Chapter 7

Summary, General Discussion and Conclusions
Summary
Over the past four decades, outcome for pediatric AML patients has improved significantly; over 90% of patients achieve complete remission after induction therapy and at least 65% show long term remissions. However, the improvements in these figures in the new millennium were marginal and this is mainly due to the persisting portion (25-40%) of patients who suffer from recurrence of the disease and subsequently show a poor clinical outcome. At best, one third of the relapsed patients will show long term survival and two thirds - who often show a poor response to re-induction chemotherapy - will eventually die from the disease. In this thesis, we set out to unravel molecular and cellular factors that are implicated in the development of relapse and determine the characteristics of relapsed AML. We aim to provide insights that may guide the future choice of therapy for the individual patient and may offer opportunities for novel treatment strategies in relapsed AML. Ultimately, prevention of relapse is the best option to further improve outcome of AML patients. These considerations are included in Chapter 1.

Initially, we determined the relevance of established AML type I/II mutations that may serve as therapeutic targets in AML patients at diagnosis and relapse, such as FLT3/ITD and RAS. In Chapter 2, we assessed the frequencies of these mutations in paired diagnosis and relapse pediatric AML specimens to study their persistence during disease progression. In 61% of the patients harboring mutations at either stage of the disease, the mutation status changed between diagnosis and relapse, particularly in FLT3, WT1 and RAS genes. Persistence or gain of poor prognosis type I/II mutations (e.g. FLT3 or WT1) at relapse was associated with a shorter time to relapse, whereas absence or loss correlated with a longer time to relapse. Moreover, a worse overall survival was observed for patients with activating mutations at relapse, which was statistically significant for FLT3/ITD and WT1 mutations. The association of mutational shifts with outcome parameters suggests that such genes play a role in disease progression after initial treatment. This observation urged us to study the frequency of established molecular markers at first relapse and evaluate their prognostic relevance after first relapse in 198 relapsed pediatric AML patients who received uniform salvage treatment according to the 'Relapsed AML 2001/01' protocol of the International Berlin-Frankfurt-Münster (BFM) Study Group (Chapter 3). Mutations in the studied panel of genes in this large cohort of pediatric relapsed AML patients were frequent. Overall, we detected receptor tyrosine kinase mutations in more than 25% of the patients. Gene mutations provided strong independent prognostic factors for dismal outcome after first relapse and were significantly associated with relapse free survival after first relapse (e.g. WT1) or with worse overall survival from the moment of first relapse diagnosis (e.g. FLT3/ITD).

In our analyses of paired diagnosis and relapse samples, we also observed FLT3/ITD mutations in diagnosis AML samples at allelic ratio’s that hardly exceeded detection limits, but nevertheless showed a marked increase in allelic ratio at relapse. In addition, with the enrichment at relapse of aberrations such as WT1 mutations, these data led us to hypothesize oligoclonal origin of genetic changes during disease progression of AML, which we explored in Chapter 4. Very infrequent blasts and stem cell subfractions from initial diagnosis samples were cell sorted in small portions of 25-50 cells, based on immunophenotypes that resembled the relapse sample. Mutational profiles of isolated fractions were compared with those of the corresponding relapse samples of seven CD34+ AML patients. At diagnosis, subfractions of the primitive CD38dim blast compartments were more heterogeneous in the distribution of mutations, when compared to the whole blast compartment in 6 out of 7 patients. Moreover, we found, in 5 of these 6 AML patients, that within the primitive CD38dim blast fraction of initial
samples, mutation profiles that had initially not been detected in the bulk of leukemic cells corresponded with the mutational profiles that dominated the bulk of leukemic blasts at relapse. These results indicate that the survival of AML relapse clones is primarily the consequence of a clonal selection of minor clones with relapse specific mutations that already exist pre-treatment. In one out of seven cases the exact relapse mutational profile did not pre-exist in clones and here the relapse clone(s) may have evolved from a genetically different clone at initial diagnosis.

