SUMMARY AND CONCLUSIONS

Acute myeloid leukemia (AML) has posed significant therapeutic challenges. Despite near myeloablative therapy and high rates of complete remission, the latter usually are temporary. Most of the patients relapse within a few years and die from their disease without chances on further intensification of chemotherapy for toxicity reasons. Identification of patients at high or low risk of relapse, either at diagnosis or early in the course of treatment, should allow treatment modification, including novel agents, to improve their outcome.

This thesis describes the studies, on a number of biological characteristics of AML blasts, which are directed toward early identification of patient populations who may benefit from such alternative therapeutic approaches. **Chapter 1** provides an outline for the studies presented. In this chapter we evaluate classical prognostic factors as well as recently established and currently investigated prognostic factors in adult AML and discuss the application of treatment strategies specifically directed at the involved targets. We propose that definition of optimal therapeutic modalities finally will be based not only on characterization of the leukemia subtype at diagnosis: characteristics of AML blasts at diagnosis may differ from those of minimal residual disease (MRD), suggesting that both classical and targeted treatment strategies should consider not only characterization of cells at diagnosis, but also characterization of the MRD cell. Moreover, under remission conditions, the MRD cell frequency itself may ultimately be used to assess the time point of clinical intervention under remission conditions.

Apoptosis is one of the key processes important for response to chemotherapy and probably also for persistence of disease in remission. In **Chapter 2** we demonstrate the applicability of the multiplex ligation-dependent probe amplification (MLPA) technique, originally developed for DNA, for simultaneous monitoring of the expression of a large (30-40) number of apoptosis related genes (RT-MLPA) in small amounts of RNA, such as obtainable from diagnosis AML subpopulations or from MRD cells. Reliable and reproducible results were obtained with cell numbers down to 300, which is relevant for the above-mentioned target populations. Other key parameters/procedures such as reproducibility, freeze-thawing, choice of house keeping genes show that the novel RT-MLPA approach enables semi quantitative gene expression studies of multiple apoptosis related genes, both in fresh and frozen-thawed blasts at diagnosis as well as in low frequency MRD blasts during follow up.

Resistance to chemotherapy has been shown to be associated with defects in both the extrinsic and intrinsic pathways of apoptosis. Such studies mostly dealt with single or low number of apoptosis related genes or proteins. We hypothesized that expression of multiple genes involved in the mitochondrial apoptosis pathway would give a more accurate characterization of blast chemotherapy resistance and would thereby allow not only more accurate prediction of treatment outcome but probably also guide the definition of new therapeutic targets. In **Chapter 3** we therefore determined whether multiple gene expression profiles established by RT-MLPA would allow such. Expression of pro- and anti-apoptotic genes in 120 AML patient samples were found to be positively correlated with high expression of both type of genes being predictive for poor overall survival (OS). This observation appears to be in contrast with the generally accepted assumption in which high expression of pro-apoptotic genes result in induction of apoptosis in leukemic cells and thereby would prolong patient survival. This observation, however, fits in a novel concept of "oncogenic addiction", which hypothesizes that

the excitation status of the whole apoptotic machinery rather than the balance between individual pro- and anti-apoptotic genes is determinative for the level of therapy resistance. In agreement with this, by applying the statistically objective approach of forward selection, an expression profile emerged with the best prognostic impact. This profile consisted of one anti-apoptotic but two pro-apoptotic genes, all three individually associated with poor prognosis. This profile identified three prognostic subgroups in the total study population. Importantly, a similar division was found within the intermediate cytogenetic risk group, thus allowing prognostification in a cytogenetically homogeneous group.

The characteristics of cells that survive chemotherapy (MRD cells) may both identify the chemotherapy resistant subpopulation putatively present in diagnosis material as well as give a clue as to the causes of outgrowth of MRD leading to relapse. In **Chapter 4** we compared apoptosis related gene expression using MLPA in purified leukemic blast cells present at diagnosis with that present at follow-up. Identification of follow-up blast cells is possible using so-called leukemia associated phenotypes (LAPs), i.e. cell surface marker combinations that are not, or in very low frequencies, present on normal non-leukemic hematopoietic cells. First, analogous to many other prognostic parameters, expression levels of both pro- and anti-apoptotic genes positively correlated with MRD cell frequency. Patients with high gene expression at diagnosis thus had higher chance on refractoriness or achievement of complete remission at a later stage of treatment. This fits into our previous findings that parameters with prognostic impact on survival do so via their impact on MRD cell frequency. In contrast to what is generally accepted, but in agreement with our previous observations on apoptosis related protein expression (Leukemia 18: 875-877, 2004), there is a clear trend towards MRD cells having lower gene expression compared to diagnosis, with no evidence for the existence of a resistant subpopulations as a left-over of therapy. These data are in concordance with the hypothesis that the microenvironment at diagnosis contains soluble factors that originate from both the micro-environmental cells and especially from AML blasts, and regulate apoptosis. The disappearance of leukemic blast cells upon therapy thus likely restores, at least partly, the normal bone marrow (soluble) microenvironment, and thereby the normal apoptosis gene expression profile. In contrast, blasts obtained form refractory patients after chemotherapy did not show an apoptosis sensitive profile, gene expression remained in the same range as was found at diagnosis.

