granulocytes and monocytes. Its crosslinking leads to myeloid cells activation and production of pro-inflammatory cytokines (Hirayasu et al. 2016). Another example is S100A10 (also known as p11) which is upregulated during the maturation of all lineages with highest levels being observed in monocytes. S100A10 forms together with annexin A2 heterotetrameric protein complex activated by calcium binding by S100A10. It is implicated in exo- and endocytosis by F-actin reorganization. Its carboxy-terminal lysine residue may bind tissue plasminogen activator as well as plasminogen resulting in plasminogen conversion to plasmin (predominant fibrinolytic enzyme). In monocytes S100A10 was proven to regulate plasmin mediated activation, migration and matrix invasion (O'Connell et al. 2010). Since it is directly upregulated by PML-RARα oncoprotein, it is believed to be involved in promyelocytic

leukemia induced coagulopathy (Huang et al. 2017). In other lineages it may regulate proliferation and cell survival (Shan et al. 2013).

Interestingly, we observed *CD79a* expression in healthy T-ly not attributable to contamination by B-ly. Although already reported on T-ALL and T-lymphoma samples (Kozlov et al. 2005; Matnani et al. 2013), CD79a on non-malignant T-ly specimens was not previously reported. Whether this mRNA expression is translated into CD79a protein or whether it is related to aberrant expression on T malignancies remains unknown. In myeloid cells, aberrant expression of this molecule is thought to play role in the promotion of tumorigenesis mediated by myeloid-derived suppressor cells (Luger et al. 2013).

5.1.2 **Leukemia specific expression changes**

In our study we observed several differences between leukemic specimens and their non-malignant counterparts. Some of the observed differentially expressed genes have already been connected to leukemogenesis or are known regulators of proliferation, apoptosis or differentiation. The biggest contrast was detected in *PAWR* with high expression in normal lymphocytes but mostly undetectable levels in pB-ALLs; on the contrary *CCDC26* was not detectable in healthy B-lymphocytes at all but expressed in most of pB-ALL specimens.

PAWR modulates WT1 expression and function and its expression was shown to be upregulated in cells undergoing apoptosis. It is believed, that its pro-apoptotic activity in B-lymphoid cells is mediated by downregulation of Bcl-2 and when expressed in pB-ALL it promotes higher chemosensitivity of leukemic cells (Boehrer et al. 2002). It is also involved in the regulation of Fas and NFκB pathways (Hebbar, Wang, and Rangnekar 2012). Interestingly, we did not observe the downregulation of *PAWR* in T-ALL. This could be possibly explained by the simultaneous expression of *NOTCH3* which competes with complex PAWR/THAP1 in binding to CCAR1 promoter. The protein complex PAWR/THAP1 induces full-length CCAR isoform expression leading to programmed cell death, whereas NOTCH3 presence induces splicing of a shorter CCAR1 isoform, which on the contrary leads to decreased T-ALL cells apoptosis (Lu et al. 2013). The potentially proapoptotic effect could then be outcompeted by the presence of NOTCH3, which is known to be highly expressed in T-ALL (Aifantis et al. 2007) as our data also confirmed.

The long non-coding RNA CCDC26, also called RAM (i.e. Retinoic Acid Modulator) is believed to modulate the initiation and course of different human cancers (Cao et al. 2018; Peng and Jiang 2016). Its polymorphisms were associated with glioma risk in several studies (Zeng et

al. 2017). The genomic region containing *CCDC26* (chr8q24) is amplified in 14 % of pediatric AML (Radtke et al. 2009); particular polymorphisms of this gene were connected to IDH-1-mutated AMLs (Lasho et al. 2012); and genome-wide analysis showed abnormalities of *CCDC26* on pediatric ALL patients with poor prognosis (Mullighan et al. 2007). Moreover, CCDC26 was demonstrated to regulate retinoic acid-induced maturation of HL60 (AML) cell line and to control myeloid leukemia (K562) cell growth (Hirano et al. 2008, 2015). The CCDC26's differentiation, apoptosis and growth regulating effects are mostly believed to be mediated by changes in tyrosine kinase signaling or Bcl2, suggesting also its possible role in therapy resistance mediation (Cao et al. 2018; Duployez et al. 2018; Hirano et al. 2015; Peng and Jiang 2016). In our data *CCDC26* was expressed in almost all malignant specimens and at lower extent in T-ly and myeloid populations; it was not expressed in B-ly at all. In contrast with published data, we did not observe any correlation between *CCDC26* and *KIT* expression in AML. This could be explained by low number of samples, wide expression range of these genes and also by possible specific molecular background that was not revealed in our analysis. These two genes have not been described in context of pB-ALL till now and may present an interesting target for new therapeutics development.

