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## ABC transporters and cholesterol metabolism in acute myeloid leukemia

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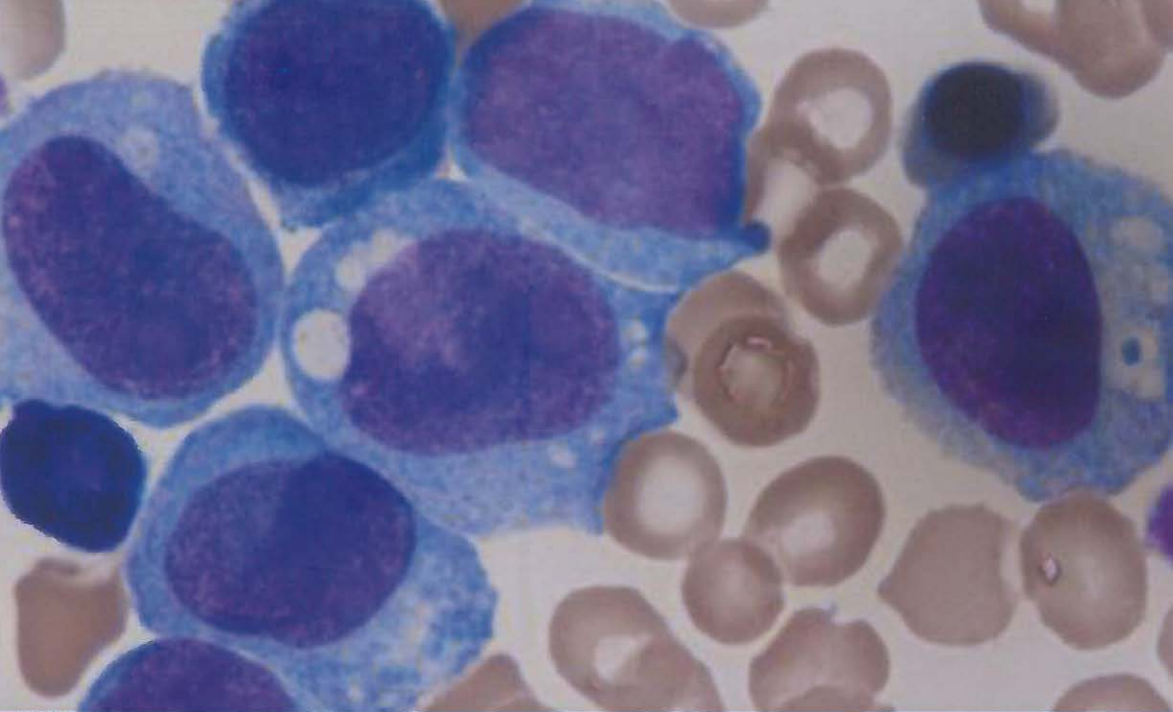
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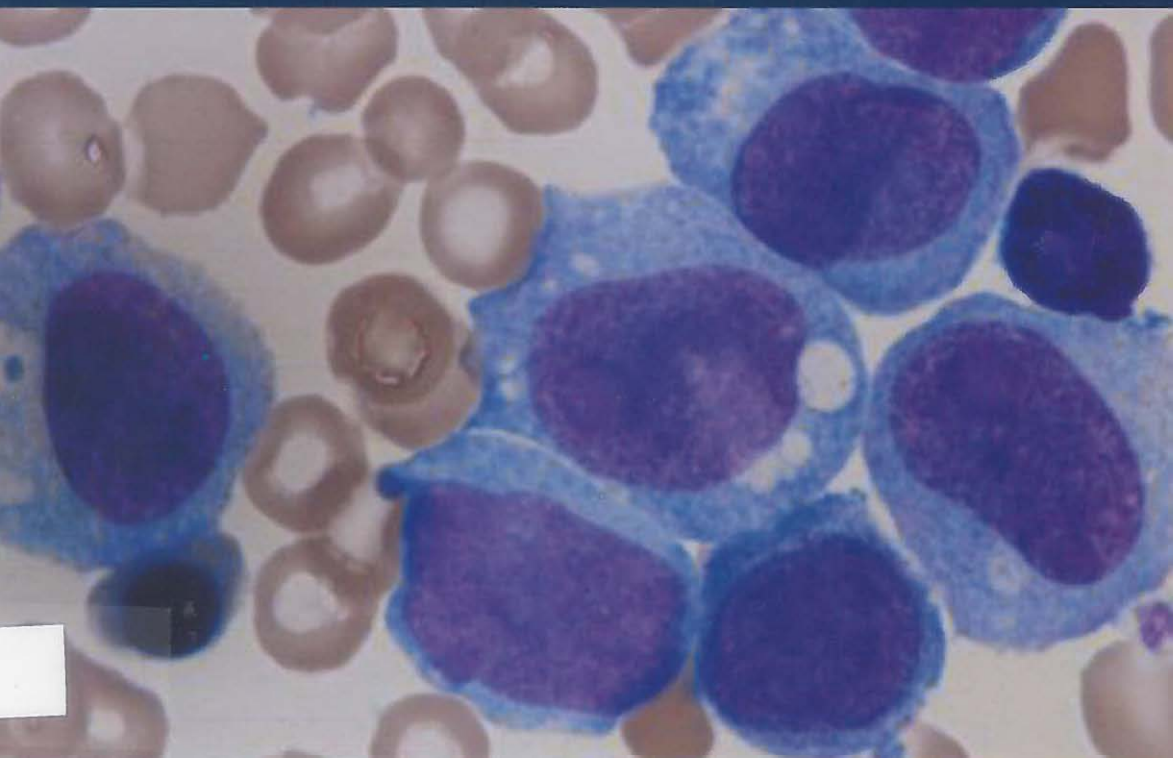
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*ABC transporters and cholesterol metabolism  
in acute myeloid leukemia*

*Susan D.P.W.M. de Jonge-Peeters*



## **ABC transporters and cholesterol metabolism in acute myeloid leukemia**

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**ABC transporters and cholesterol metabolism in acute myeloid leukemia**

1. ABCG1 komt relatief hoog tot expressie in primitieve humane beenmergcellen. (dit proefschrift)
2. Het feit dat zowel de cholesteroltransporters ABCG1 en ABCA1 als de genen die coderen voor HMGCoA reductase en LDL receptor relatief hoog tot expressie komen in de primitieve subpopulatie van normale beenmergcellen en in een subgroep van AML cellen geeft aan dat in deze cellen een actief cholesterolmetabolisme plaats vindt. (dit proefschrift)
3. Lovastatine verhoogt de chemosensitiviteit in een subgroep van leukemische primitieve beenmergcellen. (dit proefschrift)
4. Heterogeniteit in primitieve CD34<sup>+</sup>38<sup>+</sup> subpopulaties maakt het mogelijk verschillende AML patiënten te onderscheiden. (dit proefschrift)
5. De ABC transporters ABCA1 en ABCG1 spelen niet alleen een rol in cellulaire cholesterolflux, maar ook in het inflammatieproces. Dit biedt een belangrijk aangrijpingspunt voor nieuwe behandelingsstrategieën voor atherosclerose. (Ye *et al.* Curr Drug Targets 2010)
6. Statines kunnen zowel een verhoogd als een verlaagd risico kunnen geven op het ontwikkelen van een maligniteit. (Gonyeau *et al.* Pharmacotherapy 2010)
7. De leukemische stamcel wordt beschouwd als de 'Holy Grail' voor anti-leukemische therapie. (Misagian *et al.* Leukemia 2009)
8. "I am older, not elderly," said the patient with acute myeloid leukemia. (Schiffer, JCO 2009)
9. 'Education is when you read the fine print. Experience is what you get if you don't'. (Pete Seeger 1919)
10. Nothing is so practical as a good theory. (Kurt Lewin, 1890-1947)
11. 'Everything you can imagine is real'. (Pablo Picasso 1881-1973)
12. "Mama, jij bent een do(c)kter!" (Timen de Jonge)

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ABC transporters and cholesterol metabolism in acute myeloid leukemia  
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## **ABC transporters and cholesterol metabolism in acute myeloid leukemia**

### **Proefschrift**

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*Voor mijn ouders,  
Martin, Timen en Rogier*





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# Chapter 1

## General Introduction

## GENERAL INTRODUCTION

Acute myeloid leukemia (AML) is a clonal malignant disorder, characterized by the accumulation of an immature cell population in the bone marrow, resulting in a disruption of normal hematopoiesis. Remission rates with standard induction chemotherapy in patients with AML range from 70-80%. Despite intensive consolidation treatment only a limited number of AML patients achieve a long term cure. Although the presence of cytogenetic and molecular markers can predict efficacy of certain treatment regimes, it is currently not possible to accurately predict which patients will relapse.

AML is considered to be a 'stem cell' disease where alterations in normal hematopoietic stem cells (HSCs) result in a leukemic stem cell. The leukemic stem cell has the potential for unlimited replication producing daughter cells which do not differentiate normally, producing a malignant clone. The high initial response rate and high relapse rate indicate that although chemotherapeutic agents eradicate the majority of the malignant cells, the leukemic stem cell survives. It is assumed that these cells are highly resistant to the commonly used chemotherapeutic agents and that drugs which can target these resistant cells or restore their sensitivity to traditional chemotherapeutic agents are required to increase cure rates.

It has been shown that ATP-binding Cassette (ABC) transporters play an important role in this resistance to chemotherapy. ABC transporters represent a large family of transmembrane proteins, involved in the transport of diverse substrates across cell membranes. We hypothesized that the expression of ABC transporters differs between relatively resistant primitive leukemic cells and more sensitive mature subpopulations. We evaluated differential expression levels of ABC transporters in primitive subpopulation and more mature subpopulations of normal- and leukemic hematopoietic cells. Since a definitive stem cell phenotype has not yet been defined, we used a 'stem cell fraction, enriched for leukemic stem cells'. In the primitive cell fraction we indeed found increased expression of ABC transporters. Surprisingly, these were ABC transporters known to be involved in the cholesterol metabolism. The first report of a connection between cholesterol metabolism and leukemogenesis was described more than 30 years ago<sup>1,2</sup>. Only recently, aberrant cholesterol metabolism in a subset of AML cells as compared to normal hematopoietic cells, was found<sup>3,4</sup>. The subsequent aims of this thesis were to define this specific subpopulation, to investigate the role of cholesterol metabolism in primitive AML cells and to study treatment strategies modulating cholesterol metabolism, including statins and farnesyltransferase inhibitors.

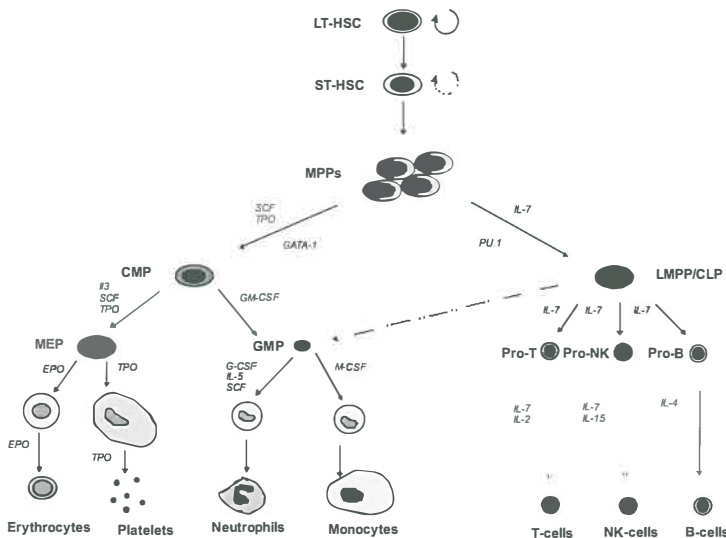
### Hematopoiesis

Hematopoiesis is a hierarchical, structured process, resulting in a continuous proliferation and differentiation of HSCs into all different blood cell types (erythroid, myeloid, lymphoid). HSCs are defined as cells that have the potential to undergo self-renewal, extensive proliferation and differentiation into multiple diverse cell types, via multipotent progenitors (Figure 1). Only a limited number (1 in  $10^5$  to  $10^6$ ) of these cells are pluripotent HSCs, characterized by their ability to

repopulate bone marrow in a transplantation setting.

The differentiation and maturation progress can be monitored by detecting changes in cytomorphology and immunophenotype, classified by the so called 'clusters of differentiation', that reflect changes in the expression profile of surface antigens. Characteristically, human HSCs and progenitors express CD34 on their cell membrane. Further characterization of the CD34 cell population can be performed by using the cell surface marker CD38. It has been shown that the CD34<sup>+</sup>CD38<sup>-</sup> cell fraction consists of the primitive cell fraction, while the more mature cell fraction is defined by CD34<sup>+</sup>CD38<sup>+</sup>. As these cells mature, they will lose this specific primitive cell marker and can also obtain new CD markers based on their lineage, such as CD15 (myeloid lineage). By using the surface antigens such as CD34, CD38, and human leukocyte antigens-DR (HLA-DR) samples can be enriched for HSC but, even the most highly enriched populations still consist of a heterogeneous cell population. Knowledge of the expression (levels) of various lineage-specific, immature, and mature markers in normal hematopoietic development provides a frame of reference for recognition of abnormal differentiation patterns.

In the past few decades, researchers have recognized a hematopoietic lineage tree (Figure 1) in which the long term-HSC (LT-HSC) gives rise to a short term-HSC (ST-HSC), followed by a multipotent progenitor (MPP). The next step is that these cells are committed to become a common myeloid progenitor (CMP) or a common lymphocyte progenitor (CLP). CMPs can subsequently further differentiate to granulocyte/macrophage progenitors (GMP) and megakaryocytic/erythroid



**Figure 1: Schematic representation of normal hematopoiesis. Model according to Passegue et al<sup>149</sup>.** LT-HSC, long term hematopoietic stem cell; ST-HSC, short term hematopoietic stem cell; MPP, multipotent progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocytic/erythroid progenitor; LMPP, lymphoid/myeloid multipotent progenitor; CLP, common lymphoid progenitor; SCF, stem cell factor; TPO, thrombopoietin; IL-7, interleukin-7; EPO, erythropoietin; G-CSF, granulocyte colony stimulating factor; GM-CSF, granulocyte-macrophage colony-stimulating factor.

progenitors (MEP), whereas the CLP differentiates a pro-B or pro-T cell. Finally mature cells like erythrocytes, platelets, granulocytes, macrophages, T-cells, NK-cells and B-cells will arise.

Recent studies in mice, however, have suggested a revised lineage tree for hematopoiesis<sup>5</sup> (Figure 1). Based on *fms*-like tyrosine kinase 3 (*FLT3*) receptor expression, it was demonstrated that MPPs that are *FLT3*<sup>-</sup> differentiate preferentially along the erythroid/megakaryocyte pathway, whereas *FLT3*<sup>+</sup> MPPs primarily give rise to lymphoid (B and T) and myeloid lineages (macrophages and granulocytes). These analyses have led to the proposal that MPPs initially undergo a binary decision to differentiate into a megakaryocyte/erythroid (MEP) progenitor and a lymphoid/myeloid multipotential progenitor (LMPP). This revised scheme suggests that both the myeloid and lymphoid lineages arise from a common progenitor and likely share one or more regulatory components such as the transcription factor PU.1. Transcription factors play a critical role in the process of lineage commitment and differentiation. Cell fate specification involves the action of multiple transcription factors that initiate and limit mixed lineage patterns and thereby enable lineage commitment<sup>6</sup>. Upregulation of the *FLT3* receptor is correlated with loss of self-renewal capacity. The regulatory proteins Gfi-1 and Bmi-1 have been shown to be necessary for HSC self-renewal, whereas C/EBP $\alpha$  and c-Myc appear to promote differentiation. GATA-1 is necessary for erythroid and megakaryocyte development whereas PU.1 is required for the generation of myeloid (macrophage and granulocyte) and lymphoid lineages. Disruption in the expression, sequence, or structure of critical transcription factors or their associated regulatory proteins can upset the delicate balance between proliferation and differentiation and lead to leukemogenesis. This has been shown, for example, by the downregulation of Notch-1 resulting in decreased PU.1 mediated myeloid signaling in turn contributing to leukemogenesis<sup>7</sup>. Growth factors are required for the survival and proliferation of hematopoietic cells at all stages of development (Figure 1). The growth factors are produced by different hematopoietic and non-hematopoietic cells, such as monocytes, T-lymphocytes, fibroblasts, osteoblasts and endothelial cells in the bone marrow. Growth factors are also produced outside the bone marrow, for example by the kidneys (i.e. erythropoietin (EPO)) and liver (i.e. thrombopoietin (TPO)). Two different types of growth factors can be distinguished. Firstly *lineage specific* growth factors that promote maturation of progenitors to mature cells (i.e. EPO that promotes the proliferation of erythroid progenitors and granulocyte colony-stimulating factor (G-CSF) that induces proliferation of granulocytes)<sup>8</sup>. Secondly, there are *pleiotropic* growth factors (i.e. stem cell factor (SCF) and interleukin-3 (IL-3)) that influence proliferation of stem- and progenitor cells and granulocyte-macrophage colony-stimulating factor (GM-CSF) that induces proliferation and activation of granulocytes and monocyte progenitors.

### Stem cell niches

In 1978, Schofield first hypothesized that 'the niche' consists of a supportive bone marrow microenvironment essential for the long-term maintenance of a stable HSC pool. The cellular components of the bone marrow microenvironment were not identified for another two

decades. More recently two specialized areas of the bone marrow were identified as distinct microenvironmental niches: 'osteoblastic (endosteal) and (peri)vascular' niches<sup>9,11</sup>. HSCs are thought to reside in these niches. Recent work showed that these two niches work together and promote either self renewal or differentiation to progenitors and mobilization in and out of the bone marrow. The endosteal niche, localized at the inner surface of the bone cavity, in conjunction with many bone-forming osteoblasts provides a microenvironment for long-term HSCs (LT-HSCs) which are capable of contributing to hematopoiesis as quiescent or slow-cycling cells. Osteoclasts also play a role in regulating this niche and are important in stem cell mobilization<sup>12</sup>. Furthermore osteoclasts mediate maintenance of HSCs at the endosteal surface by increasing the extracellular Ca<sup>2+</sup> concentrations. HSCs carry the Calcium-sensing Receptor (CaR), which is a transmembrane G-protein coupled receptor that is sensitive to the extracellular Ca<sup>2+</sup> concentration<sup>13</sup>. Genetically engineered mice lacking the CaR show a lower number of HSCs in the endosteal niche. The activity of osteoclasts is partly regulated by osteoblasts. In contrast, the vascular niche, which consists of sinus endothelial cells lining blood vessels, promotes proliferation and differentiation of actively cycling, short term HSC (ST-HSC). Recently Butler *et al* reported that endothelial cells are also involved in the self-renewal and repopulation of Notch-dependent LT-HSCs<sup>14</sup>. To maintain their long term repopulating (LTR) potential stem cells are kept quiescent and divide only sporadically. Interactions between angiopoietin-1 (Ang1) (expressed and secreted by osteoblasts) and the receptor tyrosine kinase Tie2, promote HSC quiescence and are important for the maintenance of LTR *in vivo*<sup>15</sup>, in part regulated by the Notch signaling pathway. Stromal cell-derived factor-1 (SDF1) or CXCL12 is a member of the CXC motif family of chemokines and is produced by osteoblasts and endothelial cells in the bone marrow. Binding of CXCL12 to its receptor chemokine (C-X-C motif) receptor 4 (CXCR4) is important in promoting HSC homing to- and retention in the bone marrow. In mouse knock-in studies with CXCR4, a subset of cells with abundant CXCL12 expression (named CAR, or CXCL12 abundant reticular cells) were identified. These cells are described as 'reticular' as they form a network in the marrow. Induced deletion of CXCR4 in adult mice resulted in a severe reduction in HSC numbers. These HSCs are closely associated with the CAR cells and are part of the HSC niche<sup>16</sup>. Recent data also indicate that sinusoidal endothelial cells, expressing vascular endothelial growth factor receptors (VEGFR2 and VEGFR3) are also important for bone marrow reconstitution<sup>11</sup>.

In stem cell niches, a dynamic microenvironment exist that balances stem cell activity to maintain tissue homeostasis and repair throughout the lifetime of an organism. The nature of this microenvironment is essential for the maintenance and protection of HSCs. The physiological condition of the bone marrow appears to be low in oxygen tension and limited in nutrient supply. Hypoxia, by means of its key mediator 'Hypoxia inducible transcription factor-1 alpha (HIF-1α)' is the major stimulus for upregulation of the growth factor VEGF and thereby stimulation of angiogenesis. VEGF plays a vital role in the growth and metastases of solid and hematologic malignancies. VEGF secreted by leukemic cells activates receptors on both leukemic and endothelial cells, and stimulates their proliferation<sup>17,18</sup>. Therefore, targeting angiogenesis could be a good strategy to influence the



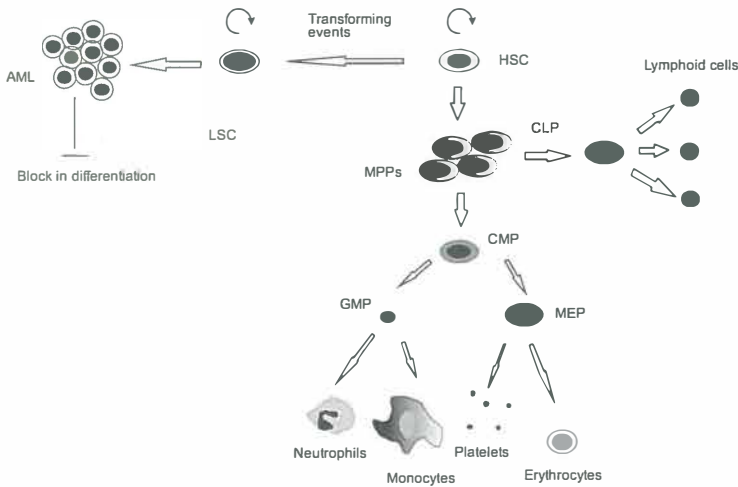
tumor/leukemia microenvironment and should be further explored in future research.

It remains unclear whether LSCs also depend on the niche for self-renewal. Both normal stem cells and LSCs depend on SDF-1-mediated CXCR4 signaling for homing and mobilization<sup>19</sup>. Leukemia is characterized by disordered growth and a failure of the normal homeostatic control mechanisms, and it has been previously proposed that this may cause leukemic cells to be independent of the niche. On the other hand, leukemia is increasingly recognized as a disease originating from HSCs or from the 'reacquisition' of stem cell (self-renewal) characteristics by more differentiated progenitors. Given the dependence of normal HSC on their niche for the maintenance of stem cell characteristics, it is conceivable that leukemic stem cells might have the same dependency on a niche. However, leukemic stem cells are also able to engraft at distinct sites other than the bone marrow microenvironment and therefore might have alternative properties giving the possibility to create and reside in new niches outside the bone marrow. Additionally, Colmone *et al.* recently described that leukemic cells also create bone marrow niches that are able to disrupt the behaviour of normal hematopoietic progenitor cells<sup>20</sup>. Disruption of normal stem cell niche function may, moreover, contribute to aging and disease onset and progression<sup>21</sup>. Consequently, very recently, a new concept was proposed by Raaymakers *et al.* demonstrating that disruption of mesenchymal cells causes malignant transformation of hematopoietic cells, supporting the concept of niche-induced oncogenesis, especially myelodysplasia or secondary leukemia<sup>22</sup>.

### Leukemogenesis

AML is, with the exception of acute promyelocytic leukemia (APL), a HSC disorder. It is characterized by a block in differentiation pattern, resulting in growth of a clonal population of neoplastic cells or blasts. These malignant blasts are usually hierarchically structured, similar to the normal hematopoietic system (Figure 2). AML encompasses a broad disease variety. According to the World Health Organization (WHO) classification six subgroups of AMLs have been identified in which genetic and clinical features of AML are combined with immunophenotypic and biological criteria of the old French-American-British (FAB) classification. (Table 1).

Lapidot, Bonnet and Dick, were the first to identify and characterize a leukemia-initiating cell population<sup>23;24</sup>. A common immunophenotype (CD34<sup>+</sup>CD38<sup>-</sup>) was identified for leukemic stem cells and their potential for self-renewal was demonstrated. Purified AML stem cells characterized by the CD34<sup>+</sup>CD38<sup>-</sup> phenotype were able to engraft in nonobese diabetic/severe combined immunodeficient mice (NOD-SCID), whereas the CD34<sup>+</sup>CD38<sup>+</sup> AML cells had properties in common with more committed cells. Recently Taussig *et al.* demonstrated greater variety in the phenotypes of leukemia-initiating cells than previously suggested. They report that the CD38<sup>+</sup> subpopulation and also the CD34<sup>-</sup> subpopulation contained leukemic hematopoietic repopulating cells<sup>25;26</sup>.



**Figure 2: A schematic model for leukemogenesis according to Chan et al<sup>50</sup>.** LSC, leukemic stem cell; HSC, hematopoietic stem cell; MPPs, multipotent progenitors; CLP, common lymphoid progenitor; CMP, common myeloid progenitor; GMP, granulocyte/macrophage progenitor; MEP, megakaryocytic/erythroid progenitor.

**Table 1. Classification of AML**

Group	Description	Genes involved
1	<b>AML with recurrent genetic abnormalities</b>	<b>Genes involved</b>
	-AML with t(8;21)(q22;q22)	<i>RUNX-RUNX1T1</i>
	-AML with inv(16)(p13.1q23) or t(16;16)(p13.1q22)	<i>CBFB-MYH11</i>
	-APL with t(15;17)(q22;q12)	<i>PML-RARA</i>
	-AML with t(9;11)(p22;q23)	<i>MLLT3-MLL</i>
	-AML with t(6;9)(p23;q34)	<i>DEK-NUP214</i>
	-AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2)	<i>RPN1-EVI1</i>
	-AML (megakaryoblastic) with t(1;22)(p13q13)	<i>RBM15-MKL1</i>
	-AML with mutated NPM1	<i>NPM1</i>
	-AML with mutated CEBPalpha	<i>CEBPA</i>
2	<b>AML with myelodysplasia (MDS)-related changes</b>	
3	<b>Therapy-related myeloid neoplasms</b>	
4	<b>AML not otherwise specified</b>	
	-AML, minimally differentiated	
	-AML without maturation	
	-AML with maturation	
	-Acute myelomonocytic leukemia	
	-Acute monoblastic/monocytic leukemia	
	-Acute erythroid leukemia	
	-Acute megakaryoblastic leukemia	
	-Acute basophilic leukemia	
	-Acute panmyelosis with myelofibrosis	
5	<b>Myeloid sarcoma</b>	
6	<b>Myeloid proliferations related to Down syndrome</b>	
	-transient abnormal myelopoiesis	
	-myeloid leukemia associated with Down syndrome	

The development of AML requires multiple genetic changes to accumulate in the 'leukemic' stem cell leading to a loss of normal hematopoietic function, which if left untreated, has a poor prognosis. In this succession of multiple events (multiple hit model) leading towards the malignant clone, mutations responsible for an enhanced self-renewal and impaired differentiation are combined with mutations causing a proliferative and/or survival advantage<sup>27</sup>. Numerous genetic alterations contribute to this leukemogenesis. One group comprises mutations (class I) that activate signal transduction pathways, resulting in enhanced cell proliferation and/or survival. Examples include mutations in *RAS*, the receptor tyrosine kinase genes such as *FLT3* or (tyrosine kinase hematopoietic receptor for stem cell factor (*KIT*)). Recently, Yang *et al.*, described that *RAS* is also involved in monocytic differentiation in juvenile myelomonocytic leukemia (JMML)<sup>28</sup>. Class II mutations affect transcription factors or components of the transcriptional co-activation complex, resulting in impaired differentiation. For example mutations in CCAAT enhancer binding factor alpha (*CEBPA*), the mixed-lineage leukemia gene (*MLL*), the nucleophosmin (*NPM1*) gene, over expression of *HOX* genes or formation of fusion genes such as *CBFβ-MYH11*. Furthermore many other (epi)genetic mechanisms, such as oncogene activation, haploinsufficiency of genes, or DNA methylation and histone deacetylation, contribute to leukemogenesis<sup>29</sup>. Multiple genetic abnormalities, in addition to cytogenetics, have allowed the further classification of AML into molecular subtypes with distinctive prognosis.

### **Molecular markers, epi- and cytogenetic differences**

Despite intensive treatment still only limited number of AML patients (with the exception of APL patients) can be cured with current therapy. Initially, AML was considered as one monolithic disease for which "one size fits all" chemotherapy was applied. This is no longer the case. AML is a genetically pleiomorphic disease. The difference between success and failure of AML treatment depends on a number of features, subdivided into those related to patient characteristics and those related to the malignant AML clone. The first subset usually predicts treatment-related mortality (TRM) and becomes more important as patient age increases while the latter predicts resistance to therapeutic modalities<sup>30</sup>. Patient related factors consist of age of the patient, co-morbid illnesses and performance status. Increasing age is an adverse prognostic factor, but age is not the most important prognostic factor for either TRM or therapy resistance. Co-morbidities tend to be more important.<sup>31</sup> AML related factors consist of white blood cell count (WBC), existence of prior MDS, previous cytotoxic therapy for another disorder, and cytogenetic and molecular genetic changes within the malignant leukemic clone at diagnosis. Cytogenetics are the strongest prognostic factor for response to induction therapy and for survival and allow us to categorize AML patients into 3 risk groups (favorable, intermediate and poor) (Table 2). Recently, in the poor risk group, it was discovered that a monosomal karyotype in AML was associated with an even worse prognosis than a complex karyotype<sup>32</sup>

However, 40-50% of patients do not have clonal chromosomal aberrations<sup>33</sup>. Until recently, all these

cases were categorized in the intermediate-risk group. Recently additional mutations have been identified that made it possible to subdivide the intermediate risk group of patients with a normal karyotype (CN-AML)<sup>34</sup>. Important molecular markers are mutations of the *NPM1* gene and *CEBPA* that have predominantly been associated with a favorable prognosis in contrary to *FLT3* that is associated with an adverse prognosis. These molecular markers will be discussed in the following paragraphs.