Apoptosis functions as a final common pathway for multiple genetic and epigenetic defects. Such upstream defects similar to the apoptotic machinery might be related to emergence of MRD and clinical outcome. Among the many factors that can be studied as to this, we chose two for which targeted treatment approaches are being developed: the genetically aberrant FLT3 mutations **(Chapter 5)** and the epigenetic event of gene methylation **(Chapters 6, 7)**
In order to refine the clinical trial design of such studies, critical early efficacy read out parameters are eagerly wanted. Up to now, remission rate, remission duration and overall survival have been used for that purpose. Apart from remission rate, MRD cell frequency assessment would offer such quick end points, especially since the latter has been shown in many studies including our own, to be an independent predictor of clinical outcome.

In **Chapter 5** we addressed FLT3 mutations which belong to the so-called class 1 mutations. The prognostically unfavourable mutations and internal tandem duplica-

tion (ITD) of the FLT3 receptor result in constitutive activation of the downstream pathways, including the apoptosis pathway, and thereby result in poor clinical outcome. The important role played by FLT3 in the survival and proliferation of blasts, and its over-expression either by mutations and probably also by increased expression of the wild type receptor in AML patients, make FLT3 an attractive therapeutic target. The mutation status of the FLT3 receptor was determined in bone marrow aspirates of 265 patients newly diagnosed with AML.
We were able to monitor MRD frequencies after consecutive courses of chemotherapy in part of these patients.
The presence of a mutation or duplication in the FLT3 gene was found to be associated with an 8.5 fold higher frequencies of MRD cells after first and a 2.6 fold higher frequency after second cycle of chemotherapy. The 8.5 fold higher frequency after first cycle, translates into a pronounced difference in median survival. Partial or complete inhibition of the downstream effects resulting from the mutations would thereby indeed translate into significant survival differences.
The efficacy of treatment can thus be determined by the MRD frequency after first cycle of chemotherapy, which enables early intervention if necessary and if possible: based on the present chemotherapy cycles this would be in the order of one month after start of therapy.
The overlap between both groups originates from FLT3-ITD positive patients performing well and wild type patients who performed poorly.
As to the first group, remarkably, in most recent experiments we found that the subgroup of FLT3-ITD positive patients who performed extremely well could be discriminated from the poorly performing FLT3-ITD positive patients by low expression of the IL2 receptor CD25. Although the molecular mechanisms of this should be revealed, this might offer the extra prognostic factor needed for proper prognosis assessment in this patient group.
As to the second group, thus far we have found no other mutations that might possibly affect tumor cell vulnerability in this FLT3 wild type group.
Another cause of poor performance, which we have not studied, is increased receptor expression. Also, at least part of the performance in this group might well be attributed to pharmacokinetic resistance: in other studies we have identified a subgroup of AML patients who have concomitant survival of normal and leukemia cells, indirectly pointing to poor availability of the chemotherapeutic drugs at target (Feller et al, Leukemia 17: 68, 2003; Feller et al, submitted).
A fourth explanation is offered by the observation that wild type patients may have become FLT3-ITD positive at relapse. Such patients have a poorer prognosis compared to patients who remain negative or even compared to patients with a shift from positive to negative (Cloos et al, Leukemia 20: 1217, 2006). Such mutations may already be present in the small stem cell compartment at diagnosis, but overlooked due to the relatively low sensitivity of mutation assays (Bachas et al, submitted).

The second type of defect concerns DNA methylation. In **Chapter 6** we have assessed the methylation status of 25 tumour suppressor genes (TSGs) in bone marrow aspirates of 119 unselected AML patients by methylation specific-multiplex ligation-dependent probe amplification (MS-MLPA). Our results indicate that concomitant methylation of genes in AML is common. The observed association between the methylation statuses was strong (0.38) and best predicted by ESR1 (estrogen 1 receptor) methylation. Methylation of ESR1 and methylation of IGSF4 and CDKN2B/p15 were found to have independent, but oppositely directed predictivity with respect to patient survival:

