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NEW DRUGS FOR LEUKEMIA

Genoa (Italy), March 15th - 17th, 2012

Organized by

DEPARTMENT OF HEMATOLOGY AND ONCOLOGY
I.R.C.C.S. A.O.U. SAN MARTINO - IST, GENOA

DEPARTMENT OF HEMATOLOGY AND ONCOLOGY
"L. and A. SERÀGNOLI"
ALMA MATER STUDIORUM UNIVERSITÀ, BOLOGNA

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ABSTRACT BOOK

Palazzo del Principe
(Galleria Aurea - Piazza del Principe, 4)

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CONTENTS

The regulation of transcription and transduction

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Human hematopoietic stem cell differentiation plasticity is mainly based on the differential gene expression occurring in the commitment phase of differentiation leading to different precursor cells and soon after to eight different types of terminally differentiated cells. The kinetic more than the hierarchical model of hematopoiesis, altogether with the microenvironment stimuli, the different stem cell niches and the differential expression of several transcription factors underlie the commitment choice of differentiation lineage of these stem cells. Particularly the study of human myelopoiesis by DNA microarrays experiments and data analysis by bioinformatics tools to develop transcriptome maps, has allowed approaches on the genome positional effects of gene expression. Several silent chromosome domains were characterized raising the problem of epigenetic imprinting of adult hematopoietic stem cells. Furthermore interference experiments based on gene transfer to overexpress or silence master regulator genes of myelopoiesis have been performed in hematopoietic stem cells using a systems biology approach. The positional effect of gene expression has been studied by 3D model during the commitment phase of myelopoiesis. Our data clarified some relevant molecular and biological aspects of human adult hematopoietic stem cell plasticity.

Leukemia stem cells: a new paradigm for targeted therapy

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Leukemia derives from the clonal expansion of a transformed hematopoietic cell, known as leukemic stem cell (LSC), which shares important properties with normal hematopoietic stem cells (HSC). The precise nature of the hematopoietic cell targeted by the leukemogenic event is not known, and it is not clear if it is a multipotent HSC or a committed progenitor cell that has re-acquired stem cell characteristics. Leukemic transformation occurs through the acquisition of specific genetic abnormalities that confer selective advantages to LSC, including unlimited self-renewal and the capacity to give origin to a hierarchy of hematopoietic cells. As for normal HSC, a sizable fraction of LSCs is quiescent, and this pool is critical for the maintenance of tumor growth. The existence of a rare subpopulation of quiescent LSC may render them refractory to anti-proliferative treatments, such as chemotherapy, and may account for disease relapse.

The molecular characterization of LSC and the identification of functions that are specific with respect to normal HSC are instrumental for designing novel therapeutic strategies aimed at eradicating leukemias. The study of specific stem cell characteristics has revealed important differences between normal and leukemic stem cells that may be exploited for targeted drugs. For example, the replicative potential of normal HSC is intrinsically restricted, and if HSC are forced to proliferate more, they are rapidly consumed. LSC, instead, have developed mechanisms such as over-expression of the cell-cycle inhibitor $p21^{Cip1/Waf1}$, to protect themselves from exhaustion through hyper-proliferation, and have acquired unlimited ability to self-renew. In terms of global gene expression, LSCs are not simply characterized by the re-activation of stem cell pathways: a direct comparison of HSC and LSC from acute myeloid leukemias have revealed important differences in diverse functional pathways. Of

particular practical interest are efforts aimed at identifying LSC-specific markers, i.e. genes and proteins that are expressed only in LSC and not in normal HSC. Further evidence suggests that LSC are at least partly dependent on specific conditions within their microenvironment (or "niche"), opening yet another possibility for therapeutic intervention.

Recent studies have demonstrated that leukemias present complex patterns of clonal architecture characterized by genetic variegation within the leukemic bulk population. Different cellular subclones within a single leukemia display the accumulation of different sets of somatic mutations. These may, in turn, lead to variable degrees of chemo-sensitivity, and to the presence of one or more chemoresistant clone(s) that could account for disease relapse. It appears, therefore, that it may be necessary to eradicate not one, but several LSC populations even within one single patient.

Diagnostic and predictive biomarkers for myelodysplastic syndromes

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Myelodysplastic syndromes (MDS) are myeloid neoplasms characterized by dysplasia in one or more cell lines, ineffective hematopoiesis, and variable risk of progression to acute myeloid leukemia (AML). The World Health Organization (WHO) classification criteria define different conditions that are heterogeneous in terms of life expectancy. In addition, risk-based stratification systems have been developed to improve prognostication of MDS, including the International Prognostic Scoring System (IPSS) and the WHO classification-based Prognostic Scoring System (WPSS). In the last few years, important steps have been made in developing novel molecular tools for diagnosis and prognostic evaluation of MDS. Somatic mutations of *TET2* represent a useful marker of clonal proliferation. Additional mutant genes, including *TP53*, *EZH2*, *ETV6*, *RUNX1*, and *ASXL1,* have been shown to be independently associated with decreased overall survival. More recently, somatic mutations in genes encoding core components of the RNA splicing machinery, including *SF3B1*, *SRSF2* and *U2AF1*, have been detected in MDS patients and shown to be associated with disease phenotype and clinical outcome.

