Internal tandem duplications in the Flt3-gene in human acute myeloid leukemia

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CHAPTER 1

General Introduction

1.1. Hematopoiesis

As mature blood cells only have a limited life span this pool of cells needs constant replenishment. In this process of blood cell formation or hematopoiesis, all mature blood cells arise via a process of proliferation and differentiation from a common pool of ancestral cells, the hematopoietic stem cells (HSC).

The first HSC responsible for all adult hematopoiesis are generated in the aorta-gonad-mesonephros (AGM) region of the embryo[1,2]. From this region the HSC migrate to the fetal liver in which definitive hematopoiesis is initiated. After birth the bone marrow (BM) is the preferred hematopoietic site.

The HSC are characterised by their ability to: a) show extensive self renewal; b) differentiate to both myeloid and lymphoid lineages i.e. they are pluripotent, and c) give long term reconstitution of the hematopoietic system of a myeloablated recipient[3-5]. Physical separation methods have shown that also within the HSC compartment a hierarchy with respect to self-renewal, turnover rate and pluripotency exists[6-9]. In a transplant setting this hierarchy might be reflected in the different periods of time in which different stem cell clones contribute to the production of mature blood cells. Murine transplantation models indicated that HSC can be divided into two main subpopulations. One consisting of cells with long-term repopulating ability, responsible for maintaining sustained multilineage repopulation and one consisting of cells with only transient, short-term, repopulating ability[10,11]. A common tool to characterise and purify HSC has been the use of immunophenotyping and fluorescence activated cell sorting (FACS). One of the major advances using this technique has been the discovery of the expression of the transmembrane glycoprotein CD34 on a population of hematopoietic cells able to reconstitute both the lymphoid- and myeloid lineages of myeloablated recipients[12,13]. CD34 is expressed on one to four percent of normal bone marrow cells. From this fraction of $CD34^+$ BM cells HSC can be further purified by depletion of CD38 or lineage marker expressing cells. Nevertheless, recent studies have shown the reversible expression of CD34 and CD38 on both murine and human HSC[14-17], suggesting that CD34 might be a marker for activated stem cells. Further investigation is necessary to determine if $CD34^+$ and $CD34^-$ HSC represent the same pool of stem cells and whether CD34 is just an activation marker.

The homeostasis of hematopoiesis is closely regulated by the bone marrow microenvironment and/or cyto- and chemokines, acting in both synergistic and antagonistic fashions.

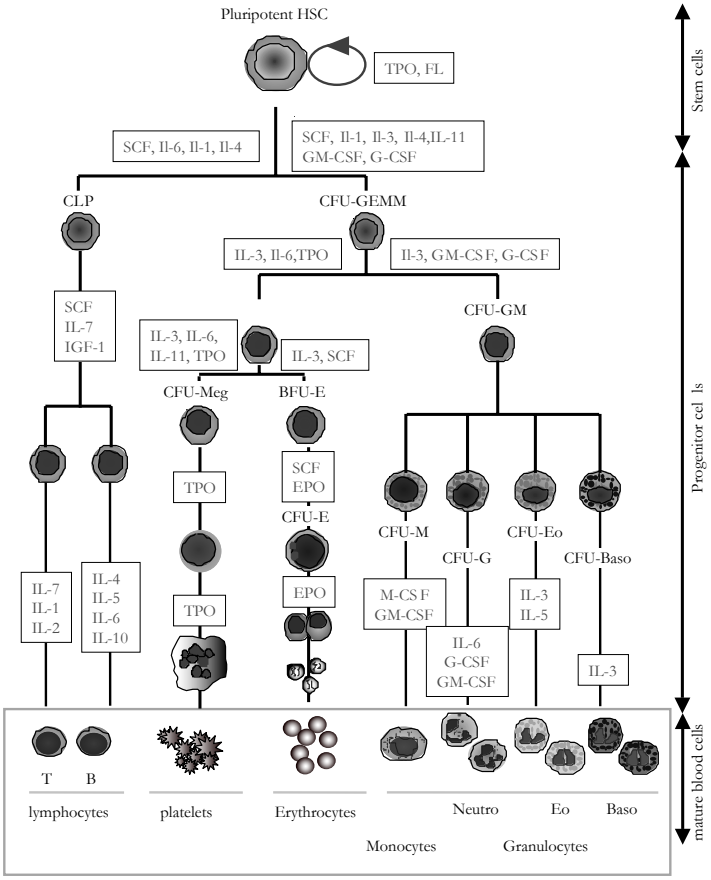

Figure 1. Schematic representation of the hematopoietic system and the growth factors involved.

All mature blood cells of the different lineages arise via a process of proliferation and differentiation directed by growth factors from a common ancestor, the pluripotent hematopoietic stem cell. Though the scheme in this figure is far from complete and only the major growth factors involved are depicted, it gives a good indication about the complexity of the hematopoietic system. In italics are depicted a number of the recognisable stages of differentiation, whilst the boxes indicate the growth factors involved. (HSC: hematopoietic stem cell; CLP: common lymphocyte precursor; CFU-GEMM: colony forming unit-granulocyte-erythroid-monocyte-megakaryocyte; CFU-GM: colony forming unit-granulocyte-macrophage; CFU-meg: colony forming unit-megakaryocyte; BFU-E: burst forming unit-erythrocyte; CFU-E: colony forming unit-erythrocyte; CFU-M: colony forming unit-monocyte; CFU-G: colony forming unit-granulocyte; CFU-Eo: colony forming unit-eosinophilic granulocyte; CFU-Baso: colony forming unit-basophilic granulocyte; IL: interleukin; TPO: thrombopoietin; FL: Flt3-ligand; EPO: erythropoietin; SCF: stem cell factor; GM-CSF: granulocyte-macrophage-colony stimulating factor; M-CSF: macrophage-colony stimulating factor; G-CSF: granulocyte-colony stimulating factor).

The cytokines or hematopoietic growth factors (HGF) acting on the hematopoietic system (Figure 1) are produced by a variety of cells such as stromal cells (fibroblasts macrophages, and endothelial cells), mature blood cells (monocytes, T- and B-lymphocytes) and hemopoietic cells proper, and are involved in the regulation of proliferation, differentiation, maturation and cell survival. HGF have in common that most display a wide range of activities (i.e. they are pleiotropic) and similar activities might be shared among different HGFs (redundancy). HGF can roughly be divided into a group of early acting cytokines such as Flt3 and SCF, mainly acting on the more primitive progenitor cells, and a group of late acting cytokines such as the colony stimulating factors (CSFs) and erythropoietin (Epo) which mainly induce proliferation and terminal differentiation of the more mature, lineage-committed, progenitor cells[18-20]. However, some HGF such as thrombopoietin (TPO), interleukin-3 and GM-CSF can act on both primitive and more committed hematopoietic cells[21-23]. Binding of the HGFs to their specific receptor will lead to activation of intracellular signalling pathways finally resulting in the activation of specific genes. The major signalling pathways involved in HGF signalling are the Ras/MAPK and the Jak/STAT pathways, though also activation of other pathways has been described[24-27]. The signalling cascade is finally tightly controlled by lineage-restricted as well as generally expressed transcription factors, co-activators and co-repressors resulting in a fine-tuned response after growth factor binding.

As outlined above, during fetal development HSC migrate from the fetal liver to the bone marrow. After birth HSC, progenitor cells as well as more mature cells can undertake targeted migration from one site in the hematopoietic system to another site. This process is considered to be a multistep process in which adhesion molecules expressed on both the hematopoietic cells and cells of the microenvironment, such as endothelial cells, are involved[28,29]. Chemokines (chemotactic cytokines, for a comprehensive review see Rollins, 1997 [30]) or growth factors bound to proteoglycans expressed on the extracellular matrix can activate β1- and β2-integrins on HSC and progenitor cells, resulting in adhesion and transendothelial migration[31-33].

Whilst its production is not restricted to the hematopoietic microenvironment alone, stromal cell derived factor-1 alpha (SDF-1) appears to be the major chemokine released by BM-microenvironment[34,35], acting on lymphocytes and hematopoietic progenitors. In SDF-1 or CXCR-4 (i.e. the SDF-1 receptor) knockout models hematopoietic precursor cells do not shift to the bone marrow during foetal development, whilst in vitro SDF-1 has been shown to activate integrins on these precursor cells and induce their

migration[36,37]. The chemokines Macrophage Inflammatory Protein-3β (MIP-3β) and Secondary lymphoid-tissue chemokine (SLC) both binding to CCR-7 originally were also described to be chemokines for HSC. However, in contrast to SDF-1 that attracts multiple types of HSC and hematopoietic progenitor cells, both SLC and MIP-3β were mainly found to be attractors for macrophage progenitors[38,39].

1.2. Leukemia

1.2.1. Leukemogenesis

The balance between hematopoietic cell proliferation, differentiation, maturation and cell survival is crucial to maintain homeostasis. In this process of hematopoietic development errors may occur, resulting in aberrant expression of genes critical in cell development leading to e.g. uncontrolled proliferation, prolonged cell survival or blockage of differentiation pathways. This may finally result in leukemia, a neoplastic disorder in which (immature) hematopoietic progenitors accumulate in the BM where they fail to fully mature and dislocate the normal progenitor cells hence bringing the normal homeostatic processes out of balance. Finally the neoplastic cells may leave the BM and lodge in other organ systems.

It has been generally accepted that leukemogenesis is a multistep process requiring multiple genetic lesions. These lesions may be acquired or congenital and involve aberrant activation of (proto-)oncogenes or inactivation of tumor-suppressor genes[40-42]. These genes encompass for example genes encoding growth factors, growth factor receptors, signalling molecules, molecules involved in DNA and RNA synthesis and transcription factors. Aberrant expression of both proto-oncogenes and tumor-suppressor genes can be caused by e.g. genetic instability, exposure to alkylating agents or radiation. This then can result in chromosomal translocations, chromosomal deletions, chromosomal inversions, aneuploidy, and critical mutations inside a gene[43-45]. Aberrant expression could also be caused by integration of a virus in a gene or in a locus controlling the expression of a particular gene[46-49]. Chromosomal translocations or inversions might result in the aberrant expression of the full-length protein or in the expression of fusion proteins which might have aberrant functional characteristics when compared to the normal protein. Aberrant expression of the oncoprotein can confer a growth advantage of the mutant progenitor cells finally resulting in them outgrowing their normal counterparts. In leukemia the genes located at the chromosomal breakpoints most often encode for transcription factors[50], such as AML-1, CBF-β, MLL and RARα[51-53]. Mutations in tumor-suppressor genes involved in leukemia generally result

in a loss-of-function mutation of these genes, which encode for negative regulators of cellular proliferation (e.g. NF1, members of the INK4 family) and regulators of apoptosis (e.g. p53)[54-56].

1.2.2. Acute myeloid leukemia

Leukemias can occur in both the lymphoid and myeloid lineages of differentiation. These leukemias are further subdivided in the more aggressive acute leukemias, characterized by uncontrolled proliferation and abnormal differentiation of immature cells, and the less aggressive chronic leukemias, commonly resulting in the expansion and accumulation of more mature elements. Since the research described in this thesis was focussed on acute myeloid leukemia (AML) this paragraph will only focus on this type of malignancy.

AML can be distinguished from other types of leukemias based on immunophenotyping, cytochemistry and cytogenetics. In the Netherlands the incidence of AML is about 2.8 per 10^5 inhabitants[57]. The incidence of AML increases with age from less than 1 per 10^5 under the age of 20 to over 10 per 10^5 in persons over 70 years of age. AML is a heterogeneous disorder which, at a morphological level, is classically subdivided in 8 subtypes based on the various degrees of lineage commitment and differentiation (FAB-classification, M0-M7)[58]. Cytogenetic analysis of leukemic samples has identified various non-random chromosomal aberrations in AML. Some of these chromosomal abnormalities correlate with specific FAB subtypes, for instance the translocation t(8;21) is present in around 40% of the FAB-M2 leukemias[52] , translocations involving the retinoic acid receptor alpha (RARα) on chromosome 17 in acute promyelocytic leukemia / FAB-M3 (t(15;17) is present in 98% of cases[59]) and inv(16) or t(16;16) involved in 80% of the FAB-M4eo cases[60,61] (Table 1). Cytogenetic aberrations can also be used to recognize subgroups of patients with distinct clinical characteristics and responses to therapy. Based on the molecular and cytogenetic aspects patients can be classified into distinct prognostic subgroups: e.g. favourable or good risk, intermediate or standard risk and unfavourable or poor risk. The favourable risk group includes around 15% of patients with an age below 60 years and is defined by the presence of t(15;17), t(8;21) and inv(16) or t(16;16). Patients presenting these mutations have an increased rate of complete remission and a relatively low risk of relapse. The poor prognosis group of AML is defined by deletions of chromosomes 5 or 7 (or the short-arms of these chromosomes), abnormalities in the long-arm of chromosomes 3 and AML with a complex karyotype (three or more cytogenetic aberrations). In some

Table 1. French-American-British (FAB) classification of AML and associated cytogenetic abnormalities.

FAB Subtype	Description	Relative Occurrence*	Associated cytogenetics	
			aberration	% of cases*
M0	Acute myeloblastic leukemia with minimal differentiation	3%	inv(3q26),t(3;3)	1%
M1	Acute myeloblastic leukemia without maturation	15-20%	-	-
M2	Acute myeloblastic leukemia with maturation	25-30%	t(8;21) t(6;9)	40% 1%
M3	Acute promyelocytic leukemia	5-10%	t(15;17) t(11;17) t(5;17)	98% 1% 1%
M4	Acute myelomonocytic leukemia	20%	11q23 inv(3q26),t(3;3) t(6;9)	~20% 3% 1%
M4eo	Acute myelomonocytic leukemia with abnormal eosinophils	5-10%	inv(16), t(16;16)	~80%
M5	Acute monocytic leukemia	2-9%	11q23 t(8,16)	20% 2%
M6	Erythroleukemia	3-5%	-	-
M7	Acute megakaryocytic leukemia	3-12%	t(1;22)	5%

* ref. Löwenberg et al., *N Engl J Med* 1999 **341:**1051-62.

studies also 11q23 mutations are considerd a marker for poor prognosis. Overall, patients with adverse risk type mutations generally have a probability of 5-year survival of no more than 20%. Patients presenting with AML with other cytogenetic abnormalities or without any apparent cytogenetic abnormalities are considered to have an intermediate risk[62-64]. In a small group of patients the disease evolves from a prior hematological disorder (e.g. Severe Congenital Neutropenia, CML or MDS)[65,66], a congenital or inherited disorder (e.g. Diamond Blackfan anemia, Fanconi's anemia)[67,68] or may be the result of to exposure to alkylating agents or topoisomerase II inhibitors

(used in treatment of other non-related neoplasias, also known as treatment-related AML)[69,70]. These leukemias are generally designated as secondary leukemias. From a scientific point of view secondary AMLs are of interest as these AMLs clearly suggest that multiple genetic aberrations are required for the full manifesation of the disease phenotype[71,72].

1.3. Flt3 and other Class III receptor tyrosine kinases in normal and malignant hematopoiesis.

The fms-like tyrosine kinase 3 (Flt3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2)[73,74] belongs to the class III receptor tyrosine kinases (RTKs) which also include c-Kit, c-Fms, and the PDGF α en β receptors[75]. Of the total number of 90 tyrosine kinases known to date 58 are of receptor, which are distributed over about 20 subfamilies or classes. All RTKs share a similar structure: an extra cellular ligand binding domain, a single transmembrane (TM) domain and an intracellular tyrosine kinase (TK) domain[76,77]. The extracellular domains of the different RTKs show a variety of conserved elements such as Ig-like domains, cysteine-rich regions and fibronectin repeats[78,79](See Figure 2). Binding of the ligand to the extracellular domain results in conformational changes that induce and stabilise receptor dimerisation. The TM domain mainly consists of a α-helical structure and anchors the receptor to the membrane in the correct orientation. TM domain linked mutations, leading to ligand independent dimerisation and constitutive activation, have been described in at least two RTK families[80]. The Class III RTKs are characterised by the presence of five Ig-like domains in their extracellular region and a split catalytic intracellular tyrosine kinase domain (fig. 2). The genomic loci encoding for the intracellular catalytic domains overall show a conservation in exon/intron boundaries, exon size and sequence, which suggests they have arisen from a common ancestor[81-83]. Recently, the Flt3 gene has been shown to be comprised of 24 rather than 21 exons, typical for the other class III members. Seven instead of four exons encode for the first three Ig-like domains of Flt3[84]. With the exception of PDGFRα almost all class III RTKs play an important role in hematopoiesis: Flt3 and c-Kit are both required for the survival, proliferation and differentiation of HSC[85,86]; c-Fms, the receptor for M-CSF, is crucial for the proliferation and differentiation in the monocyte-macrophage lineage[87].

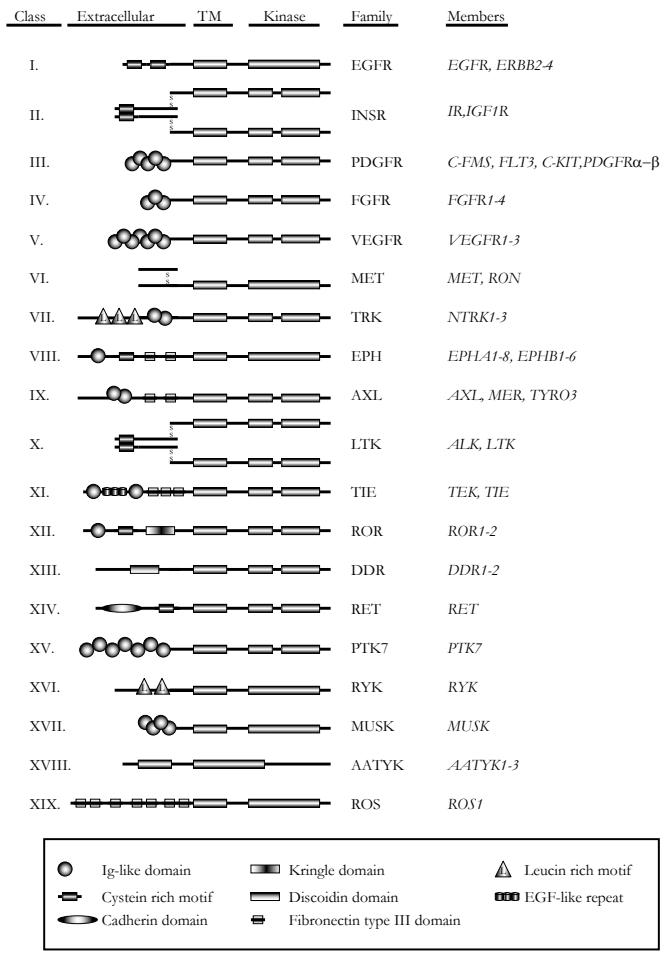

Figure 2. Schematic diagram of the receptor tyrosine kinase families.
For 19 (I-XIX) of the around 20 receptor tyrosine kinase classes the schematic structure of the protein is depicted. No known structural data of the other families were available. On the right side of each diagram the family members are depicted. The nomenclature of the families is as follows: EGFR, epidermal growth factor receptor; INSR, insulin receptor; PDGFR, platelet derived growth factor receptor; FGFR, fibroblast growth factor receptor; VEGFR, vascular endothelial growth factor receptor; MET, Common name, no abbreviation, also known as hepatocyte growth factor receptor; TRK, Tropomyosin receptor kinase; EPH, ephrin receptor; AXL, anexelekto; LTK, leukocyte tyrosine kinase; TIE, tyrosine kinase with Ig and EGF homology domains; ROR, Retinoid-related orphan receptor; DDR, discoidin domain receptor; RET, rearranged during transformation; PTK7, protein tyrosine kinase-like receptor 7; RYK, related to tyrosine kinase; MUSK, muscle-specific tyrosine kinase; AATYK, apoptosis-associated tyrosine kinase; ROS, Common name, no abbreviation.

Though the role of the PDGFRβ in hematopoiesis is less well defined, it most probably plays an important role in megakaryopoiesis[88]. The following sections will go more into detail about the role of the individual class III members in both normal and malignant hematopoiesis.

1.3.1. Flt3

Flt3 (CD135) is expressed on multipotential HSC and progenitor cells, suggesting a critical role in stem cell development and differentiation[89]. This is supported by a number of animal studies in which targeted disruption of Flt3 results in deficiencies in both myeloid and lymphoid progenitors[90]. Analysis of Flt3 knockout mice has shown that mainly primitive B cell progenitors are reduced. Yet, normal numbers of functional B-cells are present in the periphery. Also the composition and numbers of cells in the peripheral blood and hematopoietic organs are comparable between Flt3 knockout and wildtype mice. Only in competitive repopulation assays the Flt3-deficient HSC show a less efficient repopulation in comparison with wildtype HSC, especially in the lymphoid lineage. Flt3-ligand (FL) knockout mice on the other hand show an overt reduction of leukocyte counts in bone marrow, spleen and thymus as well as in the peripheral blood. In addition these animals also show reduced numbers of both myeloid en lymphoid dendritic cells and are deficient in NK-cells. Similar to the receptor knockout mice these animals only show slightly reduced numbers of myeloid progenitors[91]. These murine studies appear to indicate that Flt3 is of a lesser importance to primitive HSC when compared to the importance to the more committed lymphoid precursors. However, recent studies have shown a difference in the function of Flt3 and c-Kit in mouse and humans[92-94]. Mouse long-term reconstituting HSC (LTR-HSC) do not express Flt3 during steady-state hematopoiesis and upregulation of the Flt3 expression coincides with a loss of self-renewal capacity. This lack of Flt3 expression on murine LTR-HSC and the high level of expression of c-Kit on these cells correlates with the observation that SCF but not FL efficiently supports survival of murine LTR-HSC. In contrast in the human system SCF only has a limited capacity to support $CD34^{+}CD38^{-}$ HSC, which expresses low levels of c-Kit[95-97]. Recently, Sitnicka et al.[98] have shown that whilst only a fraction of the $CD34^{+}$ long-term culture initiating cells express Flt3, most of the CD34+ cells capable of inducing multilineage repopulation in the NOD/SCID human-chimera model are $Flt3^{+}$.

Upon ligand binding the Flt3-receptor dimerises causing auto-phosphorylation of certain tyrosine residues in the cytoplasmatic domain of the receptor. Although no specific tyrosine residues have been mapped which mediate interaction with specific signal transducing molecules, activation of Flt3 results in direct association with Grb2 and Socs1, phosphorylation of Cbl, Shc, SHIP, Shp2, Gab1, Gab2 and activation of the MAPK pathway[99-101]. Whether the STAT pathway is activated remains unclear. In 2000 Hayakawa et al.[102] published that STAT-5 is not phosphorylated following activation of wildtype Flt3, and STAT-5 activation was only observed after constitutive activation of a mutant Flt3. In contrast, Zhang et al.[103] showed that upon receptor activation STAT-5a, but not STAT5b or STATs 1-4, was phosphorylated. They also demonstrated that the activation of STAT5a required the kinase activity of Flt3. Moreover, they showed a selective role for STAT5a in the proliferative response of primary hematopoietic progenitor cells following FL stimulation. Addition of FL to a CFU-C assay, an assay for hematopoietic progenitor cells, only resulted in an increased number of colonies when marrow from STAT-5a wildtype, STAT5b wildtype or STAT5b knock-out animals was used. When STAT-5a knock-out bone marrow was used this increase was not observed.

Data from a number of groups have suggested a pathogenic role for Flt3 in leukemia. Flt3 expression has been documented in most cases of AML[104,105] and exogenous FL has been shown to induce proliferation of AML cells *in vitro*[106]. Likewise some AML-cells have been reported to express both Flt3 and FL, indicating a possible autocrine or paracrine stimulation[107]. Finally long-term over-exposure to FL has been shown to induce leukemia in mice, albeit after a long latency period[108].

In 1996 Nakao et al.[109] were the first to report a novel mutation in the Flt3 gene in a small number of AML patients. These mutations were shown to be internal tandem duplications (ITDs), mainly involving a tyrosine rich stretch at the end of exon 14 (587-NEYFYVDFREYEYD-560, see Abu-Duhier et al.[84] for revised exon numbering of the Flt3), coding for the juxtamembrane (JM) domain of the receptor (see Figure 3, overview of the Flt3 gene). Also, some ITDs involving exon 15 have been published. All ITDs were in frame and resulted in an elongated JM domain. This mutated receptor has been reported to result in ligand-independent dimerisation and phosphorylation, leading to a constitutive activation in 32D, BA/F or COS-7 cells transfected with Flt3/ITD[110,111]. Yet, Fenski et al.[112] observed no correlation between ligand-independent phosphorylation and the presence of ITDs in AML-patients. It is possible that, analogous to the c-Kit JM mutation reported in the FMA mastocytoma cell

line[113], the ITD disrupts a negative regulatory domain. Kelly et al.[114] showed that introduction of Flt3/ITDs into a murine bone marrow transplant model resulted in a myeloproliferative phenotype characterised by leukocytosis and splenomegaly with extramedullary hematopoiesis in spleen and liver. As these ITDs were insufficient to induce leukemia in this model, these data suggest that other co-operating mutations are necessary for the development of a leukemic phenotype.

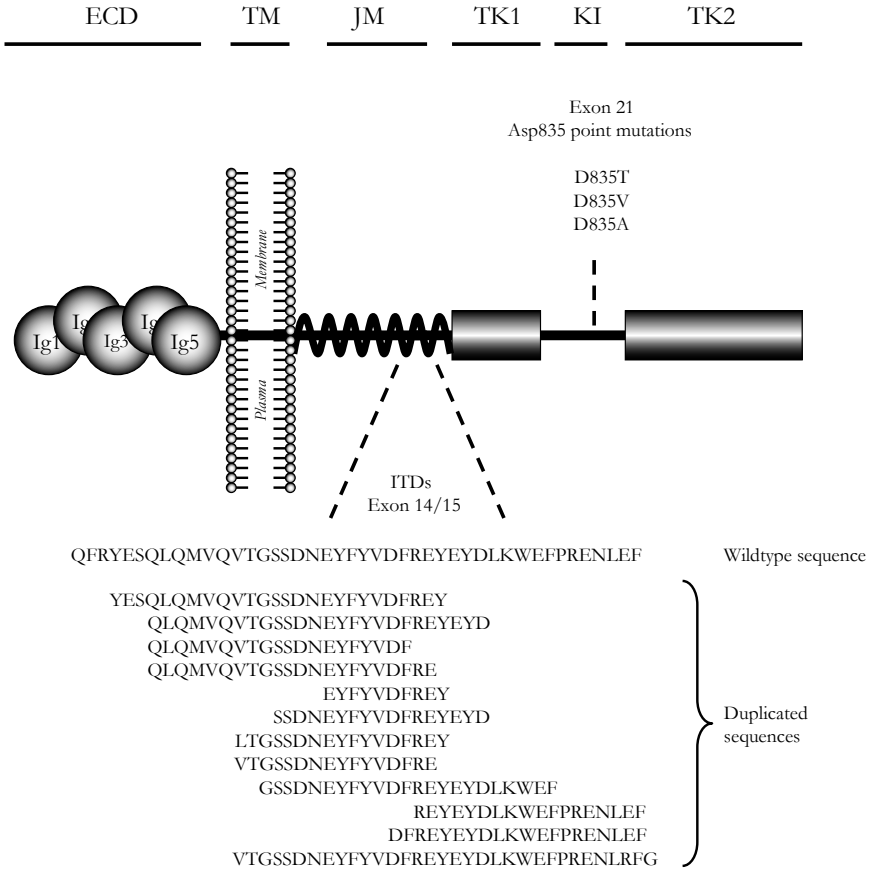

Figure 3. Overview of the Flt3 monomer spanning the plasma membrane.
This figure depicts the 5 extracellular Ig-like domains (Ig1-5) in the ligand binding domain of the extracellular part of the receptor (ECD), the transmembrane domain (TM), the juxtamembrane domain (JM) and the two intracellular tyrosine kinase domains (TK1 and TK2) separated by the kinase insert (KI). The positions of the two known types of mutations involved in AML, i.e. internal tandem duplications (ITDs) in the JM domain and point mutations in the KI, are indicated. Of each type of mutation some examples are presented.

We and others have shown Flt3/ITD mutations to be present in a high number (17 to 27%) of AML cases and to correlate with high peripheral white blood cell counts. The presence of these mutations correlates also with an unfavourable patient prognosis in both adult and pediatric AML[116-120] (See also, Chapter 3 of this thesis). Absence of the wildtype allele in Flt3/ITD AML predicts a more inferior disease free and overall survival[121]. Whereas the number of Flt3/ITDs appears to be higher in acute promyelocytic leukemia[122] the mutation is not correlated with a specific FAB-class or any known cytogenetic aberration. The presence of Flt3/ITDs has also been shown in 3-5% of MDS cases[123] and rare cases of biphenotypic leukemias[124]. No Flt3/ITDs have been reported in normal individuals and in juvenile or adult CML patients.
Notwithstanding that ITDs are the most common Flt3 mutations, other mutations in the flt3-gene have been reported as well. The observation of Glover et al.[125] that mutation of the Asp802 in the activating loop of c-Fms and similar mutations in the activating loop of c-kit[113] result in factor-independent growth of the mouse progenitor cell line FDC-P1, led two independent groups to investigate mutations in Asp835 of Flt3[126,127]. Both groups reported the presence of point mutations in this codon in about 7% of AML patients. Of these single base mutations the replacement of aspartic acid to valine is the most common, though other mutations involving the hydrophobic amino-acids tyrosine and alanine have been reported[122,128]. Although all Asp835 mutations were shown to result in constitutive activation with the ability to transform 32D cells[127] the effects of these mutations on the signal transduction pathways remain to be elucidated. In contrast to the ITDs in Flt3, the Asp835 mutations have not been linked to increased white blood cell counts and their influence on patient prognosis currently remains unclear[129].

1.3.2. c-Kit

Human c-Kit (CD117) encodes the receptor for stem cell factor (SCF, also known as: Kit ligand, Steel Factor or Mast Cell Growth Factor). The gene encoding c-Kit was identified as the human counterpart of v-kit, a viral gene from the HZ4 feline sarcoma virus[130]. Within the human hematopoietic system c-Kit is expressed on approximately 70% of the CD34+ cells in the bone marrow, including the more committed lineage-restricted progenitor cells and the more primitive long-term culture initiating cells[131,132]. Cloning of the loci for Dominant white spotting (*W*) and Steel (*Sl*) present in natural mouse mutants led to the identification of mouse c-kit and it's ligand[133,134]. Loss-of-function mutations at either the *W* or *Sl* locus results in reduced thymic cellularity, de-

pletion of mast cells and erythroid progenitors resulting in macrocytic anemia in these animals. In addition there is hypopigmentation and male infertility. The ligand of c-Kit is most commonly expressed as a bivalent dimer of which both membrane bound and soluble isoforms exist[135]. The ligand for c-Kit is known as stem cell factor as it supports the survival and self-renewal of HSC. In synergy with Epo it stimulates the proliferation of erythroid progenitors, and in synergy with TPO and other cytokines it promotes megakaryocytic growth and differentiation[136].
There is ample evidence that mutations in c-Kit are involved in a number of human hematopoietic malignancies. The most common mutation found in the c-Kit gene is an amino-acid substitution at codon 816 in the juxtamembrane domain, substituting an aspartic acid for valine[137]. This mutation, resulting in constitutive activation of the receptor has been reported in patients with mastocytosis[138] and in patients with germ cell tumors[139] or myelodysplasia[140]. More intriguingly, these and other mutations of the same codon have also been linked to AML with an inv(16) or t(8;21) karyotype[141]. In 1999 Gari et al.[142] reported a series of exon 8 deletion-plus-insertion mutations with a loss of Asp419 (located in the fifth Ig-like domain), present in one third of patients with AML-M4eo and inv(16). In AML with t(8;21) Care et al.[143] reported the presence of Asp419 and Asp816 mutations in 2.1% and 10.6% of cases respectively. The apparent link between mutations in c-Kit and AML1-CBFβ has led to speculation about the role of these proteins in the pathology of AML. Recent studies[144] have shown that mutation of transcription factors in it self is not sufficient to induce a leukemic phenotype, it could well be that the additional mutations in c-kit function as a second-hit with anti-apoptotic or proliferative effects.

1.3.3. c-Fms

The human c-Fms (CD115) is the cellular homologue of the viral v-fms. In addition to the cells of the monocyte/macrophage lineage in the hematopoietic system it is expressed on pre-B cells, placental throphoblasts, neurons in the CNS and microglial cells[145-147]. The importance of c-Fms in the monocyte/macrophage lineage has been demonstrated in the CSF-1 mutant *op/op*-mouse[148]. This osteopetrotic mouse has an impaired bone remodelling due to the absence of osteoclasts. In these mice also other types of phagocytes, including macrophages, are depleted from liver, kidney, spleen and gut. However, macrophages for the lymph nodes remain relatively normal. A similar, yet more pronounced phenotype is observed in c-Fms deficient mice. Enforced expression of Bcl-2 in monocytes from the *op/op* mice can rescue macrophages and

partially reverses the osteopetrosis, suggesting that CSF-1/c-Fms signalling provides a survival signal to cells[149].
In 1990 a number of groups reported the presence of point mutations in c-fms in around 18% of AML cases and 15% of MDS cases[150,151]. Recent studies using more sensitive techniques failed to corroborate these results[152]. On the other hand, allelic loss of c-Fms has been reported in refractory anemia and in 5q- associated MDS[153]. Yet, these studies can not exclude the role of other genes located on this arm of the chromosome. Altogether, the role of c-Fms in leukemogenesis for the time being remains controversial.