FLT3 is a receptor tyrosine kinase mostly expressed in early lymphoid and myeloid precursors which is involved in differentiation, proliferation and apoptosis regulation of hematopoietic cells. We showed its expression to be generally higher in pB-ALL compared to B-ly; it was also highly expressed in AML (in comparison with non-malignant myeloid populations), but not in T-ALL. This is in line with already published data (Carow et al. 1996). After its activation, FLT3 further transduces the signal to multiple signaling pathways including Ras/Raf, PI3K or MAPK pathways; it induces transcription of STAT5, phospholipase Cγ and others, thus promoting proliferation and survival of leukemic blasts. It is well known that activating *FLT3* mutations, particularly in AML, are related to adverse prognostic impact (Meshinchi et al. 2001).

Interestingly, we observed higher *EPOR* expression in *ETV6/RUNX1* rearranged subset of pB-ALL. This is believed to be directly induced by *ETV6/RUNX1* transcript and to mediate proliferation and apoptosis-protection of leukemic cells (Inthal et al. 2008). Although the presence of the EPOR on the leukemic cell surface has not been clearly proven, its functionality was shown by activation of JAK2 pathway signaling in *ETV6/RUNX1* positive AL cells in presence of EPO (Moya et al. 2010).

In the subset of pB/MY-ALs expression of *CEBPA* was observed to be higher than in pB-AL or B-ly. The expression reached similar levels as in AML. Other members of CEBP family did

not reach expression levels of healthy or malignant myeloid populations in pB/MY-AL. This is in line with the role of CEBPA as a master regulator of myeloid versus lymphoid differentiation.

5.2 Lineage switch from pB-ALL to monocytoid lineage

In our cohort we observed ~4 % of pB-ALL with an abundant population of monocytoid cells appearing at the beginning of the induction treatment. Although these cells presented with monocyte characteristics (immunophenotype as well as expression profile), evaluation of Ig/TCR rearrangements proved them to be of the same clonal origin as the B-lymphoblasts. Change of the phenotype from B-lymphoblast to monocytoid population, i.e. lineage switch occurred gradually, thus, an intermediate population was also observed. Interestingly, at time of diagnosis, proportion of monocytoid cells observed in samples of SW-AL patients was not different from the number of monocytes in pB-ALL. Also, the immunophenotype of evolved monocytoid cells was not different from normally present monocytes. Even their expression profile resembles more of the normal monocytes than myeloid leukemic blasts.

Till now, only few reports were available describing such switching phenomenon. Within our cohort, all SW-AL cases were $CD2^{(partly)pos}$, however not all $CD2^{(partly)pos}$ pB-ALL finally emerged the lineage switch and fulfilled the SW-AL definition. CD2 is a member of immunoglobulin superfamily and is usually found on T-ly and NK cells, where it posses function of cell adhesion molecule and binds to a surface glycoprotein LFA-3 (Makgoba, Sanders, and Shaw 1989). Few works also report superficial CD2 expression of specific subset of monocytes or healthy B-lymphocytes (Cheng et al. 2006; H. and Jen-Yih 2018) . Our mRNA expression data do not confirm this observation for B-ly, since CD2 expression was mostly very low or undetectable; as for healthy Mo, we observed low CD2 expression on mRNA level, which was however notably lower comparing to T-ly. Whether and to what extent is this expression translated also to membrane CD2 expression in Mo was not evaluated. Aberrant CD2 expression was described in ~5-10 % of pB-ALL (Uckun et al. 1997) which is in line with our observation.