The above findings and the reported karyotypic and immunophenotypic differences between diagnosis and relapse demonstrate that relapsed AML is biologically different from the disease at initial diagnosis. Hence, this difference between diagnosis and relapse samples may at least in part explain the specific functional characteristics of blasts of relapsed AML, for example the different responses to therapy at either stage of the disease. To elucidate relapse specific deregulated biological pathways, we performed genome wide expression profiling of the paired initial diagnosis and corresponding relapse samples of 23 pediatric AML samples (Chapter 5). According to cluster analysis, the gene expression profile of the relapse sample did not resemble that of the initial diagnosis sample in 11 of these patients. This was more frequently the case in patients that also showed mutational shifts between diagnosis and relapse when compared to patients without mutational shifts. Among the differentially expressed genes, many were related to impaired differentiation and encompassed genes involved in chromatin remodeling. The observed differential gene expression patterns were significantly associated with specific transcription factor pathways such as CEBPA, GFI1 and SATB1. Not all patients in the above studies showed mutational shifts or differential gene expression patterns between diagnosis and relapse. We reasoned that in these patients the relevant characteristics that allow relapse development, such as drug resistance were already present at initial diagnosis. Therefore we correlated the genome wide gene expression profiles of 73 pediatric AML patients to ex vivo cellular resistance data for drugs that are frequently used in the clinic - cytarabine, daunorubicin and etoposide-and a more experimental nucleoside analog (cladribine) (Chapter 6). The number of genes that correlated significantly with gene expression profiles varied per drug. For individual drugs, expression of known and new genes or pathways were observed for which expression was linked to drug resistance. For example, we observed a high expression of MLL genes that correlates to ex vivo Ara-C resistance and found an association of topoisomerase 2 alpha (TOP2A) expression with ex vivo VP16 resistance. Despite different genes of which expression correlated with ex vivo resistance to the different drugs, Ingenuity pathway analysis showed that for daunorubicin, cladribine and etoposide the observed gene expression patterns were in part the result of similar upstream regulation via CD40 ligand signaling. CD40 activation plays a role in neoplastic growth of various types of cancer and increased serum levels of soluble CD40 are found in several hematological malignancies and especially in multiple myeloma and AML. Moreover, activation of the CD40 pathway is associated with in vitro drug resistance in hematological malignancies, e.g. in multiple myeloma or acute lymphoblastic leukemia. The CD40 pathway is a target of novel anti-cancer therapeutics that are currently under investigation.
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General Discussion and Conclusions

In pediatric AML approximately 5-10% of the patients will not achieve a complete remission because of refractory disease, which is associated with dismal outcome.23 Another 30% of the patients show a poor response to initial therapy, a high burden of minimal residual disease25,26 and relapse. When treated at relapse, the patients show a similar poor response and most of them die. In this thesis we have set out to unravel factors that play a role in relapse development and to determine characteristics of relapsed AML that can ultimately be used to improve therapy for these patients and ideally to prevent relapses.

Development of relapse

As mentioned in the introduction of this thesis, a first hit (genetic lesion) can turn a normal myeloid precursor cell into a pre-leukemic cell. During initiation of the leukemia and subsequent progression of the disease, multiple additional hits will occur. These hits may occur in a non-linear fashion and give rise to different leukemic stem cell clones and their progeny (Figure 4, Introduction), hence generating heterogeneity of the leukemia. In what probably is a dynamic evolutionary process, in which clonal populations are likely to expand and regress in space and time, leukemic cells acquire the aberrations that are required to survive until initial treatment or thereafter until relapse. During clonal evolution, different types of selective forces put pressure on the survival of a (pre-) leukemic clone, for example the patient's immune system, changes of the microenvironment, extramedullary localization, chemotherapy and allogeneic transplanted donor (stem) cells. Clonal evolution in cancer30-33 and leukemia34,35 is extensively reviewed elsewhere. Changes in molecular aberrations of the genotype (e.g. cytogenetics, epigenetics) and/or changes in the phenotype (e.g. cell surface protein expression) between diagnosis and relapse have been described and are likely the result of this process of evolution. Genotoxic chemotherapy may have a dual role as it will act as a strong selective force, yet it may generate additional genetic lesions and an increased heterogeneity in the pool of leukemic cells from which relapse initiating cells may expand.

Below, the novel data as presented in Chapters 2-6 of this thesis will be discussed on the basis of the multi-hit model of leukemogenesis and the generally accepted cancer stem cell concept and in the context of recent insights in the clonal evolution of AML. In addition, options for treatment according to our data will be discussed.