1.3.4. PDGFR

The PDGF receptors α and β are two highly related RTKs. Whilst the Tk-domains have around 80% sequence homology the c-terminal domains only share around 27% homology[154]. The ligands for PDGFR-β, PDGF AB or BB are potent stimulators of mesenchymal cell proliferation and differentiation and play a fundamental role in wound healing[155]. PDGFR-β has also been detected on a number of hematopoietic cells, including multipotent stem cells, mast cells, B and T-lymphocytes and NK-cells[156,157]. Yet, PDGF is only able to stimulate the growth of primitive progenitors and erythroid precursors in unsorted bone marrow cultures, most likely by stimulating the release of other factors from stromal cells[158]. In 1994 Golub et al.[159] reported a t(5;12)(q31;p13) occurring in a substantial fraction of chronic myelomonocytic leukemia (CMML). This translocation resulted in the TEL/PDGFRβ fusion protein. The pathogenicity of this fusion gene is dependent on PDGFR activity as PDGFR-specific kinase inhibitors block the action of the fusion protein *in vitro*[160]. Apart from this mutation also other oncogenic fusion proteins involving PDGFRβ (e.g. t(5;10)(q33;q21) and t(5;17)(q33;p13)) have been reported in hematological malignancies[161-163].
Recently, it has been suggested that the hypereosinophilic syndrome may result from a novel, imatinib sensitive, fusion tyrosine kinase - FIP1L1-PDGFRα - that is a consequence of an interstitial chromosomal deletion[164]. In contrast to the other Class III RTKs, no point mutations have been reported which result in oncogenic variants of the PDGFRs.

1.4. Outline of this thesis

Late in 1996, the paper of Nakao et al.[109] in which ITDs in Flt3 were first presented aroused our interest. As little was known about the biological characteristics and clinical relevance of Flt3/ITD we performed some pilot cultures which showed that AML samples with Flt3/ITD had a reduced *in vitro* proliferation when compared to Flt3-Wildtype (Flt3/WT) AML. This led us to further characterise these mutations, the main aim of the experiments described in this thesis. After an optimisation of the culture conditions we investigated the effects of the presence of Flt3/ITDs on the nucleated cell production in long-term, stroma supported, cultures of AML cells, either without the addition of exogenous cytokines or in the presence of a combination of IL-3 and G-CSF or a combination of IL-6, SCF, TPO and FL (Chapter 2 of this thesis). We also investigated the impact of Flt3/ITDs on the proliferation of AML cells in short-term *in vitro* cultures in response to a panel of cytokines.

Focussing on the leukemic progenitor cell population we investigated the outgrowth of Flt3/ITD AML in the immunodefiecient NOD/SCID mouse, a model which allows for the *in vivo* outgrowth of human AML progenitors[165,166], whilst *in vitro* the leukemic progenitor cell subsets were assayed using the leukemic cobblestone area forming cell assay (L-CAFC)[167] (Chapter 3 of this thesis). As Flt3/ITDs were present in around 25% of our AML samples, and thus potentially clinically very interesting, we also investigated the clinical characteristics of Flt3/ITD AML with regard to initial therapy response, leukemia free survival and overall survival (also Chapter 3 of this thesis). In view of the reduced proliferation we observed in both short- and long-term culture, we speculated that Flt3/ITD might exert its effects on patient prognosis by inducing an anomalous organ distribution rather than inducing an increased proliferative potential. Recently it has been shown that in both normal hematopoietic cells and AML the CXCR-4 expression correlates with the SDF-1 induced chemotaxis[168,169], suggesting that SDF-1/CXCR-4 might be involved in the trafficking of leukemic cells. Hence, we analysed the SDF-1 induced chemotaxis and the expression of CXCR-4 on Flt3/ITD and Flt3/WT AML (Chapter 4).

In chapter 5 we investigated whether exclusion of Flt3/ITD AML would lead to a better correlation between the cytokine-induced proliferation and patient prognosis. In this study we specifically focussed on further distinguishing the prognosis of patients who, based on cytogenetics, were currently assigned to the intermediate risk group, the

group of patients who might benefit most of further risk assessment.
One of the assays used throughout this study has been the the transplantation of human AML cells in the NOD/SCID mouse model. The nonobese diabetic (NOD) / LtSz scid/scid strain appears to be most useful in allowing the engraftment of human AML cells. However, the large variability in ability to engraft and the levels of engraftment reached have not been explained. To address these issues we have investigated the NOD/SCID repopulating ability of 27 newly diagnosed AML samples (Chapter 6 of this thesis).

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CHAPTER 2

Human acute myeloid leukemia cells with internal tandem duplications in the Flt3-gene show reduced proliferative ability in stroma supported long-term cultures

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2.1. Abstract

Recently in-frame internal tandem duplications have been reported within the regions coding for the juxtamembrane through the first tyrosine kinase domain of the Flt3 gene. These duplications have been reported to lead to autophosphorylation of the receptor. In this study we investigated the effect of such mutations in the Flt3 gene on the in vitro proliferation of human acute myeloid leukemia cells.
The mutations were detected in 10 out of 59 AML bone marrow samples analysed and were not restricted to a specific FAB-class or cytogenetic aberration. PCR analysis of those samples showed all mutations to be present in exon 11 of the gene.
Whilst samples without a mutation of the Flt3 gene showed an increased cell production in response to either Il-3 and G-CSF or Il-6, SCF, TPO and Flt3l in long-term stroma supported cultures, mutant samples failed to do so.
As we could not find a relation between the absence of a response and either FAB-class or cytogenetic aberrations we interpret these results as an indication that the internal tandem duplications in the Flt3 gene are the prime cause of this unresponsiveness. Though our study does not explain the mechanism by which these mutations cause this unresponsiveness it does suggest that AML cells need a wildtype Flt3 for optimal in vitro proliferation.

2.2. Introduction

In normal hematopoiesis proliferation and differentiation is regulated by highly specific growth factor / growth factor receptor interactions[1,2].
The fms-like tyrosine kinase 3 (Flt3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2)[3-5] belongs to the group of receptor tyrosine kinases (RTKs), which also include the receptors c-kit (the receptor for Kit-ligand or stem cell factor) and c-fms (the receptor for CSF-1). The RTKs have similar structural characteristics such as the presence of five Ig-like domains in their extracellular region. Signals generated by ligand-induced dimerisation of these receptors involve tyrosine phosphorylation of certain regions of the receptor and activation of cellular tyrosine kinases.
Whilst a large proportion of acute myeloid leukemias (AML) has been found to express c-kit and c-fms[6-8] and these are absent on blast cells of acute lymphoid leukemia, the potential role of this class III receptor superfamily is still unclear.
Flt3 ligand (Flt3l) and Flt3 are both expressed on B-ALL and AML cells[9-12] as wells as on normal progenitor cells[13-15].

Recently internal tandem duplications (ITDs) were reported in the part of the flt3 gene coding for the juxtamembrane (JM) through first tyrosine kinase (TK-1) domains of the protein. Flt3/ITD's appeared to be present in 17-20% of AML patients[16,17] as well as in leukemic transformation of myelodysplasia[18], including 20% of patients with acute promyelocytic leukemia (APL)[19]. However these types of mutations have not been found in chronic myeloid leukemia (CML) or in CML blast crisis[20], nor have they been reported in cells of normal individuals.
Kiyoi et al.[21] have shown that elongation of the JM domain causes ligand-independent dimerisation of the mutant receptor, thus resulting in a constitutive activation. This effect was seen in both mutant/mutant and in mutant/wildtype dimers of the receptor. Immunoprecipitation studies of those mutant receptors showed evidence for a conformational change in the C-terminal region of the mutant receptor.
In the present study we investigated whether Flt3/ITD's would affect the proliferative capacity of leukemic cells on stroma-supported cultures. In addition we also investigated whether a possible effect could be abrogated or enhanced by the addition of cytokines including Il-3 and G-CSF or Il-6, SCF, TPO and Flt3l.
We found that the presence of the mutation in 10 of 30 AML-samples investigated correlated with a significant reduction in the ability to generate non-adherent cells during the entire culture period. This effect was most profound during weeks two and three of the culture period when most active proliferation was observed, while it was not affected by the cytokine combinations tested. We also found that both the mutation and its effect in the cultures were not correlated to either a specific FAB classification or cytogenetic background of the sample.

2.3. Materials and methods

Patient samples

Bone marrow cells were obtained after informed consent of patients with AML. Cases were classified according to the French-American-British committee (FAB)[22]. Mononuclear cells were isolated by ficoll separation followed by T-cell depletion, typically resulting in a population containing over 95 percent blasts. After isolation cells were subjected to controlled freezing and stored in liquid nitrogen. Before use cells were thawed by stepwise dilution in Iscoves modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands) containing 1% BSA. After thawing viability varied between 70 and 90 percent as assessed by dye exclusion.

Determination of Flt3 mutants

All AML samples were analysed for mutations in exons 11 and/or 12 of the Flt3 gene by using a modified PCR procedure as described elsewhere[16] using the following primer sets: 11F (5'-CAATTTAGGTATGAAAGCC-3') , 11R (5'-CAAACTCTAAATTT-TCTCT-3') covering the complete exon 11 and 12F (5'-TGTCTTTGCAGGGAAG-GTTAC-3') and 12R(5'-GTACCTTTCAGCATTTTGAC-3') covering exon 12 of the gene. These exons 11 and 12 allowed us to cover the whole JM and the first part of the TK-1 domain where most of the reported mutations are located.

Genomic DNA was prepared using a standard procedure[23]. Briefly DNA from around 10^4 cells was amplified in a total of 50 µl reaction mixture, containing 1x PCR buffer, 10 pM of each primer, 10 mM dNTP's and 0.5 U Taq polymerase (Supertaq, SpheroQ, Leiden, The Netherlands). Preheating of the samples at 95°C was followed by amplification for 35 cycles consisting of: 1 min. at 43 °C, 1 min. at 72 °C, 1 min. at 95 °C. Following these cycles a final extension at 72 °C was performed for 10 min.

PCR products were stained with SYBR green I (Molecular Probes, Leiden, The Netherlands) and resolved on 3% agarose gel.

As a confirmation samples positive for a flt3-mutation were reanalysed in a second PCR using the primers 11F and 12R.

Sequencing of patient samples with a mutated Flt3 gene

If both PCR reactions using the 11F/11R and 11F/12R combinations resulted in an abnormal PCR product, this was purified from the gel using a JETsorb kit (Genomed, Bad Oeyenhausen, Germany). This purified PCR product was subjected to a second PCR using fluorescent ddNTP's after which sequencing was carried out using an automated DNA sequencing system (ABI PRISM 310 genetic analyzer, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Long-term culture of AML cells

To asses the optimal conditions for long term in-vitro growth of the AML, we cultured the samples under the following conditions: A) serum free Stem Cell Growth Medium (SCGM, Biowithaker, Alkmaar, The Netherlands) without stromal support; B) SCGM with support of a stromal layer formed by the murine FBMD-1 cell line[24] and C) LTC-medium consisting of IMDM, supplemented with 10% heat inactivated fetal calf serum (Summit Biotechnology, Fort Collins, Co.), 5% horse serum (Gibco), β-mercaptoethanol ($5x10^{-5}$ mol/l) and hydrocortisone-21-hemisuccinate (10^{-6} mol/l,

Sigma) in the presence of a stromal layer. All of the above media were supplemented with penicillin (100U/ml) and streptomycin (100 μg/ml).
rhIl-3 (8 ng/ml), rhG-CSF (20 ng/ml) and rhIl-6 (100 ng/ml) were a generous gift of the Genetics Institute, Cambridge, MA, USA, rhSCF (50 ng/ml) and rhFlt3l (50 ng/ml) were a gift of Amgen, Thousand Oaks, CA and rhTPO (10 ng/ml) was a gift of Genentech. All cultures were performed in 6-well cluster plates (Costar, Cambridge, MA) at 37°C and 10% CO_2 in a humidified atmosphere. Volumes were standardized at 2 ml/well. Weekly half of the medium was replaced and non-adherent nucleated cells were counted.

Detection of AML cells in culture

If numbers of cultured cells produced allowed us, we determined their leukemic or normal hematopoietic background. This was done by May-Grunwald-Giemsa staining of cytospin preparations taken from the cultures. The lack of terminal differentiation and their strong resemblance to the original cell inoculum strongly suggested a leukemic origin.

Statistical analysis

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il.). Significance of differences was determined by the non-parametrical Mann-Whitney U test. Values were considered significant if p-values were below 0.05.

Table 1: Patient distribution

Patient No.	Sex	Age	FAB	Cytogenetic aberrations	Flt3/ ITD
1	male	70	M5		+
2	male	69	M0	-y	+
3	male	65	M2		+
4	male	73	M2		+
5	female	50	M2	t(8:21)	+
6	male	62	M5		+
7	female	67	M2	2q-	+
8	male	50	M5		+
9	female	34	M5	+8	+
10	male	29	M3	t(15:17),+8	+
11	female	68	M2	+8	-
12	female	69	M4	7q-,+8	-
13	female	77	M0	7q-,+8	-
14	male	50	M2	+8	-
15	male	74	M0	-5	-
16	female	35	M2	t(8:21)	-
17	male	40	M1		-
18	male	62	M0		-
19	male	19	M1		-
20	female	21	M3	t(15:17)	-
21	female	69	M1		-
22	female	28	M2		-
23	female	61	M2	+4,+13,+14	-
24	male	39	M2	t(8:21)	-
25	male	16	M4		-
26	female	78	M4		-
27	female	46	M5		-
28	female	54	M5	7q-	-
29	male	51	M5		-
30	male	49	M5	+8	-

A total of 30 AML patient samples were included in this study. Patients were selected to give a good representation through out the FAB classes. Cytogenetics were determined for all samples, cytogenetic abnormalities are included if observed.

Ten patients were selected on basis of the presence of an internal tandem duplication in exon 11 of the Flt3 gene as determined by exon 11 specific PCR.

2.4. Results

Screening for the Flt3 mutation

A total of 59 bone marrow samples from patients with AML were screened for the presence of a Flt3 mutation. A total of 10 patients (17%) were found to be positive. All mutations found were in the exon 11 region of the gene whilst none were found in exon 12 (data not shown). All patients, except for patient 2 who had a homozygous mutation, showed a heterozygous mutation with both normal and mutated alleles detectable by PCR. The mutation was randomly distributed over the FAB classes and did not show a correlation with any known cytogenetic aberration.

As we were not aware of any reports on the presence of these types of mutations in the normal population we also checked 46 healthy volunteers. No mutations were found in these samples, thus reducing the possibility that these mutations reflect gene polymorphisms.

In this study the 10 AML-patients with the mutation and 20 AML-patients without such a mutation were more extensively studied. Patient details are shown in Table 1.

The sequence analysis of the Flt3 mutant samples (Table 2) shows tandem duplications varying in length from 10 to 26 amino acids (30-78 bp). All mutations were restricted to the JM domain of the receptor. Four out of nine mutant samples show the same location for the start of the mutation whilst all others differ in their starting position. Patient number 7 starts with a valine to leucine mutation caused by a G to C point mutation in the DNA.

All ITD's have the duplication of the putative SH2 binding motif YFYV as a common factor. Patients 2,3,6 and 10 also have a duplication of a second putative SH2 binding motif YEYD. The involvement of these motifs in the binding of SH2-containing signalling molecules by Flt3 however remains to be elucidated.

Selection of the optimal culture conditions

To determine the optimal conditions for long-term culture the cells were grown in serum-free medium, with or without support of an adherent FBMD-1 stromal layer or in LTC-medium (including 10% FCS and 5% HS) on a stromal layer. As shown in Fig. 1, after three weeks of culture, the stroma-supported cultures produced more cells than did the stroma-free cultures. This suggests a supporting role for stroma in maintaining long-term cultures of AML cells. In contrast to the cultures with stromal support, in stroma-free cultures cell production ceased between week 4 and 5 of culture. Stroma-supported cultures maintained production until the end of the 8-week

observation period (data not shown). As the most optimal growth was observed in serum-containing stroma-supported cultures we performed all subsequent studies under these conditions.

Table 2: Sequence analysis of the Flt3/ITD's

Patient No.	duplicated amino acid sequence	Length
1	EYFYVDFREY	10 AA
2	QLQMVQVTGSSDNEYFYVDFREYEYD	26 AA
3	QLQMVQVTGSSDNEYFYVDFREYEYD	26 AA
4	QLQMVQVTGSSDNEYFYVDF	20 AA
5	QLQMVQVTGSSDNEYFYVDFRE	22 AA
6	SSDNEYFYVDFREYEYD	17 AA
7	*L*TGSSDNEYFYVDFREY	17 AA
8	not done	
9	YESQLQMVQVTGSSDNEYFYVDFREY	26 AA
10	GSSDNEYFYVDFREYEYDLKWEF	23 AA

Exon 11 wild type
QFRYESQLQMVQVTGSSDNE[YFYV]DFRE[YEYDLK]WEFPRENLEF

Of the 10 patients positive for Flt3/ITD's by PCR the exact nature of the mutations was determined by sequence analysis of exon 11 of the Flt3 gene. We were able to analyse 9 out 10 patients. This table shows the duplicated amino acid sequence found in these patients. *L* in patient 7 depicts a valine to leucine mutation in the duplicated sequence. Boxed A.A. sequences in the wild type sequence represent possible SH2 binding domains which might be involved in binding of SH2-containing cytoplasmic signalling proteins.

Effect of the Flt3 mutation on the production of non-adherent cells

At week one of culture only few samples showed a non-adherent (NA) nucleated cell production above the input value of $1x10^5$ cells/well (Table 3). With increasing culture time the various samples showed an increased variability in cumulative NA cell production. This is most clear in Il-3 and G-CSF containing cultures at week eight where the lowest cumulative NA cell production, (patient 3) was equal to the input value whilst the highest (patient 11) was $3.9x10^{11}$ cells/well. In the absence of exogenous cytokines we observed a distinct group of samples with a significant ($0.030 > p > 0.001$) higher

Table 3: Cumulative non-adherent nucleated cell production

Patient	No GF			Il-3, G-CSF			Il-6, SCF, TPO, Flt3l		
No.	*1	3	8	1	3	8	1	3	8
1	@0.1	0.2	2.2	0.3	0.3	-	0.7	1.1	47
2	0.1	0.1	2.8	0.1	0.1	-	0.1	0.1	7
3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
4	0.1	0.3	8	0.1	0.2	-	0.1	0.8	2400
5	0.1	0.9	20	0.1	1	740	0.1	1.6	16
6	0.1	1.4	24	0.1	2	210	0.1	1	43
7	0.1	1.5	8.5	0.1	2	25	0.1	0.3	28
8	0.1	0.1	7.7	0.1	0.73	140	0.2	0.3	10
9	0.1	0.1	14	0.1	0.1	14	0.1	0.9	110
10	0.1	2.4	-	0.1	3.4	460	0.1	3.7	480
11	0.3	4	13000	0.2	37	390000	0.1	36	-
12	0.1	8.7	21000	0.1	12	66000	0.1	29	6600
13	0.1	2.7	35000	0.1	11	-	0.1	38	14000
14	0.1	10	33000	0.1	4.8	34000	0.1	6.2	-
15	0.1	1.6	17	0.1	5.4	7800	0.2	2.4	4400
16	0.1	0.5	49	0.1	0.9	50	0.1	0.8	54
17	0.1	0.3	12	0.2	3.3	10	0.1	1.3	4.1
18	0.1	0.3	9.1	0.1	0.3	14	0.2	0.5	13
19	0.1	0.7	9.6	0.2	1.1	12	0.6	1	8.7
20	0.1	0.3	13	0.1	1.1	14	0.1	1.5	25
21	0.1	11	-	0.2	72	-	0.1	56	-
22	0.1	0.8	-	0.1	0.4	-	0.1	0.7	-
23	0.1	0.3	37	0.1	1.9	15	0.1	0.4	22
24	0.1	0.2	24	0.1	5.2	54	0.13	1.1	22
25	0.1	0.2	19	0.1	2.7	35	0.15	0.8	31
26	0.1	2.1	53	0.1	23	579	0.60	65	493
27	0.1	3.5	210	0.1	6.3	320	0.1	4	33
28	0.1	6.5	56	0.7	16	50	3.6	5.6	40
29	0.1	0.8	-	0.1	0.9	43	0.1	35	54
30	0.1	0.8	-	0.1	0.2	146	0.1	3.1	107

Cultures were initiated with 10^5 nucleated cells/ well. Weekly half of the medium was replaced and non-adherent nucleated cells were counted. *culture time (weeks); @ cumulative non adherent cell production per well ($x10^{-6}$); - time point not reached due to loss of culture (infection or detachment of the stromal layer)

cumulative NA cell production. This group was comprised of patients 9 to 14 which all had a trisomy of chromosome 8 as a common aberration. At weeks three and eight the cumulative NA cell production by the Flt3 mutant samples was significantly reduced in the groups without addition of growth factors and in those containing Il-3 and G-CSF ($0.05 > p > 0.001$). This reduction was also observed in the groups cultured with Il-6, SCF, TPO and, although only significantly at week three.

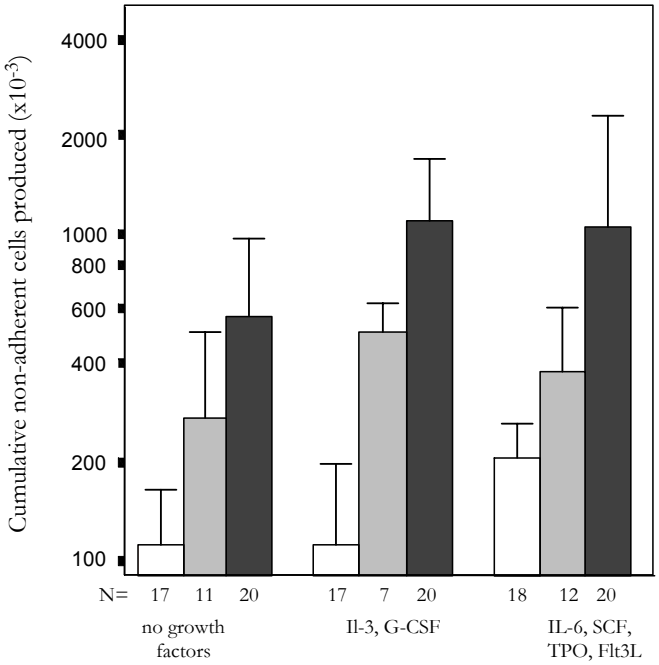

Figure 1: Effect of culture condition on the cumulative non-adherent nucleated cell production during three weeks of culture.

All cultures were initiated with 10^5 nucleated cells/ well. Cells were either cultured in: SCGM without support of a stromal layer and without serum (open bars); SCGM with support of a stromal layer but without serum (grey bars) or in IMDM with support of a stromal layer and serum (black bars). Values represent mean +/- SEM, N depicts the number of AML samples tested.

The weekly NA cell expansion, which is defined as the fold-increase between two weekly counts (Fig. 2), enables a better understanding of the kinetics of the cell production. Expansion is maximal on weeks 2 and 3, irrespective of the growth factors used. At this time point the expansion in the non-mutant population is significantly higher than in the mutant population ($0.05 > p > 0.02$) when cultured either with the addition of Il-3 and G-CSF or without cytokine addition. No significant differences were found in the Il-6, SCF, TPO and Flt3l stimulated groups at week 3 but at week 2 the mutant population showed less expansion than the non-mutant population ($p=0.03$). The analysis was repeated excluding the trisomy 8 samples as these are observed to grow more vigorously than the other samples (see table 3). Though this exclusion reduced the significance ($0.05>p>0.04$, data not shown) the differences between non-mutant and mutant groups remained.
A selection of the samples according to their FAB classification (Fig. 3) allowed a further analysis of the responses to cytokine addition between the mutant and non-mutant groups. Although the effect of cytokine addition was visible throughout all FAB classes we also noted different cytokine responses between samples of specific FAB classes. In cultures that included Il-3 and G-CSF we observed a large expansion of the

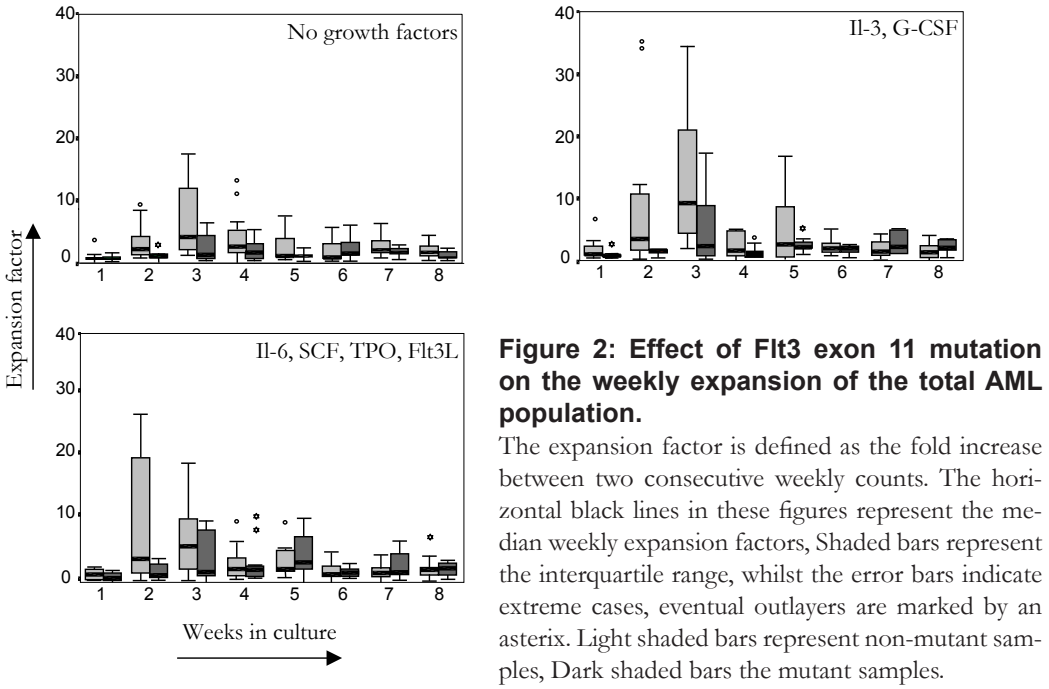

Figure 2: Effect of Flt3 exon 11 mutation on the weekly expansion of the total AML population.

The expansion factor is defined as the fold increase between two consecutive weekly counts. The horizontal black lines in these figures represent the median weekly expansion factors, Shaded bars represent the interquartile range, whilst the error bars indicate extreme cases, eventual outlayers are marked by an asterix. Light shaded bars represent non-mutant samples, Dark shaded bars the mutant samples.

non-mutant samples in the M1, M2 and M5 groups. Remarkably the Flt3 mutants within these groups did not respond. In the two-week cultures that contained Il-6, SCF, TPO and Flt3l a clear response of the non-mutant samples was seen in FAB classes M0, M1, M2 and M4. The mutant samples showed no significant response to the addition of cytokines in any of the FAB classes.
As the subdivision of AML into FAB classes is mainly based on morphological and cytological criteria, we also classified the samples according to their cytogenetic aberrations. As is apparent from Table 1, most cytogenetic aberrations are present in a low frequency. To allow for analysis we tentatively defined groups that contain multiple aberrations, i.e.: (A) samples without any known aberrations; (B) samples exclusively containing one or more deletions; (C) samples exclusively containing additions; (D) translocations; (E) samples containing combinations of additions and deletions and (F) samples containing additions and translocations. Most samples were present in the groups with either no or a single type of aberration.
At week two of culture, proliferative responses to cytokine addition were almost exclusively observed in the non-mutant samples within the cytogenetics groups B, C, D and E (Fig. 4). Group B responded dramatically to Il-3 and G-CSF (median increase 18 fold) whilst group E showed a large response in the presence of Il-6, SCF, TPO and Flt3l. Groups C and D responded more or less equally to both cytokine combinations. Again, in none of the groups the mutant samples responded to cytokine addition. After three weeks of culture only limited response to cytokine addition was observed in the different cytogenetic groups. However in group A, lacking cytogenetic aberrations, the response to cytokines increased when compared to week two. Again no significant responses of the mutant groups were seen with the exception of cytogenetics group C when cultured in the presence of Il-6, SCF, TPO and Flt3l and in group D where the mutant samples showed a higher median expansion compared to the non-mutant samples at this time point.

2.5. Discussion.

To our knowledge this is the first report suggesting a proliferative defect of AML cells carrying internal tandem duplications in exon 11 of the Flt3 gene. While non-mutant samples showed increased NA cell production in response to cytokine addition especially in week two and three of stroma-supported cultures, Flt3 mutant cells failed to do so. Other receptor mutations have been reported to have severe effects on proliferation or differentiation as well. As an example, specific truncations of the cytoplas-

mic domain of the human G-CSF-receptor gene cause almost complete abrogation of granulocytic differentiation and are associated with a propensity of affected individuals towards myelodysplasia and leukemia[25,26].

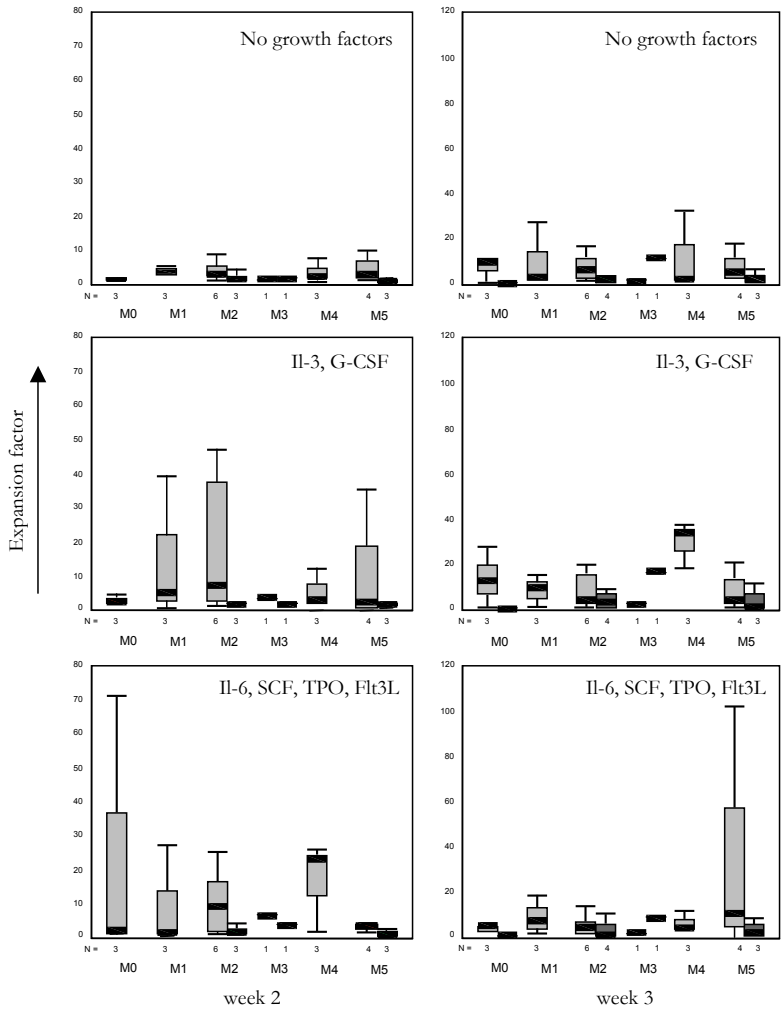

Figure 3: Effect of the Flt3 exon 11 mutation during weeks 2 and 3 of culture in relation to FAB-classification.

The horizontal black lines in these figures represent the median weekly expansion factors, Shaded bars represent the interquartile range, whilst the error bars indicate extreme cases, eventual outlayers are marked by an asterix with the sample number.

Numbers below the x-axis depict the number of cases within each group.

Light shaded bars represent non-mutant samples, Dark shaded bars the mutant samples.

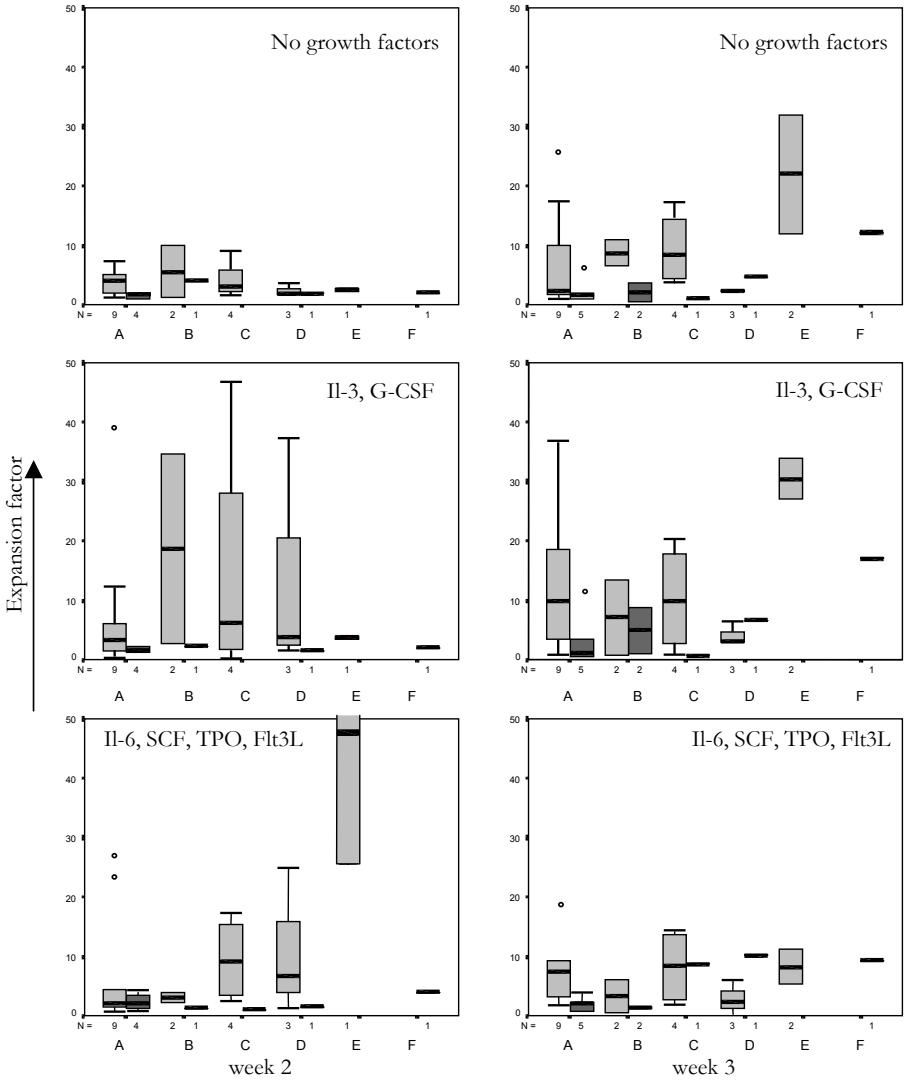

Figure 4: Effect of the Flt3 exon 11 mutation during weeks 2 and 3 of culture in relation to their cytogenetic background.