Next, we tried to decipher the molecular background leading to the lineage switch. We did not observe any common cytogenetic changes and most of the patients bore normal karyotype. None of our patients was MLL rearranged, yet this aberrancy is often described in lineage infidelity cases (Tirtakusuma et al. 2013). Since Yeoh et al. (2002) reported a subgroup of $CD2^{pos}$ pediatric pB-ALL associated with *ERG* deletion, which was further confirmed by Zaliova et al. (2013) we performed SNP arrays as well as MLPA and breakpoint-specific PCR to reveal, whether *ERG* deletions could be a common feature in SW-AL. Although ERG deletions were

found in 4 SW-AL cases we did not observe any shared genetic hits in terms of copy number variations. We next searched for *IKZF1* variations, since this transcription factor, knowing to be master regulator in lymphoid development, was reported to mediate the lineage switch from B-ly to monocytes in vitro and it also mediates glucocorticoid resistance in pB-ALL (Marke et al. 2015; Reynaud et al. 2008). *IKZF1* alterations were more common in SW-AL cases comparing to pB-ALL, however majority of patients did not bear any. This, together with presence of *IKZF1* alterations in non-switching cases excludes *IKZF1* alterations from being the main cause of the lineage switch.

Several models also show *CEBPA* upregulation leading to transdifferentiation of B-ly into myeloid lineage (Cirovic et al. 2017). From all myeloid genes, *CEBPA* was the only one upregulated already in B-lymphoblasts subpopulation (compared to normal B-ly or pB-ALL) that did not present any other aberrant myeloid features. Other myeloid differentiation regulating genes, such as *PU.1* or *CSF3R*, presented with gradually increasing expression only after transition into SW-Intermediate and SW-Monocytoid subpopulation occurred. B-lymphoid regulators, such as PAX5, EBF1 or FOXO1 on the contrary decreased their expression during the switch towards the monocytoid lineage. *CEBPA* expression may be regulated on different levels. Chromosomal translocation t(14;19)(q32;q13), that involves CEBPA leads to its upregulation in pB-ALL (Chapiro et al. 2006); however, this translocation was not observed in our cohort. Epigenetics plays important role in *CEBPA* expression regulation. Increased methylation of CpG islands in the promoter region may lead to decreased gene transcription. In our cohort we indeed observed hypomethylation of *CEBPA* promoter in majority (14/16) of SW-AL cases which explains its increased expression.

The lineage switch occurred during the initiation of induction treatment, which in all involved protocol includes glucocorticoids. Therefore, we sought to recapitulate the changes *in vitro*. *In vitro* prednisolone treatment of B-lymphoblasts from SW-AL patients indeed lead to monocytic transdifferentiation as observed in patients with gradual loss of B-lymphoid and increase of myeloid features. This observation, together with the detection of clonal Ig/TCR rearrangements further supports the leukemic origin of monocytoid population evolved by transdifferentiation from B-lymphoblasts. Different therapy sensitivity of monocytoid cells may also contribute to increased proportion of monocytoid population within the course of induction treatment. Another explanation of this phenomenon is the presence of bilineal leukemia with slower clearance on monocytoid population; this however is less probable in context of gradual phenotypical changes, clonal relatedness of SW-B, SW-I and SW-Mo populations as shown by

Ig/TCR rearrangements and recapitulation of the changes *in vitro* when $CD14^{pos}$ cells were not observed at the beginning of the cell culture.

Since the monocytoid population is not to be distinguished from normal monocytes, flow cytometry may underestimate the blast proportion and cause misleading results in MRD evaluation. Discrepancies between FC and Ig/TCR in pB-ALL were described in literature, in some cases even with concurrent monocytosis (Neale et al. 2004), suggesting that above described lineage switch might be present leading to confusion in MRD evaluation.

5.3 Tracking mutation dynamics in MDS patients treated with AZA

Several studies describe particular gene mutations present in MDS patients that could affect their response to hypomethylating agents' treatment (Bejar et al. 2011) suggesting that specific variants may promote AZA tolerance or sensitivity. Evolution of clonal architecture, a broadly accepted concept observed in AML (Ding et al. 2012), has also been suggested for MDS (Pellagatti et al. 2015).