**Table 2. AML risk groups**

Risk	Subsets
<b>Favorable</b>	t(15;17)(q22;q12~21); <i>PML-RARA</i> t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i> , $WBC \leq 20 \times 10^9/l$ Inv(16)(p13.1q22) or t(16;16)(p13.1;q22); <i>CBFB-MYH11</i> CN-X-Y*, Mutated (Mt) <i>NPM1</i> without <i>FLT3-ITD</i> CN-X-Y, Mt <i>CEBPA</i> (biallelic)
<b>Intermediate-I**</b>	CN-X-Y, Mt <i>NPM1</i> and <i>FLT3-ITD</i> CN-X-Y, Wild-type (Wt) <i>NPM1</i> and <i>FLT3-ITD</i> CN-X-Y, Wt <i>NPM1</i> without <i>FLT3-ITD</i>
<b>Intermediate-II</b>	t(8;21)(q22;q22); <i>RUNX1-RUNX1T1</i> , $WBC \geq 20 \times 10^9/l$ CN -X-Y, $WBC \geq 100 \times 10^9/l$ t(9;11)(p22;q23); <i>MLL2-MLL</i> Cytogenetic abnormalities not classified as favourable or adverse
<b>Poor</b>	Inv(3)(q21;q26.2) or t(3;3)(q21;q26.2); <i>RPN1-EVI1</i> t(6;9)(p23;q34); <i>DEK-NUP214</i> t(v;11)(v;q23); <i>MLL</i> rearranged -5 or del(5q); -7-abn(17p); complex karyotype***

\* Cytogenetically normal or only loss of X or Y chromosome

\*\* Includes all AMLs with normal karyotype except for those included in the favorable subgroup; most of these cases are associated with a poor prognosis

\*\*\* Complex karyotypes: presence of 3 or more chromosome abnormalities in the absence of t(8;21), inv(16) or t(16;16) and t(15;17)

Nowadays, the most common genetic alterations in patients with AML are mutations in the *NPM1* gene which are detectable in 24-35% of all cases and in 43-62% CN-AML patients<sup>35-37</sup>. In adult AML, the incidence of a *NPM1* mutation increases with age up to 60 years. In patients over 60 years of age the incidence seems to decrease again. Clinically, *NPM1* mutations are associated with specific features, including predominance of female sex, higher bone marrow blast percentages, elevated lactate dehydrogenase (LDH) levels, higher presenting WBC and platelet counts, and high CD33 but low CD34 antigen expression. The difference in clinical characteristics at diagnosis between *NPM1*<sub>c</sub><sup>+</sup> and *NPM1*<sup>wr</sup> AML are not only related to the *NPM1* mutational status but also to the interaction with coexisting gene mutations such as the internal tandem duplication of the *fms*-like tyrosine kinase 3 gene (*FLT3-ITD*)<sup>38</sup>. The second most common genetic change and currently the most important prognostic factor in patients with CN-AML, is *FLT3-ITD*<sup>39</sup>. AML with *FLT3-ITD* are seen in 15-34% of pediatric and adult patients. AML patients with a *FLT3-ITD* mutation are characterized by certain pre-treatment features such as increased WBC count, higher percentages of blood and

bone marrow blasts, and a more frequent diagnosis of *de novo* rather than secondary AML<sup>40</sup>. The presence of *FLT3*-ITD provides a major independent adverse prognostic indicator, associated with an increased risk of relapse and poorer overall survival. There is also evidence that outcome may be more related to the level of the mutated allelic burden, rather than to its presence, or to the insertion site of the ITD. The worst outcome is observed for patients with high *FLT3*-ITD allelic ratios (high *FLT3*-ITD load)<sup>39/41</sup>. Unlike *FLT3*-ITD mutations, *FLT3*-TKD<sup>+</sup> (tyrosine kinase domain) mutations have no consistent unfavorable outcome probably in part to the limited number of patients studied. As discussed earlier, in most studies, the presence of *NPM1* mutation has been associated with higher CR rates and better event-free survival (EFS). However, approximately 40% of patients with *NPM1* mutations also carry *FLT3*-ITD that corresponds with an unfavorable prognosis, mainly dependent on the *FLT3*-ITD load (table 2). Mutated *NPM1*<sub>c</sub><sup>+</sup> without *FLT3*-ITD represents a favorable prognostic marker (table 2), similar to well known good risk cytogenetics such as inv(16) or t(8;21).

The transcription factor CCAAT enhancer-binding protein  $\alpha$  (CEBP $\alpha$ ) is a key molecule in the mediation of lineage specification and differentiation of multipotent myeloid progenitors into mature neutrophils. CEBP $\alpha$  function is dysregulated in approximately 10% of patients with AML, by mutations in the N- or the C terminus of the CEBP $\alpha$  gene. These AML patients demonstrate distinct clinical features such as higher peripheral blood blast counts, lower platelet counts, less lymphadenopathy, or extramedullary leukemia. In addition, CEBP $\alpha$  mutations are less frequently associated with *FLT3*-ITD or *TKD* mutations<sup>42</sup>. Whether the presence of *FLT3*-ITD impacts on prognosis in patients with mutated CEBP $\alpha$ , remains to be defined<sup>43</sup>. Numerous studies propose that CEBP $\alpha$  mutation predicts a relatively favorable outcome in AML (table 2) and only recently it was suggested that only the biallelic mutation (homozygous or compound heterozygous) predict this favorable outcome<sup>44/45</sup>. The currently available data indicate that impaired CEBP $\alpha$  function contributes directly to the development of AML, whereas to re-establish CEBP $\alpha$  function represents an interesting target for new therapeutic strategies in AML<sup>46</sup>.

It is now recognized that not only (cyto)genetic, but also epigenetic alterations are important in the disease course. These epigenetic alterations might induce loss of gene function, not accompanied by alterations in primary DNA sequence, as result of DNA methylation, histone modification or non-coding RNA. Consequently, they have been considered as optimal targets for what is now known as epigenetic therapy (as discussed further on)<sup>47</sup>.

### Standard therapeutic interventions

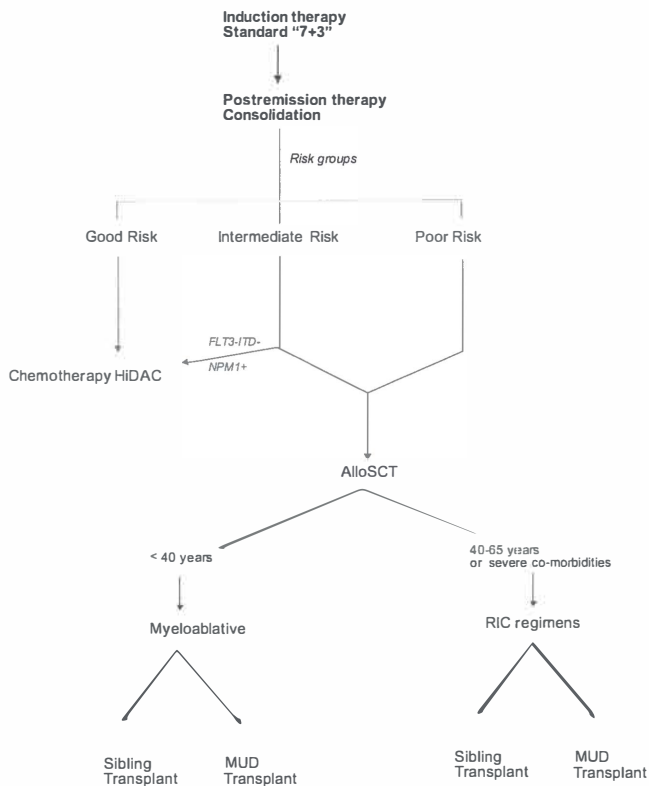
Standard (chemo)therapy-schedules differ for AML patients aged 18-65 years in comparison to elderly  $\geq 65$  years of age. For the younger patient group induction chemotherapy consists of a schedule commonly referred to as "3+7", containing 3 days of anthracyclines (choice of anthracycline may differ between different countries) and 7 days of cytarabine (Ara-C) (200 mg/m<sup>2</sup>, but dose may vary). This has been the standard initial treatment for AML for the past 3 decades<sup>48</sup>. Remission rates with standard induction chemotherapy in patients with AML range

from 70%-80%. Attempts to increase response rates by adding additional (cytotoxic) agents to the standard induction therapy have failed so far<sup>49:50</sup>. Priming the AML cells with hematopoietic growth factors has been used as an alternative approach to enhance the cytotoxic effects. However, no consistent results have been obtained although the use of the CXCR4 inhibitor in a mice model provided recently promising results<sup>51:52</sup>.

Postremission therapy in AML patients in CR promotes elimination of minimal residual disease (MRD), improved survival and increased cure rates. This includes the following standard strategies: intensive consolidation chemotherapy, autologous peripheral blood stem cell transplantation (ASCT) of allogeneic stem cell transplantation (alloSCT), or combinations. The treatment choice depends on the pre-treatment risk stratification. Patients belonging to the good risk group are treated with 1-2 courses of consolidation chemotherapy that includes mostly high-dose Ara-C (HiDAC)<sup>53</sup>. Consequently, in view of the favorable prognosis, these patients should not undergo a stem cell transplantation<sup>54</sup>. Patients belonging to the intermediate (lack of favorable genotypes or mutated *NPM1* without *FLT3*-ITD, or mutated *CEBPA*, or *FLT3*-ITD<sup>34</sup>) and poor-risk group are treated with 1-2 consolidation courses followed by an allogeneic SCT (alloSCT). If the alloSCT cannot be applied than an ASCT might be performed for patients for the intermediate-risk group. Some large clinical trials show only improved EFS, but not OS in this intermediate risk group<sup>55-57</sup>.

AlloSCT comprises either transplantation of a HLA identical related donor or HLA matched unrelated donor (MUD). Preferably, matched siblings should be considered for alloSCT<sup>58</sup>. Furthermore, alloSCT is considered to be the postremission strategy with the lowest rate of relapses, especially due to the immunological graft versus leukemia (GVL) effect<sup>59</sup>. Koreth *et al.* recently described improved EFS and OS in AMLs, belonging to the intermediate and unfavourable risk group, treated with alloSCT compared with conventional therapy. Unfortunately the benefits of this regimen will be limited by the highest treatment related mortality (TRM: <15-50%) of all three regimes especially if a myeloablative consolidation scheme is applied. The degree of TRM highly depends on numerous risk factors associated of the patient (age, comorbidity), the AML (subgroup, disease stage) or the transplant itself (i.e. time interval from diagnosis to transplant, donor type, donor-recipient sex combination serum cytomegalovirus (CMV) status of recipient and donor and non-HLA genetics)<sup>60</sup>. To reduce the transplant related mortality alternative approaches have been developed i.e. the nonmyeloablative or reduced-intensity conditioning (RIC) for alloSCT. This regimen reduces the nonrelapse mortality of the alloSCT, by applying less intensive conditions, for patients who otherwise could not tolerate a conventional myeloablative transplant<sup>61</sup>. Consequently, nowadays, AML patients >40 years will be treated with a RIC alloSCT and this new approach makes it possible that older patients up to 65-70 years can also be treated with an alloSCT<sup>62</sup>. The nonmyeloablative and RIC regimens depend more on donor cellular immune effects and less on the cytotoxic effects of the conditioning regimen to eradicate the underlying disease. This approach is based on the induction of host tolerance to donor cells followed by the administration of scheduled donor T-lymphocytes infusions. At this time, prospective studies are underway to compare this approach with conventional myeloablative

allogeneic stem cell transplantation<sup>63</sup>. Due to the lack of suitable HLA-identical donors, umbilical cord blood (UCB), can be an alternative HSC source<sup>64</sup>. The advantages of UCB include immediate availability, greater tolerance of HLA disparity, lower incidence of severe GVHD, safety to donors, easy harvesting and less risk of transmitting infection<sup>65</sup>. In children, so far, comparable 5 year EFS was found compared to normal allele-matched BMT<sup>66</sup>. Registry based studies in adult AML patients have established umbilical cord blood transplantation (UCBT) as a safe, feasible and most likely effective alternative to bone marrow transplantation (BMT) when a matched sibling donor is not available. However information on disease specific outcomes is very limited<sup>67</sup>. In adults, the major limitation is the low cell doses, leading to delayed engraftment and consequently a higher risk of infection and TRM. Furthermore it's not feasible to collect additional donor HSCs for patients experiencing either graft failure, or donor lymphocytes for recipients who relapse after initial UCB allografting<sup>65</sup>. By using double UCB units for adult AML patients, the low cell dose barrier, can effectively be overcome, leading to an improved hematological engraftment. A schematic overview of all treatment strategies is provided in Figure 3.



**Figure 3: Schematic overview of AML treatment strategies in patients, aged 18-65.**

HiDAC, high-dose cytarabine; NPM1, nucleophosmin1; FLT3-ITD, FLT3-internal tandem duplications; AlloSCT, allogeneic stem cell transplantation; RIC, reduced-intensity conditioning; MUD, matched unrelated donor. UCBT, umbilical cord blood transplantation, can be an alternative HSC source in selective cases.

Unfortunately, at this time most AML patients who received CR, will relapse within 3 years of diagnosis. During relapse, chemotherapy alone will rarely be curative, so ASCT or alloSCT will be necessary. This has variable success rates<sup>61,68,69</sup> The most important factor to predict success for a second remission is the duration of the first remission. If the initial remission was > 12 months, success percentages for a second CR can be 40-50%, in comparison to only 10-20% if initial remission was < 12 months<sup>70</sup>.

### AML and the elderly

Older patients, from a clinical practice perspective of view, may be divided according to whether they are 60 to 74, or 75 years of age or older. Importantly, AML is especially diagnosed at older age, more than half of patients diagnosed with AML are over 60 years of age. Unfortunately, older patients have not benefited as much from standard therapies as the younger patient-group. Often, elderly cannot tolerate intensive chemotherapy, experiencing an increased risk of morbidity and mortality leading to TRM rates of 10-25%<sup>71-73</sup>. Furthermore, poor therapeutic results are also believed to be due to intrinsic biological differences of AML. An increased frequency of unfavorable cytogenetics have been observed, there is a higher expression of a multidrug resistance (MDR)-phenotype and the AML more frequently develops from a precursor myelodysplastic syndrome. However, recently Juliusson *et al.* reported that remission induction chemotherapy provided better quality of life and longer survival than supportive care alone in the elderly group, based on data from a large, unselected Swedish National Acute Leukemia Registry<sup>31</sup>. Among older patients, a standard combination of daunorubicin plus Ara-C induces a CR in 40-50% of cases with a 2- 5 year OS rates of respectively ~ 10% and 2%<sup>73</sup>. High-dose daunorubicin was given in older patients with AML resulting in higher rates of CR (73% vs. 51%), EFS (29% vs. 14%) and OS (38% vs. 23%) without additional toxic effects. This benefit was especially observed in the good risk group of 60-65 year old patients<sup>74</sup>. Age and cytogenetics have a close relationship<sup>57,75,76</sup> Adverse cytogenetic abnormalities increase with increasing age. Consequently, increasing age is a strong independent prognostic factor for failure to achieve CR and OS<sup>77,78</sup>. For this subset of older patients, CR rates are 30% or less, and OS is less than 5%. AML presenting at older age shows a different gene expression profile compared to younger AML patients suggesting that AML at the older age is a separate entity<sup>79</sup>. Central in the aging of cells is cumulative cellular and genomic damage caused by endogenous as well as exogenous factors, in general leading to an activation of tumor suppressor pathways. Recently, de Jonge *et al.* demonstrated that the tumor suppressor gene CDKN2A (encoding p16<sup>INK4a</sup>) was down regulated in AML cells of patients with increasing age, compared to normal CD34<sup>+</sup> cells. Patients belonging to the favorable risk group showed no down regulation of p16<sup>INK4a</sup>, in agreement to the younger patient group. So down regulation of p16<sup>INK4a</sup> was found to be an independent unfavorable prognostic factor for OS in a subdivision of older AML patients. Therefore, differences in gene expression between AML samples of older and younger patients are not merely a reflection of the age of the patient, but also related to leukemogenesis<sup>80</sup>. Furthermore, molecular markers are



important as well at older age. The favorable impact of, for example, mutated  $NPM1_c^+$  on survival endpoints also seems to hold up among patients of older age<sup>30</sup>. There is growing evidence that AML with a favorable genetic profile such as mutated  $NPM1_c^+$  may benefit from dose escalation during consolidation. In addition a recent study suggested that AML with mutated  $NPM1_c^+$  without  $FLT3$ -ITD may benefit from the addition of all-trans retinoic acid (ATRA) to intensive induction and consolidation therapy. However this has to be verified in a larger study<sup>81</sup>. Furthermore alloSCT using reduced-intensity conditioning has become a promising therapeutic modality in the elderly patient (median age 58 years, range: 50-73 years)<sup>82,83</sup>.

In patients aged 75 or older with a performance status of 2 or 3, comorbidities, or organ dysfunction, an alternative to standard-dose induction is needed. This alternative does not come from low-dose Ara-C (LDAC) which was associated with a limited survival advantage compared to hydroxurea<sup>84</sup>. More than ever there is a need to appropriately discriminate elderly patients likely to benefit from intensive chemotherapy from those who will be candidates for less aggressive treatments. Therefore knowledge of the risk-group before start of treatment is of great relevance. Delay in treatment initiation to determine the risk-group upfront may have more advantages than disadvantages in this group of elderly AML patients<sup>85</sup>.

### **Novel non-chemotherapeutic agents**

Based on moderate current treatment results and the multiple hit model in leukemogenesis, single agent treatment will probably not be that successful. Therefore double or multiple (non) chemotherapeutic agents, utilizing various strategies of achieving cell death, may have to be combined to achieve good treatment results. Several novel, non chemotherapeutic agents, are currently under investigation. A selection will be discussed next.

#### *FLT3* inhibitors

The poor prognosis of the frequently mutated  $FLT3$ -ITD in AML patients has made  $FLT3$ -ITD an attractive molecular target for new therapeutics. Three different approaches are currently being studied in preclinical setting: anti- $FLT3$  antibodies, heat-shock protein (HSP) inhibitors and tyrosine kinase inhibitors (TKIs). The TKIs lestaurtinib (CEP 701), midostaurin (N-benzoylstauroporin: PKC412), tandutinib (MLN518), sunitinib (SU11248) and sorafenib (BAY43-9006) have also been tested in clinical trials. *In vitro* they showed cytotoxicity to leukemic cells. *In vivo*, as a single agent, they usually only cause a transient improvement in peripheral blood and bone marrow blast counts. Pilot clinical studies that combine  $FLT3$  inhibitors with standard chemotherapy in AML patients show promising results. In one study 34 first relapse AML patients with  $FLT3$ -ITD were randomized to receive either only open-label chemotherapy, or open-label chemotherapy in combination with lestaurtinib. 13 of 17 patients receiving lestaurtinib achieved plasma  $FLT3$  inhibitory activity (measured by means of a pharmacodynamic plasma inhibitory activity (PIA) assay)<sup>86</sup> of at least 85% (The threshold for optimal cytotoxic effects) and 10 patients showed a clinical response, of whom 5

patients a CR<sup>87</sup>. Clinical response was defined as either complete remission (CR, a decrease in marrow blasts to less than 5%), partial remission (PR, more than 50% decrease in marrow blasts to 5%-25%), hematological response (HR, disappearance of blasts from the peripheral blood), or bone marrow response (BMR, reduction in marrow blasts by more than 50% from diagnosis without hematologic recovery and hematologic response). Currently phase III studies are ongoing.

#### *Farnesyltransferase inhibitors*

*Ras* proto-oncogenes regulate the growth and differentiation of many cell types and are also involved in the mevalonate pathway as described below. There are three functional *Ras* genes: N-(from a neuroblastoma cell line), K-(Kirsten) and H(Harvey) *Ras*. Point mutations of N- and K-*Ras* genes are frequently present in AML (approximately 10-15 and 5% of all AML patients respectively) and result in constitutively activation of the *Ras* pathways<sup>88</sup>. Farnesyltransferase inhibitors inhibit farnesylation of *Ras* and other small GTP-binding polypeptides. Pro-apoptotic (Bim and Bax) and anti-apoptotic genes such as BCL<sub>xL</sub> and BCL<sub>2</sub> may also be involved<sup>89</sup>. Furthermore Akt, mTOR, MAPkinases, BAK and PUMA seem to be involved as well as the activation of caspase proteases in programmed cell death<sup>90,91</sup>. While farnesyltransferase inhibitors apparently can inhibit the prenylation of *Ras* isoforms during *in vitro* studies, reduced prenylation of other proteins such as RhoB, CENP-E, CENP-F and Rheb may also contribute to observed anti-proliferative effects and induce apoptosis<sup>92-97</sup>. Momentarily, clinical trials with tipifarnib in mostly elderly AML patients with poor risk features, are in progress. Preliminary data with this drug show varying results<sup>98-100</sup>. Two phase II trials, with tipifarnib in (older) adults with AML *de novo* and poor risk features, showed promising results. Tipifarnib is well tolerated and those patients that experience a clinical response may have a survival advantage<sup>98,99</sup>. In contrary the only phase III study published by Harousseau *et al.* compared, tipifarnib with best supportive care, including hydroxyurea in elderly patients (age 70 years and older) with AML *de novo* and they found no increased survival benefit in the tipifarnib treatment group<sup>100</sup>.

#### *Clofarabine*

The exact mechanism of action of the second-generation purine nucleoside analog clofarabine (2-chloro-9-[2'-deoxy-2'-fluoro-b-D-arabinofuranosyl]adenine) is still unknown. Clofarabine was designed to incorporate the favorable qualities of fludarabine and cladribine and to diminish their often dose-limiting neurotoxicity. In comparison to cladribine and fludarabine, clofarabine more completely inhibits both ribonucleotide reductase and DNA polymerase, versus one or the other. Unlike fludarabine, clofarabine is active *in vitro* in non-dividing cells and in cells with a low proliferation rate<sup>101</sup>. Clofarabine can induce the apoptotic pathway as part of its cytotoxic effect on cells. Very recently a phase II study of clofarabine monotherapy in previously untreated older adults (112 patients, median age 71 years) with AML and unfavorable prognostic factors showed acceptable toxicity and fairly good results with an overall remission rate (ORR) of 46% (38% CR, 8% CRp)<sup>102</sup>. In addition Burnett *et al.* published recently, similar results in two consecutive phase

II studies<sup>103</sup>. Currently, the HOVON 102 trial is ongoing. This trial randomizes AML patients (18-65 years) to receive either chemotherapy alone or combined with clofarabine. In comparison with former FLAG (fludarabine, Ara-C and G-CSF) regimens, less toxicity can be expected and as shown *in vitro*, hopefully better treatment results<sup>104</sup>.

#### *Anti VEGF/VEGFR agents*

Increased expression of pro-angiogenic factors such as VEGF and basic fibroblast growth factor (bFGF) have been detected in patients with AML compared with those in healthy donors, and higher plasma VEGF levels have been associated with a worse prognosis in patients with AML<sup>105</sup>. Bone marrow biopsies obtained from AML patients showed increased microvessel density (MVD) compared with healthy donors and upon successful remission induction, reductions in MVD and VEGF/VEGFR expression has been observed<sup>106</sup>. VEGF may be (over)expressed by AML cells and VEGFR by AML blasts and endothelial cells. The interaction (autocrine and paracrine) between VEGF and VEGFR seems to protect AML cells from chemotherapy induced apoptosis. Anti-VEGF-R antibodies, VEGF signaling inhibitors and kinase blockers are currently under investigation as a therapeutic strategy to overcome chemoresistance.

As described above, increased MVD has been demonstrated in bone marrow biopsies from AML patients. The bone marrow contains a variety of blood vessels (i.e. arterioles and sinusoids), that have different functions in bone marrow maintenance and hematopoiesis. Arterioles control the blood flow into the bone marrow compartment and only recently, studies suggest that bone marrow sinusoids not only serve as a channel to the bloodstream but also as vascular niches for HSCs, hematopoietic progenitors and megakaryocyte precursors<sup>107</sup>.

#### *DNA demethylating agents and histone deacetylase*

Epigenetics is defined as heritable alteration in gene expression that are not accompanied by changes in primary DNA sequence<sup>108</sup>. Two common epigenetic modifications include DNA methylation and various histone modifications. These mechanisms probably play a role in carcinogenesis by silencing tumor suppressor genes. DNA demethyltransferase inhibitor agents (DNMT, like azacitidine and decitabine) and histone deacetylase inhibitors (HDAC, like valproic acid, phenylbutyrate, MG-0103 and vorinostat) give re-expression of tumor suppressor and proapoptotic genes. The DNMT inhibitor azacitidine alone prolongs EFS and OS in high risk myelodysplastic syndrome (MDS), but also in AML subgroups (bone marrow blast count at presentation (20-30%)<sup>73</sup>. Currently, the HOVON 97 trial is ongoing where elderly AML patients are randomized in azacitidine or no maintenance therapy as post-remission treatment.

Only recently, a phase II clinical trial with decitabine was conducted in older patients (> 60 years) with previously untreated AML, who were not candidates for intensive chemotherapy. 53 Patients (36% presented with antecedent hematologic disorder or therapy related AML and 30% with complex karyotypes) received low-dose decitabine at 20 mg/m<sup>2</sup> intravenously on day 1 to 10 and

47% achieved CR after a median of three cycles of therapy. Given the DNA hypomethylating effect of decitabine, the relationship of clinical response and pretreatment level of *miR-29b* previously shown to target DNA methyltransferases was studied. Higher levels of *miR-29b* were associated with clinical response. Therefore further research is needed to verify whether levels of *miR-29b* can predict effect in older AML patients to decitabine treatment<sup>109</sup>.

Monotherapy of single HDAC inhibitors presented so far provided unsatisfactory results, so that dual targeting of both HDAC and DNMT was hypothesized to be a rational approach in AML treatment.<sup>110</sup> Several clinical studies combining DNA demethylating agents with HDAC inhibitors show promising results. In example, Ravandi *et al.* found that especially in patients with AML and high risk MDS with chromosome 5 and 7 abnormalities, azacitidine and decitabine showed superior OS compared to intensive chemotherapy<sup>111</sup>. On the other hand, this concept was challenged in a recent study in which reversal of promoter methylation following treatment with combination of DNMT (5-azacytidine) and HDAC inhibitors (entinostat, MS-275) was observed in both clinical responders and non-responders, indicating that the mechanism behind their clinical efficacy remains controversial. Recently, a pan-HDAC inhibitor panbinostat in combination with a histone methyltransferase EZH2 inhibitor, showed pro-apoptotic effects *in vitro* in primary AML cells, but not in normal CD34<sup>+</sup> cells<sup>112</sup>. Further research in large patient cohorts is needed, to confirm these data.

#### *mTOR inhibitors*

The PI3-K/Akt/mTOR cascade is strongly involved in proliferation and apoptosis. Currently one member of this pathway, mTOR (mammalian target of rapamycin), is being extensively studied for therapeutic intervention in AML. Rapamycin (sirolimus), an antibiotic, already used as an immunosuppressant<sup>113</sup>, also effectively inhibit mTOR when complexed with the FK506 binding protein 12 (FKBP12)<sup>114</sup>. Rapamycin has been demonstrated to effectively suppress leukemic cell lines and arrest the cell cycle at the G1 phase and thereby suppresses the constitutive phosphorylation of down-stream targets of mTOR. In preclinical trials, sirolimus dramatically increases the cytotoxicity of Ara-C and etoposide against AML blasts<sup>115;116</sup>. Currently multiple clinical trials are ongoing to evaluate mTOR inhibitors in combination with standard AML therapies for patients with poor-risk AML.

Furthermore, as described before, mutation or upregulation in one pathway does not account for AML transformation. Blasts rely on multiple dysregulated pathways to appear and survive, and to finally develop resistance to therapy, therefore to aim at several targets in a concurrent or serial fashion may be a promising approach in new therapies<sup>117</sup>.

#### **ABC transporters**

The 'holy grail' of leukemia therapy will be the targeting of the leukemic stem cell<sup>118</sup>. Therefore, this thesis focuses on certain specific properties of the primitive hematopoietic cells, namely the high

expression of members of the ABC transporter family. These ABC transporters represent the largest family of transmembrane proteins involved in the transport of a huge variety of substrates across cell membranes. ABC transporters use the energy of ATP binding to drive the transport of these molecules across the cell membranes. The ABC family of active transporters consists currently of 49 members and is divided into 7 subfamilies. An overview of the subfamilies of ABC transporter genes and a schematic representation of an ABC transporter is given in the review (chapter 3). ABC transporters fulfil a very basic role in biology of all living cells. Already well described and very important are the high expressions of specific ABC transporters (ie. ABCB1 (MDR1), ABCC1 (MRP1) and ABCG2 (BCRP)), contributing to multidrug resistance. Numerous antineoplastic agents are substrates of the MDR1 protein P-glycoprotein (P-gp), including anthracyclines, vinca alkaloids, taxanes, camptothecins, and epipodophyllotoxins. MDR1 and BCRP expression in leukemic CD34<sup>+</sup>CD38<sup>-</sup> leukemic cells correlate with chemotherapy response *in vitro*.<sup>119</sup> The drug-effluxing capacity of stem cells is an important feature for the isolation of HSCs. Some ABC transporters efflux the fluorescent dyes Hoechst-33342 and rhodamine 123 (used in murine HSC). HSCs are functionally characterized by a low accumulation of these fluorescent dyes, showing a subpopulation with low fluorescence besides the remaining cells, therefore called the 'side population'(SP) stem cells. It is suggested that purified SP cells and CD34<sup>+</sup>CD38<sup>-</sup> cells, are overlapping cell populations and belong to the stem cell pool (as discussed in the review, chapter 3).

The first-generation modulators included the antimalarial drugs quinine and quinidine, the immunosuppressive agent cyclosporine A, the coronary vasodilator nifedipine and others. Clinical studies with these agents added to chemotherapy did not increase the effect of chemotherapy and even presented with unacceptable toxicity due to their pharmacological properties intended for other clinical indications.<sup>120;121</sup> Based on the experience with first generation compounds, the second step was to identify their analogues that were devoid of the pharmacological properties of the original molecule but able to potently and specifically inhibit P-gp. This led to multiple trials with the most studied second-generation P-gp modulator valspodar (PSC-833), selected on the basis of encouraging preclinical results, showing a 10-20 fold higher potency than cyclosporine A, together with lower renal toxicity and lack of immunosuppressive activity<sup>122</sup>. However, in several clinical trials valspodar showed disappointing results. The second generation P-gp modulator appeared not to be active enough and in some cases increased (hepato)toxicity and even mortality were reported<sup>123;124</sup>. Momentarily third-generation modulators are under investigation<sup>125</sup>, of which zosquidar is one of the most potent P-gp inhibitors described to date. It selectively inhibits P-gp at nanomolar concentration *in vitro* and *in vivo* and there is evidence that it is not an inhibitor of the MRP family or BCRP<sup>126</sup>. However, the low effectivity of the P-gp modulators so far suggests that current MDR modulators may be ineffective due to the presence of multiple transporters, which can efflux the same drug. Furthermore the wrong transporter can be inhibited or a pharmacokinetic interaction between the chemotherapeutic agent and the ABC transporter could contribute to the low efficacy (see the review, chapter 3).