Cytogenetic groups: (A) normal; (B) deletions; (C) additions; (D) translocations; (E) deletions and additions; (F) additions and translocations. The horizontal black lines in these figures represent the median weekly expansion factors, Shaded bars represent the interquartile range, whilst the error bars indicate extreme cases, eventual outlayers are marked by an asterix with the sample number.

Numbers below the x-axis depict the number of cases within each group.

Light shaded bars represent non-mutant samples, Dark shaded bars the mutant samples.

In our study we have attempted to see whether the observed low proliferative ability and unresponsiveness of Flt3 mutant samples to exogenous cytokines is related to either FAB class or cytogenetic aberrations. As we did not observe such a relation, we interpret this as an indication that the Flt3 gene ITD's are the prime cause of the defective proliferative response rather than another defect in a specific AML subclass. We realise that all M3 and most M2 samples are included in the cytogenetic translocation group and that alternative classifications may, or may not, reveal a possible linkage of Flt3 gene ITD's with an AML subtype.

It is surprising that the cells carrying the Flt3/ITD's, in addition to Flt3L, showed unresponsiveness to a series of cytokines, including Il-3, Il-6, G-CSF, SCF and TPO. Our initial experiments indicated that AML cells could only be propagated ex vivo for a short time in serum-containing medium complemented with these cytokines, while stromal support was required for long-term cell production. Although stromal cells elaborate low concentrations of some of the cytokines employed in our study (i.e. Il-6, SCF and Flt3L), the stromal support is presumably contributing to a different presentation of these growth factors (e.g. glycosaminoglycans), or the elaboration of alternative cytokines, inhibitors and/or expression cell-adhesion molecules.

Our study does not explain the mechanism by which ITD's in the Flt3 receptor gene cause a defect in the proliferative response of mutant AML cells. It seems, however, plausible to conclude that these mutations do not only affect the response to the Flt3L, but in addition that to other cytokines. This effect of the Flt3/ITD's is unlikely caused by a defect in a common intracellular signalling cascade may rather be the result of either a disturbed synergy between Flt3 and other cytokines, or a disturbance in the sequence of cytokine signals which should lead to proliferation. As far as the limited number of analyses permit us to speculate, Flt3 mutant samples grow less well on a stromal layer in the absence of exogenous cytokines than do non-mutant samples (Fig. 3a and 4a), an observation suggesting that AML cells need a wild-type Flt3 receptor for optimal proliferation. Alternatively, if the Flt3 mutant samples would contain relatively less AML progenitor cells than do normal samples, this may lead to reduced cell production and therefore an apparently diminished cytokine response. This alternative could be substantiated by assessing the actual number of AML stem and progenitor

cells present in the starting population by leukemic CAFC and LTC assays[27,28] which will be included in a subsequent study.

Another interesting observation was that AML samples with trisomy of chromosome 8 showed a much higher proliferative activity than did other AML samples, irrespectively of the presence of a Flt3 gene ITD. A possible explanation for this observation is the over-expression of c-myc located on chromosome 8q24. Over-expression of c-myc has been described for immortalised tumor cell lines, while c-myc down-regulation in these cells often leads to differentiation[29-32]. This observation suggests that selection of a small AML subset may strongly bias the results obtained.

The presently described functional defects in AML cells caused by Flt3 gene ITD's may contribute to our understanding of how a single cytokine receptor gene defect may influence the response to other cytokines and the potential role this might have in the development and progression of leukemia.

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CHAPTER 3

Biological characteristics and prognosis of adult acute myeloid leukemia with internal tandem duplications in the Flt3 gene

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3.1. Abstract

Internal tandem duplications of the Flt3 gene (Flt3/ITDs) are present in about 18 percent of all AML cases and are therefore one of the most frequent somatic gene mutations in AML. Little is known about the role of Flt3/ITDs in leukemogenesis or their clinical relevance. In this study we compared 18 samples with Flt3/ITDs and 63 AML samples without these mutations with respect to clinical prognosis, cytokine responsiveness, progenitor cell content and repopulation in the NOD/SCID mouse. We found that in patients with a mutation CR rates are reduced ($p=0.03$) and relapse rates are increased ($p=0.01$), indicating the prognostic importance of Flt3/ITDs. This is also emphasized by the finding that in patients under the age of 60 as well as in older patients the event-free survival was more unfavourable for the mutant patients ($p=0.003$ and $p=0.03$ respectively).

At diagnosis Flt3/ITD and non-mutant AML bone marrow samples did not differ in their progenitor/stem cell frequencies. Cobblestone area forming cell (CAFC) subsets showed a similar frequency distribution in mutant and non-mutant samples.

In 7-day liquid cultures, Flt3/ITD samples showed a reduced growth in response to a variety of myeloid growth factors. In contrast, Flt3/ITD samples displayed a higher ability to engraft the NOD/SCID bone marrow with leukemic cells.

Together these data show that the Flt3/ITD represents an important diagnostic marker for patient prognosis, and that the presence of these mutations is associated with altered proliferative ability of progenitors in vivo and in vitro.

3.2. Introduction

The fms-like tyrosine kinase 3 (Flt3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2)[1-4], belongs to the group of class III receptor tyrosine kinases (RTKs), which also include the receptors c-kit and c-fms. Signals generated by ligand-induced dimerisation of these receptors involve tyrosine phosphorylation of certain regions of the receptor and activation of cellular tyrosine kinases. Whilst a large proportion of acute myeloid leukemias (AML) have been found to express class III RTKs[5-8] their potential role in leukemia is still unclear. Flt3 ligand (Flt3l) and Flt3 are both expressed on precursor-B ALL and AML cells[9-12] as well as on normal progenitor cells[13-16].

In 17-20% of AML patients in the Japanese[17] and west-European[18] population, as well as in patients with leukemic transformation of myelodysplasia[19], internal tandem du-

plications (ITDs) have been reported in the part of the Flt3 gene coding for the juxtamembrane (JM) through first tyrosine kinase (TK-1) domains of the protein. These types of mutations have not been found in acute lymphoid leukemia(ALL), chronic myeloid leukemia (CML), blast crisis CML, nor have they been reported in cells of normal individuals. In ALL Flt3/ITDs have only been reported in two patients which at closer examination were considered to be biphenotypic leukemia[20].

Though Flt3/ITDs are also observed in childhood AML their frequency is significantly lower when compared to adult AML. Frequencies reported for the Japanese childhood AML population vary between 11% (Kondo et al., 7 out of 64 cases studied)[21] and 5.3% (Iwai et al., 5 out of 94 cases studied)[22]. A possible explanation for the observed lower incidence in childhood AML could be that Flt3/ITDs results from genomic instability as was postulated by the latter authors.

Kiyoi et al.[23] have shown that in COS-cells, transduced with cDNA encoding for Flt3/ITDs, elongation of the JM domain and ligand-independent dimerisation of the mutant receptor occurs, thus resulting in a constitutive activation. This effect was seen in both mutant/mutant and in mutant/wildtype dimers of the receptor. Immunoprecipitation studies of those mutant receptors showed evidence for a conformational change in the C-terminal region of the mutant receptor.

Recently we have shown[18] that in comparison to non-mutant AML cells, cells with Flt3/ITDs have a reduced proliferative ability in stroma-supported cultures. This effect could not be abrogated by the addition of cytokines to these cultures. We have now investigated whether the presence of Flt3/ITDs in AML cases within the adult population correlated with (A) their short-term proliferative response to single cytokine stimulation; (B) the ability of these cells to engraft in the bone marrow of immunodeficient mice (NOD/SCID)[24-26] and (C) the frequency of leukemic progenitor and stem cells, as measured by a leukemic cobblestone area forming cell assay (L-CAFC)[27,28]. Furthermore, we have analysed whether the presence of Flt3/ITDs in AML in the Dutch population of patients predicts prognosis, as was recently published for the Japanese population[29].

3.3. Materials and methods

Patient samples

Diagnosis bone marrow samples of patients with AML were obtained after informed consent. Cases were classified according to the French-American-British (FAB) committee recommendations[30]. Mononuclear cells were isolated by ficoll separation fol-

lowed by T-cell depletion, typically resulting in a population containing over 95 percent blasts. After isolation cells were subjected to controlled freezing and stored in liquid nitrogen. Before use cells were thawed by stepwise dilution in Iscoves Modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands) containing 1% BSA. After thawing viability varied between 70 and 90 percent as assessed by dye exclusion.
After diagnosis patients were treated according to studies of the adult Dutch-Belgian Heamato-Oncology Group HOVON. Patients were treated according to protocols HOVON-29 (less then 60 yrs of age, 27 patients)[31], HOVON-4 (less then 60 yrs.,12 patients)[32], HOVON-31 (60 yrs+, 13 patients), AML-11 (60 yrs+, 12 patients)[33] protocols. Six patients received palliative treatment only and were therefore excluded form the survival analysis. In these protocols induction therapy included an anthracyclin and cytarabine and in patients less then 60 yrs allogeneic and autologous stem cell transplantation or intensive chemotherapy for consolidation were applied as post-remission therapy. In the Hovon-29 study induction therapy consisted of Ara-C (200 mg/m^2) for 7 days combined for the last three days with Idarubicin (12 mg/m^2) (Cycle I) followed by a second cycle consisting of Ara-C (6 days, 1000 mg/ m$^{2)}$ combined with Amsacrine (days 3-5, 120 mg/ m^2). Half of the patients according to randomizing received G-CSF (120 μg/m^2, s.c.) during both cycles. Within the Hovon-4 protocol the induction therapy was equal to the Hovon-29 protocol except for the fact that in cycle I Daunomycin (45 mg/m^2) was used instead of Idarubicin. For the Hovon-31 protocol induction therapy consisted of Ara-C 200 mg/m^2 for 7 days combined for the first three days with Daunomycin (45 mg/m^2) in arm 1 of the study, or Daunomycin (35mg/m^2) + PSC833 (10 mg/kg) in arm 2. Finally, in the AML-11 study induction therapy consisted of Ara-C 200 mg/m^2 for 7 days combined with Daunomycin 30 mg/kg on days 1-3.

Stratification according to karyotype-risk groups

As cytogenetic aberrations are thought to be one of the most important prognostic factors for AML[34,35] patients were stratified in three groups i.e. a good-risk group consisting of patients defined by a karyotype of t(8;21), t(15;17) or inv(16); a poor-risk group defined by del(5), del(7), or deletions of the q-arms of these chromosomes, t(6,9), alterations in the 11q23 region or the presence of multiple aberrations (more than 3) in the karyotype. All other karyotypic aberrations and the normal karyotype were included in an intermediate-risk group.

Determination of Flt3 mutants

All AML samples were analysed for mutations in exons 11 and/or 12 of the Flt3 gene by using a modified PCR procedure as described elsewhere17
The use of exon 11 and 12 specific primers allowed us to cover the whole JM and the first part of the TK-1 domain where most of the reported mutations are located.
Genomic DNA was prepared using a standard procedure36. Briefly, DNA from about 104 cells was amplified in a total of 50 µl reaction mixture, containing single strength PCR buffer, 10 pM of each primer, 10 mM dNTPs and 0.5 U Taq polymerase (Supertaq, SpheroQ, Leiden, The Netherlands). Preheating of the samples at 95oC was followed by amplification for 35 cycles consisting of: 1 min. at 43 oC, 1 min. at 72 oC, 1 min. at 95 oC. Following these cycles a final extension at 72 oC was performed for 10 min. PCR products were stained with SYBR green I (Molecular Probes, Leiden, The Netherlands) and resolved on 3% agarose gel. As a confirmation samples positive for a Flt3-mutation were reanalyzed in a second PCR using the primers 11F and 12R.

Sequencing of patient samples with a mutated Flt3 gene

If both PCR reactions using the 11F/11R and 11F/12R combinations resulted in an abnormal PCR product, this was purified from the gel using a JETsorb kit (Genomed, Bad Oeyenhausen, Germany). This purified PCR product was subjected to a second PCR using fluorescent ddNTP's after which sequencing was carried out using an automated DNA sequencing system (ABI PRISM 310 genetic analyzer, Perkin Elmer Applied Biosystems, Foster City, CA, USA).

Short-term proliferative response to single cytokine stimulation

To determine the proliferative response of AML samples to single cytokine stimulation we used a thymidine incorporation assay. As monocytes are known to produce many cytokines and thus potentially may perturb the results we removed these cells prior to their use in culture by adhesion to plastic (1hr incubation at 37°C in alpha-MEM (Gibco) + 1% BSA (Sigma, St. Louis, MO, USA). The non-adherent cells were plated in 96-well plates at a density of $2x10^4$ cells/well in serum free medium[37] and cultured for six days. The cytokines used were either hIL-3 (25ng/ml, Genetics Institute, Cambridge, MA, USA.), hGM-CSF (10ng/ml, Immunex, Seattle, WA, USA), hG-CSF(100ng/ml, Amgen, Thousand Oaks, CA, USA), hM-CSF(100 U/ml, Genetics Institute) or hSCF(100 ng/ml, Amgen). After six days the cells were incubated overnight with 0.1 µCi tritiated-thymidine (Amersham, Buckinhamshire, UK) per well and

harvested onto nitrocellulose filters. Thymidine incorporation was determined using a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden) as counts per minute (CPM). All cultures were done in triplicate.
The stimulation index was calculated by dividing the mean CPM of the triplicate cultures and the mean CPM of a 30Gy irradiated control of the sample.

Leukemic Cobblestone area forming cell assay

The cobblestone area forming cell assay was performed as described[38]. Briefly, confluent FBMD-1 stromal layers in 96-well plates (Costar, Cambridge, MA) were overlaid with AML cells in a limiting dilution setup. The cells were cultured in medium consisting of IMDM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Summit Biotechnology, Fort Collins, Co.), 5% horse serum (Gibco), β-mercaptoethanol ($5x10^{-5}$ mol/l) and hydrocortisone-21-hemisuccinate (10^{-6} mol/l, Sigma). rhIl-3 (8 ng/ml) and rhG-CSF (20 ng/ml) (both a generous gift of the Genetics Institute) were added to the medium and all cultures were performed at 37°C and 10% CO_2 in a humidified atmosphere. Input values were equivalent to $1x10^5$ nucleated cells/well and 12 dilutions three fold apart were used for each sample with 15 replicates per dilution. The percentage of cells wells with at least one phase-dark clone (Cobblestone area, consisting of at least 5 cells) beneath the stromal layer was determined weekly and leukemic CAFC (L-CAFC) frequencies were calculated using Poisson statistics[39].
Based on evidence from murine correlation studies it is assumed that both normal and leukemic cobblestone areas that are observed late in culture are indicators of primitive long-term repopulating cell in vivo, whilst early appearing clones are indicators of transiently repopulating and less primitive cells.

The NOD/SCID mouse model

Among a variety of immunodeficient mouse strains we and others have evaluated the nonobese diabetic (NOD) /LtSz scid/scid strain appears to be most useful in allowing the engraftment of human AML progenitors[40,41]. In contrast to the CB17/SCID strain, which is only defective in B- and T-cell function, this strain is characterized by an additional functional deficit in NK-cell function as well as defective antigen-presenting cells and an absence of circulating complement.
NOD/SCID mice were bred in the Erasmus animal facility and housed under specified pathogen free conditions, using laminar air flow units. Mice were supplied with

sterilized food and acidified tap water to which 100mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) was added. Both food and drink were available ad lib.
Before transplantation of the AML cells the mice received a total body irradiation of 3.5 Gy gamma radiation (^{137}Cs source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). Transplantation of the AML cells was done by injecting 3x10^7 AML cells in the lateral tail vein. After 30 days the mice were sacrificed by CO_2 inhalation in accordance with the institutional animal research regulations.
Cell suspensions were made from both femora and used for flowcytometry.

Flowcytometry

To determine the percentage of AML cells in bone marrow cell suspensions from mice that had been transplanted, cells were stained with antibodies against the human common leukocyte antigen (CD45), CD34, CD33 and CD38 (CD45-FITC, CD38-FITC, CD33-PE and CD34-APC, all obtained from Coulter Immunotech, Marseille, France) and measured using a FACScalibur (Becton Dickinson Immunocytometry systems, San Jose, CA, USA). Analysis was done using the Cell Quest software package (Beckton Dickinson). Erythrocytes were excluded from analysis by gating on forward and perpendicular light scatter whilst dead cells were excluded by staining with 7-amino-actinomycin D (7-AAD, Molecular Probes, Eugene, OR, USA). Human cells were considered to have engrafted when the percentage of CD45-positive cells in the mouse bone marrow was at least 0.5%. Because, in contrast to human umbilical cord blood cells, almost all samples expressed over 90% CD33 either in combination or absence of CD38 we considered them to be of leukemic origin. The only samples not abundantly expressing CD33 were the AML-M0 samples which, after passage in NOD/SCID, could be characterized by a high expression of CD34 (50-100 %).

Statistical analysis

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il.). Significance of differences for the proliferation, L-CAFC and SCID assays was determined by the non-parametrical Mann-Whitney U test. The Fisher's exact test for 2x2 tables was used to evaluate complete remission (CR) and relapse rates. For the survival analysis the Kaplan-Meier method of the same software package was used. The Breslow test was used to check for equality of the survival distributions. Overall survival was calculated from date of diagnosis to date of (leukemia-related) death. The leukemia-free survival (LFS) was calculated from date of diagnosis to date

of relapse or death. Patients not achieving CR we scored as having an LFS of 0.01. Patients dying from non-leukemia related causes during first CR were included until date of death. In all evaluations values were considered significant if p-values were below 0.05.

3.4. Results

Characterization of patients

A total of 81 diagnosis samples (bone marrow) from patients with AML were screened for the presence of a Flt3 mutation. Of the samples 18 patients (22%) were found to have such a mutation.

All Flt3/ITD mutations found were in the exon 11 region of the gene whilst none were found in exon 12. However, sequence analysis showed an involvement of one or more amino acids of exon 12 in two of the exon 11 mutant samples (see below). As previously shown[18,42] the mutation was randomly distributed over the FAB classes while no correlation with any known cytogenetic aberration was observed. In most of the Flt3/ITD positive patients the mutation was present as a heterozygous mutation but in 4 cases the ITD appeared as a single aberrant band on gel, indicating the presence of either as a homozygous mutation or, more likely, a hemizygous deletion of the wildtype gene.

The characteristics of 18 AML-patients with the mutation and 63 AML-patients without such a mutation included in this study are shown in Table 1.

Sequence analysis of the Flt3 mutant samples (Table 2) showed tandem duplications varying in length from 10 to 35 amino acids (30-105 bp). All mutations were restricted to the JM domain of the receptor. All ITDs included the duplication of at least one putative SH2 binding motif (YFYV and / or YEYDLK). Four patients additionally showed mutations in the amino acid (AA) sequence. These mutations were either in the starting or terminal AA of the duplicated sequence. The mutation in patient 1 was ending with a mutation of the last two AA's i.e. tyrosine and aspartate to serine and proline, respectively. The mutation in patient number 7 was valine to leucine, in patient 15 lysine to threonine, and in patient number 16 phenylalanine to leucine. Two patients showed an involvement of the first (patient 14) or first and second (patient 12) AA of exon number 12.

Table 1: Patient characteristics

		Flt3/ITD AML	Flt3/wildtype AML
Total number		18	63
Age	Mean (yr.)	52.1	50.3
	Range (yr.)	25-73	4-88
	<= 60 yr.	10	36
Gender	Male	7	34
	Female	11	29
WBC at diagnosis ($x10^{-9}$)	Mean	125	62
	Range	2-319	1-343
FAB Classification	Unclassified	0	2
	M0	1	4
	M1	2	11
	M2	4	15
	M3	2	4
	M4	3	14
	M5	6	13
Cytogenetic risk group*	Good prognosis	2	6
	Intermediate prognosis	16	50
	Poor prognosis	0	6
Background of AML**	De novo AML	14	45
	Secondary	2	9
	Relapse	2	3
	Unknown	0	6
Induction Therapy Response***	CR	8	43
	Failure	9	10

*Cytogenetic stratification was performed according to the criteria described in the materials and methods section. **Patients were devided into four classes on basis of their AML background i.e. de Novo AML, if the AML was not preceded by another hematopoeitic disorder; Secondary, if the AML originated from a previous hematological disorder such as MDS, or if the patient had a previous history of exposure to chemo- or radiotherapy; Relapse, if the sample was from a patient with a leukaemia relapse; unknown, if non of the other cases applied. *** For a total of 70 patients we were able to define their response to therapy. Patients were divided in two groups: patients who obtained complete remission (CR) and patients who either had a partial or no response to therapy (Failure).

Table 2: Sequence analysis of the Flt3/ITDs

Patient No.	duplicated amino acid sequence	Length
1	YFYVDFREYE*SP*	12AA
2	QLQMVQVTGSSDNEYFYVDFREYEYD	26 AA
3	QLQMVQVTGSSDNEYFYVDFREYEYD	26 AA
4	DFREYEYDLKWEFPRENLEF	20 AA
5	QLQMVQVTGSSDNEYFYVDFRE	22 AA
6	SSDNEYFYVDFREYEYD	17 AA
7	*L*TGSSDNEYFYVDFREY	17 AA
8	Not done	
9	YESQLQMVQVTGSSDNEYFYVDFREY	26 AA
10	GSSDNEYFYVDFREYEYDLKWEF	23 AA
11	REYEYDLKWEFPRENLEF	18 AA
12	QVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF*GK*	35 AA
13	YESQLQMVQVTGSSDNEYFYVDFRE	25 AA
14	VTGSSDNEYFYVDFREYEYDLKWEFPRENLEF*G*	33 AA
15	GSSDNEYFYVDFREYEYDL*T*	20 AA
16	QVTGSSDNEYFYVDFREYEYDLKWEFPRENLE*L*	33 AA
17	VTGSSDNEYFYVDFRE	16 AA
18	GSSDNEYFYVDF	22 AA

Exon 11 wild type
QFRYESQLQMVQVTGSSDNEYFYVDFREYEYDLKWEFPRENLEF

Of 17 out of 18 patients positive for Flt3/ITDs by PCR the exact nature of the mutations was determined by sequence analysis of exon 11 of the Flt3 gene. This table shows the duplicated amino acid sequence found in these patients. Amino acids printed in italics reflect mutations (patients 1, 7, 15 and 16) or involvement of exon 12 (patients 12 and 14). Boxed AA sequences in the wild type sequence represent tentative binding domains for SH2-containing intracellular signalling proteins.

Clinical response and prognosis of AML patients with and without Flt3/ITD

All 70 patients of whom we were able to obtain therapy response data were evaluated for their initial therapy response. From Table 1 it can be deduced that the CR rate of 8/17 (47.1%) patients in the population with Flt3/ITD was significantly reduced when compared to that of 39/49 (79.6%) patients in the population without Flt3/ITDs ($p=0.03$). At a median follow up period of 23 months in the group without ITDs

10 of the 39 (25.6%) patients who had achieved CR had relapsed, whilst 6 out of 8 (75.0%) patients with ITDs had relapsed (p=0.01).
The overall survival (OS) curves (Fig. 1A) and leukemia-free survival (LFS) curves (Fig. 1B) clearly suggest a significant reduction in CR rate and prognosis by the presence of Flt3/ITD in AML (p=0.002 for OS and 0.001 for LFS). Details for the LFS of the mutant population are shown in table 3. As we are aware that a bias can be introduced by comparing the total heterogeneous AML population, we also looked at the LFS for the two largest sub-populations that could be distinguished within our dataset, i.e. patients with de novo AML (Fig. 1C) and patients with an intermediate-risk karyotype (Fig. 1D). In both cases the differences still remained significant (p= 0.004 and p=0.008 respectively).

Table 3: Kaplan Meier Survival Table of the leukemia-free survival of the AML patients with Flt3/ITDs

Patient No.	Time to event (Months)	Cause of death	Cumulative EFS**	Standard Error of EFS**
1	0.01	Induction failure		
2	0.01	Induction failure		
3	0.01	Induction failure		
4	0.01	Induction failure		
5	0.01	Induction failure		
6	0.01	Induction failure		
7	0.01	Induction failure		
9	0.01	Induction failure		
10	0.01	Induction failure	0.4706	0.1211
11	1	Relapse	0.4118	0.1194
12	2	Relapse	0.3529	0.1159
13	7	Relapse	0.2941	0.1105
14	11	Alive		
15	12	Relapse	0.2206	0.1045
16	15	Relapse	0.1471	0.0920
17	16	Dead CR1		
18	20	Relapse	0.0000	0.0000

* The survival indicator is the status of the patient at that time point i.e.: CR = patient still in complete remission; Dead CR1 = patient died of non-leukemic disease during the first complete remission. ** EFS = Event Free Survival
No survival data could be obtained for patient nr. 8.

As also age is known to be an important prognostic factor we divided the samples in two groups according to age (Fig. 2). In both the age group of under 60 yr. (Fig. 2A) and over 60 yr. (Fig. 2B) the differences between the Flt3/ITD and non-mutant group with respect to LFS remained significant (p=0.003 and 0.03 respectively).

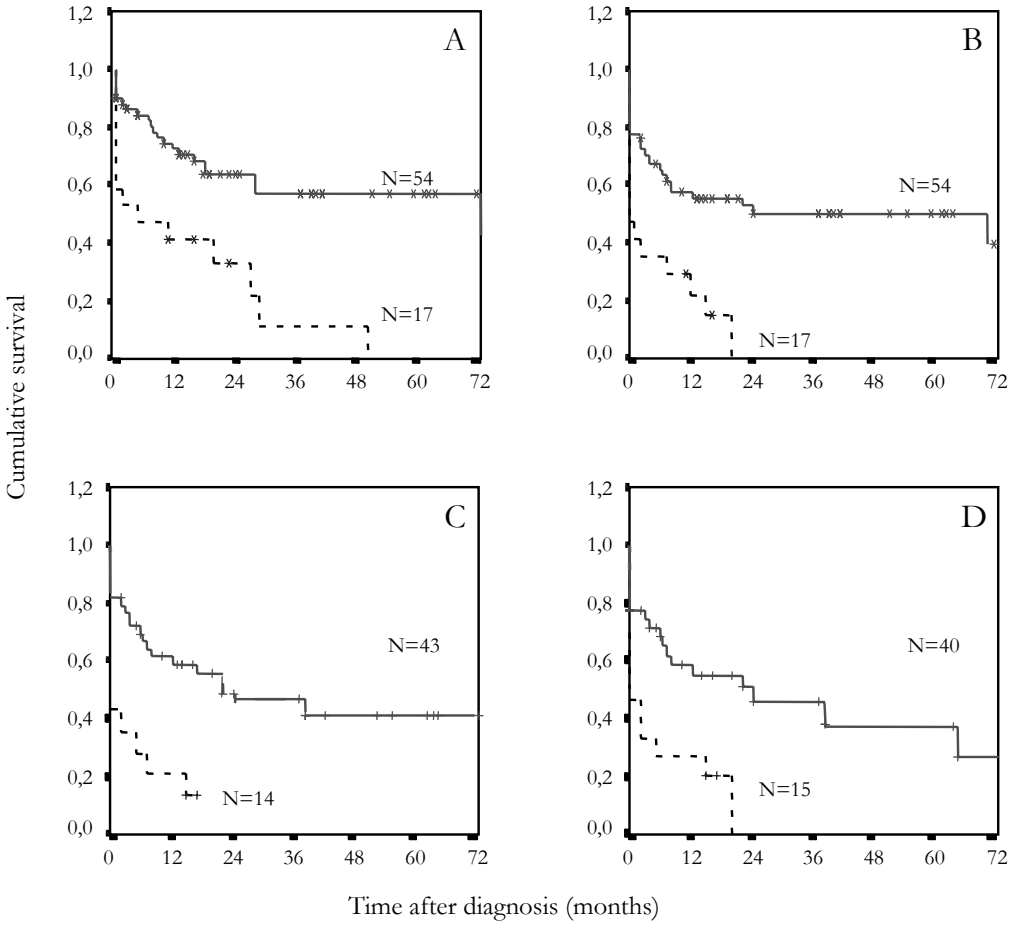

Figure 1. Kaplan-Meier survival curves of the effect of Flt3/ITDs in AML.

Solid lines, Flt3/ITD negative samples, dashed lines Flt3/ITD positive population. Censored cases are marked by an asterisk.

Panel (A) cumulative overall survival of all cases investigated; (B) cumulative leukemia-free survival of all cases; (C) leukemia free-survival based on a selection of only de novo AML; (D) leukemia-free survival of the population with intermediate-risk cytogenetics.

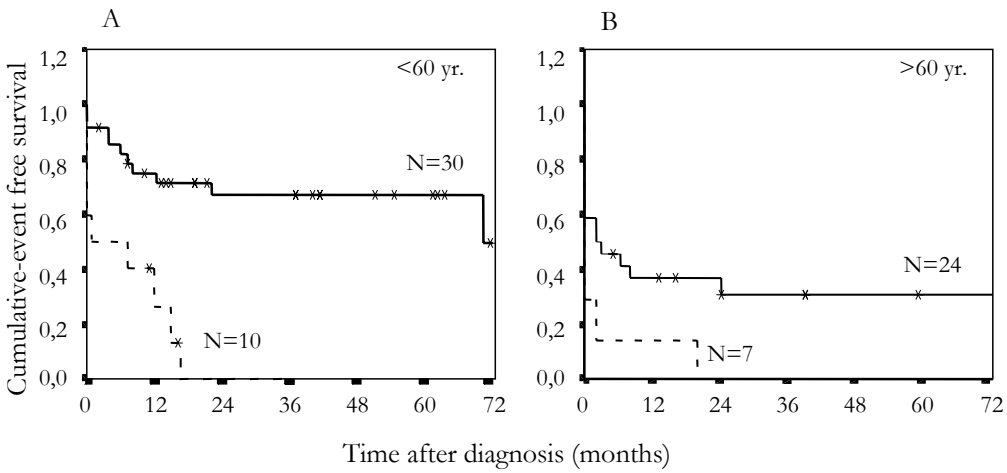

Figure 2. Leukemia-free survival in patients with and without Flt3/ITDs in patients with an age below 60 years and those older then 60 years.

Panel A, patients below 60 years of age; panel B, patients over 60 years of age. Solid lines indicate the Flt3/ITD negative samples, dashed lines represent the Flt3/ITD positive population. Censored cases are marked by a asterisk.

Short term proliferative response of AML.

We have recently shown[18] that the presence of Flt3/ITDs in AML reduces the proliferative ability of AML in stroma-supported cultures. As this was a rather unexpected finding in the light of the poor prognosis of this type of AML, we investigated whether this reduced proliferative response could also be seen in short-term cultures (7 days) without stromal support (Figure 3). We used single cytokines with a well-defined proliferative effect on the myeloid compartment of the bone marrow. As depicted by the large numbers of outlayers in both the Flt3 mutant and non-mutant samples of the de novo AML group, the cytokine response in this population is very heterogeneous. However, in almost all cases the Flt3/ITD positive samples showed a decreased response to cytokines. Statistical analysis revealed that for the combined AML population (Fig. 3A) differences were significant in response to either G-CSF, GM-CSF, Il-3 or SCF (respective p-values: 0.01 , 0.01, 0.02 and 0.03). Analyzing only the de novo AML samples (Fig. 3B) differences were significant for G-CSF, GM-CSF and SCF (respective p-values: 0.004, 0.04 and 0.05). A similar reduced response of the Flt3-mutant samples was observed when sub-dividing the samples according to FAB class or cytogenetic aberrations (data not shown).

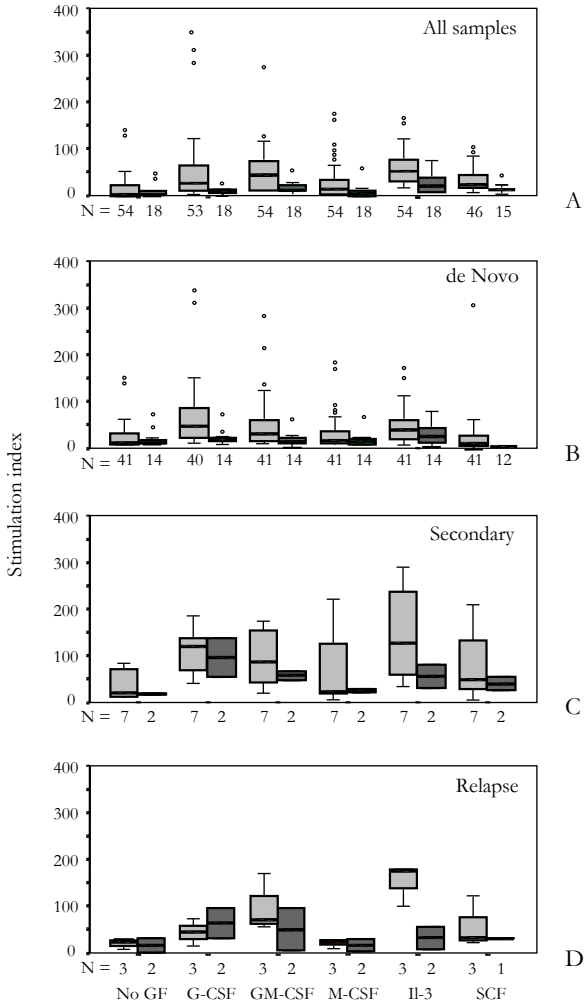

Figure 3. Effect of single cytokine stimulation on the short-term proliferation of AML cells.

Patients samples were divided into three groups on the basis of their disease history, i.e. de novo AML, Secondary or relapsed AML.

The stimulation index is defined as the fold-increase in thymidine incorporation of the tested sample over that of a 30 Gy-irradiated control of the sample.

The horizontal black lines in these figures represent the median stimulation index. Shaded bars represent the interquartile range. Error bars indicate extreme cases, whilst circles indicate outlayers.