In this work we used the massive parallel sequencing technology – TruSight Myeloid Panel (Illumina) that contains specific regions of 54 genes previously documented in myeloid neoplasms to detect dynamics of somatic mutations in consecutive BM samples from 38 MDS patients. Using in-parallel sequenced non-tumorous controls we excluded germ-line variants. Using duplicate library we also excluded falsely positive data generated by PCR and sequencing biases. Our data thus provide novel view of clonal architecture changes in MDS and its relation with possible AZA resistance.

Expectedly, >90 % of our cohort bore at least one somatic mutation with median of 3 mutations per patient. Most frequent mutations were observed in genes *CUX1*, *TP53*, *BCORL1*, *ASXL1, TET2* or *RUNX1*. Almost half of the detected mutations matched to the COSMIC database of recurrently mutated genes in cancer.

CUX1 (Cut like homeobox 1) is a DNA binding protein that regulates various gene expression (e.g. by preventing binding of positively-activating CCAAT factors to genes' promoters), differentiation, DNA repair or senescence/cell cycle progression (An et al. 2018). Its mutations and deletion are often described in myeloid malignancies, particularly in MDS. Since it plays role also in DNA repair, it is believed, that its mutations lead to increase overall mutational rate during leukemogenesis; it is also often accomplished by *ASXL1* and *SRSF2* mutations (Aly et al. 2017). We did not observe this trend in our MDS cohort possibly biased by the presence of

high-risk patients with other hits affecting their response to DNA damage such as *TP53* mutations. We did not observe any association of CUX1 mutations and/or its dynamics with presence of other mutations or particular cytogenetic changes.

TP53 and *ASXL1* (both described in section 1.2.2.1) mutations were repeatedly described as negative prognostic factors in MDS (Bejar, Stevenson, et al. 2014), although some conflicting results in context of demethylating agents treatment exist (Tobiasson et al. 2016). In our cohort these mutations were enriched in stable (ST) or increasing (INC) dynamics of their VAFs suggesting their possible role in promoting AZA tolerance. Indeed when evaluating clinical response in these patients, the subsets were enriched in SD (w HI) on extent of reaching CR. Expectedly, increasing VAF of these mutations is associated with shorter response duration. On the other hand, a positive coefficient for *ASXL1* and *TP53* from our Poisson count model suggests, that at least in part of our patients with these mutations, the disease can be successfully controlled by AZA.

Negative impact in terms of response duration as well as survival was observed for *CDKN2A* mutations. CDKN2A (Cyclin-dependent kinase inhibitor 2A, also known as p16) is a cell cycle regulator. It inhibits cyclin-dependent kinases 4 and 6 thus blocking the transition from G1 to S-phase. It is also able to block MDM2-induced p53 degradation or to block MYC mediated transcriptional activation (Eischen et al. 1999). Although its constitutional mutations were anecdotally described to mediate higher chemosensitivity (Shah et al. 2017), in many human cancers *CDKN2A* mutations or epigenetic inactivation were repeatedly described to have negative prognostic impact (Zhao et al. 2016). This is in line with our observation of shorter response duration and survival in patients bearing *CDKN2A* mutations. Similar observation was made for *EZH2*, again along with already published data (Bejar et al. 2011).

BCORL1 (BCL6 corepressor like 1) is a gene located at X-chromosome. Its protein product binds to histone deacetylases (HDAC4, HDAC5, HDAC7), interacts with other corepressor CTBP and represses the transcription (Pagan et al. 2007). It was described to be mutated in MDS and AML in ~2-10 % of cases with higher incidence in advanced disease, myeloproliferative phenotype and in patients with abnormal karyotype (Damm et al. 2013; Li et al. 2011). This may explain higher occurrence within our MDS cohort, where 9 patients out of 38 (23 %) bore *BCORL1* mutation. Most of these mutations reveal ST or INC dynamics and our Poisson count model confirms negative prognostic impact of INC dynamics on response duration, as observed by others (Damm et al. 2013). Most of the published mutations are predicted to be inactivating, suggesting a tumor suppressor role of BCORL1 in myeloid malignances (Li et al. 2011).