Their involvement in multidrug resistance is well known, but ABC transporters play many roles in normal and abnormal physiology and over-expression of some ABC transporters in specific hematopoietic fractions might be an important target for improvement of antileukemic therapy. Therefore, in this thesis we tried to elucidate one of these potential roles and found over-expression of certain 'relatively unknown' ABC transporters in hematopoietic stem cells, such as ABCG1. The ABCG1 protein is known to be highly expressed in macrophages and is a liver X-receptor (LXR) target gene, one of the major players in cholesterol homeostasis<sup>127;128</sup>. Besides the fairly unknown ABCG1 transporter, is ABCA1 a well-known participant in cholesterol efflux. ABCA1 is expressed in a variety of different tissues, with high expression levels in hepatocytes, enterocytes and macrophages<sup>129;130</sup>. Expression of ABCA1 is regulated by nuclear receptors, particularly by LXR/RXR.<sup>131</sup>

### Cholesterol Metabolism

Cholesterol is the most important sterol in mammals and functions as precursor of steroid hormones and bile salts. In normal cells, cholesterol is essential to membrane structure and the function of many membrane-bound proteins. Cellular cholesterol is either synthesized *de novo* or is derived from circulating LDL complexes via receptor-mediated endocytosis and lysosomal processing. Cholesterol homeostasis is a tightly regulated process containing of cholesterol influx (via low-density lipoprotein receptor (LDLR), efflux (by means of induction of activation of Liver X receptors (LXRs) which enhances transcription of target genes as ABCG1 and ABCA1) and synthesis. (Schematic presentation in the review: Figure 2)<sup>132</sup>. The major player in maintenance of cholesterol homeostasis is LXR. Two LXR genes are known, i.e., LXR $\alpha$  (NR1H3), which is highly expressed in the liver, and LXR $\beta$  (NR1H2) with a more diverse distribution<sup>133</sup>. LXR provides a tool for cells to 'measure' intracellular cholesterol concentrations. If LXR is activated, the LXR/RXR (retinoic X-receptor) heterodimer induces transcription of genes involved in cholesterol efflux towards acceptors such as apoA-I and high-density lipoproteins (HDL)<sup>134;135</sup>. Only very recently it was reported that mice deficient in both *Abca1* and *Abcg1*, show a leukocytosis and an expansion of the stem and progenitor population in the bone marrow<sup>136</sup>. Furthermore, apolipoprotein A-1 transgenic mice, transplanted with *Abca1*<sup>-/-</sup> and *Abcg1*<sup>-/-</sup> bone marrow, showed inhibition of HSC and multipotential progenitor cell proliferation, indicating that *Abca1*, *Abcg1* and HDL affect the proliferation of hematopoietic stem and progenitor cells<sup>136</sup>

Cholesterol can be synthesized, via the HMG-CoA reductase enzyme, in the mevalonate pathway, as well as various other by-products. These by-products include farnesyl and geranyl isoprenoids, implicated in the signaling of GTP-ases (Ras superfamily of small guanosine triphosphatases) including the small G-proteins Ras<sup>3</sup> and Rho<sup>137;138</sup>. Ras and Rho GTP-ase are involved in control of several processes such as cell proliferation, differentiation, migration, adhesion and drug efflux. A relationship between cholesterol metabolism and malignant transformation was identified more than four decades ago. Correspondingly, in 1978 an altered cholesterol metabolism in a subgroup of AML patients was found<sup>12</sup>. In the KG1a cell line it was shown that the membrane transporter

P-gp, in addition to its role in drug efflux, was involved in cellular cholesterol homeostasis<sup>139</sup>. P-gp has a direct, ATP dependent role in cholesterol transport<sup>140</sup> and has a role in the esterification of plasma membrane-derived free cholesterol in some cell types. In the presence of P-gp inhibitors, an increase in HMG-CoA reductase activity and subsequent cholesterol synthesis is also observed, suggesting a role for P-gp in cholesterol homeostasis. Connelly-Smith *et al.* report that LDL cholesterol contributes to P-gp expression and chemoresistance in primitive leukemia cells and it was suggested that the use of HMG-CoA reductase inhibitors may be of clinical value in lowering P-gp expression in AML<sup>139</sup>. In addition, recently, it was shown that some AML cells *in vitro* exposed to cytotoxic agents demonstrate an elevation of cellular cholesterol content, leading to an increase of chemoresistance, which could be reverted by the use of HMG-CoA reductase inhibitors, 'statins'<sup>3:141</sup>. Statins (used in this thesis, chapter 4 and 5) exert many anti-tumor effects. They inhibit tumor cell growth by inhibiting Ras and Rho-mediated cell proliferation<sup>142</sup>. Statins also have been reported to both stimulate or inhibit angiogenesis depending upon the tumor cell type<sup>143</sup> and the concentration of the drug<sup>144</sup>. Several experimental cancer models have shown that statins exert proapoptotic properties in a variety of tumor cells. Proposed mechanisms for statin-mediated apoptosis include an upregulation of proapoptotic protein expression (e.g., Bax, Bim)<sup>145</sup>, combined with decreased anti-apoptotic protein expression (e.g. BCL-<sub>2</sub>)<sup>146</sup>, or activation of caspase proteases involved in programmed cell death<sup>90</sup>. Furthermore it is suggested that statins repress tumor metastasis by inhibiting cell migration, attachment to the extracellular matrix and invasion of the basement membrane<sup>147:148</sup>. Various clinical trials are ongoing. In 2007, a phase I study combining pravastatine (40-1680mg/day) with conventional chemotherapy showed that statin cotreatment could be used safely<sup>141</sup>.

## OUTLINE OF THE THESIS

Most previously published studies on ABC transporters and AML focused on the total AML cell population. However, AML is a very heterogeneous disease and, moreover, predominantly considered to be a 'stem cell' disease. It has been shown that ABC transporter expression has an important role in this multidrug resistance. In order to illuminate specific stem cell properties we focused in this thesis on the differences between primitive and more mature subpopulations of AML cells. Unfortunately a definitive stem cell phenotype has not been defined yet, therefore a 'stem cell fraction enriched for leukemic stem cells' was used.

In **chapter 2** we studied the differences in ABC transporter expression between different subpopulations of normal hematopoietic cells and AML cells. We started with microarray experiments on murine stem cells (lin sca-1<sup>+</sup>c-kit<sup>+</sup>) in order to identify ABC transporters with a high expression level. Consequently, corresponding human transporters were analyzed in normal CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> bone marrow cells by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). In **chapter 3**, an overview is given of the current knowledge regarding the ABC transporter proteins in HSCs and in AML cells, especially in drug resistance. In addition, an outline

of ABC transporters involved in cholesterol metabolism is given. In AML cells, an aberrant cellular cholesterol metabolism has been reported, so our next goal was to integrate this information into a new study design. Therefore in **chapter 4** we questioned whether the cholesterol synthesis inhibitor lovastatin potentiates the cytotoxicity of chemotherapeutic agents in the primitive subpopulation of AML cells. For this we used a luminescent cell viability assay and a TaqMan PCR to measure the number of surviving cells upon exposure to statins and/or chemotherapeutics. Additionally, we tried to verify the obtained results by using a colony-forming cell (CFC) assay. In a CFC assay, hematopoietic progenitor cells, when cultured in a semisolid methylcellulose-based medium that is supplemented with suitable growth factors, proliferate and differentiate to produce clonal clusters (colonies) of maturing cells. The CFCs are then characterized, classified and enumerated *in situ* by light microscopy.

Furthermore, statins inhibit cholesterol synthesis at the level of the conversion of mevalonate and, as a consequence, also the production of various farnesyl and geranylgeranyl isoprenoid by-products. However, the statin concentration necessary to inhibit specific protein isoprenylation are 100-fold to 500 fold higher than those required to inhibit cholesterol synthesis. Therefore we investigated the further amplification of inhibitory effects of the cholesterol synthesis inhibitor simvastatin in combination with the farnesyltransferase inhibitor tipifarnib in cells of healthy individuals and leukemic cells of AML patients and also in the TF-1 and KG1A cell line as described in **chapter 5**. We used a variety of different techniques: cell viability assay and qRT-PCR (as used in chapter 4) and also annexin V and propidium iodide assays, cell cycle analysis and Western blotting to study a potentially increased effect of combined treatment with statins and farnesyltransferase inhibitors in a subset of AML patients. The results presented in this thesis are summarized in **chapter 6** along with a general discussion regarding the interpretation and implication of the results. This is followed by and indication of future perspectives in this area.

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# Chapter 2

## **Selective expression of cholesterol metabolism genes in normal CD34+CD38- cells with a heterogeneous expression pattern in AML cells**

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## ABSTRACT

*Objective.* To identify ABC transporters that are selectively expressed in normal and/or leukemic CD34<sup>+</sup>CD38<sup>-</sup> stem cells.

*Materials and Methods.* Microarray experiments on murine stem cells identified 13 ABC transporters with a high expression level. Corresponding human transporters were analyzed in normal CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> bone marrow cells by quantitative RT-PCR.

*Results.* Five ABC transporters, including ABCG1, were differentially expressed with a higher expression in CD34<sup>+</sup>CD38<sup>-</sup> cells. Besides ABCG1, known to be involved in cholesterol metabolism, expression of another major cholesterol transporter (ABCA1), some cholesterol metabolism genes (3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMG-CoAR), low-density lipoprotein receptor (LDLR)) and the transcription factor controlling ABCA1 and ABCG1 expression, liver-X-receptor-alpha (LXR-alpha) were assessed. All these genes were predominantly expressed in the more primitive sub-population, indicating a high rate of cholesterol metabolism and transport. Conversely in acute myeloid leukemia (AML) a heterogeneous expression pattern was found consisting of a considerable higher expression of particularly LXR-alpha in CD34<sup>+</sup> cells and a reverse expression pattern in a subset of AML CD34<sup>+</sup>CD38<sup>+</sup> cells.

*Conclusion.* These data suggest an active cholesterol metabolism and efflux in normal CD34<sup>+</sup>CD38<sup>-</sup> cells, while a subgroup of AMLs potentially demonstrates a hyperactive cholesterol metabolism.

## INTRODUCTION

Acute myeloid leukemia is characterized by accumulation of immature, undifferentiated myeloid cells in the bone marrow. These malignant blasts are usually hierarchically structured, similar to the normal hematopoietic system. Purified AML stem cells characterized by the CD34<sup>+</sup>CD38<sup>-</sup> phenotype are able to engraft in nonobese diabetic/severe combined immunodeficient mice, whereas no engraftment is observed with CD34<sup>+</sup>CD38<sup>+</sup> AML cells<sup>1-3</sup>. These findings indicate that CD34<sup>+</sup>CD38<sup>-</sup> cells belong to the pool of the leukemic stem cells whereas the CD34<sup>+</sup>CD38<sup>+</sup> AML cells have properties in common with more committed cells. Hematopoietic stem cells express several membrane transporters of the ATP-binding-cassette (ABC) super-family of transporter proteins, including P-glycoprotein (P-gp, ABCB1) and breast cancer resistance protein (BCRP, ABCG2), that play a role in trans-membrane transport of several substrates, including anticancer agents. In addition to ABCB1 and ABCG2, other ABC transporters have been identified in the total hematopoietic cell population, such as multidrug resistance-related protein 1 (MRP1, ABCC1) and its homologues MRP2-6 (ABCC2-6). High ABCB1 and ABCC1 expression levels represent poor prognostic factors for the outcome of AML treatment<sup>4-6</sup>. Data of most studies reported so far are based on total AML cell populations instead of the leukemic stem cell pool specifically. We have shown in previous studies that the pattern of ABC transporter expression in normal hematopoietic progenitor cells is similar to that of their leukemic counterparts<sup>7,8</sup>. The activity of ABCB1, ABCC1 and ABCG2 was higher in AML populations with high numbers of CD34<sup>+</sup>CD38<sup>-</sup> cells than in AML populations with high percentages of more mature CD34<sup>+</sup>CD33<sup>+</sup> cells<sup>9,10</sup>. Furthermore, most studies have focused on expression or activity of only a single or a limited number of ABC transporters. However, human ABC transporters have been described recently which have not yet been identified in hematopoietic cells and that might play a role in the differentiation of normal or leukemic CD34<sup>+</sup>CD38<sup>-</sup> stem cells<sup>11</sup>. We therefore decided to evaluate ABC transporter expression in normal and leukemic human hematopoietic CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> sub-populations. We made use of microarray data of murine primitive hematopoietic stem cells<sup>12</sup>: 13 murine ABC transporters were found to have a relatively high expression in these cells. In search of ABC transporters that are selectively expressed in primitive human hematopoietic CD34<sup>+</sup>CD38<sup>-</sup> cells, we verified the mRNA expression of these ABC transporters by quantitative RT-PCR in normal human bone marrow as well as in AML sorted sub-populations. Based on results obtained we looked more specifically into features of these specific genes. We demonstrate that 6 ABC transporter genes, including 2 major cholesterol transporters, are predominantly expressed in primitive CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic cells compared to more committed CD34<sup>+</sup>CD38<sup>+</sup> cells. Interestingly, a very heterogeneous pattern of ABC transporter gene expression was found in AML sub-populations.

## MATERIALS AND METHODS

### Microarray analysis

The murine studies and microarray analyses were performed and described<sup>12</sup>. BXD recombinant inbred female mice between 3 and 6 months of age were used. Bone marrow cells were flushed from the femurs and tibiae of 3 mice and pooled. The cells were stained with a panel of biotinylated lineage-specific antibodies (containing anti-CD3e, anti-CD45R, anti-CD11b, anti-TER119, anti-Gr-1), anti-Sca-1 and anti-c-kit, and purified using a MoFlo flow cytometer. Total RNA derived from pooled Lin<sup>sca-1</sup><sup>+</sup>c-kit<sup>+</sup> samples from 3 mice was isolated. Labeled cRNA was fractionated and hybridized to the U74Av2 microarray from Affymetrix (High Wycombe, UK). The data presented in this paper are based on the GNF Hematopoietic U74Av2 Cells database, which is available at [www.webqtl.org](http://www.webqtl.org)<sup>12</sup>. From the complete set of genes present on the microarray, that was published<sup>12</sup>, we selected all ABC transporter genes. The expression levels were expressed as log base 2 values of each cell. Three subgroups of expression levels were defined, expression values > 9.97 were considered as high, values between 8.0 and 9.97 as intermediate, and values < 8.0 as low expression. Based on these three subgroups we selected 7 ABC transporters with the highest expression values. Additionally, we made use of the data published at <http://www.sciencemag.org/cgi/content/full/1073823/DC1><sup>13</sup>. These murine lin<sup>sca-1</sup><sup>+</sup>c-kit<sup>+</sup> adult bone marrow and lin<sup>AA4.1</sup><sup>+</sup> + c-kit<sup>sca-1</sup><sup>+</sup> fetal liver stem cells were isolated, processed and used for microarray analysis (Affymetrix array MG-U74v2 A, B and C). From the complete set of stem cell-related genes we selected the ABC transporter genes.

### Human bone marrow samples

Normal sternal bone marrow samples were obtained after informed consent from 10 patients who underwent cardiac surgery. Mononuclear cells were enriched by Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient centrifugation and partly cryopreserved in RPMI 1640 medium (BioWhittaker, Brussels, Belgium) supplemented with 10% fetal calf serum (FCS) (Hyclone, Logan, UT) and 10% dimethyl sulfoxide (Merck, Amsterdam, the Netherlands), and stored at -196 °C. Prior to analysis, Mononuclear cells were thawed, treated with DNase (Boehringer Mannheim, Almere, the Netherlands), washed with RPMI 1640 medium, and incubated for 30 minutes in RPMI 1640 medium, supplemented with 10% FCS at 37 °C, 5% CO<sub>2</sub>. No differences were found in RT-PCR CT-levels for target and reference genes between frozen and fresh bone marrow samples.

Viability of the cells was determined by trypan blue exclusion and was > 90% in all cases.

### Patients

After informed consent, bone marrow aspirates or peripheral blood samples were collected from AML patients at diagnosis. Patients were classified at presentation of the disease according to the French-American-British (FAB) classification protocol. Leukemic blasts were enriched by Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient centrifugation and cryopreserved as described above.

To study cytogenetics, bone marrow or peripheral blood was cultured for 24 and 48 hours in RPMI 1640 supplemented with 15% FCS. The cultures were harvested and chromosome preparations were made according to standard cytogenetic techniques. The chromosomes were G-banded using trypsin or pancreatin and karyotypes were determined according to the International System for Human Cytogenetic Nomenclature (ISCN) 1995 guidelines for cancer cytogenetics <sup>14</sup>.

### Flow cytometric sorting

The normal bone marrow and AML samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 (Becton Dickinson, San Jose, CA) and a phycoerythrin (PE)-conjugated antibody against CD38. Sorting was performed using a MoFlo flow cytometer. Erythrocytes and dead cells were excluded from analysis by gating on forward and sideward light scatter. At least 30,000 CD34<sup>+</sup>CD38<sup>-</sup> and 30,000 CD34<sup>+</sup>CD38<sup>+</sup> cells were sorted. In order to obtain at least 30,000 cells in both sub-populations, CD34<sup>+</sup> leukemias were selected. No difference existed in average CD38 expression between normal and leukemic CD34<sup>+</sup>CD38<sup>-</sup> cells (Mean Fluorescence Intensity (MFI):  $0.44 \pm 0.21$  SD, range 0.25-0.71 and MFI:  $0.5 \pm 0.11$ , range 0.42-0.70).

### Cell lines

The human hematopoietic AML cell line KG-1a <sup>15</sup> and the hepatocyte cell line HepG2 <sup>16</sup> were cultured in RPMI 1640 medium, supplemented with 10% FCS.

### RNA extraction and cDNA synthesis

RNA extraction and cDNA synthesis (total RNA used: 0.5-1.0 µg) were carried out according to the manufacturer's instructions using the Rneasy Minikit (Qiagen, Hilden, Germany) and the Sensiscript-RT kit (Qiagen), respectively.

### Taqman PCR

Quantitative PCR was performed using the ABI-Prism-7700-Sequence-Detector (Applied Biosystems, Foster City, CA). Primers and probes for the human ABC transporters were used as described before <sup>17</sup>. Primers and probes for the cholesterol metabolism genes and transcription factors are presented in Table 1. Primers and probes for LXR-beta <sup>18</sup>, SREBP1A <sup>19</sup> and SREBP1c <sup>19</sup> have been described elsewhere. As endogenous controls the housekeeping genes glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and hypoxanthine-guanine phosphoribosyltransferase (HPRT) were used (Table 1). The primers were obtained from Invitrogen (Breda, The Netherlands). The probes were labeled by a 5' 6-carboxyfluorescein (FAM) reporter and quenched by 6-carboxytetramethylrhodamine (TAMRA) at its 3' end (Eurogentec, Seraing, Belgium). We used 4 µL of a 20-fold diluted cDNA in each PCR reaction in a final volume of 20 µL, containing 900 nM of sense and antisense primers, 200 nM of the Taqman probe, 5 mM MgCl<sub>2</sub>, KCl, TrisHCl, 0.2 mM dATP, dCTP, dGTP, dTTP, and dUTP, and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit; Eurogentec). The PCR program was 95 °C for 10 minutes,

**Table 1. Sequences of the PCR primers and probes used for real-time detection PCR analysis**

cDNA		Sequences
PPAR $\alpha$	Forward	5'-GTGGCTGCTATCATTGCTGTG-3'
	Reverse	5'-CACATGTACAATACCCTCCTGCAT-3'
	Probe	5'-TCGTCCTGGCCTTCTAAACGTAGGACACA-3'
PPAR $\beta$	Forward	5'-GGCCATCATTCTGTGGAGAC-3'
	Reverse	5'-CAGGATGGTGCCTGGATAGC-3'
	Probe	5'-CCAGGCCTCATGAACGTTCCACG-3'
PPAR $\gamma$	Forward	5'-GATGTCTCATAATGCCATCAGGTT-3'
	Reverse	5'-GGATTCAGCTGGTCGATACACT-3'
	Probe	5'-CCAACAGCTTCTCCTTCTCGGCCTG-3'
ApoE	Forward	5'-ACTGGGTCGCTTTTGGGATT-3'
	Reverse	5'-CTCCTCTGCACCTGCTCA-3'
	Probe	5'-ACAGTGTCTGCACCCAGCGCAGG-3'
HMGC $\alpha$ AR	Forward	5'-ACAATAAGATCTGTGGTTGGAATTATGA-3'
	Reverse	5'-GCTATGCATCGTATTATGTCAGAA-3'
	Probe	5'-CACTGCTCAAAACATCCTCTTCAAACCTTTGGA-3'
LDLR	Forward	5'-CAAAGTCTGCAACATGGCTAGAGA-3'
	Reverse	5'-GTTGTCCAAGCATTGTTGGTC-3'
	Probe	5'-CACTCTTTGATGGGTTTCTGACCAGTCC-3'
LXR $\alpha$	Forward	5'-CTTGCTCATTGCTATCAGCATCTT-3'
	Reverse	5'-ACATATGTGTGCTGCAGCCTCT-3'
	Probe	5'-TCTGCAGACCCGGCCCAACGTG-3'
SREBP2	Forward	5'-TGCAGCTACTAGCTTCAAGTCT-3'
	Reverse	5'-AATGGTGACCCGCTGTACCT-3'
	Probe	5'-CCTCAAGTCCAAAGCCTGGTGACATCC-3'
Cyp27A1	Forward	5'-TGCGGGCAGAGAGTGCTT-3'
	Reverse	5'-ACAGGATGTAGCAAATAGCTTCCA-3'
	Probe	5'-CAGGTGTCGGACATGGCTCAACTCTTCT-3'
GAPDH	Forward	5'-GGTGGTCTCCTCTGACTTCAACA-3'
	Reverse	5'-GTGGTCTGTTGAGGGCAATG-3'
	Probe	5'-ACACCCACTCCTCCACCTTTGACGC-3'
HPRT	Forward	5'-TGGACTAATTATGGACAGGACTGAAC-3'
	Reverse	5'-GCACACAGAGGGCTACAATGTG-3'
	Probe	5'-CTCCATCTCCTTCATCACATCTCGAGC-3'

followed by 40 cycles of 15 seconds at 95 °C and one minute at 60 °C.

The expression of the ABC transporter genes was standardized for the expression of GAPDH and HPRT. Since the results for both housekeeping genes were comparable, the GAPDH data are presented in the results section. Additionally, absolute CT levels for reference and target genes between normal bone marrow and leukemic patient samples were comparable. Serial cDNA dilutions of HepG2 and KG-1a cell lines were used to generate calibration curves. The expression of each gene in each sample was analyzed in duplicate. The regression coefficients of the standard curves were  $\geq 0.95$ . For the normal bone marrow samples a difference in expression of  $> 25\%$  between the CD34 $^+$ CD38 $^-$  and the CD34 $^+$ CD38 $^+$  cells was considered to be of importance.

## Statistical analysis

The paired-samples T-test was performed to calculate significant differences. Data were expressed as mean  $\pm$  standard deviation. *P* values < 0.05 were considered significant.

## RESULTS

### Microarray analysis

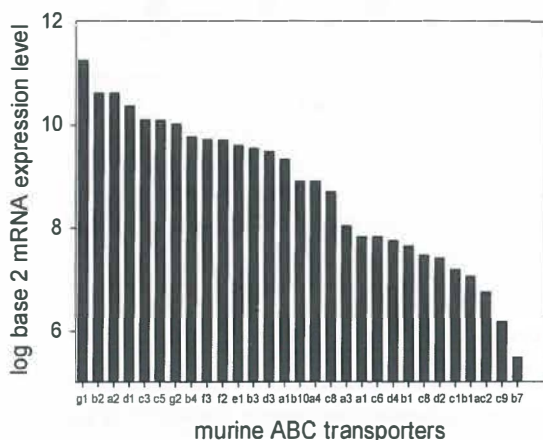
Twenty-nine (out of total of 49 identified) ABC transporter genes were represented on the MG-U74v2A Affymetrix array and were used to evaluate the expression of ABC transporter genes in murine *lin<sup>-</sup>sca-1<sup>+</sup>c-kit<sup>+</sup>* cells. The expression levels varied from very low (5.47 for *Abcb7*) to high (11.25 for *Abcg1*). Seven ABC transporter genes were expressed at a relatively high level (>9.97), i.e., *Abcg1*, *Abcb2*, *Abca2*, *Abcd1*, *Abcc3*, *Abcc5* and *Abcg2* and were selected. Eleven ABC transporter genes were expressed at an intermediate level (8.0-9.97), and 11 ABC transporter genes at a very low level (< 8.0) (Figure1).

From the data published at [www.sciencemag.org](http://www.sciencemag.org)<sup>13</sup>, we additionally selected 6 murine ABC transporter genes with a high expression profile: *Abcb1*, *Abcb11*, *Abcc1b*, *Abcd4*, *Abce1* and *Abcf2*.

### Expression levels of ABC transporter genes in CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> cells

Based on the expression levels of the ABC transporters in murine stem cells as defined above, we selected the following 13 corresponding human ABC transporter genes: *ABCG1*, *ABCB2*, *ABCA2*, *ABCD1*, *ABCC3*, *ABCC5*, *ABCG2*, *ABCB1*, *ABCB11*, *ABCC1b*, *ABCD4*, *ABCE1* and *ABCF2*.

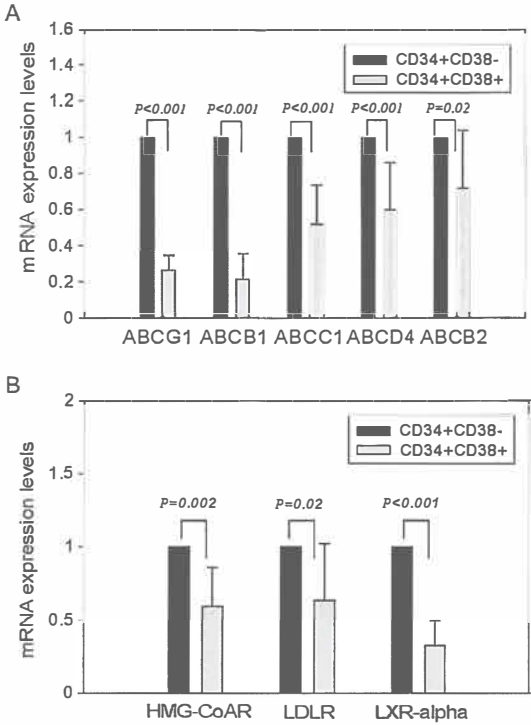
mRNA levels of these ABC transporters were measured in normal human bone marrow samples (n=10). Expression levels in the KG-1a cell line and HepG2 cell line were used as reference values. Expression of 5 ABC transporter genes was not detectable (*ABCA2*, *ABCB11*, *ABCC3*, *ABCD1* and *ABCF2*).



**Figure 1.** mRNA expression profile of 29 murine ABC transporters in *Lin<sup>-</sup>sca-1<sup>+</sup>kit<sup>+</sup>* cells, present on the microarray chip. The expression levels, shown on the y-axis, are expressed as log base 2 values of each cell of the chip. On the x-axis the 29 murine ABC transporters are plotted. Abbreviations: ABC: ATP-binding-cassette.



Three ABC transporters were expressed at similar levels in both sub-populations, i.e., *ABCC5*, *ABCE1* and *ABCG2*. Five ABC transporters were differentially expressed. In all 5 cases a higher expression was observed in the CD34<sup>+</sup>CD38<sup>-</sup> fraction compared to the CD34<sup>+</sup>CD38<sup>+</sup> fraction (Figure 2A), as reflected by the ratio of the expression levels in the CD34<sup>+</sup>CD38<sup>-</sup> versus the CD34<sup>+</sup>CD38<sup>+</sup> cells. (Table 2). *ABCB1* and *ABCC1* have been described earlier to be down-regulated upon maturation of CD34<sup>+</sup> cells<sup>20;21</sup>, but suppression of *ABCG1*, *ABCD4* and *ABCB2* gene expression during development has not been described in primitive hematopoietic CD34<sup>+</sup>CD38<sup>-</sup> cells before.



**Figure 2. mRNA expression levels of 5 ABC transporters, 2 genes involved in cholesterol metabolism (HMG-CoAR and LDLR) and of the transcription factor LXR-alpha.**

(A) The ABC transporters *ABCG1*, *ABCB1*, *ABCC1*, *ABCD4*, *ABCB2* and  
 (B) *HMG-CoAR*, *LDLR* and *LXR-alpha* were differentially expressed, they show a significantly higher expression in CD34<sup>+</sup>CD38<sup>-</sup> compared to CD34<sup>+</sup>CD38<sup>+</sup> sub-populations of normal bone marrow samples.

Abbreviations: ABC: ATP-binding cassette; HMG-CoAR: 3-hydroxy-3methyl-glutaryl-CoA reductase; LDLR: low-density lipoprotein receptor; LXR: liver-X-receptor; CD: cluster of differentiation.

**Cholesterol metabolism genes and transcription factors**

*ABCG1* plays a role in cellular efflux of cholesterol towards HDL<sup>22</sup>. Therefore we additionally measured the expression of the major cholesterol transporter *ABCA1*<sup>23;24</sup> as well as *ABCG4*<sup>22</sup>, which is also known to be involved in cholesterol efflux. *ABCA1* expression was higher in CD34<sup>+</sup>CD38<sup>-</sup> versus CD34<sup>+</sup>CD38<sup>+</sup> cells (Table 2) but *ABCG4* was not differentially expressed.

In addition, the expression of key-genes involved in cellular cholesterol metabolism, *HMG-CoAR*,

**Table 2. mRNA expression levels of ABC transporters, cholesterol metabolism genes and transcription factors**

	Ratio of expression levels in CD34 <sup>+</sup> CD38 <sup>+</sup> /CD34 <sup>+</sup> CD38 <sup>-</sup>	standard deviation	P-value
<i>ABC transporters</i>			
ABCG1	0.27	0.08	<0.001
ABCA1	0.71	0.2	0.002
ABCC1	0.52	0.22	<0.001
ABCD4	0.60	0.26	<0.001
ABCB1	0.22	0.14	<0.001
ABCB2	0.71	0.32	0.02
<i>Cholesterol genes</i>			
HMG-CoAR	0.59	0.27	0.002
LDLR	0.63	0.39	0.02
APOE	3.19	2.2	0.01
<i>Transcription factors cholesterol metabolism</i>			
LXR-alpha	0.32	0.17	<0.001
PPAR-beta	0.59	0.28	0.002

**Table 3. Clinical and cellular characteristics of AML patients**

Patient no.	BM/ PB	FAB Classi- fication.	Karyotype	CD34 <sup>+</sup> CD38 <sup>-</sup> (%)	CD34 <sup>+</sup> CD38 <sup>+</sup> (%)
1	BM	M2	49,XY,+13,+14,+21	3.5	52.5
2	PB	M2	45,XX,t(8;21)(q22;q22),-9,+12/46,XX	34.8	49.1
3	BM	M2	43,XY,-5,-7,t(1;9)(p22;p22) del(1)(p36), inv(12)(q15q23),t(11;16)(q13;q2),-17,-18 /46,XY,inv12(q15q23)	16.7	39.8
4	BM	M2	47,XX,+11/46,XX	6.4	1.0
5	BM	M2	46,XY,t(8;21)(q22;q22)	6.1	60.1
6	BM	M4	45XY,-7/46,XY	6.2	3.9
7	PB	M4Eo	46,XYinv(16)(p13q22)	16.5	27.2
8	BM	M4Eo	46,XY,der(14)t(14;22)(q32;q11), t(16;16)(p13;q22)/47,XY,t(16;16) (p13;q22),+22	12.6	20.9
9	PB	M5	45XY,inv(3)(q21q26),-7	6.3	55.4

BM, bone marrow; PB, peripheral blood

LDLR and apolipoprotein-E (APOE) and key regulatory transcription factors, sterol- response- element-binding-protein-2 (SREBP2), SREBP1a and SREBP1c were investigated. Gene expression of other transcription factors involved in control of cholesterol metabolism, such as peroxisome-proliferator-activated-receptor-alpha (PPAR-alpha), PPAR-beta, PPAR-gamma, LXR-alpha and LXR-beta were concomitantly studied. SREBP2, SREBP1A, SREBP1C, PPAR-alpha, PPAR-gamma and LXR-beta were expressed equally in both sub-populations. PPAR-beta, LXR-alpha, HMG-CoAR and LDLR, however, were predominantly expressed in the primitive CD34<sup>+</sup>CD38<sup>-</sup> sub-population (Table 2; Figure 2B). Furthermore, we studied the CYP27A1 gene, to indirectly assess cellular production

capacity of the endogenous LXR-ligand, 27-hydroxycholesterol. In contrast to our positive control, the HepG2 cell line, expression of *CYP27A1* was not detectable in CD34<sup>+</sup> cells or in the KG1A cell line. Finally, *APOE* was less expressed in the CD34<sup>+</sup>CD38<sup>-</sup> fraction compared to the CD34<sup>+</sup>CD38<sup>+</sup> cells (Table 2). From these results it appears that cholesterol synthesis and transport proceed at a high rate in the primitive CD34<sup>+</sup>CD38<sup>-</sup> cell fraction.

### Gene expression in AML blasts

Subsequently, we studied whether a comparable stem cell profile with regard to ABC transporters is observed in AML sorted cells (n=9). Different FAB-classifications were studied and patients were classified as M2 (n=5) and M4/M5 (n=4) (Table 3). The percentages of sorted cells varied between 3.5-39.8% in the CD34<sup>+</sup>CD38<sup>-</sup> fraction and between 1-60.1% in the CD34<sup>+</sup>CD38<sup>+</sup> fraction.