Light shaded bars represent Flt3/wildtype samples; Dark shaded bars the Flt3/ITD samples. The number of cases within each group is depicted below the respective bars.

Frequency analysis of AML-progenitors using the L-CAFC assay

In an attempt to investigate whether the L-CAFC content of AML with Flt3/ITDs differed from samples without such a mutation, we analyzed a panel of 27 AML-samples (10 with Flt3/ITDs and 17 without Flt3/ITD). Irrespective of the presence or absence of FLT3/ITDs we did not observe significant differences in CAFC frequencies in the AML bone marrow when the samples were subdivided on the basis of FAB classification, cytogenetics or hematological background (data not shown). As shown in Figure 4 there is no difference in the frequency of the CAFC-subsets (week-types) between mutant and non-mutant samples. Both Flt3 mutant and non-mutant samples showed a decreased frequency of the later (i.e. more primitive) CAFC subsets as compared to the week 1-2 frequencies. This is in contrast to the frequency of the various CAFC subsets in normal human bone marrow and mobilized peripheral blood, which are about similar[38].

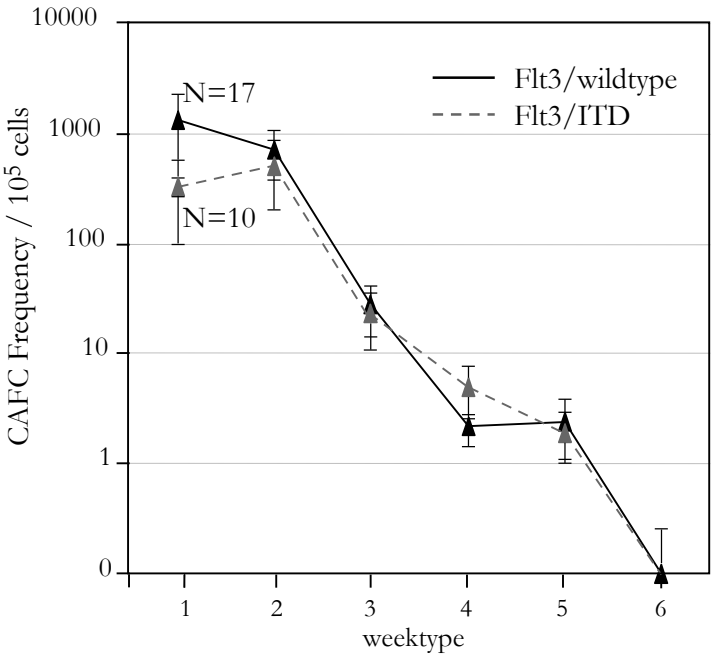

Figure 4. Effect of the presence of Flt3/ITDs on the Cobblestone Area Forming Cell frequency in AML samples.

The figure depicts the mean leukemic CAFC frequency of both the Flt3/wildtype (solid line) and the Flt3/ITD population (dashed line). Error bars indicate 1x SEM.

Not clear from Figure 4 is that there is a large heterogeneity in the CAFC frequencies of the various AML-samples. This is especially prominent at the early weeks after overlay (e.g. at week two the highest observed frequency was 4791 CAFC/10^5 cells whilst the lowest was 0.32 CAFC/10^5 cells.). This heterogeneity could not be correlated with any specific subgroup of AML (i.e. FAB class, cytogenetics or hematological background).

Outgrowth of AML cells in the NOD/SCID mouse.

The NOD/SCID mouse model was used to investigate the *in vivo* leukemia-initiating capacity of both Flt3/ITD positive and negative AML. Due to the large number of cells needed for a reproducible engraftment ($3x10^7$ cells/mouse) and the limited availability of animals we were only able to analyze a limited number of samples. Groups of 3 mice/sample were injected with AML cells and analyzed at day 30 after transplantation. We were unable to analyze samples of FAB class M3 as all samples of this type injected into NOD/SCID resulted in death of the mice within 24 hours after injection, with the highest mortality within 1 hour. This was most probably due to *in vivo* lysis of the cells.

Analysis of the percentage of engraftment in the total AML population (Fig 5A) pointed to an increased ability of the Flt3/ITD-samples to repopulate the NOD/SCID mouse bone marrow ($p=0.01$). This difference could also be observed when selecting the samples on basis of FAB classification (Fig. 5B). Whilst the non-mutant samples of the M2 and M5 type showed the lowest median repopulation of all samples investigated, the Flt3/ITD mutant samples of these FAB types showed a marked increase in human chimerism. Furthermore this figure also indicates great variability between the FAB classes where in the non-mutant population the M0 samples gave rise to the highest median level of chimerism. The most variable responses with both low and high repopulating samples were observed in the M4 FAB class. Though only a limited number ($n=4$) of Flt3-mutant samples could be analyzed, we conclude that the increased outgrowth in the NOD/SCID mice is indeed related to the presence of Flt3/ITDs as this effect can also be observed when arranging the samples according to cytogenetics (Fig. 5C) or previous hematological history (Fig. D).

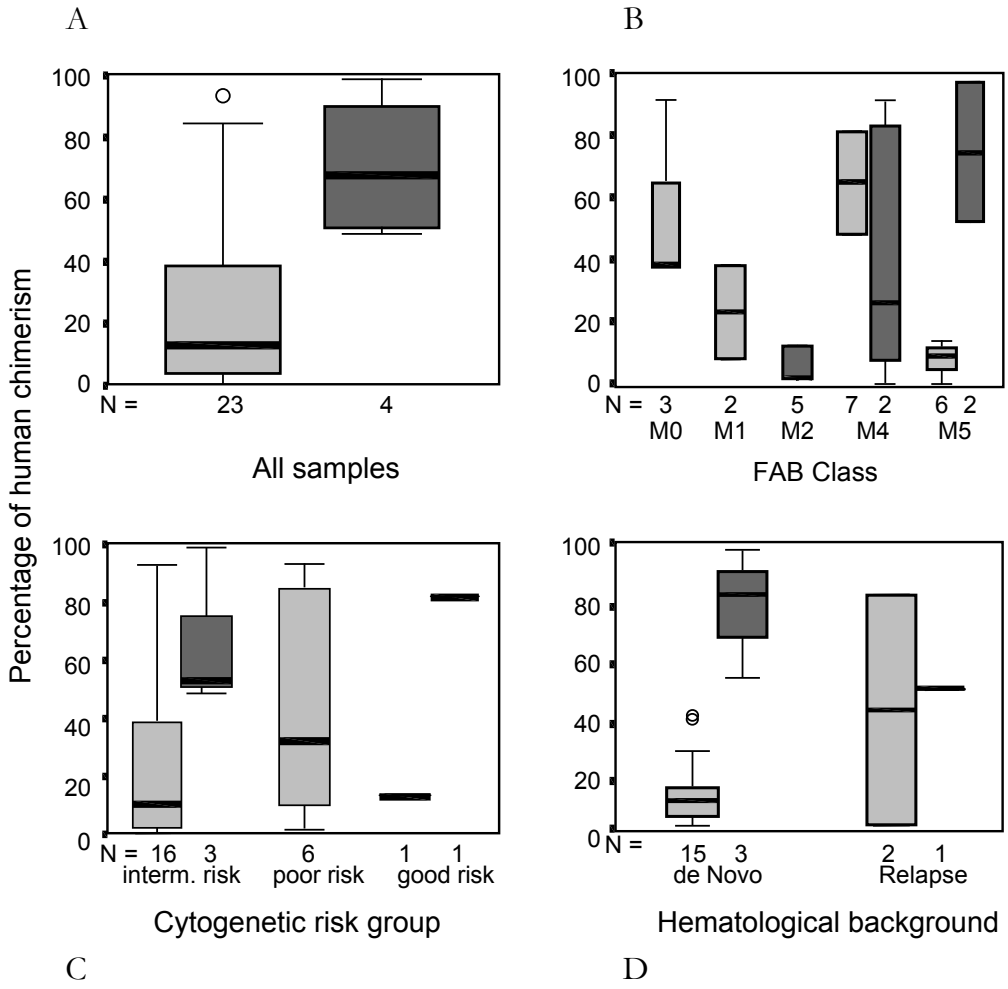

Figure 5: Outgrowth of AML in the NOD/SCID model.

NOD/SCID mice were i.v. injected with $3x10^7$ AML cells and the percentage of human AML cells in the NOD/SCID bone marrow was determined at day 30 after transplantation. Horizontal bold lines in these figures represent the median chimerism; shaded bars, interquartile range; error bars indicate extreme cases; Circles denote outlayers.

light shaded bars represent Flt3/wildtype samples; Dark shaded bars the Flt3/ITD samples. The number of cases within each group is indicated below the x-axis.

The extent of human chimerism is depicted for the total population (panel A) in relation to the FAB type (panel B), cytogenetics (panel C) or background of the AML (panel D).

3.5 Discussion

Our current study clearly demonstrates that AML patients who present with an Flt3/ITD mutation at diagnosis have a significantly poorer prognosis when compared to patients without such a mutation. This effect on the survival of AML patients is also evident in the elderly patients (>60 yr.) or in patients without any known cytogenetic aberrations. Our study on the Dutch population therefore confirms and extends the recent report on the clinical impact of Flt3/ITDs in the Japanese population[29]. However, in contrast to this study we found a significant reduction in the remission rate in the Flt3/ITD mutant population.

In our previous report[18] we have shown that Flt3/ITD mutant AML has a reduced proliferative ability in long-term stroma-supported cultures when compared to non-mutant samples. This aberrant characteristic behaviour is also observed in short-term cultures as described here. The short-term proliferation of Flt3/ITD samples is lower (though not always significant) when compared to non-mutant samples and independent of cytokines used. In contrast, Löwenberg et al.[43] have shown that a higher autonomous in vitro proliferation of AML correlates with a poorer prognosis. A similar, though less significant, correlation was also observed in cytokine-stimulated cultures[44]. It therefore seems opportune to re-evaluate a large group of AML samples, that exclude patients carrying an Flt3/ITD mutation, with respect to growth factor response and patient prognosis.

The decreased proliferation of the mutant samples is not reflected in the L-CAFC assay as all CAFC subset frequencies in the L-CAFC analysis are similar in both mutant and non-mutant populations. We also observed no differences in size of the cobblestone areas derived from either Flt3/ITD mutant or non-mutant CAFC.

Though the number of AML progenitors thus does not seem to differ between mutant and non-mutant populations we observed that the Flt3/ITD population had a better ability to engraft the bone marrow of NOD/SCID mice. One explanation for this might be that these mice provided a microenvironment, which more closely mimicked the human (in vivo) situation than did the ex vivo cultures, and favoured outgrowth of the mutant samples. Another possible explanation might be that the mutation in the Flt3 gene causes an aberrant activation of the receptor resulting in an inhibited apoptosis[9] leading to a higher surviving fraction, which is able to repopulate the mouse. Actual proof of which mechanisms are involved in the aberrant behaviour of AML with Flt3/ITD will require an in-depth molecular characterisation of both the wildtype and mutant Flt3.

In the light of the observed aberrant proliferative ability of Flt3/ITD AML it would be extremely interesting to re-evaluate current prognostic parameters based on exclusion of the Flt3/ITD samples.
In conclusion, our clinical data suggest that prognosis is strongly related to the presence of Flt3/ITDs. We therefore regard patients with Flt3/ITDs to belong to a distinct AML sub-population that seems to poorly respond to current treatment protocols and thus should have risk stratification with treatment tailored to their prognostic factors.

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CHAPTER 4

Relation between CXCR-4 expression, Flt3 mutations and unfavourable prognosis of adult acute myeloid leukemia.

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4.1 Abstract.

Recently it was shown that, analogous to normal hematopoietic cells, the level of CXCR-4 expression on AML cells correlates with SDF-1 induced chemotaxis. As we speculated that an anomalous organ distribution of AML cells could affect cell survival and thus result in an altered fraction surviving chemotherapy, we examined a possible correlation between patient prognosis and CXCR-4 expression in AML patients. We also studied chemotaxis and NOD/SCID repopulating ability of AML cells differing in CXCR-4 expression, and investigated the role of the CXCR-4/SDF-1 axis in G-CSF-induced myeloid differentiation.

We found that patients with a high CXCR-4 expression in the $CD34^+$ subset had a significantly reduced survival and a higher probability of recurrence of leukemia, resulting in a median relapse free survival (RFS) of only 8.3 months. Bone marrow cells from these patients showed an increased ability to repopulate NOD/SCID mice. Expression of CXCR-4 was significantly higher in Flt3/ITD AML than in Flt3/wt AML. Covariate analysis indicated that the negative prognostic significance of Flt3/ITDs with respect to RFS was no more apparent when analysed in conjunction with the expression of CXCR-4 in the $CD34^+$ subset, suggesting that the poor prognosis of Flt3/ITD AML patients might be subordinate to the increased CXCR-4 expression. Using a G-CSF-R expressing 32D cell line we observed that SDF-1/CXCR-4 interaction is required for the survival of myeloid differentiating cells and it also induces a block in G-CSF-induced myeloid differentiation.

These data suggest that the SDF-1/CXCR-4 axis may influence therapy responsiveness and defines unfavourable prognosis in AML.

4.2 Introduction

Stromal cell derived factor-1 alpha (SDF-1) is the major chemokine released by the bone marrow microenvironment[1,2], acting on lymphocytes and hematopoietic progenitors. SDF-1 has been shown to activate integrins on these precursor cells and induce their migration in vitro[3-5]. In SDF-1 or CXCR-4 (i.e. the SDF-1 receptor) knock-out models, hematopoietic precursor cells fail to migrate to the bone marrow during fetal development[6,7]. CXCR-4, also called LESTR or FUSIN[8,9], belongs to the G-protein coupled receptors (GPCRs). Previous studies have suggested that this receptor might play a role in malignant hematopoiesis. CXCR-4 is over-expressed and functionally active in B-CLL and might contribute to the bone marrow tropism of B-CLL cells[10]. SDF-1 has also been implicated in influencing the localisation of precursor B-ALL cells

in niches in the bone marrow microenvironment that favour their survival and proliferation[11]. In childhood ALL a high expression of CXCR-4 has been shown to predict for extramedullary infiltration[12] and might be associated with a poor outcome[13].
In acute myeloid leukemia (AML) the immature malignant cells frequently leave the bone marrow, populate the blood and lodge in extramedullary sites such as spleen and liver[14]. Recently it has been shown that analogous to normal hematopoietic cells also in AML the level of CXCR-4 expression correlates with the SDF-1 induced chemotaxis of these cells[15,16]. This indicates that SDF-1/CXCR-4 might be involved in the trafficking of the leukemic cells. We investigated whether the expression of CXCR-4 and / or the chemotactic response to SDF-1 correlates with the clinical outcome in patients with acute myeloid leukemia. We show that patients with a high expression of CXCR-4 in the $CD34^+$ subset of cells have a significantly reduced overall survival and are at greater risk of recurrence of leukemia. We also observed that Flt3/ITD AML has a significantly greater level of CXCR-4 expression as compared to Flt3/wt AML, suggesting the possibility that the poor prognosis of Flt3/ITD AML might depend on the increased CXCR-4 expression.

4.3 Materials and methods.

Patient samples.

Bone marrow samples of patients with AML were obtained after informed consent at the time of diagnosis. Cases were classified according to the French-American-British (FAB) committee recommendations[17]. Mononuclear cells were isolated by Ficoll separation followed by T-cell depletion, typically resulting in a population containing over 95 percent blasts. After isolation cells were subjected to controlled freezing and stored in liquid nitrogen. Before use cells were thawed by stepwise dilution in Iscoves Modified Dulbecco's medium (IMDM [Invitrogen, Merelbeke, Belgium]) containing 1% BSA. Post-thawing viability varied between 70 and 90 percent as assessed by dye exclusion. Patients were treated according to studies of the adult Dutch-Belgian Hemato-Oncology Cooperative Group HOVON, HOVON study AML-29 (less then 60 yrs of age, 44 patients)[18], AML-4 (less then 60 yrs., 27 patients)[19], AML-31 (60 yrs+, 11 patients) or AML-11 (60 yrs+, 6 patients)[20]. In these protocols, induction therapy included an anthracyclin and cytarabine and in patients less then 60 yrs allogeneic and autologous stem cell transplantation or intensive chemotherapy were applied as post-remission therapy. In the AML-29 study, induction therapy consisted of Ara-C (200 mg/m^2) for 7 days combined for the last three days with Idarubicin (12 mg/m^2) (Cy-

cle I) followed by a second cycle consisting of Ara-C (6 days, 1000 mg/ $m^{2)}$ combined with Amsacrine (days 3-5, 120 mg/ m^2). Patients were randomised during the first induction cycle to priming with G-CSF (150 μg/m^2, s.c.) during both cycles. Within the AML-4 protocol the induction therapy was similar to that of the AML-29 protocol, except that in cycle I Daunomycin (45 mg/m^2) was used instead of Idarubicin. Induction therapy according to the Hovon-31 protocol consisted of Ara-C 200 mg/m^2 for 7 days combined for the first three days with Daunomycin (45 mg/m^2) in arm 1 of the study, or Daunomycin (35mg/m^2) + the multidrug-resistance modulator PSC833 (10 mg/kg) in arm 2. Finally, in the AML-11 study induction therapy consisted of Ara-C 200 mg/m^2 for 7 days combined with Daunomycin 30 mg/kg on days 1-3.

Stratification according to karyotype-risk groups

As cytogenetic aberrations are considered to be important prognostic factors for AML[21] patients were classified in three groups, i.e. a favourable-risk group consisting of patients defined by a karyotype of t(8;21), t(15;17) or inv(16) and a unfavourable-risk group defined by del(5), del(7), or deletions of the q-arms of these chromosomes, t(6,9), alterations in the 11q23 region or the presence of multiple aberrations (more than 3) in the karyotype. All other karyotypic aberrations and normal karyotypes were considered intermediate-risk.

Detection of Flt3 mutants.

All AML samples were analysed for internal tandem duplications (ITDs) in exons 14 and 15 (the exons formerly designated as exons 11 and 12, see Abu-Duhier et al.[22] for revised exon numbering) of the Flt3-gene. The samples were also screened for the presence of the D835 mutation in the activation loop[23]. ITDs were detected by using a modified PCR procedure as described elsewhere[24]. The use of exon 14 and 15 specific primers allowed us to cover the whole juxtamembrane (JM) and the first part of the first kinase domain (TK-1) where most of the reported ITDs are located. Genomic DNA was prepared using a standard procedure[25]. Briefly, DNA from about 10^4 cells was amplified in a total of 50-μl reaction mixture, containing single strength PCR buffer, 10 pMol of each primer, 10 mM dNTPs and 0.5 U Taq polymerase (Supertaq, SpheroQ, Leiden, The Netherlands). Preheating of the samples at 95°C was followed by amplification for 35 cycles consisting of: 1 min. at 43 °C, 1 min. at 72 °C and 1 min. at 95 °C. Following these cycles a final extension at 72 °C was performed for 10 min. PCR products were stained with SYBR green I (Molecular Probes, Leiden, The Neth-

erlands) and resolved on 3% agarose gel. As a confirmation samples positive for an Flt3-mutation were reanalysed in a second PCR using the primers 14F and 15R.
The D835 mutation in the 2nd tyrosine kinase domain (exon 20, previously exon 17) was detected as described by Yamamoto et al.[23] Approximately 100ng of DNA was added to a reaction mix containing single strength PCR buffer, 10pMol of each primer (D835-forward: 5'-CGCCAGGAACGTGCTTG- 3', D835-reverse: 5'-GCAGCCT-CACATTGCCCC-3'), 200 μM dNTPs and 0.5 U Taq polymerase (Supertaq, SpheroQ). Preheating of the samples at 95°C was followed by amplification for 35 cycles consisting of: 1 min. at 63 °C, 1 min. at 72 °C and 1 min. at 95 °C. Following these cycles a final extension at 72 °C was performed for 10-min. Amplified PCR products were digested with EcoRV and subsequently electrophorised through four-percent agarose. Alleles containing the D835-mutation were identified by an uncut 114-bp PCR fragment whilst wildtype alleles were identified by a digested PCR product with fragments of 68 and 46 base pairs.

Migration assay.

Migration assays were performed as previously described[26]. Briefly, transwell plates (Costar Corning, Cambridge, MA, USA) of 6.5mm diameter and a pore size of 5μm were coated overnight at 4°C with 100μl of 20μg/ml fibronectin[16] (FN, Fibronectin from Bovine plasma, Sigma, St. Louis, MO, USA). Before addition of the cells, the transwells were washed three times with assay medium (IMDM + 0.5% Bovine Serum Albumin [BSA fraction V, Sigma]). After this $2x10^5$ AML cells in 100μl of assay medium were added to the upper compartment of the transwell. To the lower well 600μl of assay medium was added in the presence or absence of SDF-1 (100 ng/ml, R&D-systems, obtained through ITK-Diagnostics, Uithoorn, The Netherlands). The transwell plates were then incubated for 4 hrs. at 37°C and 10% CO_2. After incubation the number of cells that had migrated to the lower well was determined using a Sysmex cell counter (Toa Medical Electronics, Hamburg, Germany). The percentage of specific i.e. chemokine-induced, chemotaxis was calculated by dividing the number of input cells by the number of migrated cells from which the number of cells that migrated in medium alone was subtracted.
As the process of cryopreservation and the subsequent thawing is very likely a stressful event for cells, this may alter their responses when assayed immediately after thawing. We therefore compared the migratory abilities of a random selection of 7 AML samples after overnight incubation of the cells in assay-medium (37°C and 10% CO_2) with

those of the same samples assayed directly after thawing. In all conditions assayed, the chemotaxis after overnight incubation was significantly increased ($P <= 0.05$, data not shown). Therefore, all subsequent experiments were performed after overnight incubation.

In vitro proliferation and differentiation of 32D/G-CSF-R cells.

The 32D/G-CSF-R cell line[27] was cultured in RPMI 1640 medium (Gibco) supplemented with 100 IU/ml penicillin, 100 ng/ml streptomycin and 10% Fetal bovine serum and 10ng/ml of murine IL-3 (CHO-supernatant). Differentiation was started by replacing the IL-3 for 100 ng/ml G-CSF (Amgen, Thousand Oaks, CA). Cells were counted and the cell density was readjusted to $2x10^5$ cells daily. Morphologic analysis of differentiation was performed by microscopy of May-Grünwald-Giemsa stained cytospins. Specificity of SDF-1 binding was tested by blocking SDF-1/CXCR-4 interactions using CXCR-4 blocking or SDF-1 neutralizing antibodies (5µg/ml, R&D systems).

Flow cytometry.

To determine the antigen expression profile of both freshly thawed and overnight-incubated AML samples (See Migration Assay), the cells were harvested and pelleted at 1600g for 5'. Cells were washed and resuspended in PBS+1% FCS+0.1% sodium azide. Fifty microliter aliquots of this suspension, containing 1 - $2x10^5$ cells, were labelled for 30' on ice with the appropriate dilutions of αCD34-APC and αCXCR-4-PE (both from Coulter Immunotech, Marseille, France). After staining the samples were diluted to 300µl with PBS+1% FCS+0.1% sodium azide to which 2µl of 7-amino-actinomycin D (7-AAD, Molecular Probes, Leiden, The Netherlands) was added to allow for the exclusion of dead cells. Cells were measured on a FACScalibur cytometer (Beckton Dickinson Immunocytometry systems, San Jose, CA, USA) and analysed using the CellQuest software package (Beckton Dickinson).
In the 32D cell system the number of apoptotic cells was quantified by determining the number of annexin V positive cells using the Apoptest kit (NeXins research, Kattendijke, The Netherlands).

Transplantation of human AML cells in the NOD/SCID mouse.

NOD/LtSz-Prkdc^{scid} (NOD/SCID) mice were bred in the Erasmus animal facility and housed under specified pathogen free conditions, using individually ventilated cages.

Mice were supplied with sterilised food and acidified tap water to which 100mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) was added. Both food and drink were available ad lib. Housing, care and all animal experiments were done in accordance with Dutch legal regulations, which includes approval by a local ethical committee.
Before transplantation of the AML cells the mice received a total body irradiation of 3.5 Gy gamma radiation (^{137}Cs source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). Transplantation of the AML cells was done by injecting groups of 3-5 mice with $3x10^7$ AML cells in the lateral tail vein. After six weeks, the mice were sacrificed by CO_2 inhalation after which both femora were isolated and bone marrow suspensions prepared. To determine the percentage of AML cells in the bone marrow suspensions, cells were stained with antibodies against the human common leukocyte antigen (CD45), CD34, CD33 and CD38 (CD45-FITC, CD38-FITC, CD33-PE and CD34-APC, all obtained from Coulter Immunotech) and measured using a FACScalibur (Beckton Dickinson Immunocytometry systems). Analysis was done using the Cell Quest software package (Beckton Dickinson). Erythrocytes were excluded from analysis by gating on forward and perpendicular light scatter whilst dead cells were excluded by staining with 7-AAD.

Statistical analysis.

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il., USA). Differences between unrelated samples were analysed using the Mann-Whitney U-test. The Wilcoxon rank sum test was used for the analysis of paired samples. Initial screening of the correlations between the CXCR-4 expression and patient prognosis was performed by multivariate Cox regression analysis using the rank numbers of the CXCR-4 expression as a variable to test for trend. Variables which showed a significant correlation in this initial screening were studied in further detail by stratification of the patients in high and low CXCR-4 expressing categories based on the optimal cut-point for the CXCR-4 expression. In this analysis the optimal cut-point is defined as the percentage of cells expressing CXCR-4, at which the difference in patient prognosis between low and high expressing categories is largest. Patients with a CXCR-4 expression up to the cut-point were considered to be low expressing whilst all others were considered to be high expressing. To generate these categories, half of the samples were used as a training set to determine the optimal cut-point of the CXCR-4 expression. The optimal cut-points determined in the training set were then used to categorise all samples in low and high expressing categories. In

the analysis for the co-expression of CD34 and CXCR-4 we observed that the differences between the two categories were significant if the cut-point was chosen in the range between 0.4 and 1.2 percent of cells co-expressing CD34 and CXCR-4 (optimal cut-point at 1.0%). For the analysis of the fraction of $CD34^+$ cells expressing CXCR-4 we observed significant differences for cut-points in the range between 12 and 25 percent of $CD34^+$ cells expressing CXCR-4 (optimal cut-point at 20%).

For the survival analysis the Kaplan-Meier method of the SPSS software package was used. The Logrank test was used to check for equality of the survival distributions. The relapse-free survival (RFS) was calculated from date of CR to date of relapse. In this analysis, patients dying from non-leukemia related causes during first CR were included until date of death. The overall survival (OS) was calculated from date of diagnosis to date of death. Multivariate Cox regression analysis was used to identify other predictor variables present in the study population. In all evaluations values were considered significant if p-values were below 0.05.

Table 1: Clinical and hematological characteristics of patients

	Total number	90
Age	Median (yrs.)	44
	Range (yrs.)	16-88
	<= 60 yrs. (n)	72
Gender	Male	44
	Female	46
WBC	Median (10^{-9}/l)	58
	Range (10^{-9}/l)	1-319
FAB class	M0	3
	M1	18
	M2	19
	M3	5
	M4	23
	M5	22
Cytogenetic Risk*	favourable	14
	intermediate	69
	unfavourable	7

* Favourable cytogenetic abnormalities included t(8;21), t(15;17) or inv(16); unfavourable cytogenetic abnormalities included del(5), Del(7), deletions of the long arms of chromosomes 5 or 7 (5q- , 7q-), t(6;9), 11q23 abnormalities and complex karyotypes. (>3 abnormalities).Other karyotypic aberrations and normal karyotypes were included in the intermediate prognostic category.

4.4 Results

High percentages of CXCR-4 within the CD34$^+$ fraction of cells predict for an increased relapse rate in primary AML.

We analysed the expression of CXCR-4 together with the expression of CD34 in a series of 90 samples of adult patients with AML. Patient details are given in table 1. The results of the flow cytometric analysis are summarised in Table 2. On average, around one third (median 36.4%) of the ficoll fraction of marrow cells from AML patients expressed CXCR-4. As indicated by the broad range of the mean fluorescent index (MFI) we observed a large variation in the level at which CXCR-4 was expressed. In most cases, the cells expressed relatively low levels of CXCR-4, but in certain cases the cells showed an excessively high expression. While a median of 22.6 percent of cells expressed CD34, only 3.9 percent (median) of cells co-expressed CD34 and CXCR-4, indicating that the percentages of cells expressing CXCR-4 within the CD34$^+$ subset of cells was considerably less than that of the total cell population.

Table 2: Phenotypic analysis of the expression of CXCR-4 and CD34

Marker		median	range
		n=90	
CXCR-4	%	36.4	1.6 - 96.4
	MFI	39.3	6.9 - 523.3
% CD34$^+$		22.6	0.2 - 96.3
%CXCR-4$^+$CD34$^-$		23.6	0.1 - 97.3
%CXCR-4$^+$CD34$^+$		3.9	0.2 - 93.5
% CXCR-4$^-$CD34$^-$		18.8	0.3 - 97.0
%CXCR-4$^-$CD34$^+$		8.6	0.0 - 92.0

Marrow samples were labeled with fluorescently labeled monocolonal antibodies and analysed by flow cytometry. Values indicate the median percentages of cells expressing the indicated markers, MFI refers to the mean fluorescent index of the CXCR-4 expression.

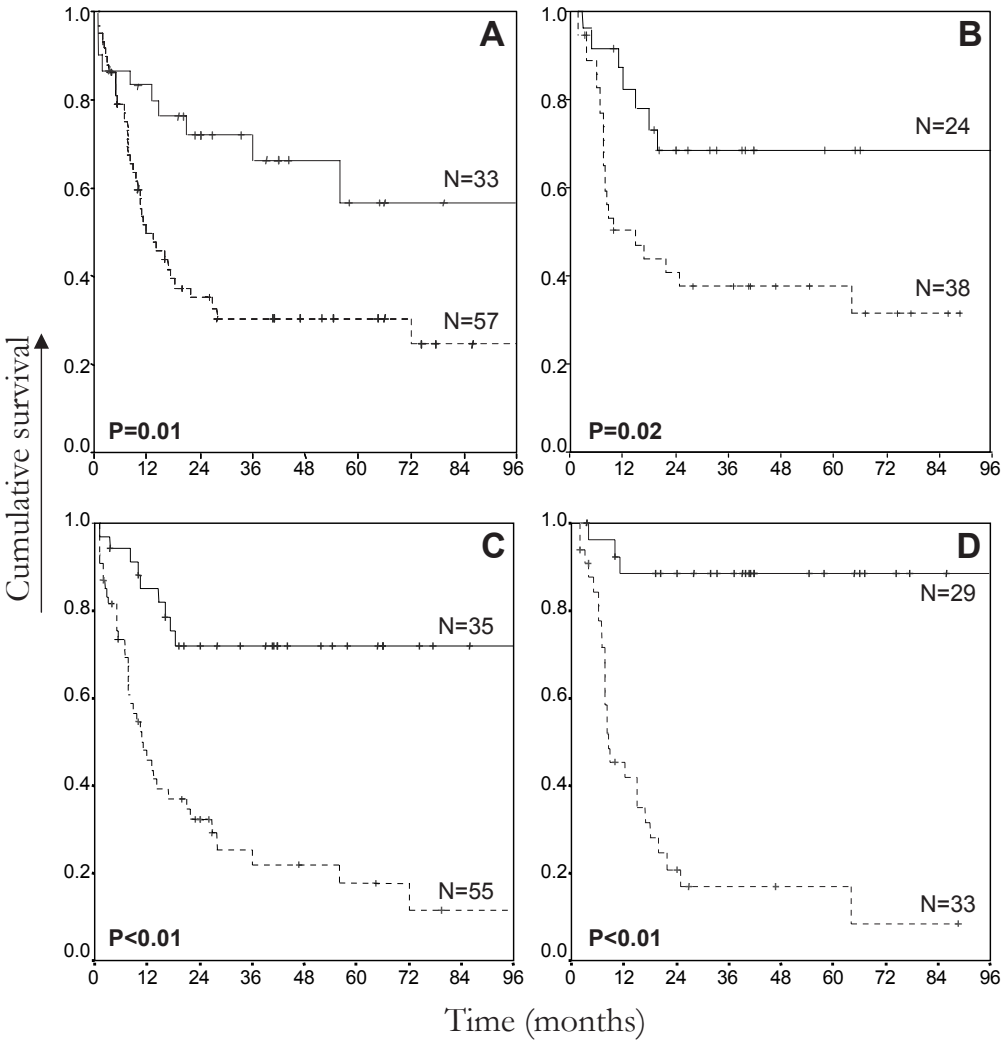

Figure 1: Overall survival and relapse-free survival of patients with primary AML in relation to the co-expression of CXCR-4 and CD34.

OS (panel A) and RFS (panel B) for AML is expressed in relation to the percentages of cells co-expressing CXCR-4 and CD34. Solid lines, patients in which < 1% of cells co-expressed CXCR-4 and CD34; dotted lines, patients in which ≥ 1% of cells co-expressed CXCR-4 and CD34. In Panels C and D, OS and RFS are given in relation to the fraction of $CD34^+$ cells expressing CXCR-4. Solid lines, patients in which < 20% of the $CD34^+$ cells expressed CXCR-4, Dotted lines patients in which ≥ 20% of the $CD34^+$ cells expressed CXCR-4.

asterisk refers to censored cases.

Initial screening using Cox regression analysis showed that the CXCR-4 expression proper only marginally correlated with patient prognosis (data not shown). Yet, the number of cells co-expressing CXCR-4 and CD34 showed a good correlation with patient prognosis and was studied in further detail (Fig. 1A+1B). At the optimal cut-point of 1% of cells co-expressing CD34 and CXCR-4, patients with comparatively high percentages of double positive cells had a reduced overall survival (OS) and a reduced relapse free survival (RFS). The impact of the CXCR-4 expression on prognosis was even more prominent when the percentage of $CXCR\text{-}4^{+}CD34^{+}$ cells was expressed as a fraction of the total number of $CD34^{+}$ cells (Fig. 1C+1D, optimal cut-point 20% of $CD34^{+}$ cells expressing CXCR-4), resulting in a median OS and RFS of 11 and 8.3 months respectively for patients with a high proportion of $CD34^{+}$ cells expressing CXCR-4. The latter values compared to a median OS and RFS of over 96 months ($p<0.01$) for patients with a small fraction of $CD34^{+}$ cells expressing CXCR-4. At a median follow-up of 18 months, 22 of 33 patients with a high CXCR-4 expression had relapsed following complete remission (relative risk of relapse: 6.1, 95% confidence interval 2.1-18.1).