TET2 (described in section 1.2.2.1) was repeatedly reported to be mutated in 10-20 % of MDS, AML and CMML patients and it was connected to higher response rate in patients treated by hypomethylating agents (Bejar et al. 2014; Itzykson et al. 2011). On the other hand, studies did not prove any impact on response duration or overall survival (Itzykson et al. 2011) as we also confirmed by our model.

RUNX1 is a transcription factors that plays important roles in different levels during hematopoiesis. In term of myeloid differentiation and malignancies its cooperation with PU.1 leading to activation of various myeloid differentiation genes (Hu et al. 2011) is of particular interest. It is suggested, that mutations of RUNX1 disrupt its functions leading to differentiation block (beside other consequences) due to repressing PU.1 functions.

Besides usual clinical, morphological and cytogenetic response, we also analyzed the molecular response to AZA treatment. In line with current data showing that permanent remission is not to be achieved by hypomethylating agents (Will et al. 2012), we proved that indeed none of our patients reached mutation free status even in clinical long term CR as defined by IWG (Cheson et al. 2006). Our data also show importance of the variants' dynamics to predict the effect of the mutation on the AZA tolerance/sensitivity. We were able to reconstruct the clonal architecture changes and coincide them with the clinical course of the disease, since significant decrease of VAF during AZA therapy (likely reflecting loss of the tumor cell burden) precludes CR or HI achievement and associates with outcome durability and survival of these patients. On the other hand, patients not responding or progressing on AZA presented with rather stable variant profile.

Furthermore, several patients showed INC dynamic of their variants long before clinical progression appeared, implicating that our approach could also bring some predictive features and thus allowing more precise control of the PG risk. Mutations dynamics during AZA treatment included occurrence of stable variants as well as those suppressed by AZA or those developing during AZA therapy. Our thought is that AZA probably selects preexisting resistant clones rather that it would induce development of new resistant mutations.

As already mentioned mutations in different genes are associated with adverse clinical outcome or are affecting response achievement or its duration (Bejar et al. 2012; Bejar, Lord, et al. 2014; Bejar, Stevenson, et al. 2014). Several genes from our study, such as *TP53* or *ASXL1* exhibited higher proportion of ST variant profile, suggesting their potential role in disease driving. Other, such as *RUNX1* or *BCOR* presented with more complicated dynamics and we consider them rather response/progression modifiers.

Our study suggests that not all variants in previously described adverse genes have necessarily negative prognostic impact. It is probably rather the type of variants and/or its combination with other mutations that matters. For example *TP53* mutations, repeatedly stated to worsen their carrier prognosis had adverse impact in about two thirds *TP53* mutated patients from our cohort. On the other hand, almost one third of patients bearing *TP53* mutation exhibited milder clinical outcome and longer OS on AZA therapy. We therefore conclude, that not only genes, but also specific gene variants associate with prognostic impact.

6 Summary

We designed simplified mRNA expression platform that evaluates 95 genes encompassing different cell fate and lineage decision regulators. Using this platform we described mRNA expression changes during the development of particular hematopoietic lineages including rare progenitor populations and we were able to define lineage specific genes that allowed grouping of particular specimens into lineage clusters. Obtained data were then used to prepare a publicly available database – leukostage.org, thus allowing the scientific community to use our observations for own needs, such as preliminary planning, own observation validation, etc. Further, we confronted described aberrant expression of particular molecules within hematologic malignancies with our data and found aberrant expression not attributable to contamination in T-ly. Other findings commonly described in AL or lymphomas were not convincingly observed. The data from non-malignant specimens' expression array measurements were also confronted with their malignant counterparts, revealing different levels of differentiation and/or survival regulators in acute leukemia. Several molecules possibly regulating malignant cell fate, such as *PAWR* or *CCDC26*, presented with striking differences in malignant vs. non-malignant expression – particularly in case of pB-ALL vs. B-ly. These were not previously described in literature to play role in pB-ALL survival.