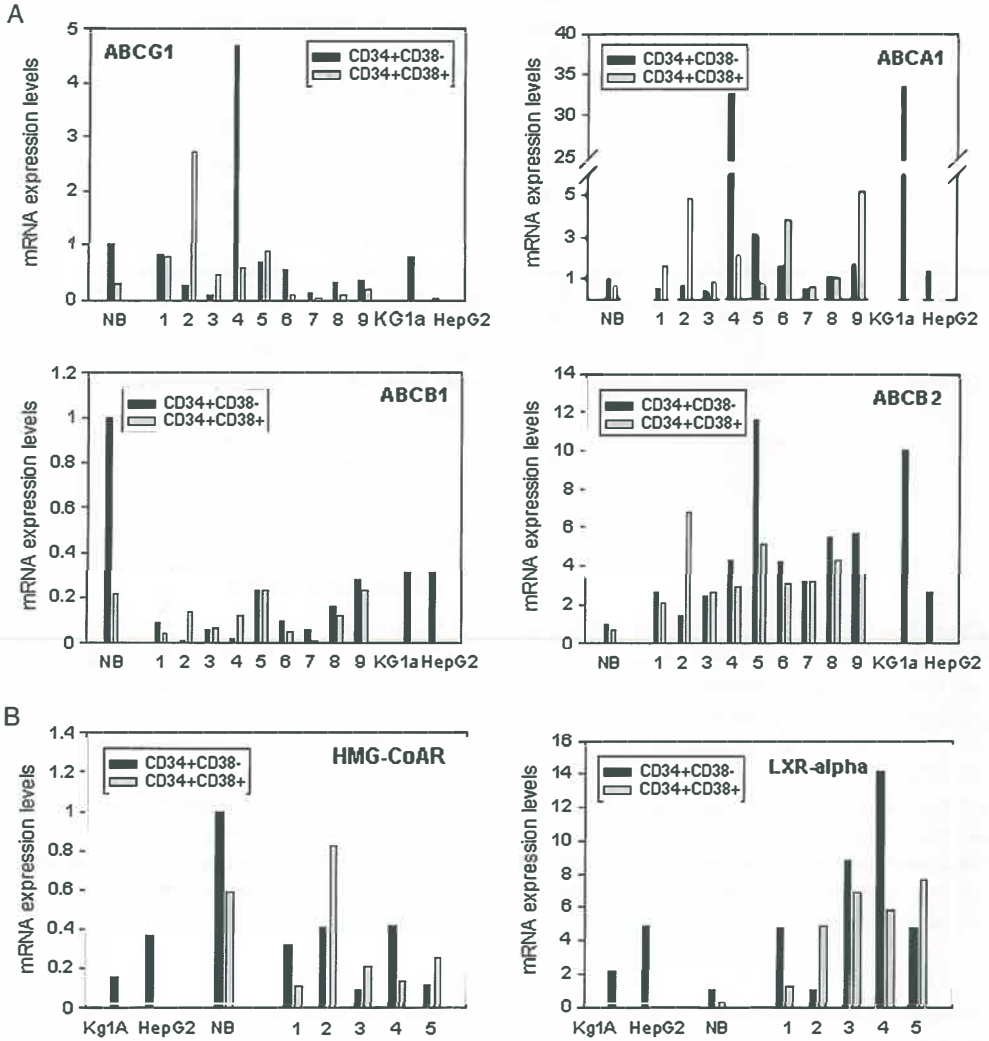
As depicted in Figure 3A, a more heterogeneous expression pattern of the ABC transporters, was observed in the AML sub-populations as compared to normal bone marrow. In general, the expression levels of ABCB1 and ABCC1 (data not shown) in the AML sub-populations were lower, while the reverse pattern was frequently noticed for ABCB2 and ABCA1. Concerning the down-regulation of ABC transporter gene expression in the CD34<sup>+</sup>CD38<sup>+</sup> cells, some AML patient samples followed the same pattern as observed with normal CD34<sup>+</sup> cells, whereas in other AML samples similar expression levels, or even a strong up-regulation was observed in the CD34<sup>+</sup>CD38<sup>+</sup> population.

Gene expression of cholesterol metabolism genes and LXR-alpha was measured in 5 of 9 AML patient samples due to lack of material. When compared to normal bone a very heterogeneous expression pattern was observed with no distinct differences for the HMG-CoAR expression between both cell fractions (Figure3B). The LDLR-expression was elevated in AML cells compared to normal CD34<sup>+</sup> cells in 4/5 studied cases (data not shown) while the LXR-alpha expression was highly increased in all studied cases (n=5) (Figure3B).

## DISCUSSION

Two ABC transporters, i.e., ABCB1 and ABCG2, have previously been described to be correlated with an immature immunophenotype in normal hematopoietic cells<sup>20;21;25</sup> and are down-regulated upon differentiation<sup>25-27</sup>. Additionally, we demonstrate that the ABC transporters ABCB1, ABCC1, ABCD4, ABCB2 and the cholesterol transporters ABCG1 and ABCA1 are predominantly expressed in normal primitive hematopoietic cells. Expression of these transporters shows a much more heterogeneous pattern in AML samples.

A specific function of ABCG1 and ABCA1 in primitive hematopoietic cells has so far not been defined. The proteins encoded by these genes promote the efflux of free cholesterol from the cells towards acceptors such as apoA-I and high-density lipoproteins (HDL) and play an important role in the regulation of cellular cholesterol homeostasis<sup>28-30</sup>. Cellular cholesterol homeostasis is maintained by tightly controlled mechanisms of cholesterol synthesis, influx and efflux (Figure4)<sup>31</sup>. Influx, especially



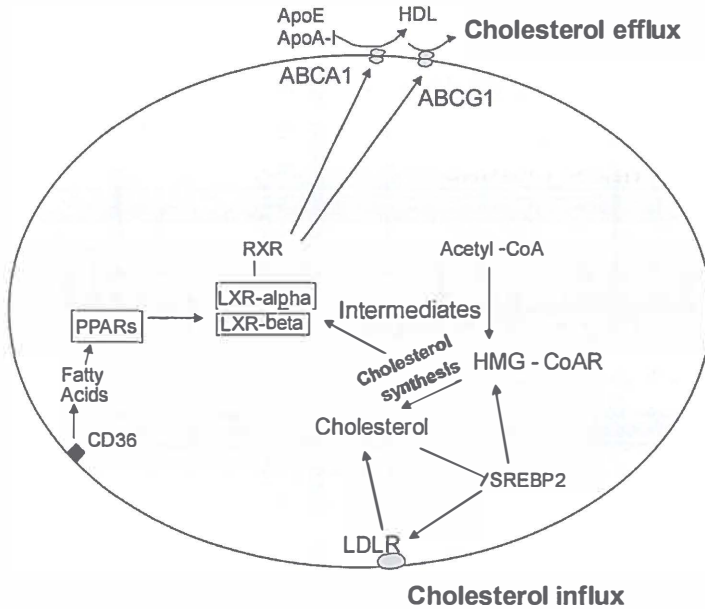
**Figure 3. mRNA expression levels of 4 ABC transporters, HMG-CoAR and LXR-alpha measured in normal bone marrow samples (NB; n=10), individual AML patient samples (n=9 or n=5) and in the cell lines KG1A and HepG2**

(A) Heterogeneous expression levels of ABCG1, ABCA1, ABCB1, ABCB2 in AML patient samples.

(A) Heterogeneous expression levels of cholesterol metabolism gene HMG-CoAR in AML patient samples and a high overall expression of transcription factor LXR-alpha in AML patient samples.

Abbreviations: ABC: ATP-binding cassette; CD: cluster of differentiation; HMG-CoAR: 3-hydroxy-3methyl-glutaryl-CoA reductase; LXR: liver-X-receptor.

LDLR-mediated lipoprotein uptake, and synthesis are mainly regulated by SREBP2 and induced when cellular cholesterol levels are low<sup>32</sup>. High cellular cholesterol levels exert a negative feedback on cholesterol influx and synthesis predominantly via SREBP2<sup>33</sup>. We observed that cholesterol metabolism genes, such as HMG-CoAR, LDLR and additional transcription factors involved in



**Figure 4. Schematic representation of cellular cholesterol homeostasis.** Mechanisms of cholesterol synthesis, influx and efflux maintain cellular cholesterol homeostasis. Influx, especially LDLR mediated lipoprotein uptake, and synthesis are predominantly regulated by SREBP2 and induced when cellular cholesterol levels are low. High cellular cholesterol levels exert a negative feedback on cholesterol influx and synthesis mainly via SREBP2. Cholesterol efflux is induced by oxysterol-dependent (intermediates) activation of LXRs, when cellular (oxy)sterol levels are high. Activation of LXR enhances transcription of target genes such as ABCG1 and ABCA1. The proteins encoded by these genes promote the efflux of free cholesterol from the cells towards acceptors such as apolipoprotein A-I (apoA-I) and high-density lipoprotein (HDL). Abbreviations: HMG-CoAR: 3-hydroxy-3methyl-glutaryl-CoA reductase; SREBP2: sterol-response-element-binding-protein-2 Acetyl-CoA: acetyl coenzyme A; LXR: liver-X-receptor; RXR: retinoid-X-receptor; LDLR: low-density lipoprotein receptor; PPAR: peroxisome-proliferator-activated-receptor; CD: cluster of differentiation; ABC: ATP-binding-cassette.

control of cholesterol efflux, i.e., LXR-alpha and PPAR-beta, were also significantly higher expressed in the CD34<sup>+</sup>CD38<sup>+</sup> fraction. Cholesterol efflux is induced by oxysterol-dependent activation of LXRs when cellular (oxy)sterol levels are high. Activation of LXR enhances transcription of target genes such as ABCG1 and ABCA1 and other genes involved in reverse cholesterol transport<sup>30;34;35</sup>. Oxysterols, oxygenated derivatives of cholesterol, are endogenous ligands of LXR. Based on reported results in human primary macrophages, potential LXR ligands in human CD34<sup>+</sup> cells could be 27-hydroxycholesterol and 24(S),25-epoxycholesterol<sup>18;36</sup>. 27-Hydroxycholesterol is the most common endogenous cholesterol-derivative in the human circulation. To assess the potential contribution of this ligand for LXR in human CD34<sup>+</sup> cells, we evaluated the mRNA expression levels of the *CYP27A1* gene. Expression of *CYP27A1* was not detectable in human CD34<sup>+</sup> cells and in the KG1A cell line. Another potential LXR ligand in human CD34<sup>+</sup> cells is 24(S),25-epoxycholesterol (24,25EC). 24,25EC is formed as an intermediate in the mevalonate pathway that is initiated by the action of HMG-CoAR. In view of the high HMG-CoAR expression in CD34<sup>+</sup>CD38<sup>+</sup> cells, it is reasonable

to assume a relatively high synthesis rate of 24,25EC in these cells. Its quantification, however, was technically hampered by the low numbers of available cells in this study.

Others have provided evidence for altered cholesterol metabolism in AML as well<sup>33;37;38</sup>. AML cells show more LDLR activity than normal hematopoietic cells do. Furthermore recent studies demonstrated an increased cholesterol synthesis in response to daunorubicin exposure in a subgroup of AML patients<sup>37;38</sup>. This was not observed with normal CD34<sup>+</sup> cells and appeared to be protective for the AML cells. It may well be that high rates of cholesterol efflux, associated with this enhanced rate of cholesterol metabolism, prevent apoptosis, facilitate cytotoxic drug-efflux and/or are crucial for maintenance of the quiescent state in this primitive sub-population.

So far the studies on ABC transporters in AML have especially focused on the total leukemic cell population. However, information of well-defined sub-populations might provide additional information concerning stem cell properties of AML cells. The AML CD34<sup>+</sup>CD38<sup>-</sup> sub-populations demonstrated distinct differences compared to normal CD34<sup>+</sup>CD38<sup>-</sup> cells. The well-established drug transporters ABCB1 (Pgp) and ABCG2 (BCRP) were found to be higher expressed in the more primitive sub-population, or equally expressed in both sub-populations, respectively. In general, an elevated ABCB2 expression level was observed in the AML cells. ABCB2 has been described to function as a peptide transporter<sup>39</sup>. The expression of ABCB1 and ABCC1 was reduced in the leukemic CD34<sup>+</sup>CD38<sup>-</sup> cells compared to normal CD34<sup>+</sup>CD38<sup>-</sup> cells. This finding is remarkable since a number of *in vivo* studies have been focused on inhibition of the efflux function of ABCB1 in AML cells to enhance drug sensitivity<sup>40;41</sup>, but so far without clinical benefit<sup>42</sup>.

The cause of the variability in ABC transporter expression in AML is unclear. It might be related to an aberrant differentiation program. Alternatively it might be a reflection of the heterogeneity of the AML stem cell compartment as demonstrated recently by Hope *et al.*<sup>43</sup>. It is unlikely that it might reflect differences between normal and leukemic cells. The CD34<sup>+</sup>CD38<sup>-</sup> cell fraction of AML patients contain mostly cells of leukemic origin as described by Haase *et al.*<sup>44</sup>.

In summary our study shows that multiple ABC transporter genes, especially those involved in cholesterol transport, and cholesterol metabolism genes are highly expressed in normal primitive CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic cells. These findings suggest that an altered cholesterol metabolism may play an important role in hematopoietic stem cell development. An overall heterogeneous gene expression pattern was observed in AML patients and interestingly, in both sub-populations of CD34<sup>+</sup> AML cells a considerable increase of LXR-alpha expression was found. This may indicate a specific role for LXR-activation in AML. Further work in subsequent studies is required to establish the importance of the obtained results.

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# Chapter 3

## **ABC transporter expression in hematopoietic stem cells and the role in AML drug resistance**

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**ABSTRACT**

ATP-binding cassette (ABC) transporters are known to play an important role in human physiology, toxicology, pharmacology, and numerous disorders including acute myeloid leukemia (AML). In AML only a few cells have properties allowing for ongoing proliferation and for expansion of this malignant disorder. These very primitive cells, referred to as leukemic stem cells, reside mostly in a quiescent cell cycle state. These cells have the capacity of self-renewal and are likely characterized by a high expression of a number of ABC transporters. In addition, over-expression of certain ABC transporters in leukemic cells has been associated with poor treatment outcome in AML patients. Therefore, to be able to improve diagnostics and therapies for AML patients, it may be important to better characterize this quiescent stem cell population. Particularly knowledge of the biology of highly expressed ABC transporters in these primitive leukemic cells might provide new insights to improve therapeutic options. This review provides an overview about ABC transporters and AML in general and particularly of the ABC transporters involved in multidrug resistance and cholesterol metabolism in primitive normal and leukemic cells.

## INTRODUCTION

Acute myeloid leukemia (AML) comprises a group of clonal hematopoietic stem cell disorders in which failure to differentiate causes accumulation of so-called blasts, frequently resulting in hematopoietic insufficiency (granulocytopenia, thrombocytopenia, anemia)<sup>1,2</sup>. Advances in our understanding of the pathophysiology of AML have not yet led to marked improvements in disease-free and overall survival of adult patients. Despite intensive treatment, only 30-40% of the AML patients between 18-60 of age can be cured and only 5-15% of the adults over the age of 60<sup>3,4</sup>.

The therapeutic approach of patients with AML has changed little over the past three decades. Conventional cytotoxic drugs such as anthracyclines (daunorubicin) and cytosine arabinoside (ARA-C) have provided the backbone therapy for induction of AML remission<sup>5</sup>, followed in recent years by idarubicin<sup>6</sup> and mitoxantrone<sup>7</sup>. This cytotoxic chemotherapy is based on the concept that the bone marrow contains two competing populations of cells (leukemic and normal cells) and that profound suppression of leukemic cells, to an extent that they can no longer be detected in marrow aspirates or biopsies, is necessary in order to permit recovery of normal hematopoiesis. Currently, increasing evidence accumulates that leukemic stem cells play an important role in the leukemogenic process<sup>8,9</sup>. A broad range of studies has indicated that AML arises from mutation(s) at the level of the hematopoietic stem/progenitor cell. These leukemic stem cells possess unique properties and are quite different from mature leukemic blasts. Leukemic stem cells apparently retain many characteristics of normal hematopoietic stem cells. To date, defining these unique properties of leukemic stem cells is a high priority for cancer research, in order to elucidate molecular mechanisms of cancer initiation and to develop new therapeutic approaches. Consequently, drug regimens designed to kill AML blasts may not be effective for destruction of the leukemic stem cell population<sup>10,11</sup>. In view of the central role of leukemic stem cells in the development and pathogenesis of AML, failure to adequately target this population is a likely cause of relapse<sup>12</sup>.

Taking into consideration the poor disease-free and overall survival rates of adults with AML, a number of clinical and biologic features that reflect the heterogeneity of AML is used to predict the probability that a patient will respond to treatment. Adverse prognostic factors include FLT-3 mutations<sup>13-16</sup>, *KIT* receptor tyrosine kinase mutations involving t(8;21) karyotype<sup>17</sup>, an age over 60 years, a poor performance score before treatment, an unfavorable karyotype, secondary AML, a white-blood cell count of more than 20·10<sup>9</sup>/l, an elevated serum lactate dehydrogenase level at presentation, a high CD34 expression, likely related to a more stem cell phenotype of the AML and often associated with features of multidrug resistance (such as high expression of ABC transporters)<sup>18-21</sup>. Favorable prognostic factors include: nucleophosmin mutations (NPM1)<sup>22,23</sup>, specific cytogenetic markers like t(15;17)<sup>20</sup> and mutations in *CEBPA*<sup>24</sup>.

In the older age group (> 60 years), there is an uneven distribution of unfavorable prognostic factors (e.g., cytogenetic abnormalities, features of drug resistance, or a history of myelodysplastic syndrome (MDS))<sup>25-28</sup>. In addition, older patients often do not tolerate intensive chemotherapy and frequently present with intercurrent medical conditions that are exacerbated by standard

antileukemic treatment. High dose chemotherapy is therefore highly unlikely to improve the clinical outcome in older patients. According to these prognostic factors and, importantly, knowing that the majority (more than 75%) of newly diagnosed patients is 60 years or older, clearly indicates a strong need for new therapeutic options to improve the poor cure rates in this large patient group<sup>32,29</sup>.

In general, there is a trend toward the modification of AML therapy to targetting of the malignant cells with molecular and immunologic therapeutic strategies. For example, multidrug resistance, one of the primary causes of suboptimal outcomes in cancer therapy, is very common in refractory leukemia. ABC transporters are involved in this process of protecting cancer stem cells from a broad range of chemotherapeutics<sup>30-35</sup>. Other than multidrug resistance, evidence has been provided to indicate an altered cholesterol metabolism in AML<sup>36-39</sup>. Particularly, AML cells, in contrast to normal mononuclear cells, often do not show efficient feedback repression of cholesterol synthesis and low-density-lipoprotein-receptor (LDLR) expression when exposed to high-sterol media *in vitro*, a feature that is associated with an increased cell survival<sup>38,39</sup>. Subsequently, indications for an active and dysfunctional cholesterol metabolism have been found at the mRNA level in a primitive sub-population (CD34<sup>+</sup>CD38<sup>-</sup>) of AML cells, protecting these cells by this critical cytoprotective lipid-signaling pathway<sup>40</sup>. ABC transporters, by promoting the cholesterol efflux, play an important role in the regulation of cellular cholesterol homeostasis. Gaining a better understanding of mechanisms of stem cell drug resistance and possibly also of the cholesterol metabolism via ABC transporters in the primitive leukemic cell population, might therefore lead to new therapeutic approaches and better antileukemic strategies.

### HEMATOPOIETIC STEM CELLS

In 1961 Till and McCulloch were the first to report experimental data indicating the existence of hematopoietic stem cells<sup>41</sup>. Hematopoietic stem cells are defined as cells that have the potential to undergo self-renewal, extensive proliferation and differentiation into multiple diverse cell types. In general human hematopoiesis is a strictly regulated process, maintaining a balance between loss and formation of cells. Only limited number of these cells are pluripotent hematopoietic stem cells. These hematopoietic stem cells represent only 1 in 10<sup>5</sup>-10<sup>6</sup> bone marrow cells and are characterized by their ability to repopulate the bone marrow of irradiated recipients<sup>42</sup>. The derivatives give rise to the briefly self-renewing multi-potent progenitors (MPPs), which then differentiate to restricted progenitors including myeloid progenitors<sup>43</sup>. The stem cell compartment was found to be variegated. Some stem cells are capable of longer-term engraftment than others<sup>44</sup>. A similar hierarchical structure has been recognized in AML<sup>45-47</sup>. Even some normal stem cell features (self-renewal, proliferation and differentiation) might be sufficient to create a malignant condition. While self-renewal is crucial to stem cell function, it is of utmost importance to gain a better insight into the mechanisms that regulate self-renewal. Understanding this regulation of normal stem cell self-renewal is also essential to appreciate the regulation of cancer cell proliferation, as cancer can be considered to be a disease of dysregulated self-renewal<sup>48,49</sup>. An emerging concept in this field implies

that stem cell function (whether normal or neoplastic) might be defined by a common set of critical genes, such as *HoxB4* (involved in expansion of hematopoietic cells)<sup>50</sup>, *Bmi-1* and  $\beta$ -*catenin* (both necessary for self-renewal)<sup>50,52</sup>. *Bmi-1* is a member of the Polycomb Group family. *Bmi-1* expression has been shown to be restricted to immature HSC from both mouse and human origin<sup>51,53</sup> as well as in the CD34<sup>+</sup> fraction of AML patients<sup>54</sup>. Evidence has been provided that the proliferative potential of *Bmi-1*<sup>-/-</sup> normal and leukemic stem cells is compromised, indicating that *Bmi-1* has an essential role in regulating the proliferative activity of both normal and leukemic stem cells<sup>54</sup>.

Interestingly, considerable evidence now indicates that stem cells play an essential role in the pathogenesis of some tumor types<sup>55-57</sup>. Many of these stem cells, referred to as 'cancer stem cells', have now been identified.

Leukemic stem cells resemble normal hematopoietic stem cells in many ways. This is highly suggestive that the malignant stem cell population can arise either from mutational transformation of normal stem cells by accumulation of genetic and epigenetic changes. Or, alternatively, other mutations might cause more differentiated cell types to acquire stem-cell like features, eventually, leading to the gain of self-renewal activity of restricted progenitors<sup>46,58</sup>, as has recently been shown for the MLL-gene<sup>59</sup>. Some signaling pathways regulating self-renewal have been found to be active in both normal stem cells and malignant cells, including the Notch<sup>60,61</sup>, Shh<sup>62,63</sup> and Wnt pathways<sup>64,65</sup>. During tumor progression these pathways have been found to be dysregulated<sup>49,66-68</sup>. In addition, leukemic stem cells share many other qualities with normal hematopoietic stem cells and it is generally acknowledged that normal hematopoietic stem cells show properties enabling a long lifespan such as relative quiescence, drug-resistance through the expression of several ABC transporters, an active DNA-repair capacity, and a resistance to apoptosis. This indicates that leukemic stem cells might also possess these resistance mechanisms.

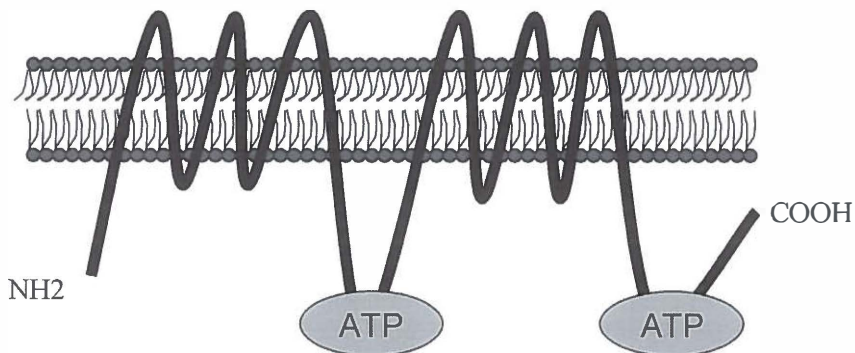
Cancer stem cells generate cells that lack the ability to differentiate into phenotypically mature cells. Some degree of differentiation often occurs, leading to the established histopathological and molecular distinctions between tumors<sup>69,70</sup>.

The leukemia-initiating cell population was identified and characterized in two papers published in 1994<sup>71</sup> and 1997<sup>72</sup>. In studies by Bonnet *et al.*<sup>72</sup> a common immunophenotype (CD34<sup>+</sup>CD38<sup>-</sup>) was identified for leukemic stem cells and their potential for self-renewal was demonstrated. Purified AML stem cells characterized by the CD34<sup>+</sup>CD38<sup>-</sup> phenotype are able to engraft in nonobese diabetic/severe combined immunodeficient mice, whereas no engraftment is observed with CD34<sup>+</sup>CD38<sup>+</sup> AML cells<sup>71-76</sup>. These findings indicate that CD34<sup>+</sup>CD38<sup>-</sup> cells belong to the pool of the leukemic stem cells whereas the CD34<sup>+</sup>CD38<sup>+</sup> AML cells have properties in common with more committed cells. Following studies have further clarified the immunophenotype of AML stem cells<sup>75,77-79</sup>. In general, it has been demonstrated that CD34<sup>+</sup>CD38<sup>-</sup> populations, which are enriched for leukemic stem cells, highly express several ABC transporters. However repopulating leukemic stem cells comprise probably only a small fraction of these populations. Until further research we can only assume that the true leukemic stem cell shows a high expression of ABC transporters and is therefore

drug resistant. A number of investigators have demonstrated the quiescent cell cycle state of the leukemic stem cell compartment, similar to their normal hematopoietic stem cell equivalents. Even though stem cells have the potential to self-renew, they are usually quiescent, spending most of their time in G<sub>0</sub>. This quiescent nature of leukemic stem cells indicates that some chemotherapeutic drugs, directed towards actively cycling populations, will generally be less effective against this dormant AML stem cell population<sup>10;11</sup>. In this review we focus on one of the specific properties of these primitive hematopoietic cells, namely the high expression of specific ABC transporters.

### ABC TRANSPORTERS

The ABC super-family of active transporters comprises 49 functionally distinct transmembrane proteins (Figure 1). They are present in virtually all cells and transport hydrophilic as well as hydrophobic compounds across plasma and intracellular membranes. Although some of these ABC transporters efflux specific (chemotherapeutic) compounds, many are able to extrude a variety of structurally dissimilar compounds. These transporters have important roles in normal physiology including the transport of nutrients and biologically active substances across the placenta, blood-brain and blood-testis barriers<sup>69</sup>. Energy derived from the hydrolysis of ATP is used to actively efflux drugs across cell membranes against concentration gradients. The 49 human ABC genes identified so far have been subdivided over seven subfamilies (ABCA through ABCG) and are expressed in both normal and malignant cells. The functional protein usually comprises 2 nucleotide-binding folds and 12 transmembrane domains<sup>60</sup>. Some ABC transporters, referred to as 'half transporters', consist of only 6 transmembrane domains and 1 nucleotide-binding fold. Nowadays, at least 14 ABC genes have been linked to genetic disorders<sup>61</sup>. Due to the various functional roles of ABC genes, they can cause a wide diversity of genetic deficiencies. An overview of the seven subfamilies of ABC transporter genes is given in table 1.



**Figure 1. Schematic figure of structure of ABCA1 transporter.**

Schematic representation of an ABC transporter.

This ABC transporter contains twelve transmembrane domains (TD) and two hydrophilic domains containing the ATP binding sites. These domains are called Nuclear Binding Domains (NBD). The N and C terminal extensions of the protein are also indicated.

**Table 1. Overview of human ABC transporters**

Name	Functional description of some family members
<p><b>ABC1: subfamily A</b> This subfamily comprises 12 full transporters.</p>	<ul style="list-style-type: none"> <li>▪ The ABCA1 protein is involved in disorders of cholesterol transport and high-density lipoproteins biosynthesis: Tangier disease (Familial HDL deficiency).</li> <li>▪ The ABCA4 (ABCR) protein is a retina specific ABC transporter, transporting vitamin A derivatives.</li> </ul>
<p><b>MDR/TAP: subfamily B</b> The ABCB subfamily contains 11 members, both full transporters and half transporters.</p>	<ul style="list-style-type: none"> <li>▪ P-glycoprotein (P-gp, MDR1, <i>ABCB1</i>) is a protein associated with acquired multidrug resistance in cancer cells. P-gp can excrete several classes of anticancer drugs from the cell, lowering the effective drug concentration inside the cell. The <i>ABCB1</i> transporter gene is highly expressed in hematopoietic stem cells and down-regulated upon differentiation.</li> <li>▪ The <i>ABCB2</i> and <i>ABCB3</i> (TAP) genes are half transporters that form heterodimers to transport peptides into the endoplasmic reticulum. These genes are associated with immune deficiency.</li> <li>▪ The ABCB4 and ABCB11 (SPGP) proteins are both located in the liver and are involved in the secretion of phospholipids and bile acids, respectively. A defective <i>ABCB4</i> and <i>ABCB11</i> gene was found in progressive familial intrahepatic cholestasis.</li> <li>▪ The proteins of the four half-transporters, ABCB6, ABCB7, ABCB8 and ABCB10 are situated in the mitochondria, where they function in iron metabolism.</li> </ul>
<p><b>CFTR/MRP: subfamily C</b> The ABCC subfamily contains 12 full transporters with a diversity in functions, including ion transport, cell surface receptor, and toxin secretion activities.</p>	<ul style="list-style-type: none"> <li>▪ 9 <i>MRP</i>-related genes have a broad spectrum of functions. <i>MRP 2-5</i> are potentially involved in mediating drug resistance.</li> <li>▪ The CFTR protein is a chloride ion channel that plays a role in all exocrine secretions and mutations in <i>CFTR</i> cause cystic fibrosis (CF). In almost 100% of men with CF, bilateral congenital absence of the vas deferens (CBAVD) results in male infertility. Also abnormalities in sperm parameters have been identified. Nowadays over 800 disease-causing mutations of the <i>CFTR</i> gene have been identified.</li> <li>▪ ABCC8 and ABCC9 proteins are involved in modulating insulin secretion.</li> </ul>
<p><b>ALD: subfamily D</b> The ABCD subfamily contains four genes in the human genome. They encode half transporters involved in the regulation of very long chain fatty acid transport.</p>	<ul style="list-style-type: none"> <li>▪ A defective (<i>ABCD1</i>, <i>ALDP</i>) gene has been found in adrenoleukodystrophy.</li> </ul>
<p><b>OABP: subfamily E and GCN20: subfamily F</b> The ABCE and ABCF subfamilies are not known to be involved in membrane transport functions. They contain genes that have ATP-binding domains but have no TM domain.</p>	



**Table 1. (continued)**

Name	Functional description of some family members
<p><b>White: subfamily G</b></p> <p>The human ABCG subfamily consists of 6 'reverse' half transporters</p>	<ul style="list-style-type: none"> <li>▪ The mammalian <i>ABCG1</i> gene is involved in cholesterol transport.</li> <li>▪ The <i>ABCG2</i> transporter most likely plays a role in mediating drug resistance, but its clinical relevance is not yet well established. <i>ABCG2</i> is highly expressed in hematopoietic stem cells and down-regulated upon differentiation.</li> <li>▪ The <i>ABCG4</i> gene, expressed predominantly in the liver, is thought to be involved in the (brain) cholesterol metabolism.</li> <li>▪ <i>ABCG5</i> and <i>ABCG8</i> transport sterols in the intestine and liver. Mutations in either one of these genes have been observed in patients with sitosterolemia.</li> </ul>

**ABC transporters and hematopoietic stem cells**

Two members of the ATP-binding cassette (ABC) super family of transporter proteins, P-glycoprotein (P-gp; also referred to as MDR1, encoded by the *ABCB1* gene) and breast cancer resistance protein (BCRP, encoded by the *ABCG2* gene), have previously been described to be correlated with an immature immunophenotype in normal hematopoietic cells<sup>82-86</sup>. Down-regulation of these genes occurs upon differentiation. Recently, based on murine micro-array data, we demonstrated an additional number of ABC transporters, especially those involved in cholesterol metabolism, to be highly expressed at the mRNA level in normal human primitive hematopoietic cells<sup>40;87;88</sup>. The ABC transporter genes *ABCG1*, *ABCA1*, *ABCB1*, *ABCC1*, *ABCD4* and *ABCB2* are predominantly expressed in the immature CD34<sup>+</sup>CD38<sup>-</sup> sub-population and are down-regulated upon differentiation into the CD34<sup>+</sup>CD38<sup>+</sup> sub-population. Accordingly, only recently, de Grouw *et al.* published expression data, containing all 45 ABC transporters. They found 22 ABC transporters to be differentially expressed. All were lower expressed in the CD34<sup>+</sup>CD38<sup>+</sup> sub-population compared to the CD34<sup>+</sup>CD38<sup>-</sup> sub-population<sup>89</sup>.