Flt3/ITD AML shows an increased CXCR-4 expression.

To be able to more accurately define the significance of the observed correlation between disease outcome and CXCR-4 expression we performed a multivariate Cox regression analysis with the fraction of $CD34^{+}$ cells expressing CXCR-4, the presence of Flt3-mutations, cytogenetic abnormalities and age as variables in the regression analysis. For the analysis of Flt3-mutations all samples were analysed by PCR for the presence of Flt3/ITDs and Flt3/D835 mutations. Of the 90 samples, 22 were shown to be positive for the presence of Flt3/ITDs and three contained the Flt3/D835 mutation. As the number of Flt3/D835 mutations was too low to allow for a separate statistical evaluation and the impact on patient prognosis is not as clear as for the Flt3/ITDs, these samples were not analysed separately. In multivariate Cox regression analysis the percentage of $CD34^{+}CXCR\text{-}4^{+}$ cells and also age, unfavourable cytogenetics and the presence of Flt3/ITDs were significant predictive factors as regards OS and RFS (Table 3). In addition, when the fraction of $CD34^{+}$ cells expressing CXCR-4 was considered, the presence of Flt3/ITDs lost its impact on patient prognosis (Table 4).

As this indicated that the presence of Flt3/ITDs was not an independent predictive factor in this analysis, we further analysed this subtype of AML. Analysis of the flow cytometric data showed that the CXCR-4 expression (median expression 72.1 percent vs. 28.1 percent, $p<0.01$) was increased in Flt3/ITD AML when compared to Flt3/wt AML. On the other hand, the number of $CD34^+$ cells was reduced (median expression 9.5% in Flt3/ITD AML compared to 42.1% in Flt3/wt AML, $p= 0.03$), resulting in a significantly greater fraction of $CD34^+$ cells expressing CXCR-4 in Flt3/ITD AML.

Table 3: Prognostic impact of the co-expression of CD34 and CXCR-4 and other prognostic variables as regards overall survival and relapse free survival.

Variable	Overall survival		Relapse free survival	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
	n= 90		n=62	
Co-expression of CD34 and CXCR-4	3.19 (1.62-6.27)	0.001	3.92 (1.57-9.8)	<0.001
Presence Flt3/ITDs	3.01 (1.59-5.66)	0.001	2.86 (1.2-6.7)	0.015
Unfavourable cytogenetics*	2.26 (0.93-5.47	0.014	3.00 (1.01-9.34)	0.047
Age	1.02 (1.00-1.03)	0.024	0.99 (0.98-1.02)	0.490

To more accurately define the significance of the observed correlation between prognosis and the number of cells co-expressing CXCR-4 and CD34, we performed a multivariate Cox regression analysis with age, the presence Flt3-mutations and cytogenetics as variables in the regression analysis. Results are presented as relative risks with the 95% confidence intervals and respective p-values.

n=the number of samples included in the analysis.

* See legend table 1

Table 4: The prognostic impact of Flt3/ITDs in relation to the fraction of CD34+ cells expressing CXCR-4

Variable	Overall survival		Relapse free survival	
	Relative risk (95% CI)	p	Relative risk (95% CI)	p
	n= 90		n=62	
CXCR-4 within the CD34+ subset	5.12 (2.38-11.01)	<0.001	13.4 (3.87-25.0)	<0.001
Presence Flt3/ITDs*	1.69 (0.91-3.15)	0.054	1.54 (0.67-3.54)	0.327
Unfavourable cytogenetics*	1.74 (0.73-2.47)	0.314	1.50 (0.81-2.63)	0.048
Age	1.02 (1.00-1.04)	0.035	0.57 (0.27-2.61)	0.370

We performed a multivariate Cox regression analysis with age, the presence Flt3-mutations and cytogenetics and the fraction of CD34+ cells expressing CXCR-4, as variables in the regression analysis. Whereas age and unfavourable cytogenetics maintain ther independent prognostic value, Flt3/ITDs do not have a significant impact on patient prognosis when analysed in conjunction with the fraction of CD34+ cells expressing CXCR-4.
n=the number of samples included in the analysis.
*See legend table 1

The percentages of CXCR-4+ cells within the CD34+ cluster correlates with the outgrowth of AML cells in the NOD/SCID mouse model.

It has been shown that SDF-1/CXCR-4 interactions may play an essential role in the homing and high level multilineage engraftment of hematopoietic stem cells from healthy donors in the NOD/SCID human-mouse chimera model[28,29]. We and others have also shown that Flt3/ITD AML has a significantly increased SCID repopulating ability when compared to Flt3/wt AML[30,31]. As levels of CXCR-4 expression appear to be increased in Flt3/ITD AML, we set out to analyse whether the levels of CXCR-4 expression could be correlated with the NOD/SCID repopulating abilities of both Flt3/ITD and Flt3/wt AML. Due to the relatively high cell numbers needed to achieve reproducible and stable levels of chimerism in this model[32], we were only able to analyse 21 AML samples (15 Flt3/wt and 6 Flt3/ITD).
As shown in Table 5, at six weeks post transplantation there is a large variation in the percentages of AML cells repopulating the bone marrow of the NOD/SCID mice

Table 5: Outgrowth of human AML cells in NOD/SCID mice

	% human AML cells	FAB	Flt3 status	Phenotype of the original AML sample			
				% CXCR-4$^+$	% CD34$^+$	% CD34$^+$ CXCR-4$^+$	% of CD34$^+$ expressing CXCR-4
1	0.5	M5	ITD	6.24	95.2	5.9	6.2
2	0.9	M2	wt	27.0	86.8	11.3	13.0
3	0.9	M2	wt	13.0	75.6	8.0	10.6
4	2.5	M5	wt	5.9	1.7	0.2	12.0
5	3.1	M4	wt	11.7	46.3	9.1	19.76
6	4.6	M5	wt	51.2	76.0	28.7	37.7
7	8.3	M1	wt	18.2	92.3	16.3	16.3
8	9.2	M5	wt	33.4	82.9	28.8	34.8
9	9.7	M2	wt	34.2	95.2	25.6	26.8
10	11.8	M5	wt	39.3	11.4	9.6	84.5
11	13.4	M5	wt	24.0	1.2	0.9	75.0
12	14.4	M4	wt	51.2	52.6	26.9	51.2
13	15.1	M4	ITD	98.5	1.0	0.3	26.8
14	26.6	M4	wt	16.8	5.7	2.5	43.9
15	30.1	M2	ITD	90.1	0.2	0.2	100
16	37.6	M0	wt	4.6	1.6	0.6	40.5
17	38.5	M1	wt	23.0	0.7	0.5	70.4
18	38.5	M0	wt	92.6	96.3	83.7	86.9
19	48.6	M2	ITD	56.2	1.5	1.0	66.7
20	81.7	M2	ITD	59.3	26.5	8.0	30.3
21	97.8	M5	ITD	73.2	0.3	0.2	72.0

15 Flt3-wildtype and 6 Flt3/ITD samples were analysed in the NOD/SCID mouse model. Three to five mice per group were injected with $3x10^7$ AML cells. After six weeks the mice were sacrificed and bone marrow cells harvested. The percentage of human AML cells in the bone marrow was analysed by flowcytometry and compared to the CXCR-4 and CD34 expression patterns of the samples before grafting.

(median 12.6% human cells, range 0.5% - 97.8%). Correlation analysis showed that the level of human chimerism in the NOD/SCID mice correlated with both the overall expression of CXCR-4 (correlation coefficient 0.588, p= 0.01) and the expression of CXCR-4 within the $CD34^+$ cluster of cells ((correlation coefficient 0.708, p= 0.001, Figure 2).

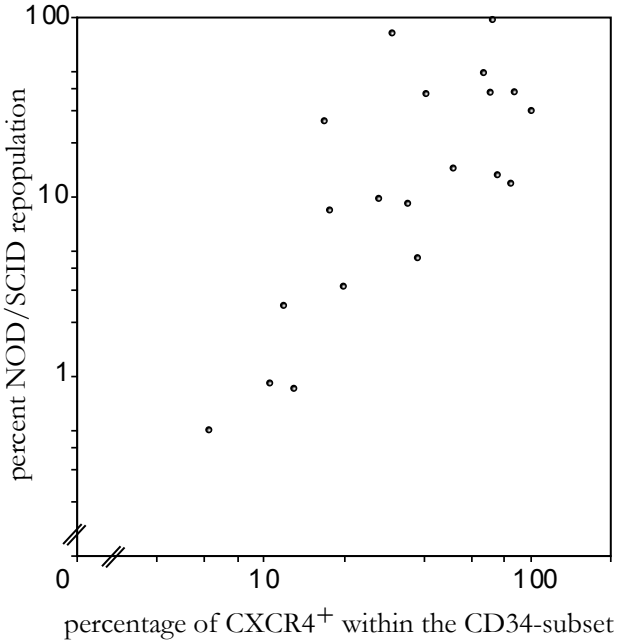

Figure 2: Correlation between the percentage of $CD34^+$ cells expressing CXCR-4 and the outgrowth in NOD/SCID.

Samples from 21 AML patients were injected in NOD/SCID mice ($3x10^7$ cells per mouse, 3-5 mice per group). After 6 weeks the mice were sacrificed and the number of human AML cells in the bone marrow was determined by flowcytometry. The outgrowth of AML cells in NOD/SCID was then correlated with the percentages of $CD34^+$ cells expressing CXCR-4.

Chemotaxis in response to SDF-1 does not correlate with patient prognosis or with the percentage of CXCR-4 expression in the CD34⁺ cluster of cells.

Recently Voermans et al.[16] reported that, similar to normal hematopoiesis, also in AML there is a linear correlation between chemotactic response and the expression levels of CXCR-4. To investigate if the migratory abilities correlated with patient prognosis we analysed the migratory abilities in response to SDF-1 in a subset of 60 AML samples. After four hours of incubation the median percentage of cells that had migrated in the presence of SDF-1 was 13.3 percent, which exceeded the comparative 2.5% value in medium alone ($p<0.01$). Correlation analysis revealed a low but significant correlation between chemotaxis and the overall CXCR-4 expression as well as the percentage of $CXCR\text{-}4^{+}CD34^{+}$ cells (correlation coefficient 0.326, $p= 0.01$ and 0.368 $p= 0.004$ respectively, data not shown). However, the SDF-1 induced chemotaxis did not correlate with patient prognosis.

SDF-1/CXCR-4 interactions are required for the survival of differentiating 32D cells, and induce a block in myeloid differentiation.

It has been shown that low levels of SDF-1 may enhance myeloid progenitor cell survival in vitro[33,34]. Further, the expression of CXCR-4 undergoes complex lineage-dependent regulation during myeloid differentiation[35]. We therefore investigated the effect of SDF-1 on the survival and G-CSF induced differentiation of the myeloid cell line 32D/G-CSF-R, a subclone of the 32D cell line known to express CXCR-4[36]. To study whether SDF-1/CXCR-4 interactions influenced cell survival and neutrophil differentiation, 32D/G-CSF-R cells were cultured with G-CSF (100 ng/ml) and G-CSF+ SDF-1 (100ng/ml). G-CSF induced a rapid increase in the number of terminally matured neutrophils between day 6 and 12, which was suppressed in the presence of SDF-1 (Figure 3). In order to check for the specificity of the SDF-1 inhibition through binding to CXCR-4 we performed the same experiment in the presence of a CXCR-4 blocking-antibody. Instead of rescuing the impairment of differentiation the CXCR-4 blocking antibody induced apoptosis in the 32D system. As induction of apoptosis by the blocking antibody was also seen in the absence of exogenously added SDF-1 (data not shown) these data strongly suggest the presence of SDF-1 in the culture medium where it is may provide a survival signal to the differentiating 32D cells. In order to exclude the possibility that the observed apoptosis was due to non-specific toxicity we also verified the effect of inhibiting SDF-1/CXCR-4 interactions by the addition of a

SDF-1 neutralising antibody. Both the SDF-1 neutralising antibody and the CXCR-4 blocking antibody induced a strong increase in apoptotic cells during the six days of culture (Fig 4.). Incubation of a CXCR-4 negative cell line (i.e. KG-1) with the CXCR-4 and SDF-1 specific antibodies did not result in induction of apoptosis, excluding the possibility that the antibody preparations contained aspecific cytotoxic activity

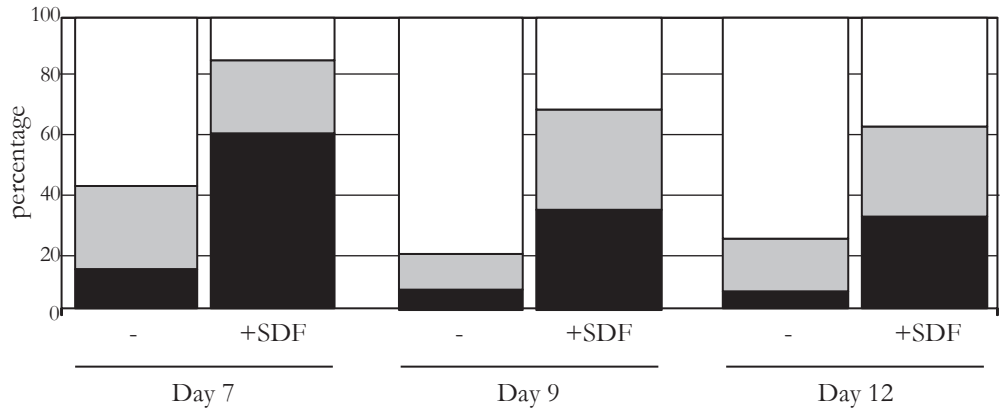

Figure 3: Effect of the addition of SDF-1 on the G-CSF-induced differentiation of 32D/G-CSF-R cells.

Differential countings after 7, 9 and 12 days of culture in the presence of G-CSF (-) or G-CSF + SDF-1 (+SDF). The white parts of the histograms indicate the percentage of terminally matured neutrophils, the grey parts indicate the percentage of intermediately matured granulocytic cells and black parts indicate the percentages of blast cells in the cultures.

4.5 Discussion

In the past few years our understanding of the presence and prognostic value of specific genetic defects (such as Flt3/ITD, AML-1-CBFβ, PML-RAR and various others) in human AML has increased. Notwithstanding this progress, there is a large subset of AML patients with prognostic heterogeneity and no detectable molecular or chromosomal aberrations. As it has been suggested that SDF-1/CXCR-4 interactions might be involved in the trafficking of leukemic cells, with possible prognostic implications, we investigated the expression of CXCR-4 and the chemotactic response to SDF-1 in relations to the clinical outcome of patients with newly diagnosed AML.

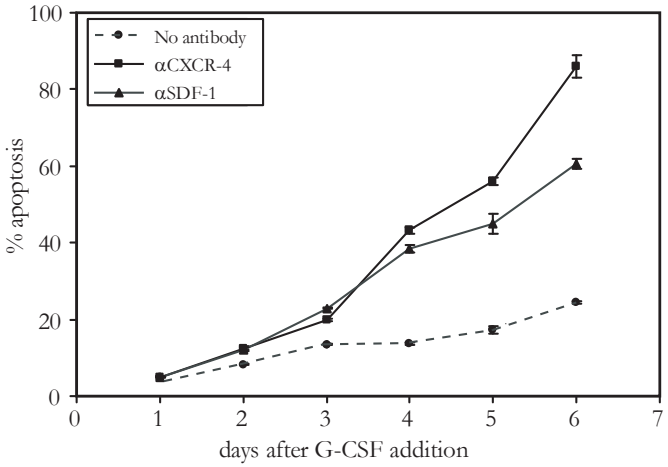

Figure 4: Blocking of SDF-1/CXCR-4 interactions induces apoptosis in 32D cells.
On day 0 cultures were initiated with $2x10^5$ 32D/G-CSF-R cells/ml. Cells were counted and cultures were readjusted to $2x10^5$ cells/ml on a daily basis. Cultures were either performed in the presence of G-CSF, G-CSF + a CXCR-4-blocking antibody (5 μg/ml) or G-CSF+ a SDF-1 neutralizing antibody. The percentage of cells in apoptosis (y-axis) was determined by a flowcytometric analysis of the number of annexin-V^+7-AAD^- cells. The x-axis shows the number of days after the start of the culture.

While the expression of CXCR-4 proper had a borderline prognostic value, the percentage of $CD34^+CXCR\text{-}4^+$ double positive cells and the fraction of $CD34^+$ cells expressing CXCR-4 appeared to be significant negative predictors of both overall survival and relapse free survival. In multivariate analysis the predictive value proved to be independent of other previously established prognostic markers such as age and cytogenetic abnormalities. A particularly interesting observation of the studies presented

here, is that Flt3/ITD AML was characterised by an increased expression of CXCR-4 (both in the number of cells expressing CXCR-4 and the level of CXCR-4 expression per cell). The difference in CXCR-4 expression between Flt3/ITD and Flt3/wt AML was also reflected in the fraction of $CD34^+$ cells expressing CXCR-4, which with a median value of 52.7% was significantly more in Flt3/ITD AML when compared to the median value of 19.8% in Flt3/wt AML ($p < 0.01$). We also observed that in Flt3/ITD AML the expression of CXCR-4 correlated with the mutant to wildtype ratio present in the respective samples and that activation of the wildtype-Flt3 resulted in an up-regulation of the CXCR-4 expression (data not shown), suggesting a link between the expression of CXCR-4 and Flt3/ITDs. Furthermore, whilst Flt3/ITDs had a significant negative impact on the RFS when analysed as a single variable (data not shown), the impact of Flt3/ITDs was no longer apparent when it was analysed in conjunction with the fraction of $CD34^+$ cells expressing CXCR-4. Taken together these data might suggest that the poor prognosis of Flt3/ITD AML depends on the increased CXCR-4 expression in this type of AML.

In order to investigate whether the correlations between the CXCR-4 expression and prognosis could be explained by a different organ distribution of AML cells, we analysed the *in vitro* migratory behaviour of these cells in response to SDF-1, the ligand of CXCR-4. We observed that AML with high percentages of CXCR-4 expression showed an increased chemotactic response in culture. However, the low correlation coefficient between CXCR-4 expression and chemotactic response would suggest that factors other than the expression of CXCR-4, such as the expression of adhesion molecules and cell cycle induction, might play a role in determining the migratory abilities. Ponomaryov et al.[37] observed an increased expression of SDF-1 in the bone marrow following conditioning with DNA-damaging agents (ionising radiation, cyclophosphamide and 5-FU), this resulted in an increase in CXCR-4 dependent homing to the bone marrow, which consequently facilitated engraftment of hematopoietic stem cells. Though not shown to be true for leukemic stem cells, these observations on normal bone marrow cells fit well with our observation of a significant correlation between the expression of CXCR-4 and the outgrowth of AML cells in the NOD/SCID mouse model.

Apart from its chemotactic activity, SDF-1 has also been suggested to play a role in hematopoietic (stem) cell survival and function by suppression of apoptosis and promoting the G0/G1 transition[37-40]. The observation that blocking of CXCR-4 induces apoptosis in the 32D system may suggest that also in myeloid cells SDF-1/CXCR-4

interactions provide strong anti-apoptotic signals. However, in the 32D cell system high levels of SDF-1 induced a partial block in differentiation. This raises the possibility that increased expression of CXCR-4 contributes to leukemogenisis by inducing a block in differentiation. Recently Jorda et al.[41] have observed that Cb2, another $G_{\alpha i}$-GPCR and a frequent proviral target in Cas-Br-M-MuLV-induced myeloid leukemias, produces an arrest in myeloid differentiation in the 32D system, suggesting a more general role of $G_{\alpha i}$-GPCRs in leukemogenesis.

In conclusion, our data indicate that a high expression of CXCR-4 in the CD34 cluster of cells defines unfavourable prognosis of AML. Little is known about the role of CXCR-4 / SDF-1 in the development, therapy responsiveness and disease progression of AML. Which of the three possible mechanisms, i.e. an increased homing, a block in differentiation and anti-apoptotic signalling, by it self influences disease prognosis remains to be seen. To this end, these data warrant a more extensive study into the role of CXCR-4 in the pathobiology of acute myeloid leukemia.

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CHAPTER 5

Improved prognostic significance of cytokine-induced proliferation in vitro in patients with de novo acute myeloid leukemia of intermediate risk: impact of internal tandem duplications in the Flt3 gene

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5.1. Abstract

The heterogeneity of acute myeloid leukemia is reflected in many clinical, biological and genetic features that are used to predict the response to therapy. On the basis of chromosome aberrations patients can be stratified in groups reflecting either good or poor prognosis. However, the majority of patients fall in an 'intermediate risk' group. Internal tandem duplications in the hematopoietic growth factor receptor Flt3 have been shown to separate a subset of high-risk patients from intermediate or low risk cases.

In an attempt to further characterize the heterogeneity of prognosis among the cytogenetic intermediate risk group of AML, we investigated the overall survival, failure-free survival, initial therapy response and relapse rates of 103 patients with *de novo* AML in relation to autonomous proliferation and the proliferative response to a panel of 10 cytokines in a short-term thymidine incorporation assay.

To exclude perturbation of the responses by other (known) risk factors our final intermediate risk population was comprized of patients with intermediate risk cytogenetics, having an age of 60 years of younger and not showing tandem duplications in the Flt3 gene.

Among this intermediate risk group, only the responses to M-CSF and Il-1α were found to be predictive for therapy outcome. Results obtained by a 7-day culture with these cytokines revealed two subpopulations characterized by a good and a poor prognosis, respectively. The complete remission rates in these subpopulations were similar, but the relapse rates, failure-free survival and overall survival differed. If further study extends and supports our data, it should be considered to include these patients in the poor risk arms of treatment protocols and offer them intensified treatment or bone marrow transplantation.

5.2. Introduction

The term acute myeloid leukemia (AML) encompasses a range of clonal hematopoietic disorders with aberrant proliferation and differentiation. The malignant clone outgrows the normal hematopoietic cells resulting in an accumulation of immature cells in the hematopoietic tissues. Over the past decade progress in both therapy and diagnosis have improved the outlook for certain AML patients. However, despite these improvements the overall survival rate is still only 40 percent in patients with an age of less than 65 years whilst for patients with an age of 65 years and beyond this survival is below 10 percent[1]. A number of clinical, hematological and (cyto)genetical features

reflecting the heterogeneity of the disease are used to predict the outcome of therapy. Adverse prognostic factors include (a) an age over 60 years, (b) AML resulting from a prior hematological disorder such as MDS and (c) a high white blood cell count[2]. Adverse cytogenetics, e.g. deletions of chromosome 5 or 7 or deletions of the long arm of these chromosomes, expresses a considerable prognostic value. In contrast, the cytogenetic translocations t(8:21), t(15:17) or inv(16) define a group of patients with good prognosis. Other patients are classified in an intermediate or standard risk group.

The fms like tyrosine kinase 3 (Flt3), also known as stem cell tyrosine kinase-1 (STK1) or fetal liver tyrosine kinase-2 (FLK-2)[3-6], belongs to the group of class III receptor tyrosine kinases (RTKs), which also include the receptors c-kit and c-fms. Signals generated by ligand-induced dimerisation of these receptors involve tyrosine phosphorylation of certain regions of the receptor and activation of cellular tyrosine kinases. While a large proportion of AML cases has been found to express class III RTKs[7-10], their potential role in leukemia development is still unclear. Recently it has been reported that internal tandem duplications in the Flt3 gene (Flt3/ITDs) occur in 17-20% of AML patients and are significantly associated with a poor patient prognosis[11-13]. Except in AML, these duplications have also been found in patients with leukemic transformation of myelodysplasia[14], but not in patients suffering from acute lymphoid leukemia, chronic myeloid leukemia (CML), blast crisis CML, or in normal individuals. We have shown that AML cells with Flt3/ITDs have a reduced proliferative ability in both long-term stroma-supported cultures[15] and short-term stroma-free cultures[11]. The apparent contrast between this reduced proliferative ability in vitro and the poor prognosis of AML patients with Flt3/ITDs led us to investigate whether exclusion of Flt3/ITD samples would lead to a better correlation between either autonomous or cytokine-induced proliferation, and patient prognosis. Specifically, we have examined the possibility to further distinguish the prognosis of patients that are currently assigned to the 'intermediate risk' group on the basis of cytogenetics. For this purpose we have correlated the *in vitro* autonomous proliferation in combination with the proliferative response to a panel of ten cytokines to (a) response to remission induction treatment, (b) relapse probability, (c) overall survival (OS) and (d) failure-free survival (FFS) of 103 patients with *de novo* AML.

5.3. Materials and methods

Patient samples

Diagnosis bone marrow samples of patients with AML were obtained after informed consent. Cases were classified according to the French-American-British (FAB) committee recommendations [16]. Mononuclear cells were isolated by ficoll separation followed by T-cell depletion, typically resulting in a population containing over 95 percent blasts. After isolation cells were subjected to controlled freezing and stored in liquid nitrogen. Before use cells were thawed by stepwise dilution in Iscoves Modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands) containing 1% BSA. Post-thawing viability varied between 70 and 90 percent as assessed by dye exclusion.
After diagnosis patients were treated according to studies of the adult Dutch-Belgian Heamato-Oncology Group HOVON, i.e. the HOVON-29 (less then 60 yr. of age, 45 patients)[17], HOVON-4 (less then 60 yr., 44 patients)[18], HOVON-31 (60 yrs+, 11 patients) and the AML-11 (60 yrs+, 3 patients)[19] protocols. In these protocols induction therapy included an anthracyclin and cytarabine, while in patients less then 60 yr. allogeneic and autologous stem cell transplantation or intensive chemotherapy for consolidation were applied as post-remission therapy. In the HOVON-29 study induction therapy consisted of Ara-C (200 mg/m^2) for 7 days combined with Idarubicin (12 mg/m^2) for the last three days (Cycle I) followed by a second cycle consisting of Ara-C (6 days, 1000 mg/ $m^{2)}$ combined with Amsacrine (days 3-5, 120 mg/ m^2). Half of the patients according to randomising received G-CSF (120 μg/m^2, s.c.) during both cycles. Within the HOVON-4 protocol the induction therapy was equal to the HOVON-29 protocol except for the fact that in cycle I Daunomycin (45 mg/m^2) was used instead of Idarubicin. For the HOVON-31 protocol induction therapy consisted of Ara-C 200 mg/m^2 for 7 days combined for the first three days with Daunomycin (45 mg/m^2) in arm one of the study, or Daunomycin (35mg/m^2) + PSC833 (10 mg/kg) in arm two. Finally, in the AML-11 study induction therapy consisted of Ara-C 200 mg/m^2 for 7 days combined with Daunomycin 30 mg/kg on days 1-3.

Stratification according to karyotype-risk groups

As cytogenetic aberrations are thought to be important prognostic factors for AML[20,21] patients were stratified in three groups i.e. a good-risk group consisting of patients defined by a karyotype of t(8;21), t(15;17) or inv(16) and a poor-risk group defined by del(5), del(7), or deletions of the q-arms of these chromosomes, t(6,9), alterations in the 11q23 region or the presence of multiple aberrations (more than 3) in the karyo-

type. All other karyotypic aberrations and a normal karyotype were included in an intermediate-risk group.

Determination of Flt3 mutants

All AML samples were analysed for mutations in exons 11 and 12 of the Flt3 gene by using a modified PCR procedure as described elsewhere[22]. The use of exon 11 and 12 specific primers allowed us to cover the whole JM and the first part of the TK-1 domain where most of the reported ITDs are located.
Genomic DNA was prepared using a standard procedure[23]. Briefly, DNA from about 10^4 cells was amplified in a total of 50 μl reaction mixture, containing single strength PCR buffer, 10 pMol of each primer, 10 mM dNTPs and 0.5 U Taq polymerase (Supertaq, SpheroQ, Leiden, The Netherlands). Preheating of the samples at 95°C was followed by amplification for 35 cycles consisting of: 1 min. at 43 °C, 1 min. at 72 °C and 1 min. at 95 °C. Following these cycles a final extension at 72 °C was performed for 10 min. PCR products were stained with SYBR green I (Molecular Probes, Leiden, The Netherlands) and resolved on 3% agarose gel. As a confirmation samples positive for an Flt3-mutation were reanalyzed in a second PCR using the primers 11F and 12R.

Short-term proliferative response to single cytokine stimulation

To determine the proliferative response of AML bone marrow samples to single cytokine stimulation we used a thymidine incorporation assay. As monocytes are known to produce many cytokines and thus potentially may perturb the results, we removed these cells prior to their use in culture by adhesion to plastic (1hr incubation at 37°C in alpha-MEM [Gibco, Breda, The Netherlands] + 1% BSA [Sigma, St. Louis, MO, USA]). FAB M5 samples were excluded from this treatment as the leukemic cells from this monocytic leukemia also adhere to the plastic. The non-adherent cells were plated in 96-well plates at a density of $2x10^4$ cells/well in serum free medium[24] and cultured for six days. The cytokines used were either hIl-1α (100 U/ml), hIl-7 (100U/ml), hGM-CSF (10ng/ml) all a generous gift from Immunex (Seattle, WA, USA); hIl-11 (10ng/ml), hM-CSF (100 U/ml), hIL-3 (25ng/ml) all a gift from Genetics Institute, Cambridge, MA, USA; TNFα (1000U/ml, Boehringer, Ingelheim, Germany), hEPO (1U/ml, Janssen Cilag, Berchem, Belgium), hG-CSF (100ng/ml) or hSCF (100 ng/ml) both a gift from Amgen (Thousand Oaks, CA, USA). After six days the cells were in-

cubated overnight with 0.1 μCi tritiated-thymidine (Amersham, Buckinhamshire, UK) per well and harvested onto nitrocellulose filters. Thymidine incorporation was determined using a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden) as counts per minute (cpm). All cultures were done in triplicate.

Statistical analysis

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il., USA). The usual χ^2 test was used to evaluate complete remission (CR) and relapse rates. For the survival analysis the Kaplan-Meier method of the same software package was used. The logrank test was used to check for equality of the survival distributions. Overall survival was calculated from the date of diagnosis to the date of (leukemia-related) death. The failure-free survival (FFS) was calculated from the date of diagnosis to the date of relapse. Patients not achieving CR were scored as having an FFS of 0.01. Patients dying from non-leukemia related causes during first CR were included until date of death. Patients not showing any event were considered "censored" observations at the date of last follow up.

Initial screening of the correlations between growth factor responses and prognosis was performed by multivariate Cox-regression analysis using the rank numbers of the growth factor responses as a variable to test for trend. Growth factors which showed a significant correlation in this initial screening were studied in further detail by stratification of the patients in high and low responders based on the optimal cut-point for the cytokine response. In this analysis the optimal cut-point is defined as the amount of thymidine incorporation, measured as counts per minute, at which the difference in patient prognosis between low and high responders is largest. Patients with a growth factor response up to and including the cut-point were considered to be low responders whilst all others were considered to be high responders.

Multivariate Cox-regression analysis was also used to identify other predictor variables present in the total study population and for the calculation of the relative hazards of these variables. In all evaluations values were considered significant if p-values were below 0.05.

5.4. Results

Characterization of patients.

Bone marrow samples from 103 patients with primary AML were screened for the presence of an Flt3/ITD. Of the 103 samples, 25 patients (25%) were found to have such a mutation. All Flt3/ITDs were in the exon 11 region of the gene, none in exon 12. As previously shown[15,25] the mutation was randomly distributed over the FAB classes and there was no apparent correlation with any known cytogenetic aberration. In most of the Flt3/ITD positive patients the mutation was present as a heterozygous mutation but in 5 cases the ITD appeared as a single aberrant band on gel, indicating the presence of either a homozygous mutation or, more likely, a hemizygous deletion of the wildtype gene.

The characteristics of these 25 AML patients with an Flt3/ITD and the 78 AML patients without such mutations included in this study are shown in Table 1.

Table 1. Patient characteristics

		Flt3/ITD AML	Flt3/wildtype AML
Total number		25	78
Age	Mean (yr.)	43	25
	Range (yr.)	18-73	16-76
Gender	Male	11	42
	Female	14	36
WBC ($x10^{-9}$)	Mean	64.5	25.0
	Range	1-253	1-158
FAB Classification	Unclassified	0	1
	M0	0	2
	M1	3	12
	M2	5	19
	M3	2	4
	M4	8	20
	M5	7	20
Cytogenetic Risk group*	Good prognosis	4	15
	Intermediate prognosis	21	52
	Poor prognosis	0	11

* Cytogenetic stratification was performed according to the criteria described in the Materials and Methods section.

Short-term proliferative response of primary AML.

We have recently shown[11] that the presence of Flt3/ITDs in AML correlates with a reduced proliferative ability of AML in short-term cultures (7 days) without stromal support. We have now extended the analysis to 10 cytokines (Table 2). In almost all cases the Flt3/ITD positive samples showed a tendency for a decreased response to cytokines as compared to those from non-mutant cases. However, impaired cytokine responsiveness of Flt3/ITD AML-blasts was only significant for G-CSF and GM-CSF (respective p-values: 0.02 and 0.05). Within the Flt3 wildtype population, the strongest responses were observed for Il-3, GM-CSF or G-CSF and the lowest median responses were seen when AML cells were stimulated with Il-7, Il-11 or TNFα (all of them even showed a median <0, indicating inhibition). None of the cytokine responses revealed a correlation with age, white blood cell counts at diagnosis or cytogenetics (data not shown). Correlations could neither be detected between proliferative ability and FAB classification except for an increased autonomous proliferation in the FAB M5 samples. This observation can be explained by the fact that in the preparation of the FAB M5 samples the plastic adhesion step was omitted, as explained in the Materials and Methods section, thus allowing for the presence of cytokine-producing monocytes.

Table 2. Response of primary AML to growth factor stimulation.