We described the new subset of pediatric AL with switch towards monocytoid lineage during the induction treatment that was discovered in Childhood leukemia investigation Prague laboratory. These ALs present at time of diagnosis as pB-ALLs with CD2 membrane expression; they further transdifferentiate into monocytoid population during the initiation of the treatment. We optimized an 8-color flow cytometry panel (8-CSAC), which allowed us to prospectively screen all diagnostic pB-AL samples incoming to our laboratory and predict the possibly occurring switching phenomenon. The relatedness of B-lymphoblasts and monocytoid population (which is immunophenotypically undistinguishable from normal monocytes) was proven by the presence of B-lymphoblastic clonal Ig/TCR rearrangements (as used for MRD evaluation) in the monocytoid population. Trying to further characterize these switching AL we did not find any common molecular/cytogenetic hit. Using the simplified mRNA expression array mentioned above we compared all SW-AL subpopulations with non-malignant and different malignant specimens and identified increased *CEBPA* expression already in B-lymphoblasts. Knowing this molecule to be a master regulator in myeloid cell fate we hypothesized *CEBPA* could play an important role in the

transdifferentiation process. Since it is known that epigenetic changes present an important level of *CEBPA* expression, we evaluated the methylation status and confirmed hypomethylation of *CEBPA* promoter which is in line with observed increased mRNA levels of *CEBPA*.

We also tested another unit from clonal hematopoietic disorders spectrum –MDS. Using TruSight Myeloid Illumina panel we evaluated the effect of azacitidine treatment on changes of clonal architecture during the disease course in high risk MDS patients. We confirmed presence of "typical" myeloid neoplasms mutation in our cohort and observed several dynamics patterns – stable, increasing, decreasing and more complex (mainly decrease with further increase over time) dynamics. These changes mostly correlated with the disease course, suggesting possible role of particular mutations in mediating AZA tolerance. We did not observe molecular complete remission in our patients, again confirming a clinical observation that AZA does not lead to a sustainable remission. Following the mutations dynamics, we were also able to predict the oncoming clinical relapse. Our statistical model allowed us not only to predict the prognostic impact of presence/absence of particular genes mutation but also to include the VAFs dynamics into this prediction. We thus confirmed that some genes, such as *CDKN2A*, indeed impaired the patients' prognosis in terms of PFS and OS, however what probably is more important is the particular variant.

7 Souhrn

Navrhli a optimalizovali jsme platformu k vyšetření mRNA exprese 95 vybraných genů, které regulují diferenciaci, přežití či proliferaci buněk v procesu krvetvorby. Díky této platformě, která nám umožnila vyšetřit i vzácné populace progenitorů v kostní dřeni, jsme popsali změny v mRNA expresi jednotlivých (myeloidních a lymfoidních) linií a jejich vývojových stádií. Získaná data jsme použili k vytvoření veřejné databáze - leukostage.org, která umožňuje vědecké komunitě použití všech získaných informací, kupř. k validaci vlastních výsledků, ke srovnání s jinými populacemi apod. Vedle clusterů liniově specifických transkripčních faktorů či jiných regulačních molekul jsme pozorovali i aberantní expresi některých znaků u zdravých populací T-ly podobně, jako to bylo již popsáno u T-ALL či T-lymfomů. Dále jsme srovnali mRNA expresi vybraných genů mezi zdravými progenitory a leukémiemi jednotlivých linií. Pozorovali jsme rozdílnou expresi několika genů regulujících diferenciaci, apoptózu či proliferaci, přičemž nejmarkantnější byly rozdíly mezi B-ly a pB-ALL v expresi genů *PAWR* a *CCDC26*, které dosud v patogenezi pB-ALL nebyly popsané a představují tak možný nový cíl v hledání terapeutik.