The *MDR1* gene is well known for its ability to confer drug resistance. Furthermore, extra data was reported, suggesting additional common effects of ABC transporter function on hematopoietic stem cell development. Enforced ABC transporter function was described to alter proliferative and developmental fate of hematopoietic stem cells<sup>90;91</sup>.

The drug-effluxing capacity of stem cells is an important feature for the isolation of hematopoietic stem cells. Some ABC transporters efflux the fluorescent dyes Hoechst-33342 and rhodamine 123. In comparison to stem cells, most cells accumulate these fluorescent dyes. Due to these properties, stem cells can be sorted based on a prevailing low level of these fluorescent dyes. During flow cytometric analysis with Hoechst-33342, a specific population of cells can be sorted, which is referred to as 'side population' (SP) cells<sup>92</sup>. A large percentage of the hematopoietic stem cells can be found in the SP cell fraction<sup>69;93</sup>. It is suggested that the SP cells and the CD34<sup>+</sup>CD38<sup>-</sup> cells are overlapping cell populations<sup>84</sup>. Purified SP cells, like purified CD34<sup>+</sup>CD38<sup>-</sup> cells, are able to engraft in nonobese diabetic/severe combined immunodeficient mice. These findings indicate that these

cells are pluripotent and belong to the stem cell pool. SP cells were also present in the bone marrow of more than 80% of AML patients studied<sup>93</sup>. These SP cells have been found to export lipophilic antileukemic drugs indicating a potential clinical significance.

### ABC transporters and multidrug resistance

Drug resistance in cancer cells remains one of the primary causes of suboptimal outcomes in cancer therapy. Cells exposed to toxic agents can develop resistance by a number of mechanisms including decreased uptake, accelerated detoxification, defective apoptosis pathways, alteration of target proteins, or increased efflux which lowers the effective drug concentration inside the cell<sup>81:94</sup>. Several of these pathways can lead to multidrug resistance, which renders the cells resistant to several structurally (un)related drugs.

The tumor cells that recur after an initial phase of treatment are normally multidrug resistant. High expression of specific ABC transporter proteins contributes to multidrug resistance via the ATP-dependent drug efflux, a process that serves to protect cells from cytotoxic drugs. Currently, 13 genes (*ABCA2*, *ABCB1*, *ABCB4*, *ABCB11*, *ABCC1-6*, *ABCC10*, *ABCC11* and *ABCG2*) (Table 2) are associated with chemotherapeutic drug transport and drug resistance<sup>94,96</sup>, but *ABCB1*, *ABCG2* along with *ABCC1* represent the three principal multidrug resistance genes that have been identified in tumor cells. Some 25 years ago, MDR1 was discovered. MDR1 overexpression in mice provided a laboratory

**Table 2. Resistance to cytostatic agents conferred by the ABC transporter proteins**

Gene	Protein/alias	Cytostatic agent
<i>ABCA2</i>		estramustine
<i>ABCB1</i>	P-gp/MDR1	colchicine, doxorubicin, idarubicin, epirubicin, etoposide, vinca alkaloids, paclitaxel, topotecan, bisantrene
<i>ABCB4</i>	MDR2	paclitaxel, vinblastine
<i>ABCB11</i>	BSEP	paclitaxel
<i>ABCC1</i>	MRP1	doxorubicin, daunorubicin, epirubicin, mitoxantrone, vinca alkaloids, etoposide, teniposide, irinotecan (CPT-11), colchicine, camptothecins (SN-38), methotrexate
<i>ABCC2</i>	MRP2	vinca alkaloids, cisplatin, doxorubicin, epirubicin, methotrexate, etoposide, CPT-11, SN-38
<i>ABCC3</i>	MRP3	doxorubicin, methotrexate, etoposide, teniposide, vincristine, cisplatin
<i>ABCC4</i>	MRP4	6-mercaptopurine, 6-thioguanine and metabolites, methotrexate (nucleoside monophosphates), methotrexate
<i>ABCC5</i>	MRP5	6-mercaptopurine, 6-thioguanine and metabolites (nucleoside monophosphates)
<i>ABCC6</i>	MRP6	etoposide
<i>ABCC10</i>	MRP7	docetaxel, paclitaxel, vinca alkaloids
<i>ABCC11</i>	MRP8	5-fluorouracil
<i>ABCG2</i>	MXR/BCRP	mitoxantrone, topotecan, doxorubicin, daunorubicin, epirubicin, topotecan, SN-38, bisantrene, irinotecan, imatinib, methotrexate, CPT-11

model for the clinically observed occurrence of multidrug resistance<sup>97</sup>. MDR1 is able to cause a high degree of resistance to amphipatic drugs, such as paclitaxel (taxol), anthracyclines, and Vinca alkaloids. Cytosine arabinoside, an important drug in the treatment of AML is not transported by MDR1<sup>98;99</sup>. Considering the mechanism of pumping, three mechanisms have been suggested. First, the efflux pump in which substrates are excreted through channels formed by transmembrane domains<sup>100</sup>. Second, the flippase model in which the hydrophobic parts of the drugs allow their rapid introduction in the membrane. The hydrophilic parts inhibit rapid flipping of the drug to the inner leaflet of the membrane, delaying entry into the cell and consequently lowering the effective drug concentration inside the cell<sup>101;102</sup>. And third, the vacuum cleaner model has been suggested in which substrates, detected in the lipid bilayer, are removed from the cell before reaching the cytoplasm<sup>103</sup>. MDR1 was discovered to be an important cause of multidrug resistance<sup>104-106</sup>. Several agents have been identified that can block and compete with P-gp drug efflux in vitro: e.g. verapamil, cyclosporin A, cyclosporin D, tamoxifen, and the phenothiazines<sup>97-99;107</sup>. Consequently, these compounds have been proposed as reversal agents<sup>107;108</sup> and tested in clinical trials<sup>109;110</sup>. So far treatment results with MDR1 inhibitors were not convincing as will be discussed in the next paragraph.

Tumors will use any drug resistance mechanism available and as frequently demonstrated, MDR1 is not the only cellular defense-system.

In 1998, BCRP was identified in multidrug resistant breast cancer cells. BCRP functions as a homodimer. The range of drugs to which BCRP can confer resistance is less broad than for P-gp and includes mitoxantrone, topotecan derivatives, anthracyclines, bisantrene and etoposide<sup>108;111</sup>. The MRP1 (ABCC1) protein is found both on the plasma membrane and on membranes of intracellular compartments and confers resistance to doxorubicin, daunorubicin, vincristine, colchicines and several other compounds, very similar to the substrates for MDR1. MRP1 transport activity, however is dependent on the presence of glutathione (GSH)<sup>112</sup>, and is thought to involve co-transport of formation of glutathione conjugates prior to efflux.

Conventionally, advances in our understanding of multidrug ABC transporters have been made through biochemical analyses. Lately, however, fundamental advances have been made with the elucidation of three-dimensional structures of several multidrug transporters. To gain an even better understanding of drug and transporter interactions, information about several conformational changes associated with drug translocation should be obtained. Finally to confirm relevance of biochemical and structural analysis, functional analysis is needed in order to develop new ideas to circumvent multidrug resistance and improve anti-cancer therapeutics<sup>113;114</sup>.

#### *ABC transporters and multidrug resistance in AML*

In patients with AML, multidrug resistance can be present intrinsically at diagnosis or can arise during chemotherapy as well as at relapse. MDR1, the best characterized of the human drug efflux pumps, has been shown to be associated with poor treatment outcome in AML patients; it is an independent

adverse prognostic factor for response and survival in *de novo* diagnosed AML. The expression of MDR1 was found to be higher in secondary leukemias and those developing from MDS than in primary disease states<sup>115;116</sup>. The clinical importance of MDR1 expression during the development of AML (refractory / relapsed disease) remains an open question<sup>117</sup>. It may very well be that new additional resistance mechanisms can be acquired by AML blasts in relapsed disease<sup>99</sup>. In addition, MRP1 has been found to contribute to drug resistance in AML<sup>26;104;105;116;118-120</sup>. Alternative transporter proteins, such as the MRP1 homologues MRP2 to MRP7 and BCRP, are still under investigation and have not yet been proven to play a role in drug resistance in AML<sup>34;100</sup>. Early studies indicated an increase of MRP1 RNA levels in AML cells with disease progression. MRP1 RNA expression was found to be higher in patients with relapsed AML compared to those at diagnosis<sup>121-123</sup>. Recent larger studies, however, have been unable to detect any correlation between MRP protein expression and clinical response in AML patients<sup>116;124;125</sup>.

In AML cells, a relatively high expression of BCRP mRNA is observed in approximately 30% of high-risk AML cases and this did not correlate with the expression of MDR1, suggesting a potential independent role for this transporter in drug resistance in leukemia<sup>126</sup>. BCRP protein expression is also correlated with an immature cellular phenotype<sup>34;127</sup>. Over the past twenty years, information obtained from cell biological studies has increased our understanding of how malignant cells become multidrug resistant. Reports linking overexpression of MDR1 to adverse treatment outcome in adult AML provided the evidence necessary to implicate this multidrug resistance phenotype as an important biologic target for pharmacologic modulation. However, despite the promising theories, treatment results with first generation MDR1 inhibitors, such as verapamil, quinidine and cyclosporine A, were not convincing<sup>109;110</sup>. Large multicentered (inter)national randomized trials of second generation MDR modulators such as PSC-833 (Valspodar) with or without cytotoxic agents have not shown any clinical benefit for the patient<sup>128;129</sup> as well. In view of RNA expression data described<sup>40;89</sup>, there seems to be a high redundancy of ABC transporters in normal and AML primitive hematopoietic cells. Therefore these studies might have failed because of the presence of additional transporters. To date multifunctional modulators have been developed that inhibit more than one transporter, such as VX710 (Biricodar)<sup>130;131</sup> and GF120918 (Elacridar)<sup>132</sup>. Another potential reason could be that the wrong transporter was inhibited. Most studies evaluating cells with the SP phenotype have shown that stem cells overexpress ABCG2, rather than ABCB1, which has been the transporter targeted in most clinical studies<sup>133</sup>. Alternatively, a pharmacokinetic interaction between the chemotherapeutic agent and the ABC transporter inhibitor could be another explanation<sup>134</sup>. The interference with clearance of several anticancer drugs that are susceptible to Pgp-mediated efflux<sup>135;136</sup> often necessitates dose reductions of these drugs, administered in PSC-833 containing regimens<sup>137-139</sup>. To date, new third generation MDR1 inhibitors have been developed that are potent, for instance LY 335979 (Zosuquidar.3HCL trihydrochloride)<sup>140</sup> and less toxic, such as XR9576 (Tariquidar)<sup>141</sup>, currently ongoing in clinical trials. Results of clinical trials of agents exclusively inhibiting ABCG2 are not yet reported.

De Grouw *et al.* found largely similar patterns of ABC- transporter expression in normal and leukemic CD34<sup>+</sup>CD38<sup>-</sup> hematopoietic cells. Based on these data they suggest that by modulation of these transporters in AML as a treatment approach, not only leukemic but also normal residual hematopoietic stem cells will be targeted, resulting in an increased bone marrow toxicity<sup>89</sup>. Compared to these data we observed a more heterogeneous expression pattern of the ABC transporters in the AML CD34<sup>+</sup>CD38<sup>-</sup> subpopulations as compared to normal hematopoietic cells. The cause of this variability in ABC transporter expression in AML is unclear. It might be related to an aberrant differentiation program. Alternatively it might be a reflection of the heterogeneity of the primitive AML cell compartment as demonstrated recently by Hope *et al.*<sup>79</sup>. It is unlikely that this variability reflects differences between normal and leukemic cells. The CD34<sup>+</sup>CD38<sup>-</sup> cell fraction of AML patients contain mostly cells of leukemic origin as described by Haase *et al.*<sup>142</sup>.

ABC transporters have a very basic role in biology in all living cells. Therefore it is important to realize that ABC transporters may have functions in normal physiology that still remain to be established. In this review we would like to focus on one of these potential functions concerning a crucial biochemical mechanism, i.e. the protein-mediated lipid translocation.

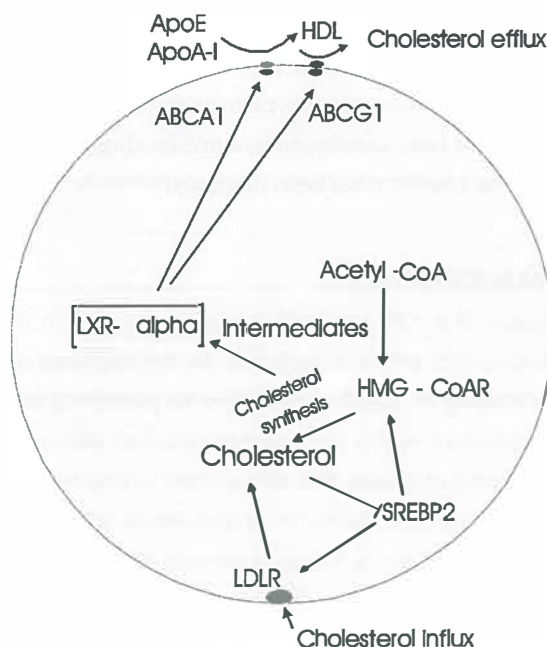
### **ABC transporters and cellular cholesterol metabolism**

Next to an important involvement of ABC transporters in multidrug resistance, an increasing number of ABC transporters are found to play a role in lipid transport. Defects in these transporters are involved in several congenital or acquired diseases in humans.

Cellular cholesterol homeostasis is maintained by coordinated regulation of cholesterol synthesis, influx and efflux (Figure 2)<sup>143</sup>. Cholesterol is synthesized via the mevalonate pathway. HMG-CoA reductase (3-hydroxy-3-methyl-glutaryl-CoA-reductase) is the rate-controlling enzyme in this pathway leading to the synthesis of cholesterol as well as of various other products, such as farnesyl and geranyl isoprenoids that are involved in the signaling of GTP-ases (Ras superfamily of small guanosine triphosphatases), including the small G-proteins Ras<sup>38;144</sup> and Rho kinase<sup>145-149</sup>. Ras and Rho kinase are involved in control of several processes such as cell proliferation, differentiation, migration, adhesion and drug efflux.

Cholesterol efflux from a variety of cells, including macrophages, is induced by activation of Liver X receptors (LXRs), of which two isoforms (alpha and beta) have been identified. Activation of LXR enhances transcription of target genes such as *ABCA1* and *ABCG1* and other genes involved in reverse cholesterol transport<sup>150;151</sup>. *ABCA1* and *ABCG1* have been described to mediate cellular cholesterol efflux from macrophages<sup>150;152</sup> as well as from a variety of other cell types. A specific function of these transporters in hematopoietic stem cells has so far not been defined. Interestingly, only recently it was reported that *Abcg1*<sup>-/-</sup> macrophages underwent accelerated apoptosis<sup>153</sup>.

The proteins encoded by the *ABCA1* and *ABCG1* genes promote the efflux of free cholesterol from the cells towards acceptors such as apoA-I and high-density lipoproteins (HDL)<sup>151;154</sup>. The cholesterol transporters *ABCG1* and *ABCA1* have recently demonstrated to be highly expressed in the immature



**Figure 2. Schematic figure of cellular cholesterol homeostasis.** Mechanisms of cholesterol synthesis, influx and efflux maintain cellular cholesterol homeostasis. Influx, especially low-density lipoprotein receptor (LDLR) mediated lipoprotein uptake, and synthesis are primarily regulated by sterol response element binding protein-2 (SREBP2) and induced when cellular cholesterol levels are low. High cellular cholesterol levels exert a negative feedback on cholesterol influx and synthesis primarily via SREBP2.

Cholesterol efflux is induced by oxysterol dependent (intermediates) activation of liver-X receptors (LXRs), when cellular (oxy)sterol levels are high. Activation of LXR enhances transcription of the target genes ABCG1 and ABCA1. The proteins encoded by these genes promote the efflux of free cholesterol from the cells towards acceptors such as apolipoprotein A-1 (apoA-I) and high-density lipoprotein (HDL). Abbreviations: HMG-CoAR: 3-hydroxy-3-methyl-glutaryl-CoA reductase; Acetyl-CoA: acetyl coenzyme A; ABC: ATP-binding cassette.

CD34<sup>+</sup>CD38<sup>-</sup> population and to be down-regulated upon differentiation<sup>40</sup>. The down-regulation of *ABCG1* and *ABCA1* gene expression in the more mature sub-population of normal hematopoietic cells suggests changes in cholesterol metabolism upon maturation. A relationship between cholesterol metabolism and cancer was identified forty years ago<sup>155</sup>. In 1978, Goldstein and Brown were the first to report an altered cholesterol metabolism in AML<sup>36</sup>. AML cells show more LDLR activity than normal peripheral white blood cells and bone marrow cells do. Additionally, AML cells compared to normal cells often do not show efficient feedback repression of cholesterol synthesis and LDLR when exposed to high-sterol media, a feature that is associated with an increased cell survival.

Interestingly, it was recently shown that a subgroup of AMLs (58%) demonstrated an acute cholesterol response, i.e. an increase in cellular cholesterol content, to cytotoxic drugs *in vitro*<sup>39</sup>. These AML cells were further characterized by an increased AML chemosensitivity upon blocking this acute cholesterol response<sup>144</sup>. The cholesterol response was not observed in normal CD34<sup>+</sup> cells and appeared to be protective for AML cell survival. Additionally, expression of the cholesterol

transporters *ABCG1* and *ABCA1* shows a very heterogeneous pattern in sorted AML samples (CD34<sup>+</sup>CD38<sup>-</sup> versus CD34<sup>+</sup>CD38<sup>+</sup>) as compared to normal hematopoietic cells<sup>40</sup>. These results suggest that a subgroup of AML cells has an active, perhaps dysfunctional, cholesterol metabolism, protecting this subgroup of AML cells. Consequently, a role for cholesterol synthesis inhibitors to improve standard antileukemic treatment has been suggested<sup>38;39;156-160</sup>.

## CONCLUSIONS / FUTURE PERSPECTIVES

It is generally acknowledged that ABC transporters play many roles in normal and abnormal physiology. Already well described and very important are the high expressions of specific ABC transporter proteins contributing to multidrug resistance by protecting the cells from cytotoxic drugs. However, current treatment results with MDR modulators, have not been convincing so far. Additionally, over-expression of certain ABC transporters in specific primitive hematopoietic cell fractions might be an important handle for improvement of antileukemic treatment as well. Therefore, it is of utmost importance to appreciate and illuminate the numerous functions of the ABC transporters that for a large part still remain to be established in AML. In this review we mainly focused on one of these potential functions and tried to elucidate the involvement of ABC transporters in sterol transport. Drug-resistant myeloid leukemia cell lines show high HMG-CoAR levels, which can be inhibited by statins<sup>39</sup>. An increased sensitivity of AMLs to statin-induced apoptosis has been described<sup>157;161</sup> and co-administration of mevastatin with cytotoxic drugs resulted in strongly reduced leukemic cell survival *in vitro*<sup>144</sup>. In general, statins are well-tolerated and known to have only few side-effects. Consequently, a promising novel application constitutes the combination of these widely used drugs, statins (e.g. simvastatin, mevastatin), with chemotherapy. The efficacy of this combination therapy should be further elucidated by a clinical controlled trial of e.g. simvastatin in conjunction with an anthracycline-containing regimen. Additionally, the role of cholesterol synthesis inhibitors has been suggested in other (non) hematological malignancies, such as multiple myeloma and non-Hodgkin's lymphoma and a phase 1-2 clinical trial have been performed<sup>162-164</sup>. Subsequent clinical trials using these cholesterol synthesis inhibitors are momentarily ongoing.

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# Chapter 4

## **Variability in responsiveness to lovastatin of the primitive CD34+ AML subfraction compared to normal CD34+ cells**

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## ABSTRACT

In the present study we questioned whether the cholesterol synthesis inhibitor lovastatin potentiates the cytotoxicity of chemotherapeutic agents in the primitive CD34<sup>+</sup> subpopulation of AML cells. AML mononuclear cells (n=17) were sorted in CD34<sup>+</sup> and CD34<sup>-</sup> fractions and compared to normal CD34<sup>+/+</sup> cells (n=7). The percentage of surviving cells upon exposure to lovastatin (25-100 μM) and/or chemotherapeutics (cytarabine or daunorubicin) was determined with a luminescent cell viability assay. The results demonstrate that the primitive CD34<sup>+</sup> subpopulation of normal and AML cells displayed a higher sensitivity to lovastatin than the more mature CD34<sup>-</sup> subpopulation. The combination of lovastatin and chemotherapeutics resulted in a more pronounced inhibitory effect on both subpopulations. In contrast to the homogeneous results in normal CD34<sup>+</sup> cells, a distinct heterogeneity in lovastatin sensitivity was found in AML samples. Therefore a group of normal (n=11) and abnormal (n=6) responders were identified based on a reduced or increased cell survival compared to normal CD34<sup>+</sup> cells. This distinction was not only observed in the CD34<sup>+</sup> AML subfraction, but also in CD34<sup>+</sup>CD38<sup>-</sup> AML cells. In the abnormal responder group, 50% of patients presented with unfavorable cytogenetics and significant higher peripheral blast cell counts, which coincided with poor treatment results. In summary the findings indicate that the primitive subfraction of CD34<sup>+</sup> AML cells is in the majority of cases affected by lovastatin treatment, which is potentiated when combined with chemotherapeutics. Heterogeneity of the response observed in AML patients allowed identification of a subgroup with poor prognosis.

## INTRODUCTION

Acute myeloid leukemia (AML) is characterized by accumulation of immature myeloid cells in the bone marrow. These malignant cells are usually hierarchically structured, similar to the normal hematopoietic system. We have reported that, according to this hierarchical structure, ABC transporters are expressed in a differentiation-dependent manner<sup>1</sup>. Specifically, in primitive CD34<sup>+</sup>CD38<sup>-</sup> cells a high expression of a number of ABC transporters genes was noticed in comparison to the more differentiated CD34<sup>+</sup>CD38<sup>+</sup> cells. Notably, in the leukemic counterpart a much more heterogeneous pattern of ABC transporter gene expression was observed<sup>1</sup>. Recently, de Grouw *et al.*<sup>2</sup>. published expression data for all 45 ABC transporters in AML cells. They found 22 ABC transporters to be lower expressed in the CD34<sup>+</sup>CD38<sup>+</sup> sub-population compared to the CD34<sup>+</sup>CD38<sup>-</sup> sub-population. In addition to the two major cholesterol efflux transporters (ABCA1 and ABCG1), two genes critical in cholesterol metabolism ((3-hydroxy-3-methyl-glutaryl-CoA-reductase (HMGCoAR) and low-density-lipoprotein-receptor (LDLR)) were highly expressed in normal CD34<sup>+</sup>CD38<sup>-</sup> cells, indicative for an active cholesterol metabolism<sup>1,3</sup>.

In AML cells an aberrant cellular cholesterol metabolism has been demonstrated, including a higher LDLR activity<sup>4</sup>. Interestingly, it was recently shown *in vitro* that cells isolated from a subgroup of AML patients demonstrated in their leukemic cells “an acute cholesterol response”, i.e., a rapid increase in cellular cholesterol content in response to cytotoxic agents<sup>5</sup>. These AML cells were further characterized by an increased chemosensitivity upon blocking this acute cholesterol response by pre-treatment of cells with a statin<sup>4</sup>. Statins act as competitive inhibitors of the enzyme HMGCoA reductase and hence block cellular cholesterol synthesis. Consequently, a role for these cholesterol synthesis inhibitors to improve standard antileukemic treatment has been suggested<sup>4,6-10</sup>. In the present study we focused on a subpopulation of AML cells (CD34<sup>+</sup>), i.e., cells that have more in common with the more primitive leukemic progenitor/stem cell compartment<sup>11</sup>, and questioned whether this subpopulation of cells is especially prone for the effects of lovastatin and chemotherapeutic agents. Our data demonstrate a higher sensitivity of the primitive CD34<sup>+</sup> subpopulation for lovastatin compared to the more mature CD34<sup>-</sup> subpopulation of normal as well as AML cells. Heterogeneity of the response observed in AML patients allowed identification of a subgroup with poor prognosis.

## MATERIALS AND METHODS

### Normal CD34<sup>+</sup>/<sup>-</sup> hematopoietic cells

Normal mobilized peripheral blood cells were collected either from healthy donors or from patients awaiting autologous stem cell transplantation, undergoing G-CSF treatment, in accordance with institutional guidelines. CD34<sup>+</sup>, CD34<sup>+</sup>14<sup>+</sup>15<sup>+</sup> (CD34), CD34<sup>+</sup>CD38<sup>-</sup> and CD34<sup>+</sup>CD38<sup>+</sup> cells were obtained by means of the MoFlo flow cytometer (Dako, Colorado, Inc. USA).

## Patients

After informed consent, bone marrow- or peripheral blood cells were collected of AML patients at diagnosis. The Human Subject Review Board of the University Medical Center Groningen (The Netherlands) approved the protocol. Patients were classified according to the WHO classification<sup>12</sup>. Mononuclear cells were enriched by Ficoll-Isopaque (Nycomed, Oslo, Norway) density gradient centrifugation and cryopreserved in RPMI 1640 medium (BioWhittaker, Brussels, Belgium) supplemented with 20% fetal calf serum (FCS) (Hyclone, Logan, UT) and 20% dimethyl sulfoxide (DMSO, Merck, Amsterdam, the Netherlands), and stored at  $-196^{\circ}\text{C}$  as described<sup>13</sup>.

## Materials

Mevinolin (L-154, 803-00G17) in the lactone form was obtained from Merck, Sharp & Dohme Research Laboratories (Rahway, NJ, USA), converted to its sodium salt, and a stock solution of mevinolin (lovastatin) at a concentration of 4 mg/mL was prepared as previously described. The cytotoxic agents daunorubicin (Aventis Pharma BV, Hoevelaken, The Netherlands) and cytarabin (Mayne Pharma (Benelux) SA-NV, Brussels, Belgium) were used.

## Flow cytometric sorting

The normal and AML samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 (Becton Dickinson, San Jose, CA, USA) and a phycoerythrin (PE)-conjugated antibody against CD14 and CD15 or CD38. Sorting was performed using a MoFlo flow cytometer. Erythrocytes and dead cells were excluded from analysis by gating on forward and sideward light scatter. For RNA extraction, at least  $3 \times 10^4$  CD34<sup>+</sup> and  $3 \times 10^4$  CD34<sup>+</sup>CD14<sup>+</sup>CD15<sup>+</sup> cells were sorted. No difference existed in average CD34 expression between normal and leukemic cells mean fluorescence intensity (MFI).

## Luminescent Cell Viability Assay and Taqman PCR

This assay was used for determining the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells.

The amount of ATP is directly proportional to the number of cells present in the culture. The ATP measurement was carried out according to the manufacturer's instructions using the Cell Titer-Glo<sup>®</sup> Luminescent cell viability assay. (Promega, Madison, WI, USA). We prepared 96-well plates with 100  $\mu\text{L}$  RPMI 1640 medium supplemented with 10% Fetal Calf Serum (FCS), and sorted, by the MoFlo flow cytometer, either  $10^4$  CD34<sup>+</sup> cells or CD34<sup>+</sup>CD14<sup>+</sup>CD15<sup>+</sup> cells per well. The wells were incubated with different concentrations of lovastatin (25, 50 and 100  $\mu\text{M}$ ) with and without different concentrations of either daunorubicin (0.01-0.5  $\mu\text{M}$ ) or cytarabin (0.0001-0.1 mg/mL) and analyzed after 20 hours. All experiments were performed in duplicate. No difference existed in baseline average and median ATP levels between CD34<sup>+</sup> and CD34<sup>-</sup> cells.

RNA extraction, cDNA synthesis and quantitative PCR were performed as described before<sup>13</sup>.

### Colony-forming cell assay

10<sup>3</sup> normal or AML CD34<sup>+</sup> cells were sorted into IMDM containing 10% FCS, 20 ng/mL interleukin-3 (IL-3), 100 ng/mL c-Kit ligand, 100 ng/mL Flt-3 ligand and 100 ng/mL thrombopoietin (TPO) as described. 24 hours after treatment with different concentrations lovastatin, cytarabine and daunorubicin, cells were plated into MethoCult H4230 (StemCell Technologies, Vancouver, Canada) supplemented with 20% IMDM, 20 ng/mL IL-3, 20 ng/mL G-CSF, 20 ng/mL IL-6, 20 ng/mL cKit ligand and 1U/mL EPO (Cilag; Eprex, Brussels, Belgium).

### Statistical analysis

Friedman's and Mann-Whitney's nonparametric tests were performed to calculate significant differences, *p* values of < 0.05. SPSS 14.0 statistical software<sup>14</sup> was used to analyze the data. Additionally we looked for additive and synergistic effects of the combination treatment of lovastatin and chemotherapeutics. Additivity was defined as an increased inhibitory effect on cell survival of the combination exposure compared to the single exposure of lovastatin or chemotherapeutics. Synergism was defined by two individual agents (lovastatin and chemotherapeutics) acting together and creating an inhibitory effect on cell survival greater than predicted by knowing only the separate inhibitory effects of the individual agents.

## RESULTS

### Patient characteristics

The AML patients (n=17) studied, median age of 51 (range 19-79), were classified according to the WHO classification: AML with cytogenetic abnormalities (n=5), AML with prior dysplastic syndrome (n=2) and the additional group consisting of M1 (n=4), M2 (n=3) and M4/M5 (n=3). The clinical and cellular characteristics of the patients are shown in Table 1. The median peripheral blast cell count at diagnosis was 48.6 x 10<sup>9</sup>/L (range 2.2-200). The majority of the patients were treated according to ongoing HOVON protocols, i.e., for patients <60 years the HOVON SAKK AML-42 study<sup>15</sup> and for patients >60 years the HOVON 43 study<sup>16</sup>. Palliative treatment was given to patients that were ineligible for intensive chemotherapy (n=2). These patients were treated with 6-mercaptopurine<sup>17</sup>. Eleven patients reached complete remission (CR) on protocol. In 6 patients CR was not attained due to progression (n=2) or to treatment related toxicity (n=4). The median EFS of all patients attaining CR was 13 months (range 1-37).