Diagnostic and predictive biomarkers for AML

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Differences in outcome among patients with acute myeloid leukemia (AML) reflect the biological heterogeneity of this disease. Cytogenetic and molecular genetic changes have been shown to be the most important tool for prognostic stratification. As a matter of fact in the current World Health Organization (WHO) classification, more than half of AML cases are categorized on the basis of their underlying genetic defect, in part defining distinct clinicopathologic entities.
Patients with cytogenetically normal AML (CN-AML) still represent a heterogeneous group that is currently considered to have intermediate prognostic significance. In the past decade, molecular studies have shown that mutations or altered expression of many genes may affect both the complete remission rate and the long-term outcome of patients with AML, not only in CN-AML, but also within specific cytogenetic subgroups of AML. FLT3 (fms-like tyrosine kinase receptor-3) internal tandem duplication and high expression of brain and acute leukemia, cytoplasmic gene (BAALC) have been associated with poor prognosis in CN-AML, whereas nucleophosmin gene (NPM) mutations have been considered favorable molecular markers, as long as they are not associated with FLT3-ITD. Furthermore, novel therapies are being developed that target some of these genetic and epigenetic changes, such as the use of tyrosine kinase inhibitors and demethylating agents.
Minimal residual disease analysis (MRD) may also be useful for the risk assessment of AML especially in order to evaluate the chemosensitivity and the impact of the therapy. In the setting of AML, MRD evaluation can provide relevant prognostic information but, with the exception of acute promyelocitic leukaemia, its clinical value in deciding therapy has yet to be demonstrated. Many studies have shown that leukaemia-associated immunophenotypes can be defined in all patients with AML. Therefore, flow cytometric methods (FCM)

are feasible for detecting the persistence or the reappearance of a residual clonal population. Moreover, a number of large studies have shown that remission status after induction and consolidation therapy as assessed by FCM is a strong prognostic predictor in AML. In the setting of AMLs with recurrent genetic abnormalities, monitoring the fusion transcripts might be the most specific and sensitive tool to evaluate MRD. In AML patients lacking specific genetic markers, the Wilms' tumour gene 1 (WT1) has been extensively used. In the subset of NPM-mutated denovo AML patients, the study of NPM mutations has been proposed as a means to assess response to therapy and monitor MRD, but it has not yet become the standard method.
Large cohorts of patients will need to be analyzed for all of the markers to evaluate their interaction and their precise prognostic value. Finally, the study of these markers in the context of novel therapies could be a useful tool to identify biomarkers that allow the definition of distinct clinicopathologic entities that benefit from a particular treatment approach.

Diagnostic and predictive biomarkers for ALL

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During the last decade, a tremendous technologic progress based on genome-wide profiling of genetic alterations, structural DNA alterations and sequence variations has allowed a better understanding of the molecular basis of pediatric and adult B and T- acute lymphoblastic leukemia (ALL), contributing to a better recognition of the biological heterogeneity of ALL and to a more precise definition of risk factors. The identification of cytogenetic abnormalities provides prognostic information, markers for therapy and targets for drug development, however experimental models[1-2] have established that they alone are insufficient to induce leukemia and that cooperative mutations are required. The advent of high-resolution genome-wide analyses of gene expression, DNA copy number alterations and loss of heterozygosity, have led to the detection of many novel genetic abnormalities refining the prognostic models for ALL. Using single nucleotide polymorphism (SNP) array, many groups detected in B-ALL multiple submicroscopic genetic abnormalities not evident on cytogenetic analysis, commonly less than one megabase in size and targeting, in most of the cases, a single or few genes implicating in key cellular pathways, such as lymphoid development (*IKZF1, PAX5, EBF1, VPREB1*), cell cycle regulation and tumor suppression (*CDKN2A/CDKN2B, PTEN, BTG1, RB1*), lymphoid signaling (*BTLA, CD200*), drug responsiveness (*NR3C1*) and DNA mismatch repair (*MTOR, HERC1, PRKCZ* and *PIK3C2B*)[3-9]. Deletion of a single *IKZF1* allele or mutations of a single copy were firstly detected in 15% of all cases of pediatric B-cell ALL and in more than 80% of Ph+ lymphoid leukemia cases and were significantly associated with an increased relapse rate and adverse events[10-11]. Deletions of *CDKN2A* and *CDKN2B* were recently identified in 29% and 25% of *BCR-ABL1*-positive ALL patients, respectively. Deletions were significantly associated with higher white blood count and with poor outcome in terms of overall survival,