Tuto platformu k hodnocení mRNA exprese jsme použili i u nově popsané skupiny akutních leukémií – AL s liniovým přesmykem. Leukémie s liniovým přesmykem byly diagnostikovány původně jako pB-ALL a v průběhu indukční terapie došlo k transdiferenciaci B-lymfoblastů do monocytoidních buněk, průtokovou cytometrií nerozeznatelných od normálních monocytů. Tato monocytoidní populace nicméně nesla identické Ig/TCR (pacient specifické) přestavby, jaké byly pozorovány v původních B-lymfoblastech a které byly použity i k hodnocení minimální reziduální choroby. V laboratoři CLIP byl optimalizován 8barevný panel, který umožnil vyšetření nově diagnostikovaných pB-ALL průtokovou cytometrií s následnou predikcí možného liniového přesmyku. Společnou vlastností leukémií s liniovým přesmykem byla membránová pozitivita CD2; na molekulární či cytogenetické úrovni jsme nenašli společné změny. Expresní profilování však odhalilo zvýšenou expresi *CEBPA*, klíčového transkripčního faktoru regulujícího myeloidní diferenciaci, již ve stadiu B-lymfoblastů, tedy ještě před liniovým přesmykem. Lze tedy předpokládat, že právě CEBPA sehrává významnou roli v patogenezi přesmyku do monocitoidní populace. Jelikož je exprese *CEBPA* významně regulována epigenetickými mechanizmy, hodnotili jsme i metylaci CpG ostrůvků *CEBPA* promoteru. V souladu s pozorováním zvýšené *CEBPA* exprese jsme pak odhalili hypometylaci těchto ostrůvků. Lze tedy předpokládat, že právě tyto epigenetické změny hrají v regulaci CEBPA a v patogenezi liniového přesmyku významnou roli.

Ve spektru klonálních onemocnění krvetvorby jsme se věnovali i myelodysplastickému syndromu, kterého patogeneze se pojí s mutacemi různých genů. Pomocí sekvenování nové generace za použití TruSight Myeloid Illumina kitu jsme prokázali mutace alespoň v jednom z 54 vybraných genů u více než 90 % pacientů s vysoce rizikovými MDS léčených azacitidinem. Opakované odběry kostní dřeně v průběhu terapie nám umožnili vyhodnotit i dynamiku a vývoj klonální architektury jednotlivých pacientů. Pozorovali jsme několik vzorců vývoje mutací – stabilní mutace, mutace se vzrůstající frekvencí, mutace s klesající frekvencí a komplexnější změny (především pokles frekvence s následným vzestupem). Tyto změny ve většině případů korelovaly s průběhem onemocnění, což naznačuje roli některých mutací na udržení AZA tolerance. U našich pacientů, včetně těch, kteří klinicky dosáhli dlouhodobé kompletní remise, jsme nezaznamenali kompletní molekulární remisi na AZA léčbě. Sledování mutací a jejich vývoje nám umožnilo předpovědět blížící se relaps onemocnění, jelikož mu často předcházel vzestup alelické frekvence původních mutací a to ještě ve stádiu klinické remise. Vytvořili jsme statistický model, který jednak hodnotil prognostický vliv ne/přítomnosti mutace konkrétního genu a jednak dle dynamiky dokázal predikovat vliv jednotlivých mutací na základě jejich dynamiky. Na základě tohoto modelu jsme zjistili, že některé mutované geny zhoršují prognózu pacientů, nicméně vliv konkrétních mutací a jejich dynamiky je rovněž důležitý.

8 Author's contribution

The author wrote the thesis and under kind supervision of Ondřej Hrušák and Tomáš Stopka performed following procedures: setup of gene expression platform, cell subsets sorting of healthy and most of leukemic samples, cell cultures, nucleic acid extractions, reverse transcription and quantitative real-time PCR, analysis of mRNA expression data, sequencing library preparation including quality control for part of the samples, patients' data collection for MDS cohort, and contributed significantly to Leukostage database built and MPS data analysis.

Other members of the CLIP and Stopka lab helped or performed the following: help with cell subsets sorting (Martina Vášková, Tomáš Kalina), help with Leukostage database built (Karel Fišer, Alena Dobiášová), help with LeukoStage Database manuscript preparation (Martina Vášková), SW-AL discovery, subpopulation sorting, immunophenotyping and data collection (Ester Mejstříková), SW-AL subpopulation sorting, *in vitro* studies, methylation analysis (Lucie Slámová), Ig/TCR analysis (Eva Froňková), SNP analysis, *IKZF1* and *ERG* alterations (Eva Froňková, Markéta Žaliová); help with cell subsets sorting and MPS library preparation for MDS project (Karina Vargová, Ľubomír Minařík), help with data analysis (Vojtěch Kulvait, Michal Pešta, Karina Vargová).

My mentors, Ondřej Hrušák and Tomáš Stopka helped me with experimental design, reviewed and discussed the results and revised this thesis as well as research manuscripts.