### **The CD34<sup>+</sup> subpopulation of normal and AML hematopoietic cells is more sensitive to lovastatin treatment than the more mature CD34<sup>+</sup> subpopulation.**

To evaluate whether differences exist in statin-sensitivity between primitive and more differentiated cells, normal CD34<sup>+</sup> and CD34<sup>+</sup>14<sup>+</sup>15<sup>+</sup> sub-fractions (n=7) were sorted from the mononuclear cell fraction from normal peripheral blood stem cells. A clear discrepancy between both fractions was observed upon lovastatin exposure. The CD34<sup>+</sup> subpopulation demonstrated a reduction of cellular

Table 1 Clinical and cellular characteristics of AML patients

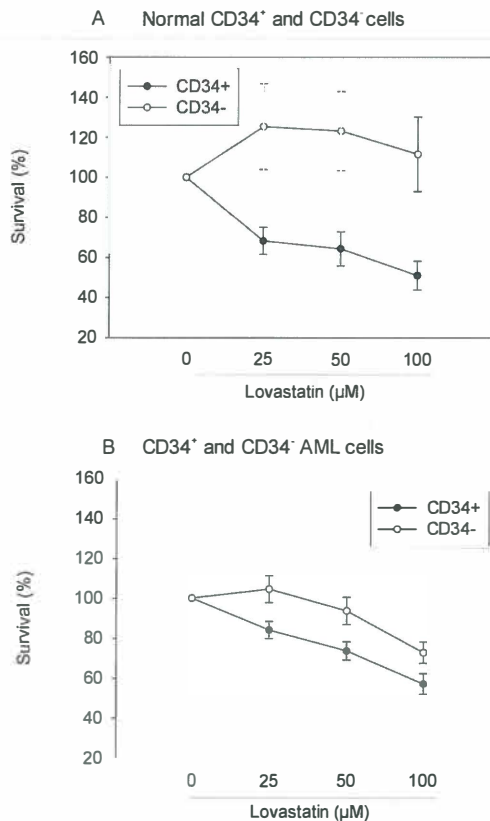
AML's	Age (Years)	Leucocytes presentation (x10 <sup>9</sup> /l)	FAB Class.	Cytogenetics*	Treatment† (it/pt)	Response‡	EFS§ (Months)	OS¶	% CD34 <sup>+</sup> in AML mononuclear cell fraction	(Ab)normal responder to Lovastatin in CD34 <sup>+</sup> cells**	Risk-group stratification††
1	59	128	M1	N	it	CR	18	1	24	N	Intermediate
2	68	4	M5b	N	it	CR	15	0	64	N	Intermediate
3	51	33	M1	N	it	CR	37	1	63	N	Intermediate
4	73	6	M2	N	it	CR	12	1	28	N	Intermediate
5	79	96	M5b	N	pt	NR	-	0	15	N	Intermediate
6	19	10	M2	N	it	CR	13	1	23	N	Intermediate
7	43	24	M5	N	it	CR	1	0	28	N	Intermediate
8	64	11	M5	inv. 16	it	CR	8	1	20	N	Good
9	48	16	M1	N	it	CR	8	0	17	N	Intermediate
10	49	50	M4/M5	N	it	CR	2.5	0	7	N	Intermediate
11	55	2	M2	N	it	CR	15	0	56	N	Intermediate
12	44	66	M5	3q-,5q-,+8	it	NE <sup>tox</sup>	0	0	8	A	Poor
13	54	48	M5a	N	it	NE <sup>tox</sup>	0	0	85	A	Intermediate
14	67	63	M1	T(6;9), Trisomy 13	it	NR	0	0	35	A	Poor
15	19	102	M5	inv. 16	it	CR	21+	1	60	A	Intermediate
16	42	200	M1	inv. 3q, -7, -10	it	NR	0	0	87	A	Poor
17	38	96	M1	N	pt	NR	-	0	92	A	Intermediate

\*Cytogenetics: N: normal; inv.: inversion; †Treatment: it: intensive treatment; pt: palliative treatment; ‡ Response: CR: Complete remission; NR: non-responder; NE: not evaluable due to treatment related toxicity. §For event-free survival (EFS), a plus following the value indicates still no relapse as at September 12, 2007.

¶OS: Overall survival: 0: death and 1: alive \*\* (Ab)normal Responders: N: Normal responders were defined as AML-CD34<sup>+</sup> cells demonstrating a similar effect on cell survival as normal CD34<sup>+</sup> cells. A: Abnormal responders were defined as AML-CD34<sup>+</sup> showing a dissimilar effect on cell survival to normal CD34<sup>+</sup> cells. †† Risk group stratification is based on (un)favorable cytogenetics combined with peripheral blood blast cell counts.

ATP to 64% at a dose of 50  $\mu\text{M}$  lovastatin compared to 123% in the CD34<sup>-</sup> fraction ( $p=0.008$ , Figure 1a).

A similar experimental set-up was used for the AMLs ( $n=17$ ). From the total AML cell fraction the CD34<sup>+</sup> sub-fraction was separated from the CD34<sup>-</sup> fraction by MoFlo. The percentage of CD34<sup>+</sup> cells varied strongly between the different AML samples as depicted in Table 1 (median 41% (range 7%-92%)). Subsequently, the CD34<sup>+</sup> and CD34<sup>-</sup> fractions were exposed to varying concentrations of lovastatin (25-100  $\mu\text{M}$ ) for 20 hours and the effects on cell survival were assessed. As depicted in Figure 1b, a significant difference in sensitivity for lovastatin was observed for the AML CD34<sup>+</sup> versus CD34<sup>-</sup> fraction. At 50  $\mu\text{M}$  a median reduction of 30% in survival (range minus 24-54) was observed for the CD34<sup>+</sup> cells compared to 12% (range minus 51-42) in the CD34<sup>-</sup> fraction ( $p=0.029$ ). A similar pattern was also observed at 100  $\mu\text{M}$  (43% versus 23%,  $p=0.029$ ). Subsequently we compared the AML subpopulations with the normal CD34<sup>+</sup> and CD34<sup>-</sup> cell fractions and observed a significant increased lovastatin sensitivity of the AML CD34<sup>-</sup> fraction compared to the normal the CD34<sup>-</sup> subpopulations ( $p=0.005$ ) (Figure 1). This was not noticed for the AML- and normal- CD34<sup>+</sup> cell fractions ( $p=0.095$ ). Based on the pattern of lovastatin sensitivity of the normal CD34<sup>+</sup> cells, two

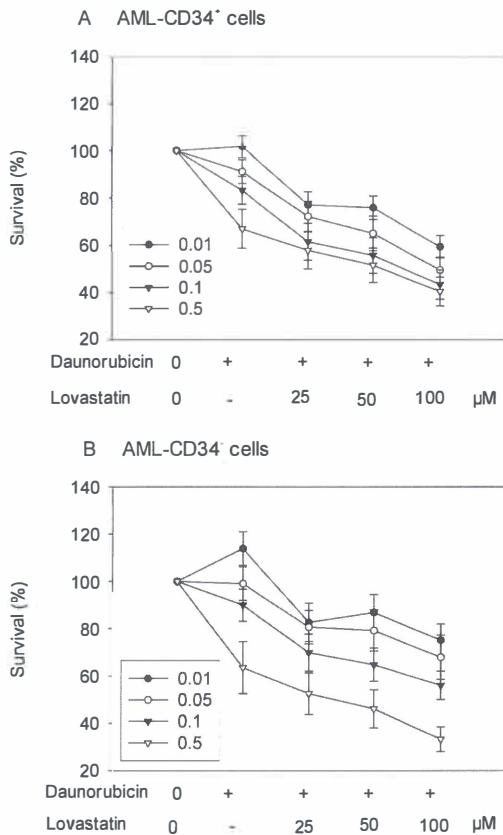


**Figure 1.** Cell survival of CD34<sup>+</sup> and CD34<sup>-</sup> normal (A:  $n=7$ ) and AML cells (B:  $n=17$ ) upon incubation with increasing lovastatin concentrations (25-100  $\mu\text{M}$ ). SEM are indicated.

subgroups within the AMLs could be distinguished. Eleven AML-CD34<sup>+</sup> samples had a response pattern comparable to normal CD34<sup>+</sup> cells, whereas 6 showed a reduced lovastatin sensitivity (Figure 3 and Table 1). A comparable pattern was observed for the AML CD34 fraction.

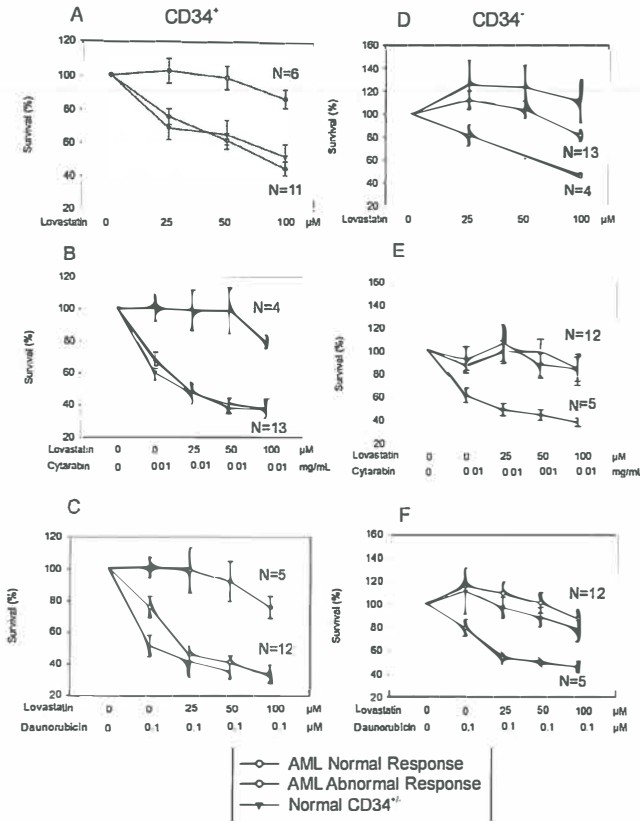
### The combination of lovastatin and chemotherapeutics potentiates cell death.

To evaluate whether the cytotoxic effects of two frequently used cytostatic agents, cytarabine and daunorubicin, might be promoted by co-treatment with lovastatin, the AML CD34<sup>+</sup> and CD34<sup>-</sup> subfractions were incubated with varying concentrations of lovastatin (25, 50 and 100  $\mu$ M) in the presence or absence of varying concentrations of daunorubicin (0.01-0.5  $\mu$ M) or cytarabine (0.0001-0.1 mg/ml) for 24 hours. A dose-dependent decrease in cell survival was seen as depicted in Figure 2 which was augmented by lovastatin treatment. A median survival reduction of 21% (range minus 28-64%) was accomplished by exposing the AML CD34<sup>+</sup> cells to 0.1  $\mu$ M daunorubicin, which was 43% (range minus 56-78%) when the CD34<sup>+</sup> AML cells were exposed to the combination of daunorubicin and 25  $\mu$ M lovastatin (Figure 2A).



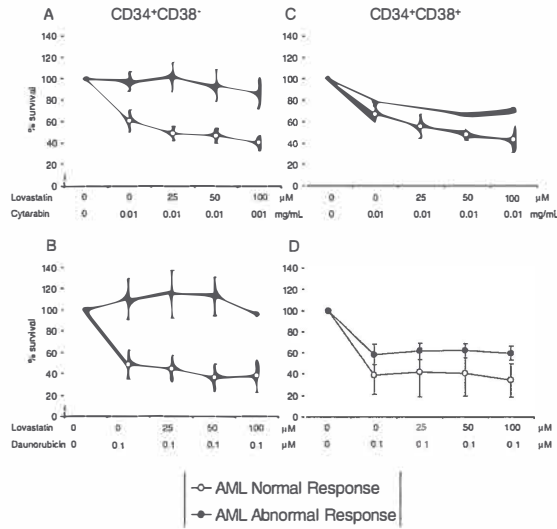
**Figure 2. Effects of increasing concentrations of chemotherapeutic drugs on in vitro survival of subpopulations of AML cells.** CD34<sup>+</sup> (A) and CD34<sup>-</sup> (B) AML cells of all patients (n=17) were incubated with lovastatin in combination with variable concentrations of daunorubicin (0.01-0.5  $\mu$ M). SEM are indicated.

The combined use was slightly more effective in the CD34+ subpopulation compared to the CD34- subpopulation (Figure 2B) (median 43% versus 36%) but the difference did not reach statistical significance. Comparable results were obtained for cytarabin (data not shown). However, also here within the group of AMLs, different subgroups could be distinguished when looking at cell survival. Most AML-CD34+ cells with a reduced statin sensitivity demonstrated also a diminished susceptibility for the combined use of statins plus cytarabin or daunorubicin (Figure 3). To investigate whether the observed effects were also noticed in the most primitive AML cell population, AML CD34+ cells were sorted into the CD34+CD38- cell fraction and the CD34+CD38+ fraction (n=7). Four AML samples belonged to the normal responder group and three samples to the abnormal responder group. As depicted in Figure 4, the same distinction in responsiveness to cytostatic agents was observed in the absence or presence of lovastatin. Especially in the CD34+CD38- subfraction the discrepancy between normal versus abnormal responders was most noticeable (e.g. for ARA-C in combination with 25 μM lovastatin  $p=0.01$  for CD34+CD38- versus  $p=0.2$  for CD34+CD38+; for DNR  $p=0.05$  versus  $p=0.5$ ).



**Figure 3. Effects of increasing concentrations of lovastatin in combination with chemotherapeutic drugs on in vitro survival of CD34+ (A-C) and CD34- (D-F) normal and AML cells. Three groups are demonstrated in graphs 3A-F. Two AML patient groups (normal responders (n=13) and abnormal responders (n=4); in total: n=17) and a normal CD34+/- group (n=7) are shown. SEM are indicated.**





**Figure 4.** Effects of increasing concentrations of lovastatin in combination with chemotherapeutic drugs on *in vitro* survival of sorted CD34<sup>+</sup>CD38<sup>-</sup> (A,B) and CD34<sup>+</sup>CD38<sup>+</sup> (C,D) AML cells. Two groups are shown: normal responders (n=4) and abnormal responders (n=3). SEM are indicated.

To verify whether similar results can be obtained in a clonogenic assay, normal and AML CD34<sup>+</sup> cells were exposed to lovastatin and/or cytostatic agents for 24 hrs and subsequently cultured in CFC medium. However, CD34<sup>+</sup> cells from 6 out of 7 AML samples did not show any *in vitro* colony formation in the CFC assay. Normal CD34<sup>+</sup> cells (n=5) showed an inhibition of 12% and 8% with 25 μM lovastatin or daunorubicin respectively, which increased to 36% inhibition when both agents were used ( $p=0.01$ , data not shown). Comparable results were obtained with cytarabine (28% vs 60%,  $p=0.04$ ).

Given the observed variability in effects of lovastatin on the CD34<sup>+</sup> AML cells, we questioned whether this might be explained by a difference in expression or modulation of downstream targets. Therefore, the ABCG1 and ABCA1 mRNA expression levels were studied both in the responder and non-responder AML group and compared to normal CD34<sup>+</sup> cells. A non-significant higher ABCG1 mRNA expression was observed in the normal responder AML group compared to the abnormal responder AML group and normal CD34<sup>+</sup> cells. In addition the lovastatin-induced ABCG1 mRNA expression was studied in the different groups. No statistically significant difference was found between normal CD34<sup>+</sup> cells (0.4 fold down-regulation, range 0.29-0.36) versus the normal and abnormal AML responder group, 0.2 (range minus 0.16-0.53), and 0.5 fold (range 0.16-0.70) down-regulation respectively.

#### In vitro response pattern and clinical outcome

In view of the observed *in vitro* difference for statin sensitivity we questioned whether these findings might correspond to patient characteristics or patient outcome. In the AML responder group a

normal or favorable cytogenetic pattern was found in 100% of the patients, while in the abnormal responder group an unfavorable cytogenetic pattern was present in 50% of the patients. In the responder group the median peripheral blast cell count at presentation was  $16.6 \times 10^9/L$  (range 2.2-128.3) and in the abnormal responder group  $81.4 \times 10^9/L$  (range 48.6-200,  $p=0.02$ ). Based on these factors three prognostic risk groups can be defined, i.e., favorable risk (favorable cytogenetics in combination with a peripheral blood blast cell count  $< 20 \times 10^9/L$  at diagnosis), poor risk (those with poor-risk cytogenetics) and intermediate risk group (all others)<sup>18,19</sup>. In the responder group 100% of the patients belonged to the good or intermediate risk group while in the abnormal responder group this value was only 50% (Table 1). These findings correlated with treatment results. Complete remission on intensive chemotherapy was attained in 91% of the patients in the AML normal responder group with an Event Free Survival (EFS of 12.5 months (range 1-37). In the abnormal responders CR was attained in 17% of the patients with a EFS of 0 months (range 0-15).

## DISCUSSION

In the present study, we analyzed the *in vitro* effect of lovastatin on the survival of AML subpopulations in the absence or presence of conventional applied chemotherapeutics. So far *in vitro* studies on statins in AML have been focused on the total AML cell population.<sup>6,9</sup> However, information of well-defined sub-populations might provide additional information especially regarding the primitive CD34<sup>+</sup> AML cells that are responsible for the ongoing *in vivo* propagation<sup>19,20</sup>. By studying two different subpopulations of AML cells (primitive CD34<sup>+</sup> cells and more mature CD34<sup>-</sup> cells) we were able to demonstrate a significant difference in lovastatin susceptibility. The inhibitory effects of lovastatin were especially observed on the primitive CD34<sup>+</sup> subpopulation. The effects on the more mature CD34<sup>-</sup> subpopulation were less pronounced, suggesting that the primitive cell fraction is more dependent on cholesterol synthesis which is in line with the high expression of ABC transporters in these cells. In addition there were increased although variable inhibitory effects by the combined use of lovastatin and cytotoxic agents in both the AML - and normal- CD34<sup>+</sup> cells. Interestingly we found that some AML CD34<sup>+</sup> cells followed a response pattern similar to normal CD34<sup>+</sup> cells while 35% of the AML CD34<sup>+</sup> cells showed no change. This could not be reversed in a number of cases by co-exposure the cells to chemotherapeutic agents. Interestingly, the distinction between normal and abnormal responders for the AML-CD34<sup>+</sup>

fraction was most pronounced in the CD34<sup>+</sup>CD38<sup>-</sup> cell fraction, a population that is enriched for leukemic stem cells<sup>21</sup>. The difference in responsiveness between the normal- and abnormal-responder AML groups could not be explained by a difference in the percentage of AML CD34<sup>+</sup> cells. In addition, no difference in susceptibility for lovastatin-mediated up-regulation of ABCG1 mRNA was observed which is a major downstream target of lovastatin. It is more conceivable that the difference is linked to additional cellular characteristics of the AML cells belonging to both groups. Patient characteristics demonstrated that the abnormal responder group had a significant higher peripheral blast cell count at presentation. Moreover the group was characterized by a high

frequency of poor-risk cytogenetic abnormalities. The cause of the difference in susceptibility is not resolved but might be linked to differences in the farnesylation or geranylgeranylation pathways that are also affected by lovastatin<sup>3,6</sup>. It has been demonstrated that statins disrupt the localization and function of isoprenylated molecules in the cell membrane<sup>3,6</sup>. This change will result in an altered GTPase function of the Ras or Rho signaling that has an important impact on cellular processes such as proliferation and survival<sup>22,23</sup>. Alternatively, the difference may be linked to an altered expression of anti-apoptotic proteins between both AML groups. It has been described that over-expression of Bcl-xL protects against statin-induced apoptosis in murine tubular cells<sup>24</sup>. Both of these possible explanations are currently under evaluation.

In summary, these results demonstrate distinct differences in lovastatin susceptibility between CD34<sup>+</sup> and CD34<sup>-</sup> in the normal and leukemic counterpart and between different AML subtypes. Additional studies will clarify whether the described *in vitro* assay can be used for *in vivo* identification of patients that might benefit from application of statins in combination with chemotherapeutic agents.

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# Chapter 5

## **Combining simvastatin with the farnesyltransferase inhibitor tipifarnib results in an enhanced cytotoxic effect in a subset of primary CD34+ AML samples**

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## ABSTRACT

*Purpose:* To demonstrate whether the inhibitory effects of the cholesterol synthesis inhibitor simvastatin on human CD34<sup>+</sup> acute myeloid leukemia (AML) cells can be further promoted by combining it with the farnesyltransferase inhibitor (FTI) tipifarnib.

*Experimental design:* Normal CD34<sup>+</sup>, AML CD34<sup>+</sup> and CD34<sup>-</sup> sorted subfractions, and AML cell lines (TF-1 and KG1 A), were exposed to simvastatin and tipifarnib. **Results:** Both simvastatin and tipifarnib demonstrated a cytotoxic effect on AML cell lines, which was additive when used in combination. In primary sorted CD34<sup>+</sup> AML cells a heterogeneous response pattern was observed upon treatment with simvastatin when analyzing cell survival. A group of normal (n=12) and abnormal (n=10) responders were identified within the AML CD34<sup>+</sup> subfraction when compared to normal CD34<sup>+</sup> cells. This distinction was not observed within the AML CD34<sup>-</sup> cell fraction. When the CD34<sup>+</sup> AML cells were exposed to simvastatin and tipifarnib, a significant enhanced inhibitory effect was shown exclusively in the normal AML responder group, while the AML CD34<sup>-</sup> cell fractions all showed an enhanced inhibitory effect. The observed heterogeneity in AML responsiveness could not be explained by differences in effects on cholesterol metabolism genes or ERK phosphorylation in response to simvastatin and tipifarnib treatment.

*Conclusion:* The results suggest that combined treatment with statins and FTIs may be beneficial for a subset of AML patients that can be defined by studying the AML CD34<sup>+</sup> fraction.

## INTRODUCTION

Acute myeloid leukemia (AML) is a clonal hematopoietic disease characterized by the accumulation of immature myeloid blasts in the bone marrow. A minor tumor subpopulation with self-renewal potential, referred to as 'leukemic stem cells' (LSC) is responsible for the sustained expansion of the leukemia<sup>1,2</sup> So far, these LSCs are phenotypically characterized by CD34<sup>+</sup>CD38<sup>-3,4</sup>, but a recent study has challenged this view suggesting that LSCs might also belong to the CD34<sup>+</sup>CD38<sup>+</sup> cell fraction<sup>5</sup>. Cholesterol synthesis and the processing of LDL (low density lipoprotein) is hyperactive in AML<sup>6,7</sup>, as indicated by high mRNA levels of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG-CoAR) and LDL receptor (LDLR) as well as LDL uptake studies. These findings are in line with the high expression of key genes of cholesterol metabolism in CD34<sup>+</sup>CD38<sup>+</sup> AML cells<sup>8</sup>. In addition, AML cells possess several mechanisms to protect them against the cytotoxic effects of chemotherapeutics, including a rapid increase in their cellular cholesterol levels following exposure to chemotherapeutic drugs<sup>9,10</sup>. Therefore, interfering with this protective mechanism potentially offers the opportunity to improve standard anti-leukemic treatment.

Statins, targeting HMG-CoAR, are widely used plasma cholesterol-lowering drugs. Statins inhibit cholesterol synthesis at the level of the conversion of mevalonate and as a consequence also inhibit the production of various by-products of the mevalonate pathway. These by-products include farnesyl and geranylgeranyl isoprenoids that are involved in the signaling of GTP-ases including the small G-protein Ras<sup>9,11</sup>. Ras GTPases must be transferred from the cytoplasm to the plasma membrane by isoprenylation to allow them to function as signal transducers<sup>12-14</sup>. Inhibiting farnesylation is of interest, since farnesylated proteins, particularly the protein products of the Ras gene family, are frequently activated in AML e.g. by Ras mutations or due to the autocrine or paracrine production of growth factors<sup>15</sup>. However, statin concentrations necessary to inhibit specific protein isoprenylation are 100- to 500-fold higher than those required to inhibit cholesterol synthesis<sup>16</sup>.

Specific inhibition of Ras farnesylation can also be realized by using the Ras inhibitor tipifarnib. Tipifarnib is an oral nonpeptidomimetic Ras inhibitor, which selectively inhibits intracellular farnesyltransferase (FTase). Both statins as well as tipifarnib have been investigated as single agents in AML patients<sup>17-21</sup>, resulting in modest response rates. Since alternative prenylation by geranylgeranyltransferase may bypass the inhibitory effect of tipifarnib<sup>22</sup> and statins are capable of blocking both geranylgeranylation and farnesylation, it is tempting to speculate that the combined use might have a more pronounced anti-leukemic effect. Therefore, sorted AML CD34<sup>+</sup> cells, enriched for LSCs, were exposed to the cholesterol synthesis inhibitor simvastatin, the Ras inhibitor tipifarnib or to both compounds, and the findings were compared to normal CD34<sup>+</sup> cells. The results demonstrate that given the heterogeneous response pattern between patient AML samples, a combination treatment with statins and farnesyltransferase inhibitors (FTIs) may be beneficial for around 50% of AML patients.



## MATERIALS AND METHODS

### Normal and AML hematopoietic cells and cell lines

Normal mobilized peripheral CD34<sup>+</sup> blood cells were collected from either healthy donors or patients awaiting autologous stem cell transplantation undergoing granulocyte colony-stimulating factor (G-CSF) treatment, in accordance with institutional guidelines. After informed consent, bone marrow or peripheral blood cells were collected from AML patients at diagnosis. The Medical Ethical Committee of the University Medical Center Groningen (The Netherlands) approved the protocol. Patients were classified according to the French-American-British (FAB) AML classification<sup>23</sup>. Mononuclear cells were enriched by density gradient centrifugation (Lymphocyte Separation Medium LSM 1077, PAA Laboratories GmbH, Cölbe, Germany) and freshly used or cryopreserved in RPMI 1640 medium (BioWhittaker, Brussels, Belgium) supplemented with 20% v/v fetal calf serum (FCS; Hyclone, Logan, UT) and 20% dimethyl sulfoxide (DMSO; Merck, Amsterdam, The Netherlands), and stored at 196°C. Prior to analysis, mononuclear cells were thawed, treated with DNase (Boehringer Mannheim, Almere, The Netherlands), washed, and incubated in RPMI 1640 medium, supplemented with 10% FCS, 100 U/mL penicillin and 100 µg/mL streptomycin (ICN, Zoetermeer, The Netherlands), at 37°C and 5% CO<sub>2</sub>. Cytogenetic analysis was performed as described earlier<sup>24</sup>. The normal and AML samples were incubated with a fluorescein isothiocyanate (FITC)-conjugated antibody against CD34 and phycoerythrin (PE)-conjugated antibodies against CD14 and CD15 (Becton Dickinson, Alphen aan den Rijn, The Netherlands). Sorting of CD34<sup>+</sup> and CD34<sup>-</sup> (CD14<sup>+</sup>CD15<sup>+</sup>) was performed using a MoFlo cell sorter (DakoCytomation, Carpinteria, CA).

The human AML cell line KG1A was cultured in IMDM medium (PAA Laboratories GmbH) supplemented with 10% FCS and 2 nM L-glutamine (ICN). The human erythroleukemic cell line TF-1 was cultured in RPMI 1640 medium supplemented with 10% FCS and 10 ng/mL granulocyte macrophage colony-stimulating factor (GM-CSF; Genetics Institute Inc, Cambridge, MA). Cultures were kept at 37°C and 5% CO<sub>2</sub>.

### Reagents

Simvastatin was obtained as a sodium salt from Merck Chemical Ltd. (Nottingham, UK) and dissolved in DMSO as to obtain a 50 mM stock solution. R1 15777 (Tipifarnib, Zarnestra) was provided by Janssen Research Foundation and dissolved in DMSO to obtain a concentration of 10 mM.

### Cell viability assay, annexin V and propidium iodide assay and cell-cycle analysis

Cell viability assays were performed in duplicate according to the manufacturer's instructions using the Cell Titer-Glo Luminescent cell viability assay (Promega, Madison, WI). 96-well plates were prepared with 100 µL RPMI 1640 medium supplemented with 10% FCS, and added (cell lines) or sorted (CD34<sup>+</sup> and CD34<sup>-</sup> AML cells) up to 10,000 cells per well. The cells were incubated with different concentrations of simvastatin (5, 25 and 50 µM) with and without different concentrations of tipifarnib (0.2- 5 µM) and analyzed after 24 (cell lines) or 48 (AML cells) h.

Cell death was assessed by using an annexin V staining kit (IQ products, Groningen, The Netherlands) according to the manufacturer's recommendations. Briefly, after 48 h of treatment with different concentrations simvastatin and/or tipifarnib, cells were harvested, resuspended in 60  $\mu$ L calcium buffer containing 3  $\mu$ L of annexin V-FITC and incubated for 20 min at 4°C in the dark. Cells were washed with 2 mL calcium buffer and subsequently resuspended in 200  $\mu$ L containing 1.7  $\mu$ L propidium iodide (PI, Sigma). Binding of FITC-conjugated annexin V and PI was measured by fluorescence-activated cell sorting (FACS) analysis on a FACScalibur (Becton Dickinson). Data were analyzed using Winlist 3D (Verity Software House, Topsham, ME).

Cell-cycle analysis was performed by determining the DNA content of cells by staining with PI (IQ products) in sodium citrate (1 mg/mL; Sigma-Aldrich, Zwijndrecht, The Netherlands) containing 100  $\mu$ g/mL RNase A (Sigma-Aldrich), 20  $\mu$ g/mL PI and 0.1% Triton X-100 (Sigma-Aldrich) for 60 min at room temperature. PI fluorescence was analyzed by FACS analysis (FACS Calibur, Becton Dickinson). Cell cycle distributions were calculated with ModFit LT (Verity Software House).

### Western Blotting

TF-1 cells were treated in GM-SCF-free medium for 48 h with simvastatin and/or tipifarnib, after which GM-CSF (10 ng/mL) was added for 15 minutes. Unsorted mononuclear AML cell fractions were cultured in RPMI 1640 medium supplemented with 10% FCS and treated for 24 h. Whole cell extracts were obtained by lysing  $5 \times 10^5$  cells in boiling Laemmli sample buffer for 5 min. Samples were separated by 10% SDS-PAGE and transferred to a PVDF membrane (Millipore, Bedford, MA) in Tris-buffer using a semidry electro blotter from Bio-Rad Laboratories (Veenendaal, The Netherlands). Membranes were probed with antibodies according to the manufacturer's protocols. Antibodies used were ERK1/2 (K23, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and phospho-ERK1/2 (Thr202/Tyr204; Cell Signaling Technology Inc., Beverly, MA). Immunodetection of phospho-ERK and total ERK was performed according to standard procedures and binding of antibodies was detected by enhanced chemiluminescence. Densitometry was carried out using ImageJ.

### Quantitative real-time PCR

Total RNA was isolated using the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) and was reverse transcribed using RevertAid™ H-Minus M-MuLV reverse transcriptase (Fermentas, St Leon-Roth, Germany). Quantitative PCR was performed using the ABI-Prism-7700-Sequence-Detector (Applied Biosystems, Foster City, CA). Primers and probes for the human ABC transporters and cholesterol metabolism genes were used as described before<sup>8,25</sup>. As endogenous control the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used. The primers were obtained from Invitrogen (Breda, The Netherlands). The probes were labeled by a 5' 6-carboxyfluorescein (FAM) reporter and quenched by 6-carboxytetramethylrhodamine (TAMRA) at its 3' end (Eurogentec, Maastricht, The Netherlands). We used 4  $\mu$ L of diluted cDNA in each PCR reaction in a final volume of 20  $\mu$ L, containing 900 nM of sense and antisense primers, 200 nM of the Taqman probe, 5 mM MgCl<sub>2</sub>,

KCl, TrisHCl, 0.2 mM dATP, dCTP, dGTP, dTTP, and dUTP, and 0.5 U of AmpliTaq DNA polymerase (qPCR Core Kit, Eurogentec). The PCR program was 95°C for 10 min, followed by 40 cycles of 15 sec at 95°C and 1 min at 60°C. The expression of the genes was standardized for the expression of GAPDH. Serial cDNA dilutions of the AML samples were used to generate calibration curves. The expression of each gene in each sample was analyzed in duplicate.