The role of genetic instability in relapse development

Genetic instability is a hallmark of cancer and the result of impaired DNA repair and/or DNA damage response mechanisms that are active in normal cells. The extent to which genetic instability occurs and the type of genetic instability that is observed differs per cancer (sub-) type. The resulting accumulation of genetic defects provide the hits that allow initiation of the leukemia. To what extend genetic instability plays a role in the development of AML relapse and thereby in outcome of the patient, is largely unknown. Garson et al. were the first to report on genetic instability between diagnosis and relapse AML samples as they observed cytogenetic differences in the paired analyses of 103 diagnosis and relapsed adult AML samples. They found changes in more than 61% of the patients with a general tendency to gain a more complex karyotype (53% of the patients). Remarkably, 8% of the patients reverted their karyotype to less complex or even normal. These data were later confirmed by others in studies with the number of changes varying between 39% and 62%. With the concept of cytogenetic changes
from diagnosis to relapse in mind, Nakano et al. studied cytogenetics and the mutational status of FLT3, TP53 and RAS genes in a group of 28 primarily adult AML patients and were the first to report shifts in mutational status of these genes. They found both gains and losses of mutations in 11 out of 28 (39%) of the patients. Although the numbers were low, they showed that changes in FLT3/ITD or TP53 mutations were associated with a worse overall survival. Later studies that focused primarily on the evolution of FLT3 mutations, confirmed these shifts and their association with outcome. We performed mutational analyses in paired diagnosis and relapse samples in a fairly large group of pediatric AML patients and found mutational shifts in 36% of the patients. Gain of poor prognosis mutations was associated with a shorter time to relapse and a worse overall survival (Chapter 2). We also confirmed the results of others that certain mutations, such as NPM1 are stable. WT1 mutations were preferentially gained. These studies were broadened when next generation sequencing was employed by Ding et al. to analyze genetic changes between diagnosis and relapse samples. In 8 AML patients they found an average of 539 mutations and structural variants (non-coding regions excluded) in diagnosis and relapsed samples, most of which concerned shared aberrancies between diagnosis and relapse samples. However, in all patients, the remainder of mutations were mostly relapse specific mutations and a few were specific for the diagnosis sample. Relapse specific mutations differed among patients and involved for example genes such as IDH2, ABCD2 and SLC25A12. Interestingly, some of these genes are similar to or from the same family of molecules as the genes that we found to correlate with ex vivo resistance towards Ara-C, DNR and VP16 (Chapter 6, supplementary Table). Based on what is known about the function of these relapse specific gene mutations (e.g. in cancer metabolism or drug transport), their role in relapse development seems obvious, but often remains to be elucidated. Of note, molecular aberrations with a known relevance in leukemogenesis or disease progression for adult AML are rare in pediatric AML (e.g. DNMT3A mutations), hence it is essential that similar studies are conducted using pediatric AML samples.

The next generation sequencing studies show that in AML typically a few small insertions and deletions, a few translocations and primarily point mutations occur. The most frequent (~50% of the detected mutations) type of mutations in an AML genome at initial diagnosis are transitions of C→T and A→G. Such DNA lesions are primarily the result of deamination of bases or oxidative DNA lesions. These processes are highly mutagenic and cause genetic instability as they may stall DNA replication or result in mutated RNA transcripts. Interestingly, the frequency of C→T and A→G transitions in relapse specific mutations (~40% of the detected mutations) was significantly lower when compared to the initial diagnosis AML. In contrast, the frequencies of A→C, C→A and C→G transversions was increased by 4%-5% in relapse specific mutations. These transversions are again caused by oxidative stress or base deamination, but the underlying DNA lesions and their mutagenic effects are different and may result in for example protein-DNA cross links or stalling or blocking transcription. In vitro exposure of AML cells to the commonly used drug etoposide has also shown that new genetic lesions can be induced by therapy and are subsequently detected in surviving leukemic cells.