	Flt3 Wildtype			Flt3/ITD		
	N	Median response	Range	N	Median response	Range
Autonomous	78	649	0 – 15776	25	714	0 – 19532
Il-1α	78	150	-1833 – 35350	25	58	-1172 – 5009
Il-3	77	3208	-1907 – 32908	25	2240	-111 – 52189
Il-5	68	102	-1124 – 14465	18	23	-591 – 16492
Il-7	69	-9	-1991 – 2257	18	-4	-8 – 112
Il-11	62	-5	-3046 – 1510	15	-35	-319 – 145
TNFα	73	-140	-14209 – 12212	23	-289	-5088 – 2456
EPO	77	139	-712 – 30495	25	29	-1413 – 1333
SCF	63	838	-851 – 27859	16	676	-99 – 3546
M-CSF	78	225	-1170 – 20440	25	63	-1124 – 4735
G-CSF	77	1996	-2483 – 48333	25	702	-668 – 9658
GM-CSF	78	2039	-1164 - 20647	25	910	-708 - 15288

Responses are given as counts per minute (cpm), defined as the difference in cpm between the test sample and an irradiated control sample. All cytokine stimulation data were corrected for the component caused by the autonomous proliferation, thus explaining negative values.

Clinical response and prognosis of AML patients.

Within our total study population, multivariate analysis with the rank number of the cytokine responses as variables, only showed the autonomous proliferation to be a significant predictor for prognosis (Table 3). None of the panel of 10 cytokines tested proved to be a significant predictor. For further survival analysis patients were classified into low and high autonomous proliferation groups according to the criteria described in the Materials and Methods section (optimal cut point 3000 CPM). The latter analysis showed an even better distinction in both failure-free and overall survival between low and high autonomous responders (Figure 1).

Table 3. Significances for cox regression analysis determining the relations between the growth factor responses and failure free survival.

Variable	No. of observations	P-value
Autonomous proliferation	103	0.003*
GM-CSF induced	103	0.3
G-CSF induced	102	0.2
M-CSF induced	103	0.08
Il-lα induced	103	0.09
Il-3 induced	102	0.2
Il-5 induced	86	0.5
Il-7 induced	87	0.06
Il-11 induced	77	0.2
TNFα induced	96	0.08
SCF induced	79	0.9
EPO induced	102	0.9

For this analysis the rank number of the growth factor response was used as input variable for the regression analysis.
* values considered to be statistically significant

Table 4. Relative hazards within the total study population as calculated by cox regression analysis.

Variable	P-value	Relative Risk
Intermediate risk cytogenetics	0.06	3.2
Poor risk cytogenetics	0.001*	7.0
Age above 60 years	0.003*	2.7
Flt3/ITD	0.0002*	2.9

* values considered to be statistically significant

To be able to more accurately define the intermediate risk group within our study population we performed a multivariate analysis with age, FAB-classification, gender, the presence of Flt3/ITD and conventional cytogenetics as variables in the regression analysis. Of these variables poor-risk cytogenetics, an age over 60 years and the presence of Flt3/ITDs (Figure 2) appeared to represent independent predictors of poor prognosis (Table 4). We therefore defined our intermediate risk population as that population of patients without either poor or good risk cytogenetics, not having an Flt3/ITD with an age of 60 years or younger. Analysis of the failure-free survival of the resulting good, intermediate and poor risk populations is shown in Figure 3.
Though the autonomous proliferation was a significant predictor for survival with in the total primary AML population this parameter was not a significant predictor within the intermediate risk population. On the other hand two of the tested cytokines, i.e. M-CSF and Il-1α were shown to be significant predictors within this population (Table 5).

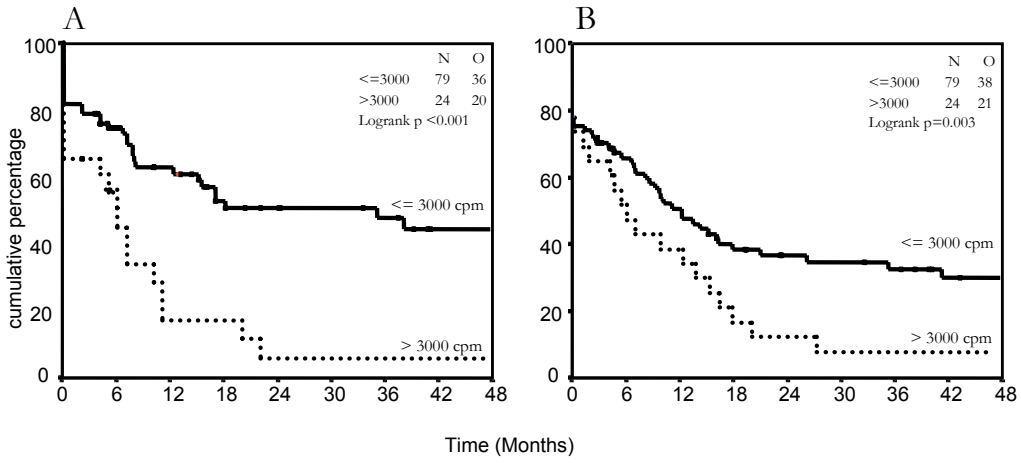

Figure 1. Cumulative failure-free (panel A) and overall (panel B) survival of the total primary AML population.

Stratification was based on the autonomous proliferation in a 7-day thymidine incorporation assay. Solid lines, population with a low (<=3000 cpm) autonomous proliferation. Dotted lines, population with a high (>3000 cpm) autonomous proliferation. In the inserts values under "N" depict the total number of samples in each population whilst the values under "O" depict the numbers of observed events in each population.

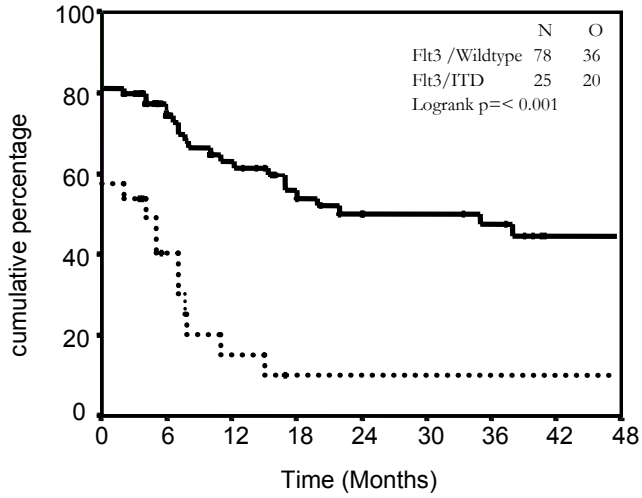

Figure 2. Cumulative failure-free survival of primary AML patients with or without internal tandem duplications.

Solid lines, Flt3-wildtype population. Dotted lines, population with Flt3/ITD. In the insert values under "N" depict the total number of samples in each population whilst the values under "O" depict the numbers of observed events in each population.

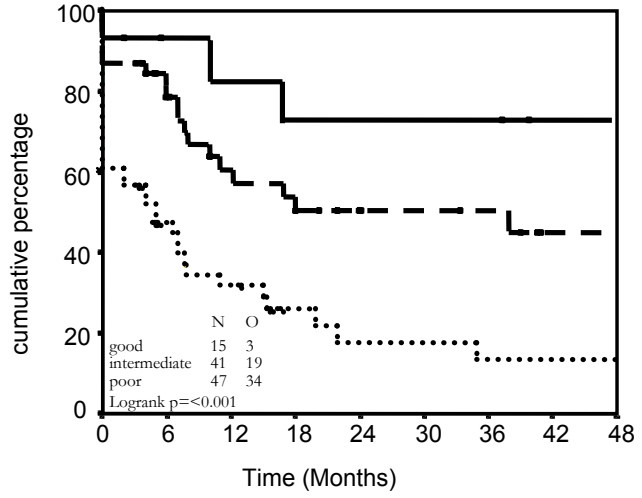

Figure 3. Cumulative failure-free survival of poor, intermediate or good risk AML.

Solid lines, Population with favorable or good risk AML. Dashed lines, population with intermediate risk AML. Dotted lines, population with poor risk AML. In the insert values under "N" depict the total number of samples in each population whilst the values under "O" depict the numbers of observed events in each population.

Table 5. Cox regression for failure free survival within the intermediate risk population in relation to growth factor responses.

Variable	No. of observations	P-value
Autonomous proliferation	41	0.1
GM-CSF induced	41	0.2
G-CSF induced	40	0.08
M-CSF induced	41	0.03*
Il-lα induced	41	0.008*
Il-3 induced	41	0.4
Il-5 induced	35	0.6
Il-7 induced	35	0.5
Il-11 induced	32	0.9
TNFα induced	37	0.9
SCF induced	33	0.4
EPO induced	41	0.3

For this analysis the rank number of the growth factor response was used as input variable for the regression analysis. The intermediate risk population studied was comprized of patients with a primary AML, having an age of 60 years or younger, having intermediate risk cytogenetics and not showing a Flt3/ITD. * values considered to be statistically significant

In univariate analysis, stratification of the patients in low and high responders at an optimal cut-point of 300 and 400 CPM for Il-1α and M-CSF respectively, proved both growth factors to be good predictors for both failure-free and overall survival (Figures 4 and 5). These figures also show the initial therapy response of high and low responders to both Il-1α and M-CSF not to be different. However, at a median observation time of 15 months, significantly different relapse rates could be observed (Table 6). Relapse rates varied form 5/21 and 4/17 (24%) for the low responder groups of M-CSF and Il-1α respectively to 5/5 (100%) for the M-CSF high responder population and 6/9 (67%) for the Il-1α high responder population.

Table 6. Therapy response of intermediate risk AML at the median observation period of 15 months.

	M-CSF induced proliferation			Il-1 induced proliferation		
	<= 400 cpm	>400 cpm	N total	<= 300 cpm	> 300 cpm	N total
No CR	3	3	16	2	4	6
CR	16	0	10	13	3	16
Relapse	5	5	6	4	6	10
N total	24	8	32	19	13	32
P-value	0.005			0.04		

The response to therapy at the median observation time of 15 months was determined for each of the indicated response groups. No Cr, patients who failed to respond to therapy or only had a partial response. CR, the number of patients who still showed a complete remission. Relapse the number of patients who had relapsed before the median observation period.

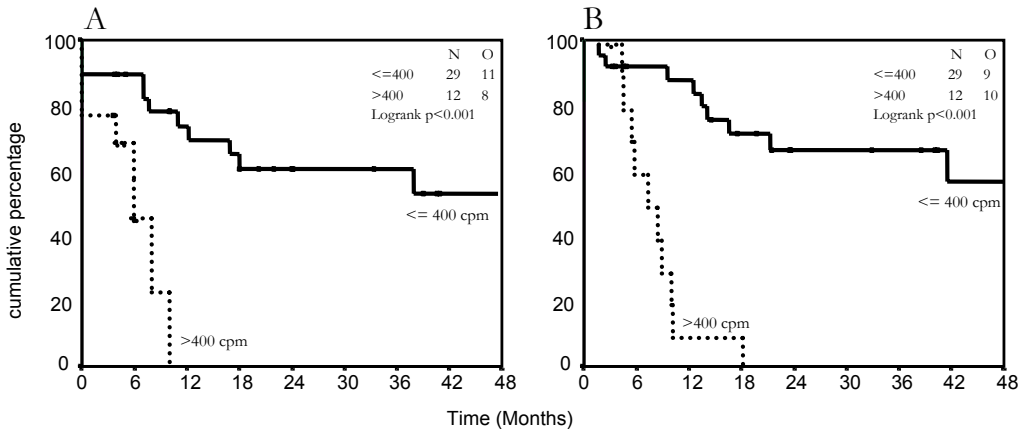

Figure 4. Cumulative failure-free (panel A) and overall (panel B) survival of the intermediate risk AML population, stratified according to the M-CSF induced proliferation.

The intermediate risk population studied was comprized of patients with a primary AML, having an age of 60 years or younger, having intermediate risk cytogenetics and not showing an Flt3/ITD.
Solid lines, population with a low (<=400cpm) M-CSF induced proliferation. Dotted lines, population with a high (>400 cpm) M-CSF induced proliferation. In the insert values under "N" depict the total number of samples in each population whilst the values under "O" depict the numbers of observed events in each population.

5.5. Discussion.

In the past few years much progression has been made in the identification of specific genetic defects, such as Flt3/ITD, AML-1-CBFβ, PML-RAR and various others, their respective role in the pathogenesis of AML and their clinical prognostic impact. Notwithstanding, there is a large population of patients who can not be stratified in specific cytogenetic risk groups. According to the conventional cytogenetic risk stratification there is a large number of patients with no detectable aberrations or with aberrations without prognostic value. Such patients are currently considered to have an "intermediate risk". This group probably is heterogeneous and includes a mixture of favourable and unfavourable risk individuals.
Hematopoietic growth factors may stimulate the *in vitro* proliferation of AML[26,27]. Correlations between the *in vitro* cytokine responsiveness and patient prognosis have been previously been reported[28-30].
Our observation that patients with a Flt3/ITD comprise a "low" cytokine responder population coupled to a poor prognosis, combined with the poor prognosis of pa-

tients with an age over 60 years, led us to define our intermediate risk population as that population of primary AML patients with an age below 60 years not having a tandem duplication in the Flt3 gene and with intermediate risk cytogenetics. Here we show that among this intermediate risk population, poor and good risk patient prognosis can be further stratified based on the short-term (7-day) *in vitro* response to either G-CSF or Il-1α. Our observation that the autonomous proliferation, though predictive in the total study population, was not predictive in the intermediate risk population could not be explained by the absence of a specific high autonomous responder, poor prognosis, population in our intermediate risk population (data not shown).
Though the survival curves of both M-CSF and Il-1α resemble each other (Figs 4-5), analysis of the individual patients showed different responses to both growth factors i.e. patients within the high responder population after Il-1 stimulation could be low responders after M-CSF stimulation and *vice versa* (data not shown).

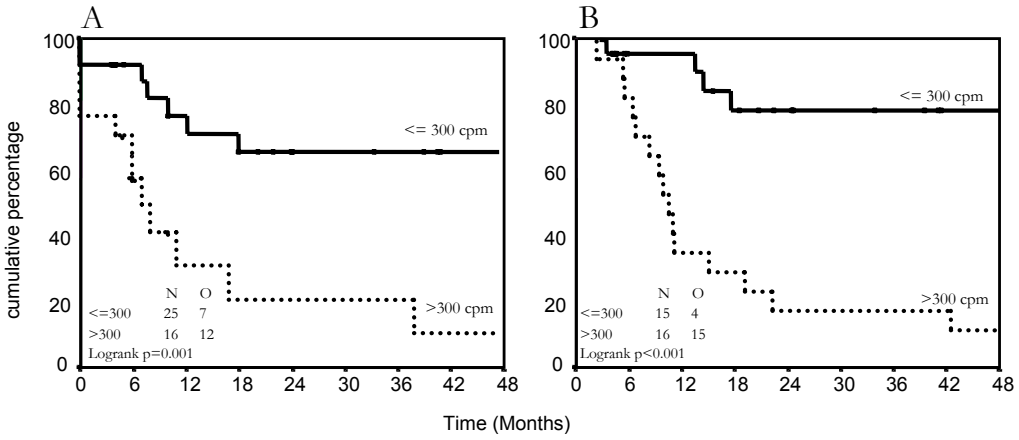

Figure 5. Cumulative failure-free (panel A) and overall (panel B) survival of the intermediate risk AML population, stratified according to the Il-1α induced proliferation.
The intermediate risk population studied was comprized of patients with a primary AML, having an age of 60 years or younger, having intermediate risk cytogenetics and not showing an Flt3/ITD.
Solid lines, population with a low (<=300cpm) Il-1α induced proliferation. Dotted lines, population with a high (>300 cpm) Il-1α induced proliferation. In the insert values under "N" depict the total number of samples in each population whilst the values under "O" depict the numbers of observed events in each population.

As calculated from the Kaplan-Meier survival analysis within the subpopulation of the redefined intermediate risk patients, the low responder groups had a four-year OS of around 60-80 percent whilst the four-year FFS of these groups varied between 60-70 percent. This is in sharp contrast with the high responder populations where these values were between 0-10% for both OS and FFS. The difference in the FFS of the intermediate risk population was also visible when calculating the median duration of the FFS (Table 7). In both cytokine combinations the median FFS for the low responder groups was over 60 months whilst this value was around 6-7 months for the high responders. In the cytokine responses that were found to be predictive, the better prognosis of the low responder group was not based on an increased initial therapy response. Rather, high responders had both a shorter FFS and an increased relapse rate. However, the biological significance in AML progression or AML relapse of both the autonomous and M-CSF or Il-1α induced response observed *in vitro* would require further study.

Table 7. Median duration of failure free survival in patients with intermediate risk primary AML.

	M-CSF induced proliferation		Il-1 induced proliferation	
	<= 400 cpm	>400 cpm	<= 300 cpm	> 300 cpm
Median duration (months)	> 60	6.0	>60	7.0
Standard Error		0.9		1.6
95% conf. interval		4.2 - 7.8		3.9 - 10.1
P-value	0.0003		0.0014	

Of patients with intermediate risk AML, i.e. those patients with an age below 60 years with intermediate cytogenetics and a wildtype Flt3 gene, the median duration of failure free survival was calculated by Kaplan-Meier analysis. Patients were stratified in to either a low or high response group based to the response to cytokines (see Results section).

As cytokine responses might be limited in time we also studied the cytokine responses after three days *in vitro* culture (data not shown). Though we did observe differences in the levels of responses (both increases and decreases were found when compared to day 7) neither of the cytokines tested at this time point was predictive for survival.
In the present study we have focussed on the total primary AML population and the population with an intermediate risk. We argued that the sub classification of the latter population into variable prognosis subgroups would be clinically most relevant. We also showed, that a poor patient prognosis is not linked to high overall cytokine re-

sponses of the patients AML-blasts, as is indicated by the significance of both M-CSF and Il-1 which only caused a modest proliferation in most samples (see Table 2). To date, the median follow-up period of our patient population is 15 months, which limits the predictive value of our experiments. Therefore, we plan to extend the observation period in the near future.

In conclusion, our present data indicate that within the intermediate risk group additional risk stratification is possible. Despite the fact that further follow-up of especially the good risk groups is required to study their disease progression over a prolonged period of time, we consider the detection of a high responder "poor risk" population in the intermediate risk group to be of importance. Whilst the initial therapy response of the patients within this population equals that of the other response groups, they are characterised by an increased relapse rate as well as a shorter overall and failure-free survival. If further study extends and supports our data, it should be considered to include these patients in the poor risk arms of treatment protocols and offer them intensified treatment or bone marrow transplantation.

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CHAPTER 6

Identification of variables determining the engraftment potential of human acute myeloid leukemia in the immunodeficient NOD/SCID human chimera model.

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6.1. Abstract

Among a variety of immunodeficient mouse strains the nonobese diabetic (NOD) / LtSz scid/scid strain appears to be most useful in allowing the engraftment of human AML. However, the large variability in ability to engraft and the levels of engraftment reached have not been explained. To address these issues we have investigated the NOD/SCID repopulating ability of 27 newly diagnosed AML samples. Patients were selected for the absence of internal tandem duplications in the Flt3 gene as we previously reported this mutation to be associated with an enhanced engraftment potential in this model.

We observed that secondary AML (n=6) had a significantly increased level of engraftment when compared to primary AML (n=21, median levels 73.3% for secondary AML vs. 8.94% for primary AML, p=0.01). Within the primary AML, a significantly higher engraftment was observed of the FAB class M0 than of FAB classes M2, M4 and M5. Within primary AML, samples of patients who failed to respond to the initial therapy gave rise to a higher level of engraftment than samples of patients who did respond to therapy. A similar observation of an increased engraftment correlating with a poorer patient prognosis could be made when applying cytogenetic risk stratification. However, within the primary AML the most important clinical parameter correlating with the level of engraftment appeared to be the patient's WBC counts at diagnosis (p=0.0000). Covariate analysis with the WBC count as a covariate could also fully explain the differences observed in the cytogenetic risk groups, or on the basis of the initial therapy response. Though large differences could be observed, the ability to engraft the NOD/SCID mice was not linked to either the autonomous or cytokine-induced proliferation in vitro. As the leukemic cobblestone area forming cell frequencies also revealed no correlation with repopulation in the NOD/SCID model, we consider it very likely that the level of engraftment reflects the in vivo proliferative ability of the AML samples assayed rather than the number of leukemia-initiating cells infused into the NOD/SCID mice. Phenotypic analysis based an the expression of CD33, CD34 and CD38 before and after passage in NOD/SCID showed that in 10 out of 16 samples investigated phenotypes were different.

In summary, in addition to the Flt3 internal tandem duplications we have identified a series of clinical parameters that determine the NOD/SCID repopulating ability of AML samples, whilst our data strongly suggest that AML in NOD/SCID does not reflect the leukemic process in the patient.

6.2. Introduction

The term acute myeloid leukemia (AML) encompasses a range of clonal hematopoietic disorders with an aberrant proliferation and differentiation. Generally, these leukemic cells are committed to the myelo-monocytic lineage though they might also be show erythroid or megakaryocytic lineage characteristics. Analysis of clonal markers[1-3] has shown that leukemic transformation may occur at the level of the pluripotent or the less primitive, committed, hematopoietic stem progenitor cell. We and others have recently shown that these primitive leukemic progenitors can give rise to long-term malignant hemopoiesis *in vitro* when supported by a feeder of murine stromal cells[4-6].
In normal hemopoiesis murine transplantation models have played a crucial role in our understanding of the most primitive stem/progenitor cells able to give multilineage engraftment. Until recently, research in normal human hemopoiesis was hampered by a lack of suitable in vivo assays. This has changed with the availability of chimeric models in which human hemopoietic progenitors exhibit multilineage engraftment when injected into sublethally irradiated immunodeficient mice[7-9].
Immunodeficient mice can also support the growth of malignant progenitors of chronic myeloid leukemia (CML) and AML[10-13]. Among a variety of immunodeficient mouse strains, we and others have evaluated, the nonobese diabetic (NOD) /LtSz scid/scid strain appears to be most useful in allowing the engraftment of human AML progenitors[14,15]. In contrast to the CB17/SCID strain, which is only defective in B- and T-cell function, this strain is characterized by an additional functional deficit in NK-cell function as well as defective antigen-presenting cells and an absence of circulating complement. Injection of AML-cells into these mice reportedly results in engraftment and development of leukemia in the murine hematopoietic tissue in a similar way to that seen in patients[16]. Though in most AML cases the cells able to initiate engraftment (SCID Leukemia-initiating cells or SL-IC) were phenotypically characterized as $CD34^{+}$ $CD38^{-6}$, some cases have been reported in which these SL-IC were $CD34^{-}CD38^{-4}$. The ability of SL-IC to engraft after a prior *in vitro* incubation with 5-FU suggests that these cells, analogous to normal hemopoiesis, belong to a more quiescent population[5].
The large variability between AML samples in their ability to engraft the bone marrow of NOD/SCID mice has thus far not been explained. Also, little is known about the correlation between engraftment in NOD/SCID and clinical or biological characteristics of the AML sample. To address several of these issues we have investigated a series of newly diagnosed AML samples in their ability to engraft in the NOD/SCID mice. These data were correlated with clinical parameters such as prognosis, cytogenetics, white blood

cell counts and FAB classification. We also looked for correlations with the Leukemic-Cobblestone Area Forming Cell (L-CAFC) content of the samples as well as the short-term in vitro proliferation in response to a panel of cytokines. As we have previously shown that AML samples from patients with an internal tandem duplication in the Flt3 gene (Flt3/ITD) represent a distinct subpopulation of AML with a greatly enhanced potential to engraft in the NOD/SCID[27] these samples were excluded from this study.

6.3. Materials and methods

Patient samples

Diagnosis bone marrow samples of patients with newly diagnosed AML were obtained after informed consent. Cases were classified according to the French-American-British committee (FAB)[17]. Mononuclear cells were isolated by ficoll separation followed by T-cell depletion, typically resulting in a population containing over 95 percent blasts. After isolation, cells were subjected to controlled freezing and stored in liquid nitrogen. Before use cells were thawed by stepwise dilution in Iscove's Modified Dulbecco's medium (IMDM, Gibco, Breda, The Netherlands) containing 1% BSA. After thawing viability varied between 70 and 90 percent as assessed by dye exclusion.
After diagnosis patients were treated according to studies of the adult Dutch-Belgian Heamato-Oncology Group HOVON and the Dutch childhood leukemia group SNWLK. Patients were treated according to protocols HOVON-29 (less than 60 yr. of age, 11 patients)[18], HOVON-4 (less than 60 yr., 3 patients)[19], HOVON-31 (60 yrs+, 4 patients), AML-11 (60 yrs+, 5 patients)[20] or SNWLK-ANLL-94 protocols (less than 15 yr. of age, 2 patients). Two patients received palliative treatment only. In these protocols induction therapy included an anthracyclin and cytarabine and in patients less then 60 yrs allogeneic and autologous stem cell transplantation or intensive chemotherapy for consolidation were applied as postremission therapy.

Stratification according to karyotype-risk groups

As cytogenetic aberrations are thought to be one of the most important prognostic factors for AML[21,22] patients were stratified in three groups i.e. a good-risk group consisting of patients defined by a karyotype of t(8;21), t(15;17) or inv(16) and a poor-risk group defined by del(5), del(7), or deletions of the q-arms of these chromosomes, t(6,9), alterations in the 11q23 region or the presence of multiple aberrations (more

than 3) in the karyotype. All other karyotypic aberrations and the normal karyotype were included in an intermediate-risk group.

Determination of the short-term proliferative response to single cytokine stimulation

To determine the proliferative response to single cytokine stimulation we used a thymidine incorporation assay. As monocytes and macrophages are known to produce many cytokines and thus potentially may perturb the results we removed these cells prior to their use in culture by adherence to plastic (1hr incubation at 37°C in alpha-MEM (Gibco) + 1% BSA (Sigma, St. Louis, MO, USA). The non-adherent cells were plated in 96-well plates at a density of $2x10^4$ cells/well in serum free medium[23] and cultured for six days. The cytokines used were hIL-3 (25ng/ml, a gift from Genetics Institute, Cambridge, MA, USA.), hGM-CSF (10ng/ml, Immunex, Seattle, WA, USA), hG-CSF (100ng/ml, a gift from Amgen, Thousand Oaks, CA, USA), hM-CSF (100 U/ml, generously supplied by Genetics Institute) or hSCF (100 ng/ml, a gift from Amgen). After six days cells were overnight incubated with 0.1μCi tritiated-thymidine (Amersham, Buckinhamshire, UK) per well and harvested onto nitro-cellulose filters. Thymidine incorporation was determined in a liquid scintillation counter (Pharmacia-LKB, Bromma, Sweden) as counts per minute (CPM). All cultures were done in triplicate.
The stimulation index was calculated by dividing the mean CPM of the triplicate cultures by the mean CPM of a 30Gy-irradiated control of the sample.

Leukemic Cobblestone area forming cell assay

The cobblestone area forming cell assay was performed as described[24]. Briefly, confluent FBMD-1 stromal layers in 96-well plates (Costar, Cambridge, MA) were overlaid with AML cells in a limiting dilution set-up. The cells were cultured in medium consisting of IMDM (Gibco) supplemented with 10% heat-inactivated fetal calf serum (Summit Biotechnology, Fort Collins, Co.), 5% horse serum (Gibco), β-mercaptoethanol ($5x10^{-5}$ mol/l) and hydrocortisone-21-hemisuccinate (10^{-6} mol/l, Sigma). rhIl-3 (8 ng/ml) and rhG-CSF (20 ng/ml) (both a generous gift of the Genetics Institute) were added to the medium and all cultures were performed at 37°C and 10% CO_2 in a humidified atmosphere.
Input values were equivalent to $1x10^5$ nucleated cells/well and 12 dilutions three-fold apart were used for each sample with 15 replicates per dilution. The percentage of wells with at least one phase-dark clone (cobblestone area, consisting of at least 5 cells)

beneath the stromal layer was determined weekly and leukemic CAFC (L-CAFC) frequencies were calculated using Poisson statistics[25].
Based on evidence from murine correlation studies it is assumed that both normal and leukemic cobblestone areas that are observed late in culture are indicators of primitive long-term repopulating cell in vivo, whilst early appearing clones are indicators of transiently repopulating and less primitive cells.

The NOD/SCID mouse model

NOD/SCID mice were bred in the Erasmus animal facility and housed under specified pathogen free conditions, using laminar airflow units. Mice were supplied with sterilised food and acidified tap water to which 100mg/l ciprofloxacine (Bayer AG, Leverkusen, Germany) was added. Both food and drink were available ad lib.
Before transplantation of the AML cells the mice received a total body irradiation of 3.5Gy gamma radiation (^{137}Cs source, Gammacell, Atomic Energy of Canada, Ottawa, Canada). Transplantation of the AML cells was done by injecting $3x10^7$ AML cells in the lateral tail vein. After 30 days, the mice were sacrificed by CO_2 inhalation in accordance with the institutional animal research regulations.
Cell suspensions were made from both femora and used for flowcytometry.

Flow cytometry

To determine the percentage of AML cells in bone marrow suspensions from mice that had been transplanted, cells were stained with an antibodies against the human common leukocyte antigen (CD45), CD34, CD33 and CD38 (CD45-FITC, CD38-FITC, CD33-PE and CD34-APC, all obtained from Coulter Immunotech, Marseille, France) and measured using a FACScalibur (Beckton Dickinson Immunocytometry systems, San Jose, CA, USA). Analysis was done using the Cell Quest software package (Beckton Dickinson). Erythrocytes were excluded from analysis by gating on forward and perpendicular light scatter whilst dead cells were excluded by staining with 7-amino-actinomycin D (7-AAD, Molecular Probes, Eugene, OR, USA).

Statistical analysis

Statistical analysis of the data was performed using the SPSS software package (SPSS Inc., Chicago Il.). Significance of differences for the proliferation, L-CAFC and SCID assays was determined by either the non-parametrical Mann-Whitney U test or the

Kruskal Wallis test for multiple independent parameters. Correlation analysis was performed using Spearmans rho test for nonparametrical correlation.

Table 1: Patient characteristics

patient No	Age	FAB classification	Cytogenetics	Initial therapy response	WBC (x 10^{-9}/l)
1	74	M0	-5,-2,-17,-18	No Cr	129
2	77	M0	7q-,+8	No Cr	214
3	62	M0	N	No Cr	39
4	19	M1	N	CR	50
5	40	M1	N	CR	72
6	79	M1	N	CR	260
7	61	M2	+4,+13,+14	No Cr	1
8	35	M2	t(8:21)	CR	16
9	28	M2	t(8:21)	CR	11
10	39	M2	t(8:21)	CR	8
11	88	M2	N	No Cr	47
12	61	M2	N	CR	2
13	66	M2	N	CR	1
14	16	M4	N	CR	1
15	69	M4	7q-,+8	CR	343
16	30	M4	t(6:11)	CR	126
17	23	M4	t(16;16)	CR	84
18	78	M4	N	No Cr	45
19	4	M4	N	CR	14
20	73	M4	N	CR	18
21	32	M4	N	CR	48
22	51	M5	+8	CR	85
23	49	M5	+8	CR	30
24	9	M5	t(10:11)	CR	3
25	54	M5	7q-	No Cr	11
26	63	M5	N	No Cr	32
27	46	M5	N	CR	44

A total of 27 samples of patients with newly diagnosed AML were included in this study. Patients were selected to give a good representation through out the FAB classes. cytogenetics were determined for all samples, cytogenetic abnormalities are included if observed.samples from AML-M3 patients are absent as we were unable to generate reliable results in the NOD/SCID using these samples.
Initial therapy response was defined as CR if the patient achieved a complete remission, No Cr if the patient failed to get in to remission or only achieved a partial remission.

6.4. Results

Variability in the outgrowth of AML cells in the NOD/SCID mouse.

The NOD/SCID mouse model was used to investigate the *in vivo* leukemia-initiating capacity of newly diagnosed AML. Due to the large number of cells needed for a reproducible engraftment ($3x10^7$ cells/mouse) and the limited availability of animals we were only able to analyze a limited number of samples (n=27). Groups of 3 mice/sample were injected with AML cells and analyzed at day 30 after transplantation. We were unable to analyze samples of FAB class M3 as all samples of this type injected into NOD/SCID resulted in death of the mice within 24 hours after injection (with the highest mortality within 1 hour). This was most probably due to *in vivo* lysis of the cells resulting in intravascular coagulation.

Patient details of the analyzable samples are given in Table 1. Analysis of the percentage of engraftment shows a clear variability between the FAB classes (Fig. 1). The M0 and M1 samples gave rise to the highest median level of chimerism whereas FAB M2 and M5 showed a low level of engraftment. As indicated by the large interquartile range there was an extreme variation within the M4 class. The median within this group was in the lower part of this range as most samples only induced a relatively low engraftment. A closer study of the clinical data indicated that the higher engraftment data in the FAB M4 class were by AML samples from patients that were likely to have had a previous history of MDS. Because we could not exclude the possibility that such so-called "secondary AML" patient samples were also included in the other FAB class groups; we re-analyzed the other groups as well (Fig. 2). Indeed we found that in all cases the secondary AML samples induced a significantly higher level of NOD/SCID engraftment as compared to primary AML samples.

When we excluded the secondary AML samples from our analyses, the highest level of engraftment was still observed in FAB class M0 and M1, however, statistical analysis showed only significant differences between classes M0 and M2, M4 or M5 ($p=0.036$, 0.025 or 0.024, respectively). As we only had six secondary AML samples in our data set we were not able to analyze this the engraftment ability of population on the basis of FAB class.A median engraftment of 8.9% was observed after pooling all primary AML samples irrespectively of FAB class (Fig. 3). In contrast, secondary AMLs showed a median engraftment of 73.3%, which was significantly different from that of primary AML samples ($p=0.01$). Based on this clearly different ability of both populations to engraft in the NOD/SCID the primary and secondary AML groups were separately analyzed in this study.