### Statistical analysis

Student's *t*-test was used to calculate differences between cell line samples; Friedman's and Mann-Whitney's nonparametric tests were performed to calculate significant differences between AML samples. Data were expressed as mean  $\pm$  SD or SEM, as indicated. All *P* values are given for two-sided tests and  $P \leq 0.05$  was considered significant. Additivity was defined as an increased effect of the combination exposure compared to the single exposure of simvastatin or tipifarnib. Normal CD34<sup>+</sup> AML responders were defined as follows: at the concentration of either simvastatin or tipifarnib at which the first significant effect was observed (i.e., 25  $\mu$ M simvastatin and 1  $\mu$ M tipifarnib) using the cell viability assay, the decrease in viability of the normal CD34<sup>+</sup> cells  $\pm$  standard deviation was considered as normal. AML CD34<sup>+</sup> cells with viabilities above this value were considered to give an abnormal response.

## RESULTS

### Patient characteristics

The 22 AML patients studied, with a median age of 52 years (range 19-79), were classified as the French-American-British (FAB) classification groups M0 ( $n = 1$ ), M1 ( $n = 6$ ), M2 ( $n = 5$ ), and M4/5 ( $n = 10$ ). The clinical characteristics of the patients are shown in Table 1. The median peripheral blast cell count at diagnosis was  $62 \times 10^9/L$  (range 2-220). The majority of patients were treated according to ongoing HOVON protocols, i.e., for patients  $< 60$  years the HOVON SAKK AML-42 study<sup>26</sup> and for patients  $> 60$  years the HOVON 43 study<sup>27</sup>. Palliative treatment consisting of treatment with 6-mercaptopurine<sup>28</sup> was given to patients who were ineligible for intensive chemotherapy ( $n = 3$ ).

### Tipifarnib and simvastatin decrease cell viability by inducing apoptosis and cell cycle arrest

Firstly, the effects of tipifarnib and simvastatin on the hematopoietic cell lines KG1A and TF-1 were assessed. With simvastatin alone, a dose-dependent decrease in cell survival up to 30% was shown (Figure 1) in both cell lines. Similar results were obtained with tipifarnib (up to 60% decrease of survival), while the combined use demonstrated an even more efficient decrease of up to 75% in cell viability, which was additive compared to either treatment alone. To define whether this decline in cell survival was due to cell cycle arrest or apoptosis, cell cycle status and cell survival were defined by PI staining and an annexin V/PI assay, respectively. Treatment of TF1 cells with tipifarnib increased the number of cells in the G2/M cell cycle phase (9% versus 27% at 0.2  $\mu$ M;  $P = 0.002$ ), and lowered the number of cells in the G0/G1-phase (51% versus 36% at 0.2  $\mu$ M;  $P = 0.049$ ) (Figure 2A). Cells in

Table 1 Clinical and cellular characteristics of AML patients

AMLs	FAB Class.	Leukocytes at presentation ( $\times 10^9/l$ )	% CD34 <sup>+</sup> *	Cytogenetics <sup>†</sup>	Normal responder to simva in CD34 <sup>+</sup> cells <sup>‡</sup>	Normal responder to simva + tipi in CD34 <sup>+</sup> cells <sup>‡</sup>
1	M2	56	29	N	Yes	Yes
2	M1	200	87	inv (3q),7-,10-	Yes	Yes
3	M5	109	1	ND	Yes	Yes
4	M5b	25	28	N	Yes	Yes
5	M5b	97	15	N	Yes	Yes
6	M4	89	43	inv (16)	Yes	Yes
7	M1	128	24	N	Yes	Yes
8	M2	7	28	N	Yes	Yes
9	M1	17	40	N	Yes	Yes
10	M1	36	80	N	Yes	No
11	M5	102	60	inv (16)	Yes	No
12	M2	2	36	N	Yes	No
13	M4/M5	50	7	N	No	Yes
14	M5	67	8	3q-, 5q+, +8	No	Yes
15	M5b	220	23	t(11;20)	No	No
16	M2	59	17	del9 q12q22	No	No
17	M2	10	23	N	No	No
18	M1	64	35	t(6;9), trisomy 13	No	No
19	M1	96	92	N	No	No
20	M5a	49	85	N	No	No
21	M0	102	90	5q-, trisomy 6	No	No
22	M5a	8	11	46N,xy +11q23	No	No

\* Percentage of CD34<sup>+</sup> cells in the AML mononuclear cell fraction; <sup>†</sup> Cytogenetics: N: normal; inv.: inversion; ND: not determined; <sup>‡</sup> Normal Responders: Normal responders (Yes) and Abnormal responders (No) to simvastatin (simva) and tipifarnib (tipi) were defined as described in materials and methods.

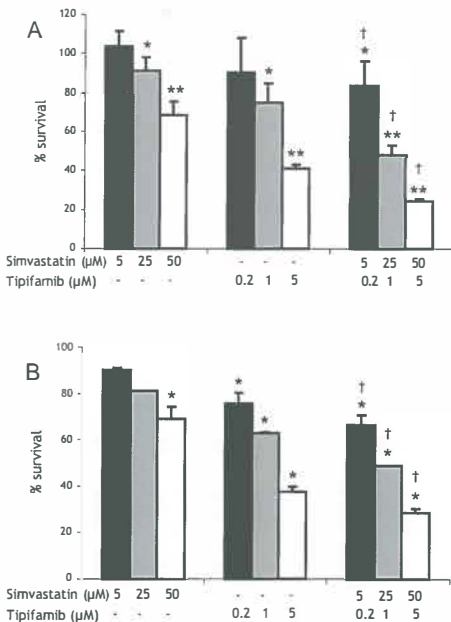
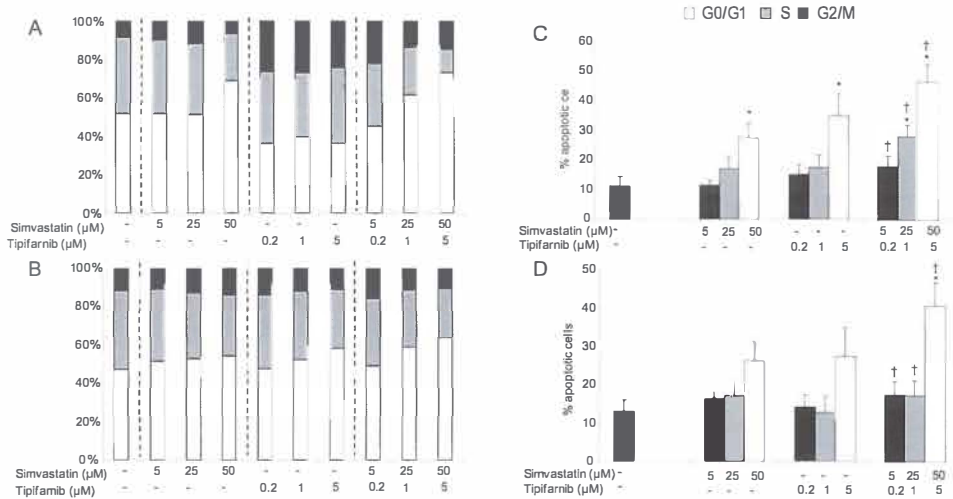


Figure 1. Cell viability of TF-1 (A;  $n = 4$ ) and KG1A (B;  $n = 2$ ) treated for 48 h with tipifarnib, simvastatin or the combination. Data are shown as the percentage of ATP levels compared to control cells. Data shown are mean values  $\pm$  SD, \* $P < 0.05$ , \*\* $P < 0.001$ ; † addition.



**Figure 2. The effect of simvastatin and tipifarnib on cell cycle status and cell death in TF-1 and KG1A cells after 48 h of treatment.** Percentage of TF-1 (A) and KG1A (B) cells in G0/G1, S and G2/M phase are shown after treatment with either tipifarnib or simvastatin alone, or after combination treatment. Data are shown as a mean of 3 independent experiments. C. The effect of simvastatin and tipifarnib treatment cell death in TF-1 (C) and KG1A (D) cells after 48 h of treatment. Data are represented as a percentage of (late) apoptotic cells. Average results of 5 and 4 independent experiments are shown for TF-1 and KG1A, respectively. \*  $P < 0.05$ ; † addition.

S-phase remained the same. Treatment with 50 μM simvastatin resulted in fewer cells in the S-phase (40% versus 24%) and more in G0/G1 (51% versus 69%; Figure 2A), but these differences did not reach significance ( $P = 0.2$ ). When combining both treatments, we observed an inhibitory effect on the cells in the S-phase ( $P = 0.02$ ), and there were more cells in G0/G1-phase ( $P = 0.09$ ) or G2/M phase ( $P = 0.05$ ) when using tipifarnib and simvastatin at a dose of 5 μM and 50 μM (Figure 2A).

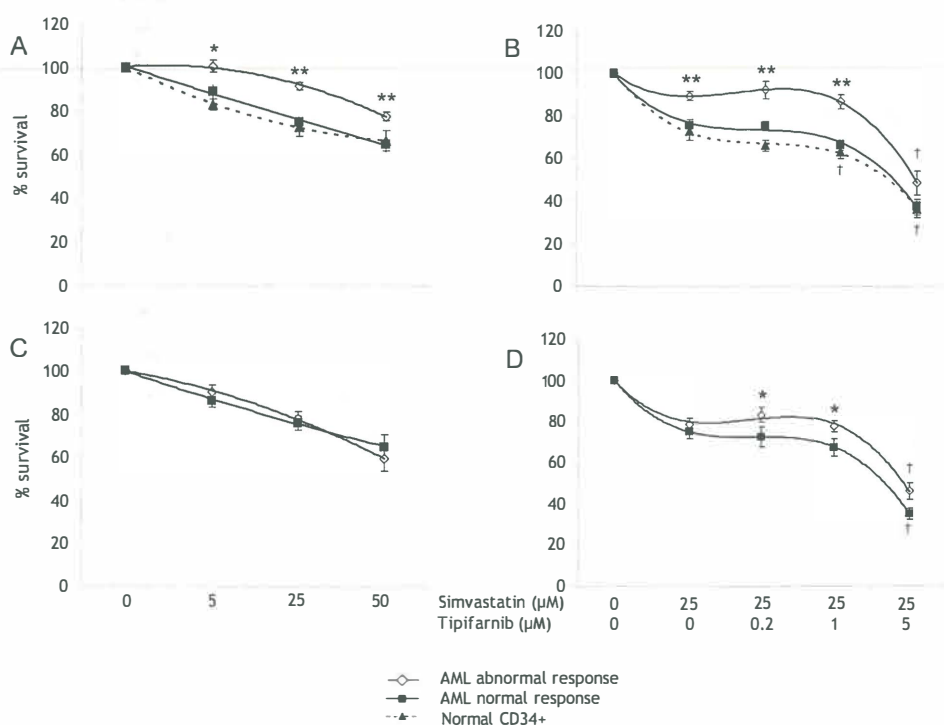
In KG1A cells we observed similar effects: combination of both treatments resulted in an increase of cells in G0/G1 phase (Figure 2B; 47% versus 64% at 50 μM simvastatin and 5 μM tipifarnib;  $P = 0.002$ ) and a decrease of cells in S-phase (41% versus 25%;  $P < 0.001$ ). Thus, treatment of TF-1 and KG1A cells with simvastatin and tipifarnib results in a G0/G1 cell cycle arrest. It appeared that this cell cycle arrest was associated with an increased number of cells in apoptosis which was especially noticed for the TF-1 cell line (Figure 2C, D). Also here we found additive effects when combining both treatments.

### The effect of simvastatin and tipifarnib on primitive CD34<sup>+</sup> normal and AML cells

To demonstrate whether in patient AML cells comparable effects can be noticed, AML mononuclear cells ( $n = 22$ ) were sorted into CD34<sup>+</sup> and CD34<sup>-</sup> subfractions and exposed to the two agents. The results were compared to the effects seen in normal CD34<sup>+</sup> ( $n = 8$ ). When exposed to simvastatin (Figure 3A) or tipifarnib (data not shown), normal CD34<sup>+</sup> cells showed a concentration-dependent decrease in cell survival, which was significant at a concentration of 25 μM simvastatin ( $P < 0.001$ ) and

1  $\mu\text{M}$  tipifarnib ( $P = 0.01$ ). Treatment of normal  $\text{CD34}^+$  cells with 25  $\mu\text{M}$  simvastatin in combination with tipifarnib demonstrated an additive inhibitory effect on cell survival compared to the effects of the separate compounds (Figure 3B). However, within the  $\text{CD34}^+$  AML cell fraction, a marked variability in responsiveness could be observed. Based on the response pattern of normal  $\text{CD34}^+$  cells, two AML subgroups could be distinguished (see materials and methods) when the  $\text{CD34}^+$  AML cells were exposed to simvastatin. 55% ( $n = 12$ ) of the AML- $\text{CD34}^+$  cells had a response pattern comparable to normal  $\text{CD34}^+$  (e.g.  $P = 0.6$  at 25  $\mu\text{M}$ ) whereas 45% ( $n = 10$ ) of the AML  $\text{CD34}^+$  cells showed reduced simvastatin sensitivity (Figure 3A; e.g.  $P = 0.003$  at 25  $\mu\text{M}$ ). In contrast, the  $\text{CD34}^-$  AML subfraction demonstrated a response pattern comparable to normal  $\text{CD34}^+$  cells, and no difference was observed between normal and abnormal responders within  $\text{CD34}^-$  AML cells (e.g.  $P = 0.6$  at 25  $\mu\text{M}$ ).

Next we studied whether the suppressive effects of simvastatin on AML cells can further be promoted by co-treatment with tipifarnib as demonstrated for normal  $\text{CD34}^+$  cells. The results demonstrate that 50% ( $n = 11$ ) of the AML  $\text{CD34}^+$  cells had a response pattern comparable to normal  $\text{CD34}^+$  cells,



**Figure 3.** Effect of simvastatin and tipifarnib on *in vitro* survival of normal and AML  $\text{CD34}^+$  (A, B) and  $\text{CD34}^-$  (C, D) AML cells. Two groups of AML patients, consisting of either normal or abnormal responders, and a control group are shown. The responder group is defined as having a decrease in cell viability which is mean  $\pm$  SD of control cells at 25  $\mu\text{M}$  simvastatin or 25  $\mu\text{M}$  simvastatin + 1  $\mu\text{M}$  tipifarnib. Normal  $\text{CD34}^+$  cells (PBSCs)  $n = 8$ ; AML normal responders  $n = 12$  (A) or  $n = 11$  (B); AML abnormal responders  $n = 10$  (A) or  $n = 11$  (B). SEM is indicated. \*  $P < 0.05$  and \*\*  $P < 0.01$  normal versus abnormal responder; †  $P < 0.01$  versus 25  $\mu\text{M}$  simvastatin alone.

while the other half of the AML CD34<sup>+</sup> cells showed reduced sensitivity for the combined treatment of simvastatin (25  $\mu$ M) and tipifarnib (1  $\mu$ M, Figure 3B;  $P < 0.001$ ). It appeared that 8 out of 10 of AMLs that were not responsive to simvastatin were also not affected when both compounds were used. When the abnormal responder group was exposed to 1  $\mu$ M tipifarnib and 25  $\mu$ M simvastatin, the decrease in cell survival was not enhanced compared to the effect of simvastatin alone ( $P = 0.5$ ). In contrast, in the AML-CD34<sup>+</sup> responder group a significant enhancement in cell death was observed when both compounds were used at low dose (1  $\mu$ M,  $P = 0.01$ ). At higher concentrations a strong reduction in cell survival was observed in normal CD34<sup>+</sup> cells as well as in the AML abnormal and normal responder group, but these concentrations are physiologically not relevant. The AML CD34<sup>+</sup> subpopulation demonstrated a comparable response pattern to normal CD34<sup>+</sup> cells and no clear distinction could be made between responders and abnormal responders when both compounds were used ( $P = 0.03$  and  $P = 0.05$  for 0.2  $\mu$ M and 1  $\mu$ M tipifarnib, respectively, Figure 3D).

### **Heterogeneity in AML response pattern is not related to differences in modulation of cholesterol metabolism genes and ERK phosphorylation by simvastatin and tipifarnib**

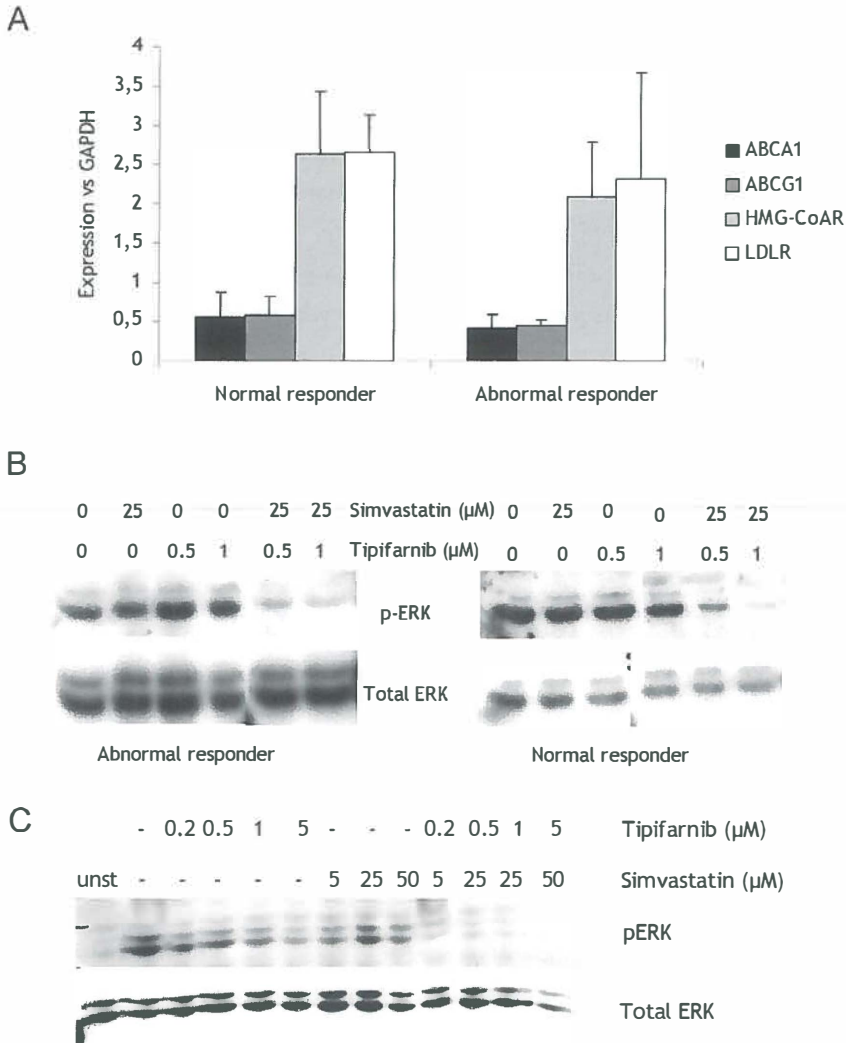
To investigate whether functionality of either simvastatin or tipifarnib was different in the normal versus the abnormal responder group, we studied important downstream targets of simvastatin and tipifarnib. The functionality of simvastatin was tested based on reported upregulation of HMG-CoAR and LDLR and a downregulation of ABCA1 and ABCG1 at the mRNA level<sup>29,30</sup>, while the downstream effects of tipifarnib were analyzed by studying the change in ERK1/2 phosphorylation, a downstream target of Ras. In the 6 AML samples (3 normal responders and 3 abnormal responders to simvastatin, tipifarnib and both drugs) studied, expression of HMG-CoAR and LDLR was almost 2.5-fold higher after treatment with 25  $\mu$ M simvastatin (Figure 4A;  $P = 0.001$  and  $P = 0.003$  respectively) and ABCA1 and ABCG1 expression was 2-fold decreased ( $P < 0.001$ ). Treatment with tipifarnib did not alter mRNA expression, and the combination of tipifarnib and simvastatin had no additive effect compared to treatment with simvastatin alone (data not shown).

Tipifarnib (0.5 and 1  $\mu$ M) did not affect ERK phosphorylation in any of the studied AMLs of the normal responder or abnormal responder group (Figure 4B). Also simvastatin did not lead to inhibition of ERK-phosphorylation. However, combining simvastatin and tipifarnib resulted in 69% of the 16 tested AML samples in a decrease of pERK expression ( $88\% \pm 10\%$  for 1  $\mu$ M tipifarnib and 25  $\mu$ M simvastatin,  $n=11$ ) irrespective of the AML used. Similar results were observed when TF-1 cells were cultured with GM-CSF and incubated with simvastatin and tipifarnib (Figure 4C).

### **In vitro response pattern and patient characteristics**

In view of the observed *in vitro* difference for simvastatin and tipifarnib sensitivity we questioned whether these findings might correspond to patient characteristics. In the group responding *in vitro* to both tipifarnib and simvastatin the median percentage of CD34<sup>+</sup> cells in the AML mononuclear cell fraction at presentation was 28% (mean 28; range 1-87) and in the non-responder group 50%

(mean 36; range 11-92;  $P = 0.08$ ) (Table 1). No correlation was found between AML responder groups and cytogenetics and peripheral blast count. The FAB classifications were distributed equally over the normal and abnormal responder group.



**Figure 4. Effect of simvastatin and tipifarnib on mRNA expression levels and ERK phosphorylation in primary AML samples and TF-1 cells.** (A) Cells were treated for 24 h with 25 μM simvastatin. mRNA expression levels of cholesterol metabolism genes in total mononuclear cell (MNC) fractions of normal ( $n = 3$ ) and abnormal ( $n = 3$ ) responder AML samples are shown. Results are displayed as mean  $\pm$  SD, compared to untreated cells, which were set to 1. Data were normalized to GAPDH. (B) Effects of tipifarnib and simvastatin on phosphorylation of ERK in AML samples. The total MNC fraction was incubated with 0.5 and 1 μM tipifarnib and/or 25 μM simvastatin for 48 h before harvesting. Anti-ERK was used as a loading control. This experiment is representative of 7 abnormal responder and 4 normal responder AMLs. (C). Effects of tipifarnib and simvastatin on phosphorylation of ERK in TF-1 cells. Cells were deprived of GM-CSF and incubated with tipifarnib and simvastatin for 48 h and stimulated with GM-CSF for 15 min. "Unst" indicates unstimulated cells. Anti-ERK was used as a loading control.



## DISCUSSION

In this study we addressed the question whether the combined use of a cholesterol synthesis inhibitor and a farnesyltransferase inhibitor might have an augmented cytotoxic effect on CD34<sup>+</sup> AML cells. The results demonstrate that in human CD34<sup>+</sup> AML leukemic cell lines, simvastatin and tipifarnib have an enhanced suppressive effect on cell survival compared to either compound alone, which was characterized by an inhibition of cell cycle progression and enhanced apoptosis. Several studies have shown that geranylgeranylated proteins, not farnesylated proteins are required for the G1 to S-phase transition<sup>31</sup> and that statins are able to block cell cycle in G1 due to geranylgeranylation rather than farnesylation<sup>32</sup>. Apparently this mechanism varies among different cell types. We observed G1 cell cycle arrest after treatment with both simvastatin and tipifarnib, whereas only limited effect was observed with either compound alone. This suggests that also farnesylated proteins are responsible for cell cycle progression in AML cells. Likewise, ERK-phosphorylation was only strongly inhibited when using both simvastatin and tipifarnib. It may be that geranylgeranylation of Ras can overcome the inhibitory effects of tipifarnib on Ras farnesylation, and both geranylgeranylation and farnesylation must be blocked in order to decrease Ras activation. This is consistent with the idea that K-Ras and N-Ras can be both farnesylated and geranylgeranylated<sup>22</sup>.

In contrast with AML cell lines, the observed inhibitory effect of simvastatin and tipifarnib was found not to be a general phenomenon for all primary AML samples. Most studies on statins have been focused on the total AML mononuclear cell fraction<sup>33-35</sup>. In the total mononuclear AML fraction a heterogeneous response to lovastatin has been observed<sup>33</sup>. Our data, demonstrate that a heterogeneous response also exists within the more homogeneous primitive AML CD34<sup>+</sup> subfraction. Two subgroups could be defined based on sensitivity to simvastatin alone. Almost 50% of the AML-CD34<sup>+</sup> showed a reduced sensitivity to simvastatin, tipifarnib, or to both compared to normal CD34<sup>+</sup> cells, while the remaining AML samples demonstrated a response pattern comparable to normal CD34<sup>+</sup> cells.

A remarkable finding was the fact that, within the AML CD34<sup>+</sup> subpopulation, no distinction could be made between normal and abnormal responders. These findings demonstrate that immature AML cells are intrinsically different from the CD34<sup>+</sup> cell population. This is in line with previous studies demonstrating that the total AML cell fraction is a heterogeneous cell population<sup>33,36</sup>, that LSC belong to the CD34<sup>+</sup> cell population<sup>1:37</sup> and that CD34<sup>+</sup> AML cells have almost exclusively self renewal properties as defined in long-term cell culture assay as well as engraftment studies in SCID mice<sup>38,39</sup>. Moreover, it demonstrates that by using the total mononuclear cell population the differences in response would have been unnoticed, especially when the total AML cell fraction comprises a low percentage of CD34<sup>+</sup> cells.

Several clinical studies have already been performed with either statins or with tipifarnib<sup>17-21</sup>. In AML, statins have been combined with intensive chemotherapy while tipifarnib has been used as single agent. Phase I and II studies have shown that only a minority of the patients were responsive

to treatment. Likewise, the results of the present study suggest that *in vitro* not all AML patients are responsive to clinically relevant concentrations of simvastatin and tipifarnib<sup>17;19</sup>, and that this distinction can be made by using exclusively the CD34<sup>+</sup> fraction. Therefore it might be useful to determine whether the *in vitro* response is predictable for clinical response, in order to be able to select patients who are eligible for treatment.

Importantly, to be able to predict response, the rationale behind the difference in susceptibility of AML cells to simvastatin and tipifarnib should be elucidated. We observed a considerable overlap between the simvastatin and tipifarnib responders, and no differences on studied downstream targets of simvastatin and tipifarnib were observed, suggesting that there might be a common mechanism of resistance.

It is conceivable that a specific set of anti-apoptotic proteins are responsible for the differences in response. It has been shown that sorted CD34<sup>+</sup> AML cells are more resistant to (spontaneous) apoptosis than the corresponding CD34<sup>-</sup> fractions, which is paralleled by higher Bcl-2, Bcl-xL, Mcl-1, Pgp and low Bax expression levels<sup>40</sup>. In addition, it has been shown that overexpression of Bcl-xL and Bcl-2 protects against statin-induced apoptosis<sup>41;42</sup>. It is unlikely that simvastatin and tipifarnib responsiveness correlates with the Ras activity status of the AML cells, since both normal and abnormal responders show a basal phosphorylation level of ERK and a decrease of ERK activity upon combination treatment, which is in line with the results of Stirewalt *et al*<sup>11</sup>, who suggested that neither Ras mutations, nor high Ras protein expression are found to be consistently associated with increased statin sensitivity. Likewise, in clinical studies using tipifarnib in AML, the Ras mutational status and inhibition of phosphorylated ERK did not correlate with clinical responsiveness to tipifarnib<sup>19;20</sup>.

So far, clinical data on the use of either agent are encouraging, but not convincing. Since we observed *in vitro* significant cytotoxic effects in an AML subgroup with clinically achievable concentrations, we predict that combining both agents *in vivo* will be advantageous in a subset of AML patients.

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# Chapter 6

## **Summary, general discussion and future perspectives**

AML is characterized by the accumulation of an immature cell population in the bone marrow. The expansion of such a leukemic clone eventually results in suppression of the normal hematopoiesis. Despite intensive treatment the perspective of AML patients for long-term cure is still poor, especially for the elderly AML patient. This points to the persisting need to unravel the mechanism involved in chemoresistance in AML patients. It is well-established that metabolic changes, such as elevated plasma levels of insulin, cholesterol, free fatty acids or triglycerides, correlate with malignant transformation of normal cells<sup>1,2</sup>. Consequently, drugs that affect, for instance, cholesterol metabolism might be of importance<sup>3,4</sup>. Statins are frequently prescribed as cholesterol lowering drugs to reduce risk for cardiovascular disease and are used at a dose of up to 1 mg/kg/day. Higher doses (2-45 mg/kg/day) are so far especially applied in clinical trials in solid tumors<sup>5</sup>. In this context, based on preclinical data statins may exert anticarcinogenic activity by inhibition of cell proliferation, induction of apoptosis and inhibition of angiogenesis<sup>6</sup>.

To further address chemoresistance and cholesterol modulation in leukemia we analyzed the effects of statins in AML, in particular their effect on the CD34<sup>+</sup> AML subfraction, since this cell population is enriched for leukemic stem cells.

In **chapter 2** we describe a high expression of 13 ABC transporters, defined by Lin<sup>-</sup>Sca-1<sup>+</sup>c-Kit<sup>+</sup>, using micro-array in murine stem cells. Subsequently, we have evaluated expression of the corresponding human ABC transporters in the normal human primitive hematopoietic CD34<sup>+</sup>CD38<sup>-</sup> stem cell fractions. Five ABC transporters, i.e., ABCB1, ABCC1, ABCD4, ABCB2 and ABCG1 appeared highly expressed in the 'stem cell enriched' fraction compared to the progenitor fraction. Some of these transporters, like ABCB1 and ABCC1, are already well-known for their involvement in multidrug resistance (MDR). Others, such as ABCG1, were not known in conjunction with MDR. ABCG1 is involved in cholesterol transport; therefore, we also assessed additional genes involved in the cholesterol metabolism and found that the ABC transporter ABCA1 and some additional key genes participating in cholesterol metabolism including LDLR, HMGCoAR and LXRA were concomitantly highly expressed. During differentiation the gene expression levels strongly declined in normal hematopoietic cells. In AML CD34<sup>+</sup> cells however, we demonstrated a heterogeneous expression pattern of cholesterol metabolism genes suggesting that the make-up or mutations in the AML cells affect the cholesterol metabolism.

In **chapter 3** an overview is given of the current knowledge of ABC transporters in general and specifically in the leukemic stem cell compartment, with emphasis on the expression of specific sterol-related ABC transporters, i.e., ABCG1 and ABCA1, which were highly expressed in primitive hematopoietic cells. It is postulated that high expression of sterol-related ABC transporters in the leukemic enriched stem cell fraction might be an important handle for improvement of leukemic therapy by interfering with cholesterol inhibitors. This is for example illustrated by the high expression of HMG-CoAR in drug resistant myeloid leukemic cell lines. The actions of the encoded protein can be inhibited by statins<sup>7</sup>. All these findings allude to an aberrant cholesterol metabolism

that might be involved in chemotherapy resistance. Therefore treatment with statins might be of help to disrupt the chemotherapy resistance and making the AML cells more prone for the effects of chemotherapy. This idea is further addressed in chapter 4.