Together these data indicate an increased and different type of genetic instability as a result of genotoxic chemotherapy. New mutations may emerge after treatment that provide AML cells with characteristics that allow therapy resistance and survival. Future research should elucidate more relapse-specific and relapse-promoting characteristics of AML cells. Genetic instability during treatment generates additional genetic heterogeneity of AML cells, thereby increasing the chance that cells emerge that are fit enough to expand and cause relapse and consequently a poor prognosis for the patient.
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The contribution of heterogeneity of immature AML cells at diagnosis to relapse development

AML is thought to be a tumor to which the cancer stem cell model applies: only a small subfraction of leukemic cells is tumorigenic\(^54\) and these cells resemble the phenotype of normal hematopoietic stem cells and their biological characteristics\(^55\). The strong clinical relevance of leukemic stem cells either at presentation\(^56–58\) or after initial therapy\(^59\), indicates that leukemic stem cells survive therapy and initiate relapsed leukemia. When AML is treated in xenograft mouse models, CD34\(^+\)CD38\(^-\) leukemic stem cells are significantly more resistant towards cytarabine treatment than AML non-stem cell fractions and they cause relapse in the mouse model\(^60\). Genetic changes between diagnosis and relapse must therefore originate from different leukemic stem cells with distinct genetics and distinct survival characteristics. Indeed, genetic instability and clonal evolution of stem cells may provide an efficient means for a leukemia to acquire drug resistance properties\(^61\). Below, it will be discussed how our data as presented in Chapter 4 and data of others show that a Darwinian like clonal evolution of immature AML cells within a hierarchical framework may be involved in relapse development (Figure 1).

Ding et al.\(^46\) used whole genome sequencing on the bulk of leukemic cells to cluster mutations according to similar allelic ratios. This allowed them to estimate the clonal architecture of the leukemia at diagnosis and at relapse and model the evolution of leukemic clones. They suggested a model in which a clone at diagnosis could evolve to expand at relapse in two ways; 1) a genetically less complex founder clone of the initial leukemia (not the dominant clone at diagnosis) gains mutations and this new clone expands at relapse (e.g. green clones in Figure 1); and 2) a subclone of a founder clone from the initial leukemia (may or may not be the dominant clone at diagnosis) – that already acquired a limited number of additional mutations before initial diagnosis-expands to cause relapse (e.g. the dark red clone in Figure 1). Since the founder clone contains less genetic lesions than its subclone(s), the first option allows an apparent loss of genetic aberrations between diagnosis and relapse, which in reality is the selection of a clone with less complex genetic characteristics when compared to the bulk of the leukemic cells at initial diagnosis. The number of detected leukemic clones at initial diagnosis varied from 2 to 5 clones. In summary, their data show that relapse always evolves from a (founder) clone of the initial leukemia and that the relapse clone is genetically different from the clones at initial diagnosis. The emergence of a completely new clone that did not resemble the diagnosis clones at all was never observed.

Data of Welch et al.\(^51\), who used next generation sequencing to analyze hematopoietic stem cells of 7 healthy individuals of different age at the genomic level, suggest that mutations accumulate in hematopoietic stem cells in a time-dependent manner. In addition they sequenced the relatively non-complex genomes of 12 t(15;17) FAB M3 AML and 12 cytogenetically normal AML patient samples of the FAB M1 sub-type and showed that mutations in patients also accumulated in an age-dependent manner and that most patients were polyclonal.

Together these two papers by Ding et al. and Welch et al. give insight into the clonal evolution of AML from diagnosis to relapse. These studies suggest indirectly that the genetic differences between diagnosis and relapse result primarily from the selection of leukemic cells by the initial treatment after which further clonal evolution may occur.

Our results (Chapter 4) add to this by the actual isolation and genetic characterization of such relapse initiating populations. We showed that leukemic cells with relapse characteristics exist prior to treatment and that they were enriched in very minor subpopulations of leukemic stem cells that were characterized not only by relapse-specific genetic lesions, but also by a relapse-specific aberrant immunophenotype.
Single nucleotide polymorphism array-based genomic profiling later confirmed in a larger set of 28 paired diagnosis and relapse AML samples and 11 patients with refractory disease that relapsed AML is invariably the consequence of re-emergence or clonal selection of a clone that exists pre-treatment within a compartment with stem cell characteristics. These data strongly suggest that relapse is the result of a chemotherapy mediated, Darwinian like selection of therapy resistant leukemic stem cells from a heterogeneous pool of leukemic stem cells at presentation (Figure 1).