good versus poor survival, respectively. When combined, they constitute a unique and powerful factor for predicting clinical outcome, both in the total AML population as well as in the intermediate cytogenetic risk group. Whether such methylation phenotype exerts prognostic impact via transcriptional deregulation of specific target genes with prognostic impact, or whether the phenotype itself represents a distinct oncogenic pathway remains to be elucidated. In **Chapter 7** we present a study on the involvement of methylation of 11 FA-genes in sporadic cases of leukemia. Out of 119 unselected adult AML bone marrow samples and 20 pediatric AML samples, the latter selected on bases of cytogenetic abnormalities that are frequently found in FA patients.
Aberrant promoter methylation of the FANCC gene was detected in diagnosis and relapse material of a single adult patient with biphenotypic AML.
FA-gene methylation was not strongly associated with the biphenotypic phenotype as we did not detect methylation in an additional group of four biphenotypic AML cases.
Surprisingly, in an additional group of 15 adult and 82 pediatric ALL samples, one adult ALL sample showed FANCL promoter methylation, while FANCC promoter methylation was found in three pediatric ALL samples. All four patients were diagnosed as having BCP-ALL with a hyperdiploid phenotype.
At the cellular level, FA is defined by genomic instability, which is in an experimental setting expressed by an increased sensitivity to DNA interstrand cross-linking agents such as mitomycin C (MMC). Colony-Forming Units assays revealed four FA-gene methylated samples to be 6.9-fold more sensitive to MMC compared to controls.
Hypermethylation of FA-genes may thus contribute to the occurrence of sporadic acute leukemia in a small portion of patients diagnosed with AML or ALL and may translate into enhanced sensitivity to cross-linkers, such as MMC.
Up to now the role of DNA cross-linking agents in the treatment of leukemia is limited in general. It is tempting to speculate on the clinical implications for such leukemia cases which might be particularly sensitive to regimens containing drugs such as cyclophosphamide.
It is unknown whether other mechanisms also contribute to impairment of the FA-pathway in sporadic leukemia's. If so, the frequency of such abnormalities may be larger than estimated in this study.

FUTURE PERSPECTIVES

In the past years development of therapeutic regimens in AML is directed at the so-called targeted therapies, which are based on exploitation of newly understood pathophysiological events which may be critical for leukemogenesis.
Such events include unbridled proliferation, failure to differentiate, stromal cell-mediated survival factors, and failure to undergo normal programmed cell death.
Our understanding of the complex mechanisms underlying cellular proliferative and apoptotic pathways, with involvement of multiple interlocking proteins, gradually increases.
Supervised analyses of gene and protein expression support the idea of alternative cooperating pathways leading to transformation. These observations not merely determine our perception on gene function, but also influence our idea of adequate targeting of gene expression pathways.
Initially the focus with regard to targeting genes, involved in for instance programmed cell death, confined itself to single target genes (e.g. like Bcl-2 in lymphoma) that were under- or over-expressed.
By the identification of pathway wide involvement based on correlations between pro- and anti-apoptotic genes this thesis provides new insights in the role of apoptosis related

genes in AML and the possible way to target such mechanism. Whether pathway activation status is limited to the apoptosis pathway or also a characteristic of other pathways remains to be elucidated.
From these observations it can be questioned whether selective down regulation of an individual gene (like XIAP or BCL-2) in the apoptosis pathway holds the best reasonable approach. The value of individual genes (like the apoptosis related genes XIAP or BCL-2) in AML may hereby lie preferentially in their use as indirect read out for efficacy or more general therapeutic approach rather than in embodying direct targets, since these genes may be the best representatives for the activation status and prognostic value of the pathway as a whole. This by no means excludes the possibility that drugs that target such specific genes, mutations and aberrations will have impact in 'knock down' studies or in clinical trails in subgroups of patients.
For the identification of such subgroups that may benefit from such targeted approaches, sophisticated algorithms are needed to attach the proper weight to an over- or under-expressed gene, activated or inactivated status of a pathway, the presence or absence of a mutation or aberration in its context.
Combining factors with complementary predictivity like expression of the presence of pro- and anti-apoptotic genes, prognostically favourable and unfavourable mutations like NPM1 and FLT3-ITD, respectively, or methylation aberrancies like ESR1 and CDKN2B/p15 or IGSF4 may increase prognostic power.
MRD frequency assessment may hereby play an important role as a short-term endpoint, covering all possible resistance mechanisms.
A remarkable observation in this and earlier studies was the "fluidity" of apoptosis gene and protein expression: entering remission is associated with a shift from an apoptosis resistant to a more sensitive phenotype. This contrasts to the more "logic" expectation of emergence of resistant (sub)populations under the influence of chemotherapy and may affect our view on how to approach minimal residual disease.
Activation of whole pathways ("oncogenic addiction") raises another intriguing possibility i.e. pathway activation is not restricted to the intrinsic apoptosis pathway, but a general feature at least in AML with poor prognostic impact.
Our unpublished data on the strong correlation between apoptosis gene expression on the one hand and methylation on the other hand, might suggest such strong overlap in prognostic factors/resistance characteristics.
Independent of the cellular resistance characteristics, there is evidence that with the present chemotherapy there is always a group of patients in which cellular sensitivity/resistance characteristics are overruled by pharmacokinetic resistance (Feller et al 2003). It will be of extreme importance to identify such patients as early as possible, since this group may well abrogate the inherent straight forward relationship that may exist between a prognostic factor on the one hand and MRD cell frequency and patient survival parameters on the other. We have argued before how this might explain poor response to therapy in patients with wild type FLT3.

The challenge for the future is to incorporate biologic discoveries into novel risk-adapted therapeutic strategies that will improve the currently disappointing cure rate. Both for the patients with treatment failure as a result of cellular resistance associated with a particular genotype or phenotype as well as for the patients that experience treatment failure as a result of pharmacokinetic resistance.
Ongoing and planned trials will assess the effects of drugs targeting biological pathways whose clinical importance may vary as a function of the unique genotype, phenotype or "pharmacokinetotype" of each case of AML.