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10 Supplement

10.1 Detailed non-commertial protocols

10.1.1 **Ficoll-gradient based PB/BM mononuclear cell separation**

1) Dilution of EDTA anticoagulated PB or BM with sterile 1x PBS in ratio 1:1
2) Carefull loading of diluted PB on Ficolle-Paque (volume ratio 1:1)
3) 30 min centrifugation at RT, 400 g, with the brake off
4) Harvesting of the mononuclear cells
5) PBS wash: dilution in 1x PBS 1:3, 5 min centrifugation at RT, 500 g

10.1.2 **FACS from BM and PB**

1) Lysis of erythrocytes by 15 min incubation in 3ml 155mM NH_4Cl solution at RT
2) 1x PBS wash
3) Adding antibodies in desired amount or dilution
4) Incubation in dark room, RT, 15min
5) 1x PBS with EDTA (2mM) wash
6) Sorting (BD FACS Aria III) into 2-mercaptoethanol for RNA extraction or 1x PBS for other purposes (e.g. for purity control)

10.1.3 **FACS from thymus**

1) Cutting the tissue in adequate amount of 1x PBS with EDTA (2mM) on ice
2) Cell release by 10min shaking on rocker, on ice
3) Filtering the cells by pipetting through 40µm cell strainer to remove cell aggregates from the suspension
4) Centrifugation for 10min, 4°C, 400g
5) Lysis of residual red cells by 15 min incubation with 155mM NH_4Cl
6) 1x PBS with EDTA (2mM) wash
7) Measurement of the cell concentration by flow cytometry
8) Dilution of the sample to 100 000 000 cells/ml
9) Adding antibodies in desired amount or dilution
10) Incubation in dark room, on ice, 15min

11) Wash with 1x PBS with EDTA (manipulation on ice, centrifugation at 4°C)
12) Sort (BD FACS Aria III) into 2-mercaptoethanol for RNA extraction or 1x PBS for flow cytometry measurement (e.g. for purity control)

11 List of publications

Related to thesis

Polgárová K, Vášková M, Froňková E, Slámová L, Kalina T, Mejstříková E, Dobiášová A, Hrušák O. Quantitative expression of regulatory and differentiation-related genes in key steps of human hematopoiesis. The LeukoStage database. Differentiation. (2016) 91: 19-28 (IF 2.461) – **Supplement 1**

Slámová L, Starková J, Froňková E, Žaliová M, Řezničková L, van Delft FW, Vodičková E, Volejníková J, Zemanová Z, Polgárová K, Cario G, Figueroa M, Kalina T, Fišer K, Bourquin JP, Bornhauser B, Dworzak M, Zuna J, Trka J, Starý J, Hrušák O, Mejstříková E. CD2-positive B-cell precursor acute lymphoblastic leukemia with an early switch to the monocytic lineage. Leukemia. (2014) 28: 609-20 (IF 12.104) – **Supplement 2**

Polgarova K, Vargova K, Kulvait V, Dusilkova N, Minarik L, Zemanova Z, Pesta M, Jonasova A, Stopa T. Somatic mutation dynamics in MDS patiens treated with Azacitidine indicates clonal selection in Patients Responders. Oncotarget. (2018) in press (IF 2016 5.168) – **Supplement 3**

Unrelated to thesis

Polgárová K, Lüthje P, Cerami A, Brauner A. The erythropoietin analogue ARA290 modulates the innate immune response and reduces Escherichia coli invasion into urothelial cells. FEMS Immunol Med Microbiol. (2011) 62: 190-6 (IF 3.078)

Behuliak M, Bencze M, Polgárová K, Ergang P, Loučková A, Kuneš J, Vaněčková I, Zicha J. Ontogenetic changes in contribution of calcium sensitization and calcium entry to blood pressure maintenance of Wistar-Kyoto and spontaneously hypertensive rats. Journal of Hypertension (2015) 33: 2443-2454 (IF 5.062)

Behuliak M, Bencze M, Polgárová K, Kuneš J, Vaněčková I, Zicha J. Hemodynamic Response to Gabapentin in Conscious Spontaneously Hypertensive Rats: The Role of Sympathetic Nervous System. Hypertension (2018) 72: 675-686 (IF 6.857)

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