Figure 1. A schematic overview of the Darwinian like branching evolution of AML. In the pre-leukemia phase, an initial hit transforms a normal myeloid precursor cell into a pre-leukemic stem cell (founder clone, blue or green, several may be generated). A driver hit (e.g. NPM1 mutation; green) will initiate the leukemia and is followed by many additional hits that likely occur before the onset of the disease. Some of the additional mutations (e.g. FLT3/ITD mutation; FLT3+, yellow) will confer properties that give progeny clones a survival advantage. After initial diagnosis, treatment puts massive selective pressure on clones and only one or a few drug resistant clones remain, grow out to minimal residual disease (MRD) and have characteristics (e.g. a WT1 mutation; dark red) that allow relapse initiation. Although therapy may induce new characteristics, most relapse initiating characteristics may already be present prior to treatment. Colors represent unique genetic and phenotypic characteristics of clones. During evolution leukemic stem cells will maintain part of their stem cell phenotype. Size indicates the proportion of the clonal population and its progeny. (Adapted from Greaves & Maley)

Reliable detection and isolation of relapse initiating leukemic stem cells now seems possible and has several important clinical implications: 1) it will allow prospective analysis of the burden of relapse initiating leukemic stem cells, prior to and early during treatment, which may provide the most relevant risk factor for outcome; 2) improved purification and subsequent biological and functional characterization of these cells will be crucial in the development of treatment strategies that aim to eradicate all leukemic clones -preferably in first line treatment; 3) valuable insights in the intra-tumor heterogeneity of individual AML patients and the dynamics of clonal populations can be obtained that may guide the timing and application of personalized treatment. To study this, single cell analyses of all clones that constitute individual leukemias at different time points after presentation will be required. An initial focus on minimal residual
disease stem cells may provide fast insights in the characteristics of the drug resistant relapse initiating leukemic stem cells.

Options to prevent AML relapse or improve its treatment

The option to improve outcome for relapsed AML patients that is closest at hand, is refinement of current therapy. Optimization of induction chemotherapy over the past decades has resulted in clear improvements in terms of improved remission rates, reduction of relapse rates and overall survival\(^2,63\). Since the late 1980s, studies of Nordic Society for Paediatric Haematology and Oncology and the BFM-AML Group have applied a number of consecutive relapse treatment protocols that showed a significantly improved remission and overall survival rates and reduced the number of patients who received palliative treatment only\(^9,11\). The International BFM Study Group recently published the first report on a randomized study for the treatment of pediatric relapsed AML patients according to their ‘Relapsed AML 2001/01’ protocol. This brought further improvements in terms of response rates, second complete remission and overall survival rates\(^5\). These studies showed the feasibility of application of structured protocols and randomized trials in poor prognosis relapsed AML patients.

Further improvements of existing salvage protocols could come from risk group directed intensified therapy. Risk factors that have been suggested to be useful in that setting are duration of first complete remission, cytogenetics at initial diagnosis and FLT3ITD mutations at presentation\(^64-66\). These studies were however performed in adult AML patients and molecular analyses were performed at initial diagnosis only. Since we showed in Chapter 2 that genetic characteristics frequently change between diagnosis and relapse and since molecular data that was assessed at first relapse (Chapter 3) provides independent prognostic variables for the subgroup of relapsed AML patients, we highly recommend a large prospective study that will evaluate clinical and molecular risk factors in the relapsed setting. This should also include cytogenetic and molecular data that was assessed at relapse.

As mentioned in the introduction of this thesis, future therapies will probably target AML according to patient specific molecular aberrations, i.e. a personalized treatment. Our data from Chapters 2-4 shows that repeated assessment of molecular aberrations at first relapse will allow accurate tailoring of such therapy. For example, a large portion of patients are eligible for kinase inhibitor treatment according to their FLT3 and KIT mutation status at first relapse. The discovery of relapse specific mutations at initial diagnosis may provide the best options for the development of new targeted therapeutics.

Since we have shown (Chapters 2-4) that genetic instability plays a role in the development of relapse, genetic instability as such may be a relapse specific mechanism of interest to target. DNA repair deficient cancers may be targeted by interfering in affected pathways and increase the damage of genotoxic chemotherapeutics\(^67\). For example, cancers that are deficient in double strand brake repair due to defects in BRCA1 or BRCA2 genes are sensitive to inhibitors of poly(ADP) ribose polymerase\(^68\). In AML, the deficient DNA repair mechanisms that cause genetic instability remain to be elucidated. Reports have implicated a defective mismatch repair mechanism, as aberrations were detected in MSH2 or MLH1 genes in up to 33%\(^69,70\) of AML patients and others observed a relatively frequent micro satellite instability\(^71-73\). Controversially, a lack of microsatellite instability was also reported in a larger group of patients\(^74\). We could not detect microsatellite instability ourselves in 25 paired diagnosis and relapse AML samples using markers of the Bethesda panel that are commonly used in colon cancer (data not shown). Additionally, defects in double strand break repair\(^75,76\), homologous
recombination\textsuperscript{77–79} or error prone repair\textsuperscript{80} have also been implicated in genetic instability in AML.