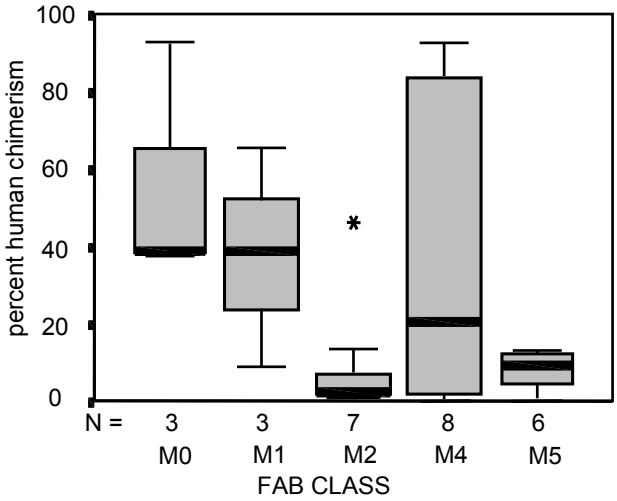

Figure 1: Distribution of the engraftment of AML in NOD/SCID mice over the FAB-classes.

The horizontal black lines in these figures represent the median observed chimerism. Shaded bars represent the interquartile range, whilst the error bars indicate extreme cases. Outlayers are marked by an asterisk. Numbers below the x-axis depict the number of cases within each group.

We also investigated whether the ability to engraft the NOD/SCID mouse could be correlated to the initial response of the patient towards therapy (Fig.4). In the primary AML samples there was a clear, though not significant, enhanced engraftment in the population who failed to achieve a complete remission (CR). Within the secondary AML samples no difference could be observed in engraftment between patients who achieved a CR and those who failed. Due to the low number of secondary AML samples in the group who failed to respond to therapy no significant differences between primary and secondary AML samples could be found. In the group of patients who achieved a CR the differences between these subtypes of AML were significant ($p=0.049$). Further subdivision of the patients achieving a CR in patients who remained in CR, and patients who relapsed within 23 months (our median observation period) no differences could be observed for both primary and secondary AML (data not shown). The observation that, at least for the primary leukemias, there was a relation between poor prognosis (i.e. therapy failure) and an increased ability to engraft the mice led us to investigate whether this observation could be related to other important clinical parameters associated with patient prognosis. Correlation analysis for the patient's age and the ability to engraft NOD/SCID revealed no relationship (data

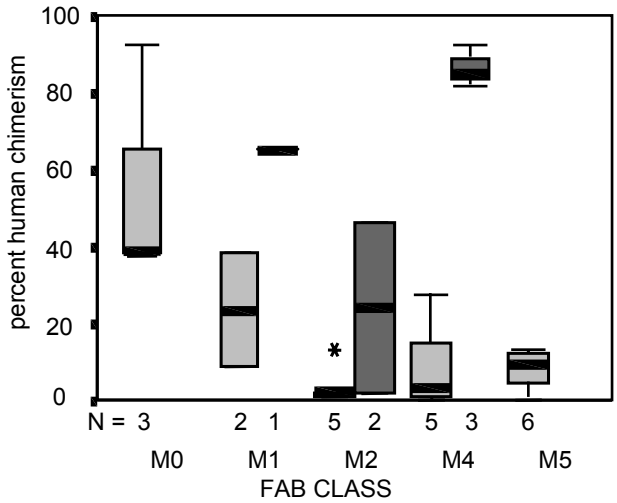

Figure 2: Distribution of the engraftment of AML in NOD/SCID over the FAB classes corrected for primary and secondary AML.

Light shaded bars represent primary AML whilst dark shaded bars represent secondary AML. The horizontal blackline indicates median values, bars represent the interquartile range, whilst the error bars indicate extreme cases. For furhter legends see figure 1

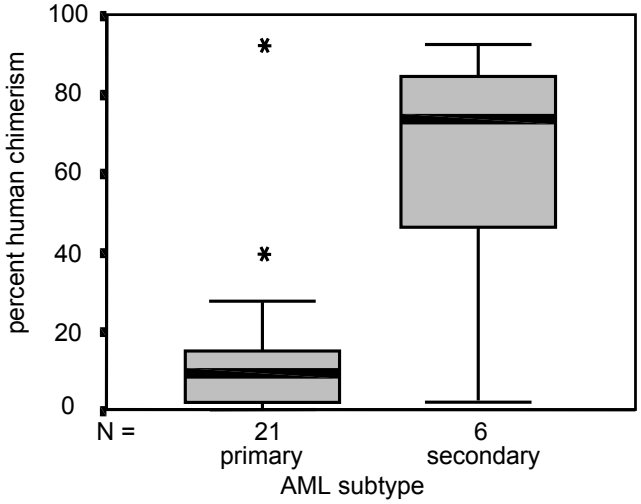

Figure 3: Differences in the overall level of chimerism between primary and secondary AML.

The horizontal black lines in these figures represent the median observed chimerism. Shaded bars represent the interquartile range, whilst the error bars indicate extreme cases. For furhter legends see figure 1

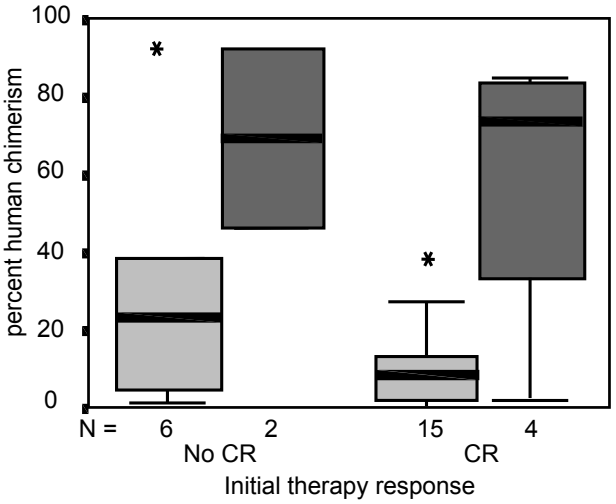

Figure 4: Relation between the level of engraftment in NOD/SCID and the initial therapy response of the patient.

CR group: Patients who achieved a complete remission in response to initial treatment, No CR group: Patients who failed to respond to therapy or only achieved a partial remission. Light Shaded bars, Primary AML, Dark shaded bars, Secondary AML. For furhter legends see figure 1

not shown). Application of cytogenetic risk stratification to the data indicated an increasing ability for the primary AML samples to engraft with a decreasing prognosis for the patient (Fig. 5). Though this relation was shown not to be significant, the most extreme responses were clearly in the poor prognosis group. As most secondary AML samples fell within the intermediate risk stratum no objective comparisons could be made within the other strata. In the intermediate risk group we could again observe a much higher level of engraftment for the secondary leukemias as compared to the primary leukemias ($p=0.026$).

Because an increasing white blood cell count (WBC) at diagnosis is also associated with poor prognosis, we performed a correlation analysis for this parameter with regard to NOD/SCID repopulation ability. Even without taking into account the presence of both primary and secondary AML this was shown to be a highly significant parameter ($p=0.0001$, data not shown). However, this correlation was only linked to the primary AML samples ($p=0.0000$ compared to $p=0.4680$ for secondary AML). As can be seen in Figure 6, low WBC levels correlate with a low level of engraftment of primary AML samples in NOD/SCID. The observation that some samples fell outside of the 95%

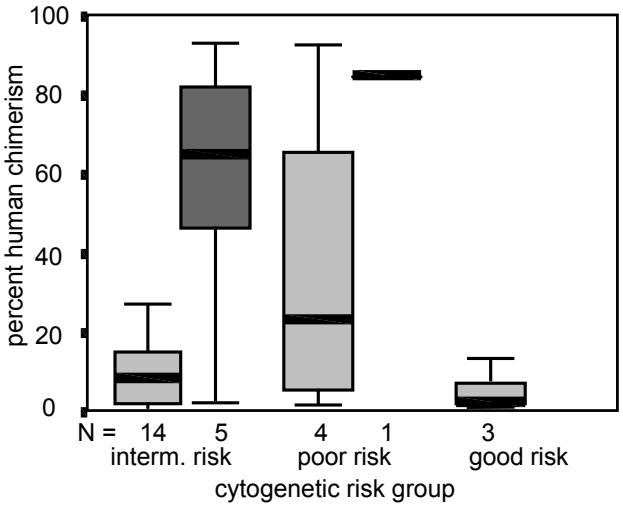

Figure 5: Distribution of the engraftment of AML in NOD/SCID over the cytogenetic risk groups.

Patients were stratified into cytogenetic risk groups according to the criteria as described in the Materials and Methods section. Light shaded bars, primary AML. Dark shaded bars, secondary AML. For furhter legends see figure 1

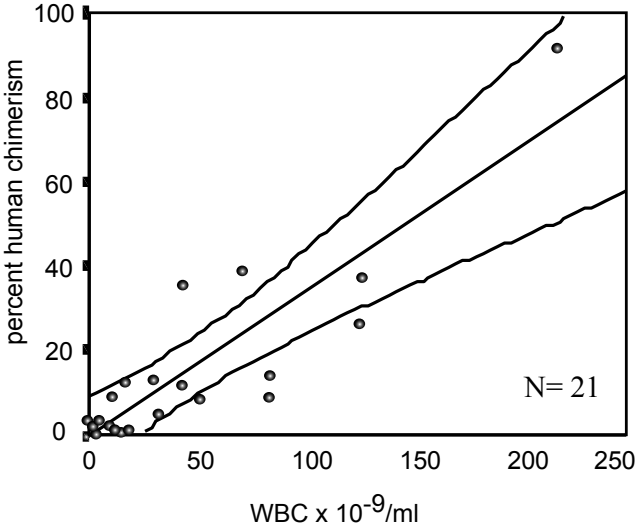

Figure 6: Correlation between the level of chimerism observed in the bone marrow of NOD/SCID infused with primary AML samples and the white blood cell count at diagnosis.

Each data point represents a single patient sample. Drawn lines indicate the fitted linear regression line and the calculated 95% confidence limits.

confidence interval suggest however that WBC counts are not the sole factor governing the NOD/SCID repopulating ability of primary AML.

Differences in surface marker expression after passage in the NOD/SCID mouse.

To investigate whether expression of the surface markers CD34 and CD38 either before or after passage in NOD/SCID could be linked to the engraftment of the mice, we performed a two-color flowcytometrical analysis. From the input phenotypes no correlations could be derived between the expression of CD34 and/or CD38 with regard to NOD/SCID repopulating ability. After passage in NOD/SCID only the $CD34^-CD38^+$ phenotype in the primary AML samples showed a low though significant negative correlation with NOD/SCID repopulation (Pearsons correlation: -.659, p= 0.014, data not shown). Correlation analysis between the phenotype of the samples before and after passage in NOD/SCID revealed no correlation (data not shown). As this suggested that for the majority of samples the phenotype of the leukemia cells differed before and after passage in NOD/SCID mice, 16 samples were subjected to a more detailed analysis using three-color flowcytometry for the expression of CD33, CD34 and CD38 (Table 2).

Table 2: Phenotype of the AML before and after passage in NOD/SCID mice.

FAB Class	patient No	input phenotype	% of total	phenotype after passage in NOD/SCID	% of total	
M0	1	$CD33^+CD34^-CD38^+$	86.2	$CD33^-CD34^+CD38^-$	65.9	*
	2	N.D.		$CD33^-CD34^+CD38^-$	98.8	
	3	mix		mix		
		$CD33^+CD34^+CD38^+$	33.5	$CD33^+CD34^-CD38^+$	40.1	*
		$CD33^-CD34^+CD38^+$	47.4	$CD33^+CD34^+CD38^+$	28.8	
M1	4	$CD33^+CD34^+CD38^+$	76.5	$CD33^+CD34^-CD38^+$	75.3	*
	5	$CD33^+CD34^-CD38^+$	97.1	$CD33^+CD34^-CD38^-$	82.1	*
	6	mix		mix		
		$CD33^+CD34^+CD38^+$	29.3	$CD33^+CD34^+CD38^+$	47.6	
		$CD33^+CD34^-CD38^+$	49.9	$CD33^+CD34^-CD38^+$	54.5	
M2	7	$CD33^-CD34^+CD38^-$	77.9	$CD33^+CD34^-CD38^+$	61.9	*
	8	N.D.		mix		
				$CD33^+CD34^-CD38^-$	26.5	
				$CD33^+CD34^-CD38^+$	40.0	

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table 2 cont'd

	9	mix $CD33^+CD34^-CD38^+$ $CD33^+CD34^+CD38^+$	 58.7 27.3	mix $CD33^+CD34^-CD38^+$ $CD33^-CD34^+CD38^-$	 49.4 34.9	*
	10	$CD33^-CD34^+$ $CD38^+$	75.8	$CD33^+CD34^-CD38^+$	83.6	
	11	N.D.		N.D.		
	12	N.D.		N.D.		
	13	N.D.		N.D.		
M4	14	mix $CD33^+CD34^-CD38^+$ $CD33^+CD34^+CD38^+$	 43.0 35.7	$CD33^+CD34^-CD38^+$	99.6	*
	15	$CD33^-CD34^+$ $CD38^-$	89.3	$CD33^+CD34^-CD38^+$	92.7	*
	16	N.D.		N.D.		
	17	N.D.		N.D.		
	18	mix $CD33^+CD34^-CD38^+$ $CD33^+CD34^+CD38^+$	 48.9 25.5	$CD33^+CD34^-CD38^+$	96.0	*
	19	N.D.		N.D.		
	20	N.D.		N.D.		
	21	N.D.		N.D.		
M5	22	$CD33^+CD34^-CD38^+$	90.2	$CD33^+CD34^-CD38^+$	90.1	
	23	$CD33^+CD34^-CD38^+$	91.4	$CD33^+CD34^-CD38^+$	90.1	
	24	N.D.		N.D.		
	25	mix $CD33^-CD34^+CD38^+$ $CD33^+CD34^+CD38^+$	 63.5 23.7	mix $CD33^+CD34^-CD38^-$ $CD33^+CD34^-CD38^+$	 54.1 27.9	*
	26	N.D.		N.D.		
	27	$CD33^+CD34^-CD38^+$	78.2	$CD33^+CD34^-CD38^+$	80.5	

A total of 27 samples of patients with newly diagnosed AML were included in this study. Patients were selected to give a good representation through out the FAB classes. cytogenetics were determined for all samples, cytogenetic abnormalities are included if observed.
samples from AML-M3 patients are absent as we were unable to generate reliable results in the NOD/SCID using these samples.
Initial therapy response was defined as CR if the patient achieved a complete remission, No Cr if the patient failed to get in to remission or only achieved a partial remission.

This analysis revealed that in 10 out of 16 samples the phenotype of the major populations changed with in vivo passage in the mice. The most frequent changes observed were a loss of CD34 expression accompanied by an increased expression of CD33 and CD38 (if those markers were not already expressed in the input population). Only in the FAB M0 class we found changes in phenotype from $CD33^{+}CD34^{-}CD38^{+}$ to $CD33^{-}CD34^{+}CD38^{-}$.

Table 3: The responses of primary and secondary AML to growth factor stimulation

	Primary AML				Secondary AML			
	in vitro proliferation index		correlation with NOD/SCID		in vitro proliferation index		correlation NOD/SCID	
Growth factors added	median	range	corr. coeff.	p-value	median	range	corr. coeff.	p-value
No Growth factor	9	1-131	-0.147	0.549	21	8-80	0.486	0.329
G-CSF	26	-10-309	-0.235	0.332	49	8-173	0.377	0.461
GM-CSF	22	1-185	-0.031	0.901	65	8-469	0.086	0.872
Il-3	36	2-172	-0.147	0.549	140	22-599	-0.029	0.957
M-CSF	9	-11-57	-0.054	0.828	10	-6-137	0.886	0.019
SCF	13	-43-278	-0.375	0.114	68	23-126	0.800	0.104

This table shows the median response and the range of primary and secondary AML to growth factor stimulation in a short-term thymidine incorporation assay. Is also shows the absence of correlation between these assays and the in vivo NOD/SCID model.
Responses are given as stimulation indexes defined as the fold-increase in thymidine incorporation between the test sample and an irradiated control sample. All values obtained from cytokine stimulations are corrected for the component caused by the autonomous proliferation, thus explaining negative values.

The extent of autonomous or cytokine-induced in vitro proliferation does not correlate with NOD/SCID repopulation.

There have been contrasting reports on the use of exogenous growth factors such as PIXY to enhance the level of engraftment in the AML-NOD/SCID model. To test if *in vitro* growth factor responsiveness or growth factor independence might be indicative for NOD/SCID repopulating ability of AML we studied the cytokine responsiveness using a tritiated thymidine assay (Table 3). All growth factor response data were corrected for the autonomous responses, i.e. the ^{3}H-TdR uptake in the absence of growth factors. Though large differences in response could be observed, especially with G-CSF and SCF, neither the growth factor-induced, nor the autonomous proliferation, proved to be indicative for repopulation of the NOD/SCID bone marrow.

The secondary AML samples generally had both a higher autonomous proliferation and a higher cytokine-induced proliferation. However, differences between primary and secondary AML were found to be not significant at a level of $P< 0.05$ (data not shown).

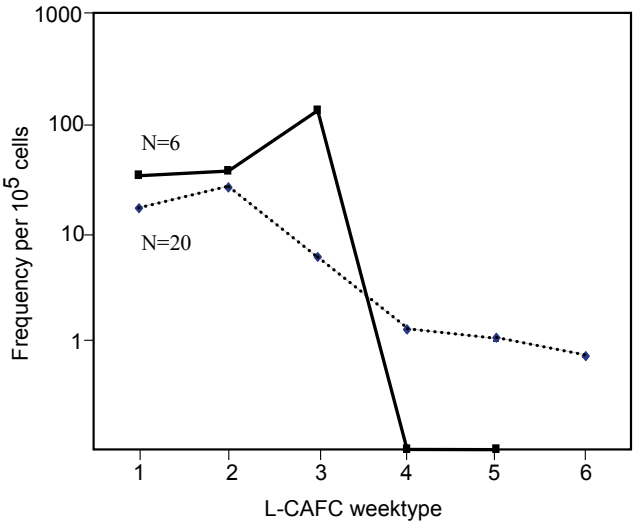

Figure 7: Median frequencies of various leukemic-CAFC weektypes.
20 primary and 6 secondary AML samples were plated in the L-CAFC assay, after which the frequency of the cobblestone area forming cells was analysed on a weekly basis. Primary AML, dotted lines. Secondary AML, solid lines.

NOD/SCID repopulation does not link with a specific leukemic cobblestone area forming cell subset.

The finding that *in vitro* incubation of AML with 5-FU depletes early weektype L-CAFC combined with the finding that these samples at least partially retain their NOD/SCID repopulating ability[5] led us to investigate whether we could find a correlation between the level of engraftment and L-CAFC frequencies. As shown in Figure 7, no significant differences were observed between the frequencies of L-CAFC week 1-2 in primary versus secondary AML. Frequencies of L-CAFC week 4-6 in secondary AML were at the lower threshold of detection, i.e. 1 per 10^6 cells and appeared to be reduced in comparison to the primary AML.

No correlations could be observed between the level of engraftment and any of the L-CAFC weektypes (Table 4), nor could we find a correlation between L-CAFC frequencies and FAB type, cytogenetics or white blood cell counts in patients (data not shown).

Table 4: Correlation analysis between the different L-CAFC weektypes and the chimerism observed in the bone marrow of NOD/SCID mice

	L-CAFC weektype	1	2	3	4	5	6
Primary AML	N	20	20	17	14	12	5
	Corr. coeff.	-0.128	-0.024	-0.183	0.133	0.421	0.200
	p-value	0.591	0.920	0.482	0.651	0.173	0.200
Secondary AML	N	6	5	5	3	3	
	Corr. coeff.	0.543	0.900	0.500	0.866	0.000	
	p-value	0.266	0.037	0.391	0.333	1.000	

This table shows the correlation coefficients and the p-values for Spearmans rho-test when analyzing for a correlation between the L-CAFC frequencies for the given weektypes and the level of human chimerism in the bone marrow of the NOD/SCID mice.

6.5. Discussion

The availability of an in vivo assay for human AML progenitors responsible for the maintenance of the malignant clone(s) is important both for our understanding of the basic biology of AML as well as for the development of new therapeutic strategies.
Over the last few years, we and other groups have shown the ability of AML cells to engraft in the NOD/SCID mice. However, one of the basic shortcomings of this model has been the low and unpredictable level of engraftment in this assay.
To our knowledge, this paper is the first to show that primary and secondary AML greatly differ in the ability to engraft the NOD/SCID mice. In addition, we show that at least in primary AML repopulating capacity was highly correlated to the patient's white blood cell counts at diagnosis. Covariant analysis of level of the engraftment and FAB class with the patient's WBC as a covariant could not fully explain our results thus suggesting there also is a FAB-specific factor involved in determining the level of engraftment. Similar covariant analysis, with WBC counts as a covariant, could however fully explain the observed correlation between the level of engraftment and the cytogenetic risk groups, i.e. poor, intermediate or good prognosis cytogenetics. The above findings might also explain a similar correlation with cytogenetics observed

by Ailles at al.[26] who did not distinguish between primary and secondary AML, nor looked at the relation with WBC. It might also explain why these authors observed different levels of engraftment in the various FAB classes.
For the primary AML we were unable to find a correlation between the NOD/SCID repopulating ability and any of the L-CAFC weektype frequencies. We could also not find a correlation between L-CAFC weektypes and any of the CD34/CD38 subpopulations, or between the CD34/CD38 subpopulations and repopulating ability. The latter two observations could be explained by assuming that both the SL-IC and L-CAFC only represent a minor subpopulation within any of the phenotypes. The absence of a correlation between NOD/SCID repopulating ability and any of the L-CAFC weektypes could imply that the SL-IC is either a subpopulation contained in any of the L-CAFC weektypes, or is a cell more primitive than the L-CAFC week 6 and thus not detectable in our assay. However, taken together our observations suggest that: A) there is a lack of correlation between L-CAFC frequencies and engraftment in the mice and B) there is a high correlation between WBC counts and NOD/SCID repopulation. This strongly suggests that the level of engraftment reached might rather reflect the proliferative capacity of the AML cells than the number of SL-IC infused. However, this proliferative capacity could not be correlated with either the autonomous or cytokine-induced proliferation in short-term thymidine incorporation studies. If the proliferative capacity of the samples is one of the main variables determining the engraftment of NOD/SCID mice this would also imply that assessing the number of SL-IC by limiting dilution in NOD/SCID can be biased by the proliferative ability of the sample under investigation.
Though it has been observed that AML cells injected into NOD/SCID have a similar dissemination pattern as in patients[16], we demonstrate here that in 10 of the 16 investigated samples the phenotype of the cells after passage in the mice differed markedly from the input phenotype. This phenotypic change of the infused cells may have been due to the fact that a subclone with the phenotype observed after passage is responsible for outgrowth, or that the AML cells undergo a different differentiation pattern in mice than in patients.
Whilst from our previous study[27] it has become clear that the presence of internal tandem duplications in the Flt3 gene in AML favours the outgrowth of these samples in the NOD/SCID model, the current study has been able to identify some additional important variables involved in determining the NOD/SCID repopulating ability of the Flt3 gene wildtype population.

Our present identification of parameters that determine the outgrowth of AML samples in NOD/SCID mice may contribute in selecting patient samples for therapy development in these immunodeficient mice.

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CHAPTER 7

General Discussion

7.1. Do Flt3-mutations occur at the level of the leukemic stem cell?

Recently the concept of leukemic stem cells[1] has gained increasing support. If the leukemia within a patient arises from such a malignant stem cell the treatment should ideally be directed against these stem cells.

Most of the studies carried out to reveal the biological characteristics of Flt3/ITD AML have been performed using cell line models, such as 32D and BAF/3, which are no proper tools to resolve the question whether Flt3/ITDs occur at the level of the leukemic stem cells or at a later stage. However, certain observations in patients allow us to address this question. The observation that in clinical AML, Flt3/ITDs often occurs at a mutant to wildtype ratio less than one strongly suggests that the Flt3/ITDs are not present at the level of the leukemic stem cell. This is further supported by the observations of Kottaridis et al.[2] who, using 44 paired samples of patients at diagnosis and relapse, observed that 6 of the 18 patients who presented with Flt3/ITD at diagnosis had completely lost the original mutation at relapse. One of these patients at relapse was found to have a mutation that had been absent at diagnosis. Four patients who at diagnosis were Flt3/wt, had acquired an Flt3/ITD at relapse. A second group[3], using another technique, studied 108 paired AML samples. In this study, 8 patients who were originally Flt3/wt had acquired an Flt3/ITD at relapse. Sixteen of the patients showed an ITD at both diagnosis and relapse, 8 of these patients had more then one Flt3/ITD. Yet, the observation of an increased outgrowth of Flt3/ITD AML in NOD/SCID mice (See chapter 2 and Lumkul et al.[4]) suggests that in a large part of the Flt3/ITD positive leukemias the mutation is present at the level of the malignant stem cell. Nevertheless, it is not clear if the observed outgrowth is solely dependent on the transplanted leukemic stem cells or that also other, more committed, cells are able to expand in this model. In chapter 4 of this thesis we show that the Flt3-induced up-regulation of CXCR-4 results in an increased SDF-1 induced chemotaxis, whilst it may possibly also contribute to cell survival by providing an anti-apoptotic signal. Both of these mechanisms could explain the increased outgrowth of Flt3/ITD AML in the NOD/SCID model. The occurrence of an enhanced survival of Flt3/ITD AML is further supported by the observations by Schnitgger[5] and Kottaridis[2] who observed that Flt3/ITD patients who relapsed, for the greater part showed an increased mutant to wildtype ratio at relapse.

However be these studies still can not definitively exclude that the poor prognosis of Flt3/ITD AML is an epiphenomenon, i.e. the observed poor prognosis is not due to the mutations themselves but by some other feature of the leukemic stem cells. Such a

mechanism could for example be a genetic instability that induces a tendency to result in the mutation of both Flt3[6] and other genes, which in turn are the true cause of the poor prognosis.

7.2. Clinical implications of Flt3 mutations in AML

7.2.1. Incidence of Flt3/ITD

To date over 20 studies, in total comprising over 5000 patients, have been published investigating the incidence and clinical implications of Flt3/ITDs in adult or pediatric AML. As is evident from the data summarised in Table 1 many studies involved a limited number of patients or only a subset of AML, which may explain the variations in incidence rates. Even more important, many of the studies represent retrospective studies using banked leukemia samples. Using banked samples one would expect to induce a bias for samples with high WBC counts or bone marrow cellularity. As most studies indicate Flt3/ITDs to be linked to high WBC counts, one expects the incidence rates as depicted in this table to be somewhat over estimated. Incidence rates in the studies can also be influenced by source material (peripheral blood, bone marrow or plasma DNA[7]) or the techniques used to amplify and detect the Flt3 mutations. Most of the studies are based on PCR amplification of genomic DNA or RNA followed by simple agarose gel electrophoresis, whilst others use a single strand conformation polymorphism analysis to detect mutations not detected in the standard gel electrophoresis. Yet, with regard to incidence of Flt3/ITDs several features are evident from the studies depicted in this table. First, the incidence of Flt3/ITDs is increased in patients with FAB-M3 / Acute promyelocytic leukemia (APL) and patients with socalled intermediate risk cytogenetics. On the other hand the incidence is decreased in patients with t(8;21) or inv(16), the so called core binding factor (CBF) leukemias.
In this context, Libura et al.[6] recently reported that Flt3 abnormalities in CBF-leukemias with AML-ETO or CBF-β-MYH11 were mainly restricted to cases with variant CBF-β-MYH11 fusion transcripts and/or atypical morphology. However, most of the presented clinical studies do not allow for the analysis of the prognostic impact of Flt3/ITDs in this specific subpopulation of CBF leukemias. Second, the incidence of the mutations in juvenile AML is reduced when compared to adult AML. However, 4 of the 6 studies involving juvenile AML have reported Flt3/ITD mutations to increase with age[8-11]. This is in contrast to adult AML where only one study reported an increasing incidence of Flt3/ITD with age[5].

Table 1: Occurrence and prognostic impact of Flt3/ITDs in AML

Study	Flt3/ITD present							Therapy	Median follow-up	Overall survival	CR Rate	Relapse Rate
	Adult AML	Elderly AML	Juvenile AML	2nd AML	FAB-M3	t(8;21) inv(16)	int. risk					
Rombouts[19]	23.7% (14/59)	22.9% (8/35)		18.2% (2/11)	33.3% (2/6)		24.2% (16/66)	Conv.	23 Mo.	↓	↓	↑
Kyoi[63]					20.3% (15/74)			ATRA	26 Mo	↔	↔	↔
Yamamoto[26]	18.9% (81/429)					11.8% (2/11)	28.0% (23/82)	Conv.	50 Mo.	↓		↑
Abu-Duhier[64]					10% (1/10)	5.8% (3/52)	22.7% (10/44)	Conv.		↓		
Stirewalt[21]		33.6% (47/140)						Conv.		↔		
Kottaridis[20]	26.6% (227/854)			27.4% (17/62)	36.8% (49/133)	8.3% (9/109)	34.2% (96/281)	Conv.	52 Mo.	↓	↓	↑
Whitman[15]							28.0% (23/82)	Conv.	20.4 Mo.	↔[a]	↔	↑
Thiede[12]	22.2% (217/979)			8.8% (3/34)	33.3% (13/39)	4.5% (4/88)	29.7% (134/451)	Intens.	12.2 Mo.	↔[b]		↑
Schnittger[5]	23.3% (234/1003)			15.6% (12/77)	35.8% (24/67)	5.4% (6/111)	39.3% (149/379)	Intens.	11.1 Mo.	↔	↔	↑
Boissel[18]	25.2% (40/159)					0.0% (0/23)	34.5% (28/79)	Intens.		↔	↔	↔
Noguera[14]					36.7% (33/90)			AIDA	> 8 yrs.	↔	↔	↔
Frohling.[65]	22.8% (119/523)			9.2% (7/76)	39.2% (20/51)	5.7% (4/70)	31.7% (71/224)	Intens.	34 Mo.	↓	↔	↑
Kainz[17]					38.1% (8/21)	7.7% (2/26)	30.2% (16/53)	Conv.		↓[c]		

To be comtinued on the next page

Table 1 cont'd

Moreno[27]	15.3% (32/208)			17.4% (4/23)	31.5% (6/19)	0.0% (0/14)	27.6 (31/112)	Conv.			↔	↑
Jilani[7]	21.2% (18/85)				25.0% (1/4)		25.4% (14/55)				↔	
Steudel[16]	26.1 (55/211)							Conv.		↔[d]		↑
Iwai[66]			5.3% (5/94)		11.1% (1/9)			Conv.		↓		
Xu[67]			13.8% (12/87)					Conv.		↓		
Kondo[10]			10.9% (7/64)		66.6% (2/3)			Conv.		↓	↔	↑
Meschinchi[9]			16.5% (15/91)					Conv. + Intens.		↓	↓	↑
Liang[11]			11.3% (9/80)		25.0% (3/12)			Conv.		↔	↔	
Zwaan[8]			11.5% (27/234)		22.2% (2/9)	2.7% (1/37)		Conv.	39 Mo.	↓	↓	↔[e]
Arrigoni[68]			27.0% (20/74)		34.5% (10/29)			Conv.		↓	↔	

[a] OS is significantly reduced in patients with deletions of the wildtype gene.
[b] OS is significantly reduced in patients with a high mutant to wildtype ratio.
[c] OS is significantly reduced in patients with intermediate risk cytogenetics.
[d] OS is significantly reduced in patients with a high mutant to wildtype ratio.
[e] relapse rates are increased in patients with a high mutant to wildtype ratio.

↓ = decreased
↔= No effect
↑= increased

Though Flt3 mutations are clearly more common in FAB-M3, they otherwise appear to be very evenly distributed over all other FAB classes. Nevertheless, some studies do report significant differences in the incidence within certain FAB classes[5,8,12]. Still, as these studies indicate different FAB classes to be involved they are most likely caused by biases in the sampled populations.
Taken together, from the data in Table 1 the occurrence of Flt3/ITDs can be estimated to be around 20% in adult AML (30% for the intermediate risk population) and around 14% in childhood leukemia.

7.2.2. The impact of Flt3/ITDs on patient prognosis

Of the 15 studies involving adult AML presented in table 1, two exclusively studied the impact of Flt3/ITDs in APL and both studies observed no effects of Flt3/ITDs on patient prognosis[13,14]. However, it should be noted that the numbers of these APL series were relatively small and thus the statistical power of these analyses is limited. Of the remaining 13 studies, 12 provided an analysis regarding the impact of Flt3/ITDs on the overall survival. Seven of these studies observed a reduced overall survival (OS) in Flt3/ITD AML in general, whilst three others only found a reduced OS in patients with either a hemizygous deletion of the wildtype allele (Whitman et al.[15]) or patients with a high mutant/wildtype ratio (Thiede et al.[12], Steudel et al.[16]). Kainz et al.[17], who studied a population with a relatively high APL frequency observed no effect of Flt3/ITDs in the total study population. Yet, when only patients of cytogenetic intermediate prognostic risk were analysed, Flt3/ITDs had a clearly negative impact on OS. The two remaining studies, which failed to detect any effect of Flt3/ITDs on OS both involved an intensified conditioning regimen[5,18]. It should however be noted that whilst the median follow-up in the study by Boissel et al. is unknown, the study by Schnittger et al. only had a median follow-up 11.1 month, which might have been too early for detecting differences in OS.
The major factor attributing to the lower survival of Flt3/ITD AML appears to be the increased relapse rates. Only in two studies involving adult AML, including our own, Flt3/ITD AML showed a reduction in the remission rate[19,20].
Even though the incidence in childhood AML seems to be reduced when compared to adult AML, also in this subtype of AML Flt3/ITDs appear to have a negative impact on patient prognosis. Similar to Adult AML, juvenile AML with Flt3/ITDs showed a reduced overall survival and an increased relapse rate.