In **chapter 4**, we questioned whether cholesterol synthesis inhibitors like lovastatin induce cytotoxicity or potentiates the cytotoxicity of chemotherapeutic agents in the primitive CD34<sup>+</sup> subpopulation of cells of AML patients. This was analyzed by means of a luminescent cell viability assay. We found a higher sensitivity to lovastatin of the primitive CD34<sup>+</sup> subpopulation compared to the more mature CD34<sup>-</sup> subpopulation of normal and AML cells. Furthermore the combination of lovastatin with chemotherapeutics (daunorubicin or cytarabine) resulted in a more pronounced, dose-dependent inhibitory effect on both subpopulations. In contrast to the homogeneous results in normal CD34<sup>+</sup> cells, we found a distinct heterogeneity in the AML group. Therefore, a group of normal ( $n = 11$ ) and abnormal ( $n = 6$ ) responders were identified based on a reduced or increased cell survival compared to normal CD34<sup>+</sup> cells response upon combined exposure of lovastatin and chemotherapeutics. The group of abnormal responders was additionally recognized by more unfavorable parameters such as adverse cytogenetics and higher peripheral blast cell counts, corresponding with poor treatment results. In summary, these findings indicate that the primitive subfraction of CD34<sup>+</sup> AML cells is in the majority of cases affected by lovastatin treatment which is potentiated when combined with chemotherapeutics. By observing a heterogeneity in the response of AML patients cells, it might be possible to identify a subgroup of AML patients with an unfavorable prognosis that is sensitive to the effect of statins.

Statins inhibit cholesterol synthesis via the mevalonate pathway and, at the same time, the production of diverse by-products of this pathway. These by-products include farnesyl and geranylgeranyl isoprenoids that are involved in the of GTPases, including the small G-protein Ras. Inhibiting farnesylation is of interest because farnesylated proteins, particularly members of the Ras family, are frequently activated in AML. However, the statin concentrations necessary to inhibit specific protein isoprenylation are 100--500 fold higher than those required to inhibit cholesterol synthesis.

In **chapter 5** we questioned whether the inhibitory effects of the cholesterol synthesis inhibitor simvastatin could be amplified in combination with the farnesyltransferase inhibitor tipifarnib. It is plausible that the combined use might have a more pronounced antileukemic effect. We found that both simvastatin and tipifarnib exhibited a cytotoxic effect on the AML cell lines KG1A and TF-1, which was additive when used in combination. In primary sorted CD34<sup>+</sup> AML cells, a heterogeneous response pattern was observed upon treatment with simvastatin when analyzing for cell survival. Two different groups (responders/non-responders) were identified within the AML CD34<sup>+</sup> subfraction when compared with normal CD34<sup>+</sup> cells. This distinction was not observed within the AML CD34<sup>-</sup> cell fraction. The distinction between responders versus non-responders was not linked to an altered expression of cholesterol metabolism genes or extracellular signal-regulated kinase phosphorylation.



In summary, we show that the combination of simvastatin and tipifarnib has an enhanced cytotoxic effect on the primitive CD34<sup>+</sup> AML subpopulation in about 50% of the patient samples, which was not noticed when using the total mononuclear AML cell population. This indicates the importance of the subdivision of the total mononuclear AML cell population into different subpopulations, based on maturity, in order to show that the primitive leukemic stem cell population can be sensitive to cholesterol modulation.

## FUTURE PERSPECTIVES

In order to approach the ultimate goal of providing a cure for AML, we still encounter several limitations in our knowledge of AML, like the definition of the leukemic stem cell (LSC), the complex interactions with the niche and the significance of ABC transporters in combination with cholesterol metabolism. In addition we come across numerous problems in the treatment of AML, such as MDR, treatment of the 'elderly' AML patients and graft versus host disease (GVHD) in the setting of allogeneic stem cell transplantation. All these items will now be addressed.

The identification of *the* LSC/ leukemia initiating cell (LSC/LIC) has not been resolved so far. Taussig *et al.* demonstrated greater variety in the phenotypes of leukemia-initiating cells than previously reported by Lapidot, Bonnet and Dick *et al.*<sup>8,9</sup>. Taussig *et al.* reported that the CD38<sup>+</sup> subpopulation and also the CD34<sup>-</sup> subpopulation contained leukemic hematopoietic repopulating cells<sup>10,11</sup>. Recently, Martelli *et al.* confirmed these results in the minor population of CD34<sup>+</sup>CD38<sup>+</sup> hematopoietic progenitors in *NPM1*-mutated AML. Furthermore, Taussig *et al.* found that both CD34<sup>+</sup> and to a lesser extent CD34<sup>-</sup> cells from a few *NPM1*-mutated AML patients, exhibited LSC activity. However, mostly CD34<sup>+</sup> but not CD34<sup>-</sup> cells generated leukemic outgrowth similar to the patient's original disease. More frequently, CD34<sup>-</sup> cells engrafted as leukemia showed more differentiated morphological and immunohistological features<sup>12</sup>. Based on the heterogeneity in the make-up of the supposed LSC, it will be particularly difficult to eradicate these cells by using monoclonal antibodies, targeted against surface antigens. Furthermore also the complex crosstalk with the surrounding stromal microenvironment/niche is currently highly debated. Some receptors like CXCR4 and CD44 are known to play an important role in this collaboration<sup>13,14</sup>. LSC survival depends largely on homing in their appropriate micro-environment. CD44, a transmembrane glycoprotein mediating cell-cell and cell-extracellular matrix interactions, was identified as a key regulator of AML LSCs<sup>15</sup>. Targeting CD44 with a monoclonal antibody (i.e., H90 and A3D8) might help to eradicate AML stem cells by blocking the homing ability and altering the stem cell fate, indicating that CD44 blockade can be a LSC-specific approach in AML treatment<sup>16</sup>. Additionally, CXCR4-SDF1/CXCL12 interaction plays an important role in regulating leukemic cells. High expression of CXCR4 in AML blasts predicted a shorter survival<sup>17</sup>. AMD3100, a CXCR4 antagonist, inhibited the transmigration and colony formation of AML blasts<sup>18</sup>. Recently, in a mouse model, administration of AMD3100 enhanced mobilization of the leukemic blasts, increased chemosensitization and improved overall survival<sup>19</sup>.

Another CXCR4 antagonist AMD3465 has been shown to impede the chemoprotective effects of stromal cell-leukemia interaction<sup>20</sup>. Furthermore, in the past two decades, hematopoietic growth factors like G-CSF, granulocyte macrophage-colony stimulating factor (GM-CSF) and interleukin-3, have been applied as priming agents to increase chemosensitization<sup>21-23</sup>. Ambiguous results with respect to survival improvements were described in different AML patient groups<sup>23</sup>. A recent study demonstrated that G-CSF-mediated stem cell mobilization was CXCR4-dependent, suggesting that release of leukemic cells from their protecting micro-environment was involved in the process of growth factor priming<sup>24</sup>.

In recent years, hypoxia has been described to be another important feature of the microenvironment of cancer cells. In solid tumors, low oxygen tension represents an independent risk factor for poor prognosis<sup>25</sup>. In leukemia, the micro-environment in the bone marrow compartment has a low oxygen tension. Hypoxia is regarded as an essential component for proper stem cell function *in vivo*. However, the exact mechanism of hypoxia mediated regulation remains to be elucidated. Accordingly, high levels of oxygen seem to negatively affect stem cells by inducing diminished self-renewal and an increase in cell-cycle entry<sup>26</sup>. Furthermore, physiological hypoxia simulation of the bone marrow induces expression and function of CXCR4 and changes in cell signaling processes, especially in the mitogen-activated protein kinase (MAPK) pathway. The MAPK pathway is of special interest for AML as the constitutive activation of MAPK is linked to proliferation and cell survival of AML cells<sup>27,28</sup>. Only recently, Fiegl *et al.* have shown that physiological oxygen conditions in the bone marrow microenvironment increases membrane cholesterol content. This promotes lipid raft formation which, in turn, leads to activation of PI3K and consequently activating both the MEK/Erk and AKT signaling pathway, surprisingly independent of Ras and RAF1<sup>13;29;30</sup>. Specific niche-targeted therapy might be a promising approach in the future by inhibiting homing mechanisms or adhesion molecules in order to reduce the growth of the LSCs.

Recently, another link between cholesterol metabolism and hematopoietic stem cells has been uncovered. High-density lipoproteins (HDLs) transport excess cholesterol from peripheral tissues to the liver and are consequently of importance in protection from atherosclerosis. Recently, Yvan-Charvet *et al.* demonstrated, a new role for HDL in regulating hematopoietic stem cell proliferation<sup>31</sup>. Several surface receptors accumulate in membrane "rafts" that contain high contents of cholesterol and glycolipids<sup>32</sup>. In hematopoietic progenitor stem cells, lipid rafts organise growth factor receptors to promote cell proliferation and migration. Excess cholesterol is removed by ABC transporters present in the plasma membrane to acceptors linked HDL particles<sup>33;34</sup>. When hematopoietic cells lack *Abca1* and *Abcg1* than an increase in lipid rafts is demonstrated due to accumulation of membrane cholesterol and leading to increased responsiveness to the hematopoietic growth factor exposure such as interleukin-3 (IL-3) and GM-CSF. These effects finally result in increased proliferation of myeloid precursor cells, but could be reversed by adding HDL<sup>35</sup>. Both the normal hematopoietic stem cell-enriched fraction and a subpopulation of the LSC enriched fraction over-express the sterol related ABC transporters (ABCA1 and ABCG1) and lipoprotein receptors such as LDLR (chapter 2).

Apolipoprotein A-1 transgenic mice, transplanted with *Abca1*<sup>-/-</sup> and *Abcg*<sup>-/-</sup> bone marrow, indeed showed inhibition of hematopoietic stem cell and multipotent progenitor cell proliferation and therefore did not develop a myeloproliferative disorder. These data provide new insights for future translational research.

MDR is one of the major problems in anti-leukemic treatment as defined above. Consequently, finding ways to overcome MDR is of great relevance. The active site of the most prominent multidrug resistant protein P-gp is lipophilic<sup>36</sup>. It is hypothesized that anticancer drugs can in part be modified by the addition of lipid moieties prior to efflux from resistant cells, as described in *Drosophila* embryos<sup>37</sup>. Statins might be an interesting tool, since they have an inhibitory function on the lipid synthesis. Further clinical studies with the administration of statins in the setting of MDR are urgently needed. We therefore performed a phase 1/2 clinical trial, including patients with AML relapsing after at least one line of chemotherapy and patients with AML not eligible for standard first line of intensive chemotherapy. This study was recently completed.

A subsequent major problem in treatment of AML patients is the relatively high age at presentation. More than half of the patients are aged 60 years or older. Formerly a large fraction of these patients referred to as 'elderly' patients were offered no other therapy than supportive care or lower-dose, rather than conventional intensive treatment schedules aimed at achieving a CR<sup>38</sup>. It might therefore be of importance to identify and separate a relatively healthy subpopulation of older AML patients that can undergo standard intensive treatment from the other group considered to be 'unfit for standard intensive chemotherapy'. Most clinical trials in AML thus far have excluded patients older than 60, and therefore their results cannot automatically be translated to the older AML patient. However, recently, Juliusson *et al.* reported, based on the large and unselected Swedish National Acute Leukemia Registry, that remission induction chemotherapy provided better quality of life and longer survival than supportive care only in the elderly group<sup>39,37</sup>. This benefit effect was especially observed in the good risk group at the age group of 60-65 years. Therefore it would be attractive to have tools to identify AML patients with different prognostic outcomes. A few predictive factors could be selected based on a large, so far unpublished, study by Kantarjian *et al.* analyzing 446 AML patients aged 70 years or older that were treated with an intensive chemotherapy regimen between 1990 and 2008<sup>40</sup>. The prognostic factors include: age >80 years, three or more genetic abnormalities, poor performance score (Easter Cooperative Oncology Group (ECOG)) 2-4, and raised creatinin levels. The more risk factors the patients have, the poorer their survival will be with intensive chemotherapy<sup>40</sup>. Furthermore, apart from supportive care or lower-dose therapy in 'elderly' AML patient, it is of utmost importance to find new treatment strategies with less side-effects and a possible chemotherapy sparing drug, i.e. statins and farnesyltransferase inhibitors. Especially the combination of statins and farnesyltransferase inhibitors should be further explored also in *in vivo* leukemic models to establish the most important cellular target for this drug combination.

As described in this thesis, allogeneic hematopoietic stem cell transplantation is currently the first choice of consolidation treatment for the intermediate and poor risk group of AML patients, aged

18 to 65 years (introduction, figure 3) after having achieved CR following intensive chemotherapy. Despite prophylaxis with immunosuppressive drugs, one of the major complications is the GVHD with a high morbidity and mortality rate. It has been demonstrated that this condition depends on donor T-cells essential for the success of the graft versus leukemia effect (GVL)<sup>41</sup>. The incidence of acute GVHD ranges from 10-80%<sup>42</sup>. Currently, the primary therapy for GVHD is corticosteroids (methyl)prednisolon in combination with the calcineurin inhibitor cyclosporine. Secondary, mycophenolate mofetil, followed in the third place by infliximab. However, only 30-75% of patients with acute GVHD respond to current therapy. Therefore there is a need for new and probably more effective treatment regimes with less side-effects<sup>43</sup>. Presently, statins are under investigation for this indication. Statins block leukocyte function-associated antigen-I (LFA-1), T-cell adhesion, proliferation, and cytokine production *'in vitro'* and *'in vivo'* in murine cells<sup>44</sup>. Recently Rotta *et al.* published data suggesting that *in vivo* treatment of donors with statins (20-80 mg/day) may be a promising strategy to prevent acute GVHD without compromising immunologic control of the underlying malignancy in the recipient<sup>45</sup>. Apart from in murine cells, statins seem to interfere with T-cell responses in humans as well. This therefore provides an excellent potential treatment strategy to protect against GVHD. Several questions still have to be answered in preclinical trials before designing prospective clinical trials.

By observing the variety between different subpopulations of AML cells and consequently by specifically targeting the multiple aspects of the LSCs, this will provide us with a better understanding of their role in leukemogenesis. Additionally, we should eventually reach our goal of eradicating the LSCs and providing a cure for AML.

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# Chapter 7

## Summary in Dutch (Nederlandse Samenvatting)



### **Bloedcelvorming (hematopoese)**

Bloedcellen worden gevormd uit een klein aantal onrijpe stamcellen in het beenmerg (hematopoietische stamcel, HSC). Deze zeldzame cellen zijn de moedercellen van waaruit de verschillende dochtercellen ontstaan. De HSC kan zich enerzijds te vernieuwen (self-renewal) of anderzijds differentiëren naar een van de vele dochterbloedcellen te weten de witte bloedcellen (leukocyten), die de afweer tegen ziekteverwekkers verzorgen, rode bloedcellen (erythrocyten) die zuurstof door het lichaam transporteren, en bloedplaatjes (trombocyten) die verantwoordelijk zijn voor de bloedstolling. Wanneer deze bloedcellen voldoende vermenigvuldigd en uitgerijpt zijn verplaatsen ze zich vanuit het beenmerg naar de bloedbaan. Dit proces van hematopoese is strak geregisseerd, waarbij zowel intra- als extracellulaire mechanismen een belangrijke rol spelen.

### **Acute myeloïde leukemie**

Acute myeloïde leukemie (AML) is een kwaadaardige stoornis van de bloedcelontwikkeling en gaat gepaard met ophoping van onrijpe myeloïde blasten in het beenmerg en het perifere bloed. Deze blasten hebben niet het vermogen zich te differentiëren tot één van de dochtercellen. Ongeveer 40% van alle leukemieën valt onder de categorie AML.

Een belangrijk probleem bij de behandeling van AML patiënten is de relatief hoge leeftijd bij presentatie. Meer dan de helft van de patiënten is ouder dan 65 jaar. Ondanks intensieve behandeling met chemotherapie zijn de perspectieven voor deze AML patiënten op langere termijn niet goed. Daarom is het van belang parameters te ontwikkelen waarmee onderscheiden kan worden welke patiënten baat zullen hebben bij de ingestelde behandeling.

### **De leukemische 'stamcel'**

De leukemische stamcel kan worden gezien als de moedercel die verantwoordelijk is voor het blijvend aanwezig zijn van de leukemische cellen in het beenmerg. Incomplete eradicatie van de leukemische stamcel zal uiteindelijk leiden tot 'relapse' (terugkeer) van de ziekte. Dit wordt veelvuldig waargenomen bij patiënten met AML waarbij meestal wel een remissie (afname van ziekte) wordt bereikt, maar niet een situatie waarbij de leukemie blijvend verdwijnt. Kennelijk zijn de huidige chemotherapeutica wel in staat tot de eradicatie van de bulk van de leukemische cellen, maar overleeft zeer waarschijnlijk het merendeel van de leukemische stamcellen. Daarom lijkt, teneinde AML in de toekomst beter te kunnen bestrijden, een betere herkenning van de eigenschappen van deze cellen van groot belang. Momenteel wordt aangenomen dat de leukemische stamcel zich voornamelijk bevindt in de pool van de meest primitieve AML cellen. Deze worden gekarakteriseerd door de aanwezigheid van het oppervlakte antigeen CD34 en het verlies van de marker CD38 (CD34<sup>+</sup>CD38<sup>-</sup>). Daarom is dit onderzoek gericht op deze subpopulatie van cellen. Dergelijke subpopulaties worden verkregen door middel van sortering via een flowcytometer. Recent zijn er echter aanwijzingen gekomen dat de leukemische stamcellen zich ook in andere, meer uitgerijpte, subpopulaties kunnen bevinden. Hier wordt momenteel verder onderzoek naar gedaan.

Inzicht in de mechanismen waarmee de stamcellen zich verdedigen tegen chemotherapie en de ontwikkeling van strategieën om deze verdediging aan te grijpen is daarom van groot belang voor de toekomstige behandeling van AML.

### **ABC transporters en cholesterolmetabolisme**

We stelden ons de vraag of de expressie van bepaalde membraaneiwitten, ATP-binding Cassette (ABC) transporters, van belang kan zijn bij de eradicatie van de leukemische stamcel. Reeds langer is bekend dat deze eiwitten vele substanties, waaronder bepaalde chemotherapeutica, de cel uit kunnen pompen. Dit kan aanleiding geven tot de zogenaamde multi-drug resistentie in tumorcellen, een situatie waarbij de tumorcellen ongevoelig worden voor de medicijnen die door deze transporters uitgepompt worden. Deze pompen blijken ook van belang te zijn (zie proefschrift) bij het reguleren van het cholesterolmetabolisme in de stamcel. In zoogdiercellen is cholesterol belangrijk voor de membraanstructuur en de functie van aan-membraan-gebonden eiwitten. Cholesterol is ook een belangrijke bouwsteen voor o.a. steroid hormonen en galzouten. Cholesterol homeostase in cellen wordt gehandhaafd door strak gereguleerd processen, dat wil zeggen cholesterol influx, efflux en synthese. Influx verloopt voornamelijk via de low-density-lipoprotein-receptor (LDLR), efflux via de activatie van Liver X receptors (LXRs) welke zorgen voor een toename van de ABC transporters ABCG1 en ABCA1 en synthese vindt plaats door het HMG-CoA reductase enzym.

Het cholesterolmetabolisme kan gemoduleerd worden door klinisch veel gebruikte cholesterol verlagende medicijnen, de HMG-CoA reductaseremmers oftewel statines. Doel van dit proefschrift is inzicht te verkrijgen in het cholesterolmetabolisme van de (leukemische) stamcel en de rol van de ABC transporters hierin.

**Hoofdstuk 1** geeft een uitgebreid overzicht van de diverse facetten van AML zowel op cellulair niveau als ten aanzien van de huidige klinische praktijk. In **hoofdstuk 2** wordt de aanleiding voor dit promotieonderzoek gepresenteerd waarbij met behulp van een micro-array studies in haematopoietische muizencellen wordt gekeken naar de expressie van verschillende ABC transporter genen. Een micro-array is een techniek die het mogelijk maakt om naar de cellulaire RNA expressie van heel veel genen tegelijkertijd te kijken. We waren benieuwd welke ABC transporter genen met name tot expressie kwamen in de primitieve haematopoietische stamcelfracties. Dertien ABC transporter genen bleken hoog tot expressie te komen in primitieve muizencellen. Vijf hiervan (ABCB1, ABCC1, ABCG1, ABCD4, ABCB2) werden ook sterk tot expressie gebracht in een menselijke primitieve stamcelfractie. Een aantal van deze ABC transporters waren reeds bekend op het gebied van de multi-drug resistentie, maar één hiervan, ABCG1 bleek betrokken te zijn bij het cholesterolmetabolisme. Aansluitend is verder gekeken naar een andere, beter bekende, cholesterol ABC transporter (ABCA1) en meerdere genen betrokken bij het cholesterolmetabolisme. Uit deze studie is naar voren gekomen dat zowel ABCA1 als

de bekende cholesterolmetabolisme genen HMG-CoAR, LDLR en LXRA ten opzichte van meer uitgerijpte cellen ook hoger tot expressie komen in de stamcelfractie. Deze data ondersteunen het belang om het cholesterolmetabolisme specifiek in gedefinieerde celpopulaties te bestuderen. In **hoofdstuk 3** wordt een overzicht gegeven van de huidige kennis van zaken met betrekking tot de ABC transporters in normale en leukemische stamcellen. Met name wordt hier aandacht gegeven aan de rol van de ABC transporters in het cholesterolmetabolisme van deze cellen, temeer daar er steeds meer aanwijzingen zijn dat AML cellen een afwijkend cholesterolmetabolisme vertonen. Ook wordt reeds gezinspeeld op de mogelijkheid om met cholesterolverlagende medicatie als statines de chemotherapie-resistentie te verstoren en de cellen gevoeliger te maken voor chemotherapie. Aansluitend in **hoofdstuk 4** proberen we door middel van de cholesterolverlager lovastatine het cholesterolmetabolisme te moduleren in cellen afkomstig van AML patiënten, en tevens deze statine te combineren met bekende chemotherapeutica als cytarabine en daunorubicine om zo mogelijk een versterkend effect te bewerkstelligen. Hiervoor hebben we gebruik gemaakt van een luminescent cell viability assay om het aantal overlevende cellen na toevoeging van desbetreffende middelen te meten. We vonden een hogere gevoeligheid en dus een lagere celoverleving voor cellen die blootgesteld waren aan lovastatine en die behoorden tot de primitieve CD34<sup>+</sup> subpopulatie. Echter de AML CD34<sup>+</sup> cellen van patiënten reageerden niet allen op dezelfde manier. Een duidelijke heterogeniteit kon worden waargenomen tussen verschillende AML patiënten. Twee groepen werden gedefinieerd: normale responders (gelijkwaardig aan de controlegroep bestaande uit patiënten zonder actuele beenmergaandoening) en de abnormale responders. Opvallend was dat de groep van abnormale responders ook getypeerd werd door de aanwezigheid van andere ongunstige parameters zoals een slechter cytogenetisch profiel een hoger blast aantal in het perifere bloed wat weer correspondeert met een slechtere therapierespons.

Statines grijpen aan in de mevalonaat pathway, maar remmen hiermee niet alleen de cholesterol synthese maar ook verschillende bijproducten van deze route, waaronder farnesyl en geranyl isoprenoiden. Deze isoprenoiden zijn belangrijk voor de modificatie van bepaalde eiwitten waaronder het Ras eiwit dat frequent geactiveerd/gemuteerd is in AML. De statine concentratie die nodig is om deze bijproducten te remmen is 100-500 maal hoger dan de concentratie die nodig is om de cholesterol synthese te blokkeren. Om deze dosisverhoging en diens gevolge de verwachte bijbehorende bijwerkingen te voorkomen wordt er in **hoofdstuk 5** voor gekozen om het effect van de cholesterolverlager simvastatine te versterken door een specifieke remmer van de farnesyl isoprenoiden, de farnesyltransferase inhibitor tipifarnib toe te voegen. Dit resulteerde in een versterkt cytotoxisch effect van simvastatine en tipifarnib op de leukemische cellijnen KG1A en TF-1, wat zich vertaalde in een remming van de celcyclus en een toegenomen apoptose. In de primitieve CD34<sup>+</sup> AML cellen konden ook hier twee verschillende responsgroepen onderscheiden worden (responders/non-responders) met een aanzienlijke overlap tussen de simvastatine en de tipifarnib responders. Dit verschil leek niet samen te gaan met een veranderde expressie van cholesterolmetabolisme genen of genen die lager gelegen zijn in de signaaltransductieroutes van

simvastatine en tipifarnib. Ook een verschil in RAS mutatiestatus kon niet worden aangetoond. Het belang van het bestuderen van een selectieve cel fractie werd onderstreept. De effecten werden uitsluitend gevonden in de CD34<sup>+</sup> AML fractie en niet in de totale AML fractie. Dit maakt het noodzakelijk dat bij toekomstige studies naar meer geselecteerde cel populaties wordt gekeken waardoor mogelijk patiëntengroepen kunnen worden onderscheiden waarin bepaalde medicamenten beter of slechter zullen werken.

In dit proefschrift is getracht in geselecteerde subpopulaties verschillende aspecten van de leukemische stamcel, waaronder het aberrante cholesterolmetabolisme, aan te pakken. Op deze manier is inzicht verkregen in hun rol in de leukemogenese. Het uiteindelijk uiteindelijke doel is de leukemische stamcel effectief en volledig te eradiceren en zodoende een genezing voor AML te vinden.



## List of abbreviations

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ABC	ATP-binding cassette
AlloSCT	Allogeneic stem cell transplantation
AML	Acute myeloid leukemia
Ang-1	Angiopoietin-1
ApoA-1	Apolipoprotein A-1
ARA-c	Cytarabin, cytosine arabinoside
ASCT	Autologous stem cell transplantation
ATRA	All-trans retinoic acid
BCRP	Breast cancer resistance protein
bFGF	Basic fibroblast growth factor
BMT	Bone marrow transplantation
CaR	Calcium-sensing receptor
CBT	Cord blood transplantation
CD	Cluster of differentiation
CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CMV	Cytomegalovirus
CR	Complete remission
CEBPA	CCAAT/enhancer binding protein $\alpha$
CXCR4	CXC chemokine receptor
DNMT	Demethyltransferase inhibitor agents
ECOG	Easter Cooperative Oncology Group
EFS	Event free survival
EPO	Erythropoietin
FAB	French-American-British
FACS	Fluorescence-activated cell sorting
FLAG	Fludarabine, Ara-C and G-CSF
FLT-3	Fms-related tyrosine kinase-3
FTI	Farnesyl transferase inhibitor
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte macrophage colony-stimulating factor
GMP	Granulocyte/macrophage progenitor
GVHD	Graft versus host disease
GVL	Graft versus leukemia
HDAC	Histone deacetylase inhibitors

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HDL	High density lipoprotein
HiDAC	High dose Ara-C
HIF	Hypoxia inducible growth factor
HLA	Human leukocyte antigen
HMG-CoAR	3-hydroxy-3-methyl-glutaryl-CoA reductase
HPRT	Hypoxanthine-guanine phosphoribosyltransferase
HSC	Hematopoietic stem cell
HSP	Heat shock protein
IL-3	Interleukin-3
ITD	Internal tandem duplication
JMML	Juvenile myelomonocytic leukemia
LDAC	Low dose Ara-C
LDH	Lactate dehydrogenase
LDLR	Low density lipoprotein receptor
LFA-1	Leukocyte function-associated antigen-1
LIC	Leukemic initiating cell
LMMP	Lymphoid/myeloid multipotential progenitor
LSC	Leukemic stem cell
LTR	Long term repopulating
LXR- $\alpha$	Liver X receptor alpha
LT-HSC	Long-term hematopoietic stem cells
MAPK	Mitogen-activated protein kinase
MDR	Multidrug resistance
MDS	Myeloid dysplastic syndrome
MEP	Megakaryocyte/erythroid progenitor
MFI	Mean fluorescence intensity
MLL	Mixed-lineage leukemia
MPP	Multipotent progenitors
MRD	Minimal residual disease
MRP	Multidrug resistance-related protein
mTOR	Mammalian target of rapamycin
MUD	HLA-matched unrelated donor
MVD	Micro vessel density
NOD-SCID	Non-obese diabetic severe combined immunodeficiency
NPM1	Nucleophosmin 1
ORR	Overall remission rate
P-gp	P-glycoprotein
PI3K	Phosphatidylinositol 3-kinases



PPAR	Peroxisome-proliferator-activated-receptor
PR	Partial remission
RAEB	Refractory anemia with excess of blasts
RAEB-T	Refractory anemia with excess of blasts in transformation
RFS	Relapse free survival
RIC	Reduced intensity conditioning
RNA	Ribonucleic Acid
RXR	Retinoic X receptor
SCF	Stem cell factor
SDF-1	Stromal derived factor 1
SREBP	Sterol-response-element-binding-protein
ST-HSC	Short-term hematopoietic stem cell
SP	Side population
TKD	Tyrosine kinase domain
TKI	Tyrosine kinase inhibitor
TPO	Thrombopoietin
TRM	Treatment related mortality
UCB	Umbilical cord blood
VEGFR	Vascular endothelial growth factor receptor
WBC	White blood cell count
WHO	World health organization

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**Susan**

## Curriculum Vitae

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Susan Dorothé Petra Wilma Maria Peeters werd op 27 april 1976 geboren in Overloon, gemeente Vierlingsbeek. Aansluitend aan het behalen van haar Gymnasium diploma aan de Katholieke Scholengemeenschap Jerusalem te Venray in 1994 werd zij tot viermaal toe uitgeloot voor de studie Geneeskunde. In die tussentijd is zij Au-pair geweest in Vancouver, Canada, heeft zij de propedeuse Pedagogiek behaald en een jaar Bewegingswetenschappen gestudeerd aan de Rijks Universiteit Groningen (RUG) om daarna nog een jaar Geneeskunde aan de Katholieke Universiteit Leuven, België te studeren. In 1998 werd zij alsnog ingeloot en is teruggekeerd vanuit België naar Groningen om daar de studie Geneeskunde te vervolgen aan de RUG. Gedurende de studie werd deelgenomen aan de Junior Scientific Masterclass. Tussen 2000 en 2001 werd wetenschappelijk onderzoek gedaan in het kader van het BORG project naar honkvastheid van inwoners in Noord-Nederland, uitgaande van de afdeling Medische Oncologie, Medische Genetica en het Integraal Kankercentrum Noord-Nederland onder leiding van Prof. dr. W.T.A. van der Graaf. Aansluitend werd gedurende de co-schappen onderzoek gedaan naar de rol van BCL<sub>XL</sub> bij het Multipel Myeloom bij de afdeling Hematologie van het Universitair Medisch Centrum Groningen (UMCG) onder leiding van Prof. dr. E. Vellenga. Na het behalen van het artsexamen in 2004 werkte zij drie jaar als artsonderzoeker op een gemeenschappelijk project van de afdelingen Hematologie, Medische Oncologie en Kindergeneeskunde onder begeleiding van Prof. dr. E. Vellenga, Prof. dr. E.G.E. de Vries en Prof. dr. F. Kuipers. Het onderzoek was gericht op 'de rol van ABC transporters en het cholesterol metabolisme in Acute Myeloide Leukemie', zoals beschreven in dit proefschrift. Het onderzoek werd mede gefinancierd door een persoonlijke beurs verkregen van KWF-Kankerbestrijding in 2005. In oktober 2007 werd gestart met de opleiding interne geneeskunde (opleider Prof. dr. R.O.B. Gans) waarvan de eerste 2,5 jaar plaats vond in Zwolle (Isala Klinieken, opleiders dr. M. van Marwijk-Kooy en dr. M.A. Alleman), alvorens in maart 2010 de opleiding te vervolgen in het UMCG. Aansluitend zal in 2013 gestart worden met het aandachtsgebied Hematologie binnen de interne geneeskunde waarbij zij uiteindelijk een combinatie van kliniek, onderwijs en onderzoek ambieert. In 2006 is zij getrouwd met Martin de Jonge en samen hebben zij twee zoons, Timen (2007) en Rogier (2008).