Although the average genome wide number of mutations is more variable in AML when compared to other cancers, AML is, with an average of 0.37 mutations per $10^6$ base pairs, a relatively genetically stable tumor type\textsuperscript{81}. Hence, DNA repair may be proficient instead of deficient in AML and a strong mutator phenotypes may be lacking. In which case carcinogens or genotoxic therapy results in faulty DNA repair, the maintenance of new mutations and induce cancer evolution. This also justifies targeting DNA repair mechanisms to sensitize tumor cells to chemotherapy.

Whatever the therapy applied, a general drug resistance may impair the effects of treatment\textsuperscript{82}. We emphasize in Chapter 6 that multiple mechanisms may contribute to drug resistance. Only a system (patient) wide approach can resolve all factors that are involved in drug resistance. Personalized approaches that take multiple factors (pharmacokinetics, drug pumps, quiescence) into account may overcome this problem.

Most important implications of our results for improvement of therapy are that it will be crucial to target the right cell. Current therapy apparently fails to eradicate minor immature populations that cause relapse. When minimal residual disease is detected after initial therapy, additional therapy should be applied that aims to eradicate these cells. Since most targeted therapeutics are also designed to eradicate the bulk of leukemic cells and not designed to target LSC, these strategies will also be hampered by drug resistance\textsuperscript{23}. Therefore, options to specifically target and eliminate LSC while preserving normal HSC are currently being developed\textsuperscript{83}. Leukemic stem cells are often characterized by the expression of leukemic stem cell specific markers\textsuperscript{84} (e.g. $CD123$, $CLL$-$1$) and this may be exploited in the development of sophisticated antibody mediated therapy\textsuperscript{85}. *In vitro* targeting and/or *ex vivo* exposure of AML cells in xenograft models via $CD44$\textsuperscript{86}, $CD123$\textsuperscript{87}, $CD47$\textsuperscript{88} and $CLL$-1 (Dr. P.Noordhuis, unpublished) shows preferential elimination of leukemic stem cells. Drugs may also specifically be delivered to LSC by using nano-scale carriers\textsuperscript{89} or devices. Efforts are also made to develop LSC -directed therapy by modulation of the immune system; LSC specific antigens can for example be used in the development of tumor cell-based vaccination strategies\textsuperscript{90,91}. Another option is to interfere with the capacity of the LSC micro-environmental niche to protect against chemotherapeutic drugs, for example by targeting the SDF-1/CXCR4 axis in combination with other drugs\textsuperscript{92}. Current research also aims to elucidate new LSC specific signaling pathways and metabolic routes that may be targeted\textsuperscript{93,94}.

**Conclusions**

Our results indicate, in concordance with recent literature, that AML shows highly instable genetics between diagnosis and relapse (Chapter 2). The genetic changes are of prognostic relevance, therefore only data acquired at relapse should be used for prognostication or risk-group stratification of relapsed AML patients and guide future targeted salvage treatment (Chapter 2/3). Relapsed AML is a distinct disease modality; it is often biologically and clinically different from AML at presentation (Chapter 2,3,4,5) and novel treatment strategies should be developed accordingly. We have shown relapse specific gene expression profiles (Chapter 5) that may offer options for such new targeted approaches. However, to overcome problems of general drug resistance it will be required to combine classical chemotherapy and novel targeted therapeutics in a personalized manner, since our gene expression studies show that multiple mechanisms lead to a drug resistant phenotype (Chapter 6).

Relapse of leukemia is caused by very minor subclones within the leukemic stem cell compartment that pre-exist prior to treatment and expand after therapy to cause relapse (Chapter 4). A sophisticated personalized and targeted approach should therefore not
only aim to target the right pathways in an individual patient, but also the right cells and
at the right time, early during initial treatment.

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