Yet, in contrast to adult AML, three of the five evaluable studies showed a significant reduction of the CR rate in juvenile AML. This might explain why the effect of Flt3/ITDs on the overall survival appears to be even more dramatic than that in adult AML. Of the total of 695 patients involved in the seven pediatric AML studies, 85 were identified as having an Flt3/ITD. Of these patients only 19 were reported to have survived. This results in an overall survival of 22% for Flt3/ITD patients compared to 58% for the Flt3-wildtype patients in these pediatric studies.
Except for our own study, only the study of Stirewalt et al.[21] specifically investigated the prognostic impact of Flt3/ITDs in elderly AML. In contrast to our study, the latter study failed to detect an effect of Flt3/ITDs in elderly patients. However, the median OS of seven months in the older age population may obscure a possible negative impact of these mutations in elderly patients.
The majority of the studies presented indicate the presence Flt3/ITDs to be a significant and independent predictor of an unfavourable clinical response. Yet, as many studies have focussed on specific subgroups of AML patients and the duration of the follow-up is quite heterogeneous, it is difficult to directly compare the overall prognostic impact. This comparison is even more troubled by the variable incidence of Flt3/ITDs in the different studies. Also, in the first series of studies published patients were only divided in Flt3/ITD positive or negative cases. Within the Flt3/ITD positive cases, the ratio wildtype and mutant Flt3, or loss of heterozygosity was not taken into account.

7.2.3. Mutant to wildtype ratio and Flt3/ITD in relapsed AML

An increasing number of studies give evidence that the relative expression of the mutant Flt3 allele vs. the wildtype allele is an important factor in Flt3/ITD AML.
In 8 patients in which the wildtype allele could not be detected, Whitman et al.[15] observed an increased peripheral WBC count and an even poorer prognosis than in patients in which the wildtype allele was detectable. An increased WBC-count and the suggestion of a poorer prognosis was also observed in the studies by Kottaridis et al.[20] and Thiede et al.[12]. Several mechanisms, such as loss of heterozygosity[15] and homologous recombinations[12] have been reported to attribute to the allelic loss of the wildtype allele. Thiede et al. also noted that patients with a mutant/wildtype ratio higher than the median (i.e. 0.78) had a poorer prognosis than patients with a lower mutant to wildtype ratio and similar observations have been reported in the pediatric AML study by Zwaan et al.[8]. Libura et al.[6] observed in a real-time quantification of Flt3 of

141 AML samples that Flt3/ITD AML could be divided into cases with a high expression, which essentially belonged to the monocytic lineage, and those with a relatively low expression, which predominantly demonstrated PML-RARα or DEK-CAN translocations. Unfortunately this study contained no data about the clinical implications of these observations and neither did the authors investigate if the transcript levels correlated with the mutant to wildtype ratio at the DNA level.
Several groups have investigated the Flt3/ITD status in paired diagnostic and relapse samples[2,3,5,22]. Even though in a small fraction of patients the mutation is lost at relapse, these studies conclude that there is a tendency for an increased mutant to wildtype ratio at relapse. Also patients who at diagnosis were Flt3/ITD negative may acquire Flt3/ITDs at relapse in similar rates as observed in primary AML. Shih et al.[3] reported that all of the 6 patients presenting with multiple mutations at diagnosis showed changes in mutation patterns at relapse, suggesting selection and outgrowth of a mutant clone or evolution of a new clone harbouring these mutations. These findings suggest that Flt3/ITDs are not just the result of genetic instability but a distinct entity, which may affect cell survival.

7.2.4. Flt3/TKD mutations

Except for the ITDs in the juxtamembrane domain also other mutations, mainly involving the tyrosine kinase domain of the Flt3 gene, have been reported.
The observation by Glover et al.[23] of mutations in Asp802 in the activating loop of c-Fms and similar mutations in the activating loop of c-kit[24] led two independent groups to investigate mutations in Asp835 of Flt3[25,26]. Both groups reported the presence of mutations in this codon in about 7% of AML patients. Of these single base mutations the replacement of aspartic acid to valine is the most common, though other mutations in this codon involving the hydrophobic amino-acids tyrosine and alanine have been reported[2,27]. Recently also mutations in the neighbouring codon I836 have been reported[28] and it has also been demonstrated that a 2-amino acid elongation between codons 840 and 841 (840GlySer) within the activating loop represents an other form of activating mutation[29].
Similar to Flt3/ITD mutations, the frequency of these so-called Flt3/TKD mutations appear to be reduced in pediatric AML[30]. As the overall occurrence of Flt3/TKDs is less frequent than that of Flt3/ITDs, only a limited number of studies (see, Table 2) have investigated the clinical implications of Flt3/TKD mutations. Whilst in most studies Flt3/TKDs express no effect on overall- and relapse free survival, Thiede et

al.[12] observed a reduced overall survival in patients with Flt3/TKDs, but failed to find any effect on the relapse rates. Nevertheless, interpretation of the results from this study is complicated by the fact that 22% of the patients who presented with Flt3/TKDs also had an Flt3/ITD. Only Moreno et al.[27] observed both a reduced overall- and relapse free survival in Flt3/TKD AML. Yamamoto[26] observed no significant effects on relapse free survival, but did point out that all Flt3/TKD AML patients who achieved a complete remission relapsed within 28 months.

Table 2: Occurrence and prognostic impact of Flt3/TKDs in AML

Study	Presence of TKD mutation	Treatment	Overall survival	Relapse Rates
Thiede[12]	7.7% (75/979)[a]	Conventional	↓	↔
Sheikha[69]	7.5% (6/80)	Conventional	↔	↔
Liang[30]	3.3% (3/91)[b]	Conventional	↔	↔
Moreno[27]	9.6% (16/166)	Conventional	↓	↓
Frohling[65]	14.2% (32/224)	Intensified	↔	↔
Yamamoto[26]	7.0 (30/429)	Conventional	↔	↔[c]

[a] 22% of the TKD mutations also contained an ITD.
[b] only juvenile patients studied
[c] all TKD mutant patients reaching CR relapsed within 28 months

↓= reduced
↔ = no effect

7.3. Mode of action of Flt3/ITDs

From studies in the 32D and BA/F3 cell line models it has been suggested that both Flt3/ITD and Flt3/TKD mutations result in ligand-independent dimerisation and phosphorylation, leading to constitutive activation of STAT5 and MAP-kinase[31-33]. This is supposed to result in the ligand-independent proliferation observed in these models. Yet, Fenski[34] reported that in only one out of four patients with Flt3/ITD AML the receptor was ligand-independently autophosphorylated. Likewise, in the same study where constitutive autophosphorylation in cell lines was demonstrated, Kiyoi et al.[35] were unable to show ligand-independent phosphorylation of Flt3 in patient samples. Whereas it appears that the presence of Flt3/ITDs is associated with

STAT5 activation in primary AML cells[36], the levels at which the phosphorylated STAT5 is expressed in these samples was shown not to be associated with the presence of Flt3/ITDs[37]. Our observation (chapter 2 and 3) of a reduced in vitro proliferation of primary AML samples is in contrast with the ligand-independent proliferation as observed in the Flt3/ITD-transduced 32D and BA/F3 cell line models. Similar observations of a reduced in vitro proliferation of Flt3/ITD positive primary AML samples have later been reported by Bruserud[38]. It therefore seems unlikely that the mutations in the Flt3-gene cause ligand-independent proliferation of the AML cells in patients. Thus, ligand-independent proliferation in the BA/F3 and 32D models could be specifically restricted to these models rather than represent an intrinsic effect of the mutated receptor. Yet, it has also been shown that Flt3/ITD transfected 32D cells are blocked in the G-CSF induced differentiation pathway[39,40]. We have shown (chapter 4 of this thesis) that Flt3/ITD AML is characterized by an increased expression of CXCR-4. Furthermore in the presence of high SDF-1 levels CXCR-4 signalling induced a partial block in the G-CSF induced myeloid differentiation. We also observed that Flt3 is able to up-regulate the expression of CXCR-4, thus explaining the link between Flt3/ITDs, which represent an activated form of the receptor, and an increased CXCR-4 expression. However, it is unlikely that in clinical AML Flt3/ITDs are the primary cause of a differentiation block. If Flt3/ITDs would induce a differentiation block one would expect all Flt3/ITDs to arise in primary AML, which is not the case. It is known that Flt3/wt AML at relapse acquire Flt/ITDs at a similar rate as observed in primary AML. Though rarely, also the opposite may be true: primary AML presenting with Flt3/ITDs can loose this mutation and at relapse present with wildtype Flt3. Kelly et al.[41] observed that mice transplanted with Flt3/ITD transfected bone marrow cells developed a lethal myeloproliferative disease within 40-50 days following transplantation. This strongly suggests Flt3/ITDs to provide proliferative or survival signals while exerting little effect on differentiation. Binding of the ligand to the wildtype Flt3 has been shown to reduce apoptosis in primary AML cells by prevention of the up-regulation of Bax[42]. Likewise, Minami et al.[43] have shown the existence of different anti-apoptotic pathways, involving the BCL-2 family member BCL-X_L, in wildtype and mutant Flt3 in the murine myeloid cell line 32D. Whilst ligand-binding to Flt3/wt-transfected cells reduced apoptosis by maintaining the phosphorylation of BAD, it did not restore the down regulation of BCL-X_L. On the other hand, in Flt3/ITD-transfected 32D cells both the phosphorylation of BAD and the expression levels of BCL-X_L were maintained. Consequently, it could be that mutations in Flt3 promote

the survival of AML cells by suppressing apoptosis. Yet, the authors of the latter study were unable to explain the mechanisms involved and treatment with antisense BCL-X_L was only partially able to reduce the anti-apoptotic effects of the Flt3/ITD.
In the same series of experiments where we showed that SDF-1/CXCR-4 interactions could induce a block in the myeloid differentiation of 32D cells, we also showed that SDF-1/CXCR-4 interactions provide a strong anti-apoptotic signal in the 32D system. It could therefore well be that the up-regulated CXCR-4 expression provides an anti-apoptotic signal which, following chemotherapy, would result in an increased survival of Flt3/ITD AML when compared to Flt3/wt AML.

7.4. Flt3/ITDs as therapeutical targets in AML

Even though the mechanisms by which Flt3/ITDs have their impact on patient prognosis are not clear, the prognostic significance of these mutations adds to the credentials of Flt3 as potential therapeutical target in the treatment of AML. The recent clinical success of the ABL-kinase inhibitor STI571 (imatinib mesylate, Gleevec, Glivec) in the treatment of CML has boosted research into the use of kinase inhibitors for other types of hematological malignancies. These tyrosine kinase inhibitors (TKIs) encompass a variety of chemical classes but all are heterocyclic compounds containing a structure which mimics the purine component of ATP[44,45]. Despite the fact that all of these compounds are likely to fit the ATP binding pocket of the receptor, some do so by a mechanism of an induced fit whilst others, such as STI571, bind in a lock-and-key manner[46,47]. Therefore not all TKIs are able to inhibit all subclasses of RTKs with the same efficiency. Even within the same RTK family, levels of inhibition might very considerably. As an example, STI571 very effectively inhibits BRC-ABL, wildtype-c-Kit, some forms of mutated c-Kit and PDGFR, however, it is not very effective in inhibiting the kinase activity of wildtype-Flt3 or mutated Flt3 (IC_{50} > 3μg/ml).
The first Flt3 inhibitors described were AG1295 and AG1296[48,49], which originally were characterised as inhibitors of the PDGFR[50]. Both of these compounds are in vitro cytotoxic to Flt3-dependent cell lines at an IC_{50} of 300nM. AG1295 has been shown to be selectively cytotoxic to primary AML blasts with Flt3/ITDs[48]. CEP-701 and CEP-5214 are two other orally available biomolecules that have been shown to more selectively inhibit wildtype-Flt3 and mutant-Flt3 (IC_{50} CEP-701 2-3 nM for Flt3 compared to > 500nM for c-Kit)[40,51]. CEP-701 has been shown to be cytotoxic to cells harbouring Flt3/ITDs both in vitro, using transfected cell line models, and in vivo, using a mouse model of Flt3/ITD leukemia[51]. SU5416, SU5614 and SU11248 also

inhibit the Flt3/ITD induced phosphorylation both in cell line models and *in vivo*[52-55], but unlike the CEP-compounds, these TKIs also show considerable activity against other RTKs like c-Kit and VEGF. CT53518 is a novel quinazoline compound that inhibits Flt3, PDGFRβ and c-kit[56]. GTP-14564, a compound with structural characteristics of the part of staurosporine that inhibits protein kinase-C by competing for ATP, has *in vitro* been tested against a wide range of kinases. It inhibits members of the class III RTKs including Flt3, c-Kit, c-FMS and PDGFRβ. In *in vitro* cell based assays the compound was able to inhibit the Flt3/ITD-induced phosphorylation with a 30 fold lower concentration as was needed for wildtype-Flt3[57]. Finally, PKC412 is a benzoylstaurosporine which originally was developed to inhibit VEGF but which is also active against Flt3[58]. Several of these compounds are currently in clinical trials (Table 3). Three of these, CEP-701, SU5416 and PKC412, have entered phase II studies of patients with relapsed or refractory AML harbouring Flt3-activating mutations[59,60.]

While the preliminary results of these trials give reason for some optimism, there are some important considerations to take into account. First, the cytotoxic activity against Flt3 of most compounds has only been shown *in vitro*, only of a minority of compounds *in vivo* data from animal models are available. *In vitro* toxicity cannot always directly be translated to *in vivo* toxicity due to for example bio-distribution, degradation and clearance of the compounds. Also binding to specific proteins could alter bio-availability *in vivo*, as for example was observed for the binding of STI-571 to Alpha1 acid glycoprotein[61]. Further, none of the TKIs are truly specific for mutated-Flt3. Though this could be seen as an advantage as most AML blasts express Flt3, it may also be a reason for concern. As part of the hematopoietic progenitors and the HSC also express Flt3 there is a possibility that such a compound could delay the recovery from therapy- or leukemia related cytopenias. Another theoretical problem would be if the drug would result in the suppression of both Flt3 and c-Kit, as this could result in an even greater depletion of these cells. Another factor to take into account is the large variability in the expression levels of the mutated Flt3. Most of the *in vitro* and *in vivo* models used for screening the cytotoxicity of the potential TKIs show high expression levels of the aberrant gene product, therefore it could well be that in patients with AML showing a relatively low expression of Flt3 mutations the drugs are less efficient in killing the aberrant cells. Furthermore, Flt3/ITD and Flt3/TKD mutations are distinctly different types of mutations, which could respond differently to TKIs. As Flt3/TKDs concern mutations in the kinase domain it might therefore be expected that some of these mutations would confer a relative resistance to TKIs. Recently,

Grundler et al.[62] have indeed observed that sensitivity for three different TKIs varied between different types of Flt3-mutations. In this study Flt3/ITD and Flt3-Ile836del expressing cells showed a dose-dependent apoptosis in response to AG1296, whilst this compound did not result in an increased apoptosis of Flt3-Asp835Tyr and Flt3-Ile836Met mutations at any of the concentrations tested. PKC412 at concentrations between 25 and 100nM rapidly induced apoptosis in cells expressing the TKD mutations, whilst Flt3/ITD mutants displayed far less apoptosis. The third TKI tested, SU5416 induced apoptosis most effectively in cells expressing the Flt3-Ile836del mutation. From the above mentioned study it is clear that some compounds are very well able to inhibit Flt3/TKD mutations but are relatively ineffective in inhibiting Flt3/ITD mutations and vice-versa. Therefore, each of the Flt3-mutations should be tested for sensitivity to the different TKIs before patients could be treated with these compounds as it would be inappropriate to treat AML patients with Flt3-mutations known to be resistant to a given inhibitor. It should also be noted that in rare cases patients present with both Flt3/ITD and Flt3/TKD mutations, which makes treatment with kinase inhibitors even more complicated. This indicates that all patients should have sequence analysis of both the juxtamembrane and kinase domains to enable selection the most effective treatment strategy.

Table 3: Flt3 inhibitors

Compound	IC_{50}	Clinical stage
AG1295[48,49]	300 nM	--
AG1296[48,49]	300 nM	--
CEP-701[40,51]	3 nM	Phase II
SU5416[52,53]	100 nM	Phase II
SU5614[55]	10 nM	Phase I
SU11248[54]	50-250 nM	Phase I
GTP-14564[57]	1μM	--
PKC412[58,60]	< 10 nM	Phase II
D-64406[70]	3 μM	--
D-65475[70]	1 μM	--
CT-53518[56]	30-100 nM	Phase I

IC_{50} refers to the drug concentration required to inhibit the Flt3-autophosphorylation by 50% as determined in a variety of different assays.

Finally, perhaps the most important factor to take in to consideration is that Flt3-mutations most probably are late-occurring events. Therefore it is not clear whether the mutations play a crucial in the survival of the Flt3/ITD AML. If this is not the case, treatment with a kinase inhibitor alone does not eradicate the leukemia.

7.5. Conclusion

With an occurrence of around 28 percent in adult AML, Flt3-mutations are to date the most frequent genetic mutations observed in AML. Although the mechanism by which mutations in the Flt3-gene influence patient prognosis is unresolved, it is clear that AML with Flt3/ITDs represent a subset of leukemia with a poor prognosis. An increasing number of studies indicate that the relative expression of the mutant gene compared to the wildtype gene is an important factor in Flt3/ITD AML. The main factor contributing to the reduced outcome of patients with Flt3/ITD AML is the increased relapse rate. Whereas the incidence of these mutations is reduced in juvenile AML, Flt3/ITDs predict for an even poorer response to therapy. In contrast to adult AML, in juvenile AML the presence of Flt3/ITDs results in both a reduction of the CR rate as well as an increased relapse rate.

While the preliminary results of clinical trials using tyrosine kinase inhibitors for the treatment of Flt3 mutant AML may pave the way to new effective targeted therapies for chemotherapy resistant AML, one should also be aware of the potential limitations and pitfalls of the approach. For instance, it is conceivable that TKIs might inhibit normal hematopoietic stem- and progenitor cells which also express Flt3 and other RTKs and the different types of mutations may correlate with the broad variability of TKI sensitivity. This would suggest the importance of sequence analysis of both the JM domain and the kinase domain in patients in order to correlate the therapeutic effect with the type of mutation. Yet, the development of Flt3 inhibitors as monotherapy is an unlikely scenario, as mutation of the Flt3 gene as a single abnormality is insufficient to cause leukemia. Since multiple mutations are likely to be present in Flt3 mutant AML, one would predict that treatment regimens combining chemotherapy and TKIs will need to be developed.

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Summary

In the process of hematopoietic development errors may occur, resulting in the aberrant activation of (proto-)oncogenes and inactivation of tumor-suppressor genes. This aberrant gene expression may finally result in leukemia, a neoplastic disorder in which immature hematopoietic cells accumulate in the BM where they fail to fully mature and dislocate the normal progenitor cells hence bringing the normal homeostatic processes out of balance.

The fms-like tyrosine kinase 3 (Flt3) is expressed on multipotential HSC and progenitor cells, suggesting a critical role in stem cell development and differentiation. In 1996 Nakao et al. were the first to report a novel mutation in the Flt3 gene in a small number of AML patients. These mutations were shown to be internal tandem duplications, mainly involving a tyrosine rich stretch at the end of exon 14 (formerly known as exon 11), coding for the juxtamembrane domain of the receptor. Since at that time little was known about the biological characteristics and clinical relevance of Flt3/ITD this led us to further characterise these mutations, the main aim of the experiments described in this thesis.

Our studies (**chapter 3**) indicate that with around 20-25 percent of adult AML expressing Flt3/ITDs, these mutations represent the most frequent genetic alterations in AML. Clinically these mutations were significantly associated with a high probability of recurrence of leukemia and reduced survival.

In **chapter 2** we investigated the effect of Flt3/ITDs on the proliferative capacity of leukemic cells in long-term culture. Using stromal support to maintain long-term cell production, we were able to show that Flt3/ITD AML samples lacked an increased non-adherent cell production in response to cytokine addition as was observed for Flt3/wt AML. This aberrant behaviour was also observed in short-term cultures using single cytokine stimulation (**chapter 3**). Our observations of a reduced *in vitro* proliferative ability of these primary AML cells are in contrast to the reported growth factor independent proliferation following introduction of Flt3/ITDs in cell line models (32D and BA/F). The decreased proliferative capacity of the Flt3/ITD samples was probably not due to a decreased AML progenitor frequency as in the L-CAFC assay both the frequencies and the size of the cobblestone areas derived from Flt3/ITD or Flt3/WT were similar. Remarkably, we observed an increased ability of FLT3/ITD AML to engraft in the immunodefiecient NOD/SCID mouse. We have shown (**chapter 6**) that primary (or de novo) and secondary AML differ greatly in their ability to engraft in the NOD/SCID model. However, as both the Flt3/WT and Flt3/ITD population were mainly comprised of patients with primary AML this could not ex-

plain these differences. We also showed that within the primary AML population there is a strong correlation between the patients WBC counts and the repopulation in the NOD/SCID mice. Even though Flt3/ITD patients in general showed an increased WBC count when compared to Flt3/WT samples, this did not explain the mechanism behind the increased repopulation. Peled et al. (*Science* 1999;**283:**845-8.) have reported that engraftment and repopulation of normal human hematopoietic stem cells in the NOD/SCID mouse is dependent on the expression of CXCR-4 which is the receptor for SDF-1. Yet, in **chapter 4** we could not find a correlation between the NOD/SCID repopulating ability and the migratory abilities in response to SDF-1 or the overall expression of CXCR-4 in both Flt3/ITD and Flt3/wt AML. However, we did observe a strong correlation between the NOD/SCID repopulating ability and the percentage of $CD34^+$ cells expressing CXCR-4. When compared to Flt3/wt AML, Flt3/ITD AML was characterised by both an increased percentage of cells expressing CXCR-4 as well as an increased expression of CXCR-4 per cell. This increased expression of CXCR-4 in Flt3/ITD AML, which represents AML with a constitutively activated form of Flt3, could be explained by our observation that activation of Flt3 by its ligand results in an up-regulation of the CXCR-4 expression. The increased expression of CXCR-4 resulted in an increased SDF-1 induced chemotaxis of Flt3/ITD AML when compared to Flt3/wt AML, which might suggest that Flt3/ITDs also alter the *in vivo* migratory behaviour of Flt3 mutant AML. In addition, the observation that blocking of CXCR-4/SDF-1 interactions induced apoptosis in the 32D system suggests that CXCR-4 also provides a strong anti-apoptotic signal. It could therefore also be that the Flt3 induced up-regulation of the CXCR-4 expression provides an anti-apoptotic signal which following chemotherapy would result in an increased survival of Flt3/ITD AML. The testing of this hypothesis would require clinical data regarding the biodistribution, survival and proliferation of Flt3/ITD AML.

Even though the mechanisms by which Flt3/ITDs have their impact on patient prognosis are far from clear, the prognostic significance of these mutations adds to the credentials of Flt3-mutations as a potential therapeutical targets in the treatment of AML. Whilst the preliminary results of clinical trials using tyrosine kinase inhibitors for the treatment of Flt3-mutant AML give reason for some optimism some important considerations have to be taken in to account. On the one hand these concern the effects of these kinase inhibitors on normal hematopoietic stem- and progenitor cells which also express Flt3 and other receptor tyrosine kinases and, on the other hand these concern the relative resistance to some of the kinase inhibitors due to the dif-

ferent types of mutations which can be present. The latter observation also indicates that all patients should have sequence analysis of both the JM domain and the kinase domain to decide for the most effective treatment strategy. Yet, the use of Flt3-inhibitors as monotherapy is unlikely, as mutation of only the Flt3-gene does not result in leukemia, multiple mutations must be present in Flt3-mutant AML. Therefore optimal treatment regimens combining chemotherapy and tyrosine kinase inhibitors will need to be established.

Samenvatting

In het proces van bloedcelvorming kunnen fouten ontstaan, die kunnen resulteren in een afwijkende activatie van (proto-) oncogenen en/of inactivatie van tumorsuppressorgenen. Deze afwijkende genexpressie kan uiteindelijk resulteren in leukemie, een neoplastische afwijking waarbij onrijpe bloedvormende cellen in het beenmerg accumuleren en de normale hemostatische processen uit balans brengen.
De "fms-like" tyrosinekinase 3 (Flt3) wordt tot expressie gebracht op multipotente HSC en voorlopercellen, wat een kritische rol in de stamcelontwikkeling en differentiatie suggereert. In 1996 waren Nakao et al. de eersten die een nieuwe mutatie in het Flt3 gen bij een klein aantal AML patiënten rapporteerden. Bij deze mutaties bleek het te gaan om interne tandemduplicaties, welke voornamelijk aanwezig waren in een tyrosinerijk gebied aan het einde van exon 14 (voorheen bekend als exon 11) coderend voor het juxtamembraandomein van de receptor. Aangezien er destijds weinig bekend was over de biologische eigenschappen en klinische relevantie van deze mutaties hebben wij deze verder gekarakteriseerd, hetgeen het voornaamste doel werd van de experimenten beschreven in dit proefschrift.
Onze studies (**Hoofdstuk 3**) geven aan dat Flt3/ITDs voorkomen bij 20-25 procent van de volwassen AML patiënten, wat betekent dat Flt3/ITDs tot op heden de meest voorkomende genetische mutatie bij deze vorm van AML is. Klinisch zijn deze mutaties significant geassocieerd met een verhoogde kans op het terugkeren van de leukemie en een verlaging van de overlevingskansen.
In **hoofdstuk 2** hebben we gekeken naar de effecten van Flt3/ITDs op de proliferatieve capaciteiten van leukemiecellen in lange-termijnkweken. Door gebruik te maken van stromale lagen om de lange-termijnkweken te ondersteunen, waren we instaat om aan te tonen dat Flt3/ITD AML, in tegenstelling tot Flt3/wt AML, geen toename in de proliferatievecapaciteiten vertoonde in respons op de toevoeging van cytokines. Dit afwijkende gedrag van Flt3/ITD AML werd ook gezien in respons op cytokine-additie in kortetermijnkweken (**Hoofdstuk 3**). Onze observatie van een gereduceerde *in vitro* proliferatie van deze primaire AML cellen staat in contrast met de gerapporteerde groeifactor onafhankelijke proliferatie ten gevolge van de introductie van Flt3/ITDs in cellijn modellen (32D en BA/F3). Deze afgenomen proliferatieve capaciteit van de Flt3/ITD monsters is waarschijnlijk niet veroorzaakt door een afname in de frequentie van AML-voorlopercellen, daar in het L-CAFC assay zowel de frequentie als de afmetingen van de cobblestone-area's gelijk waren voor zowel Flt3/ITD als Flt3/wt AML. Opmerkelijk was de observatie dat Flt3/ITD AML een verhoogde uitgroei in de immuundeficiënte NOD/SCID-muizen te zien gaf. Wij hebben laten zien (**Hoofd-**

stuk 6) dat primaire (of de novo) en secundaire AML een groot verschil vertonen in hun vermogen om in deze muis uit te groeien. Omdat zowel de Flt3/wt- als de Flt3/ITD-populatie voornamelijk bestonden uit monsters van patiënten met een primaire AML kon deze observatie het verschil in uitgroei in NOD/SCID-muizen niet verklaren. Tevens hebben we aangetoond dat er binnen de primaire AML-populatie een sterke correlatie bestond tussen de witte bloedcelaantallen gemeten in de patiënten en het repopulerend vermogen in NOD/SCID muizen. Hoewel Flt3/ITD patiënten in het algemeen een verhoogd aantal witte bloedcellen hadden in vergelijking met Flt3/wt patiënten, kon ook dit het verschil in uitgroei niet verklaren. Peled et al. (*Science* 1999;**283:**845-8.) hebben laten zien dat het aanslaan en de uitgroei van een transplantaat van normale bloedcelvormende stamcellen in de NOD/SCID muis afhankelijk is van de expressie van CXCR-4, de receptor voor de chemokine SDF-1. In **hoofdstuk 4** waren wij evenwel niet in staat om een correlatie aan te tonen tussen het NOD/SCID-repopulerend vermogen en de migratie in respons op SDF-1 voor zowel Flt3/ITD als Flt3/wt AML. Echter, binnen het cluster van $CD34^+$ cellen was er een duidelijke correlatie tussen de expressie van CXCR-4 en de uitgroei in NOD/SCID. In vergelijking met Flt3/wt AML is Flt3/ITD AML gekarakteriseerd door zowel een verhoogd percentage cellen dat CXCR-4 tot expressie bracht als door een verhoogde CXCR-4 expressie per cel. Deze relatief verhoogde expressie van CXCR-4 in Flt3/ITD AML, welk een subtype van AML met een constitutief geactiveerde Flt3 vertegenwoordigd, zou verklaard kunnen worden door onze observatie dat activatie van Flt3 door zijn ligand resulteert in een verhoogde expressie van CXCR-4 op het celmembraan. *In vitro* resulteerde de verhoogde expressie van CXCR-4 in een toename van de SDF-1 geïnduceerde chemotaxis van Flt3/ITD AML in vergelijking met Flt3/wt AML, hetgeen suggereert dat Flt3/ITDs mogelijk ook *in vivo*, in de patiënt, de migratoire eigenschappen veranderd. Voorts wijst de observatie dat blokkering van de CXCR-4/SDF-1 interactie apoptose induceert in het 32D systeem er ook op dat activatie van CXCR-4 resulteert in een sterk anti-apoptotisch signaal. Het zou daarom ook kunnen dat de Flt3-geïnduceerde verhoging van de CXCR-4 expressie leidt tot een anti-apoptotisch signaal wat uiteindelijk resulteert in een verhoogde overleving van Flt3/ITD AML na chemotherapie. Het testen van deze hypothese vereist echter klinische data met betrekking tot de biodistributie, overleving en proliferatie van Flt3/ITD AML.

Hoewel het mechanisme waarmee Flt3/ITDs de prognose van patiënten beïnvloedt verre van duidelijk is, zijn Flt3-mutaties een potentieel aangrijpingspunt voor therapie. De voorlopige resultaten van klinische trials naar het gebruik van tyrosinekinaserem-

mers geven enige reden tot optimisme, maar toch verdienen enige punten de aandacht. Enerzijds hebben deze betrekking op het mogelijke negatieve effecten van deze kinaseremmers op de normale stamcellen, die eveneens Flt3 en andere receptor-tyrosinekinases tot expressie brengen en anderzijds op de mogelijke resistentie voor sommige kinaseremmers, veroorzaakt door de diversiteit van de mutaties die aanwezig kunnen zijn. Deze laatste observatie geeft aan dat het noodzakelijk zal zijn om een sequentie-analyse, van zowel het juxtamembraandomein als het kinasedomein van de receptor, uit te voeren bij alle patiënten die in aanmerking komen voor behandeling met kinaseremmers. Dit om de meest effectieve behandelingsstrategie te bepalen. Daar de mutatie van enkel het Flt3-gen niet resulteert in leukemie, en in Flt3/ITD AML dus additionele mutaties aanwezig moeten zijn, is het evenwel onwaarschijnlijk dat Flt3-remmers als monotherapie gebruikt zullen worden. Het zal daarom dan ook noodzakelijk zijn om behandelingsstrategieën op te zetten waarin chemotherapie en kinaseremmers worden gecombineerd.

List of abbreviations

AML	Acute Myeloid Leukemia
APC	Allophycocyanin
BSA	Bovine Serum Albumin
CAFC	Cobbelestone Area Forming Culture
CD	Cluster of Differentiation
CFU-c	Colony Forming Unit-culture
CML	Chronic Myeloid Leukemia
CR	Complete Remission
CSF	Colony Stimulating Factor
EFS	Event Free Survival
EPO	Erythropoeitin
FAB	French-American-British
FCS	Fetal Calf Serum
FITC	Fluorescein-Isothiocyanate
FL	Flt3-Ligand
Flt3	Fetal Liver Tyrosine Kinase 3
G-CSF	Granulocyte-Colony Stimulating Factor
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HSC	Hematopoietic Stem Cell
IL	Interleukin
IMDM	Iscove's Modified Dulbucco's Medium
ITD	Internal Tandem Duplication
JM	Juxtamembrane
L-CAFC	Leukemic-Cobbelestone Area Forming Culture
LFS	Leukemia Free Survival
M-CSF	Macrophage-Colony Stimulating Factor
MDS	Myelodisplastic Syndrome
MEM	Minimal Essential Medium
NOD/SCID	Non-Obese Diabetic/Severe Combined Immunodeficiency
OS	Overall Survival
PBS	Phosphate Buffered Saline
PE	R-Phycoerythrin
RFS	Relapse Free Survival
RTK	Receptor Tyrosine Kinase

SCF	Stem Cell Factor
SDF	Stromal cell Derived Factor
STAT	Signal Transducer and Activator of Transcription
TK	Tyrosine Kinase
TKD	Tyrosine Kinase Deletion
TKI	Tyrosine Kinase Inhibitor
TPO	Thrombopoietin
WBC	White Blood Cell
WT	Wildtype

Curriculum Vitae

Elwin Rombouts werd op 8 januari 1966 geboren te Den Haag. Na het voltooien van zijn middelbare school (Thomas More College, te Den Haag) volgde hij de zoologische richting van het Hoger Laboratorium Onderwijs van de Hogeschool Rotterdam en Omstreken te Delft. In 1988 begon hij aan het toenmalige Radiobiologisch Instituut TNO te Rijswijk zijn afstudeeronderzoek: "de zuivering en werking van een door neonatale T-cellen geproduceerd immuunsuppresief cytokine", in de werkgroep van Prof.dr. D.W. van Bekkum. Een onderzoek waaraan hij ook na afronding van zijn opleiding nog heeft doorgewerkt. In 1993 werd de overstap gemaakt naar de afdeling Hematologie van de Erasmus Universiteit Rotterdam (het huidige ErasmusMC), waar hij gedurende de eerste jaren werkzaam was bij de werkgroep "Therapie Ontwikkeling" onder leiding van Prof.dr. A. Hagenbeek. Na het vertrek van een deel van deze werkgroep naar de universiteit van Utrecht stapte hij in september 1997 over naar de groep van Dr. R.E. Ploemacher om daar te werken aan een *in vitro* alternatief voor het leukemisch NOD/SCID model. Een spin-off van dit project leidde tot het in dit proefschrift beschreven promotieonderzoek. Sinds januari 2004 is hij werkzaam binnen de werkgroep "Immuunreconstitutie" onder leiding van Dr. E. Braakman en Dr. J.J. Cornelissen.

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Tot Slot

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