disease-free survival and cumulative incidence of relapse[7]. Four independent groups in late 2009 and early 2010 have identified that up to 50% of *BCR-ABL1*-like ALL cases have dysregulated expression of *CRLF2*, the gene encoding the cytokine receptor-like 2 factor, also known as thymic stromal lymphopoietin (TLSP) receptor. *CRLF2* rearrangements include a translocation of *CRLF2*, which is located at the pseudoautosomal region 1 (*PAR1*) of chromosome Xp/Yp, into the immunoglobulin heavy chain locus at chromosome 14q or a focal *PAR1* deletion proximal of *CRLF2* that results in a novel fusion *P2RY8-CRLF2*[12-15]. Less commonly, a missense mutation in exon 6, F232C, results in constitutive *CRLF2* dimerization[12]. All these events result in overexpression of full-length *CRLF2* on the surface of leukemic cells harboring the genetic alterations, providing a cell-surface marker amenable to detection by flow cytometry for clinical diagnostic purposes. Overall aberrant expression of *CRLF2* is found in 12.5 to 15% of B-ALL that lacks typical chromosomal rearrangements[12-15] and it is seen in a striking 50-60% of Down syndrome associated ALL[12-13,15]. In high risk B-ALL, rearrangement of *CRLF2* are frequently found together with *IKZF1* alterations and activating mutations in *JAK1* and *JAK2*, most commonly at or near R683 in the pseudokinase domain of *JAK2*[13-15], and are associated with very poor outcome[16-17]. The landscape of genetic alterations in B-ALL was extended by the identification of loss-of-function mutations (either deletions or sequence mutations) of *CREBBP*, encoding the transcriptional coactivator and histone acetyltransferase CREB-binding protein or CBP, in 19% of relapsed samples from children with B-cell progenitor ALL[18]. Although the prognostic impact remains to be determined, the observation that the *CREBBP* mutations impair regulation of glucocorticoid-responsive genes, and that the mutations are selected for at relapse, suggests that these alterations may influence the likelihood of relapse. Moreover, recently, copy number and sequence alterations of *TP53* have been identified in 12.4% of B-cell precursor ALL samples[19] and in more than half of cases (54%) *TP53* alterations were gained at relapse. Mutations were strikingly associated with non-response to chemotherapy and poor event-free survival and overall survival rates, allowing the identification of patients at high risk of treatment failure. Finally,

recently, the application of whole-genome sequencing (WGS) to the characterization of ‘‘early T-cell precursor’’ (ETP) ALL identified activating mutations in genes regulating cytokine receptor and RAS signaling (67%), inactivating lesions disrupting haematopoietic development (58%) and histone-modifying genes (48%). In conclusion, the identification of novel genetic alterations in B/T ALL allows a better classifications of leukemia patients in subgroups according to specific genetic lesion, provides new markers for risk-assessment and/or for monitoring minimal residual disease (e.g. *IKZF1*, *CRLF2*, *TP53*) and highlights novel therapeutic approaches (e.g. inhibitors of *JAK2*, *HDAC* and *NOCHT1*).

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Monosomy as general pathogenetic mechanism of leukemia

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The abnormal number of chromosomes (aneuploidy) is a recurrent feature of cancer cell karyotypes. It arises through chromosomal instability by the persistent loss and gain of whole chromosomes. Aneuploidy, including monosomy and trisomy of non-random chromosome entities, and chromosome instability are generally associated with poor patient prognosis, metastasis, and resistance to chemotherapeutics.
In leukemia, almost half of the cases display aneuploidy. The loss of one or more chromosomes, in particular, strongly predict for an adverse prognosis. Negative prognostic impact of autosomal monosomies in acute myeloid leukemia (AML) has been described in the past for monosomies of chromosomes 5 and 7, but recent studies show that any type of monosomy is associated with a dismal outcome. Even more profound is the influence of multiple (i.e. two or more) autosomal monosomies or one autosomal monosomy in combination with at least one structural chromosomal rearrangement, which results in extremely poor outcome. Consequently, since 2008, this new cytogenetic category was defined as Monosomal Karyotype (MK), associated with poor prognosis in adult AML.
Concerning the pathogenetically molecular genetic consequences of monosomies, they remain elusive. It may seem obvious that a monosomy results in hemizygous loss of the genes located on that chromosome. However, molecular cytogenetic studies revealed that a chromosome loss, such as -5, is frequently not a real monosomy, but just the result of a complex rearrangement resulting in net loss of a portion of the chromosome [i.e. del(5q)].
Accordingly, the molecular impact of the most frequent monosomies/chromosome losses in leukemia will be discussed.

Aurora and Polo Kinases

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Aurora kinases are mitotic kinases that play a critical role in chromosome segregation and cytokinesis. Of the three human isoforms A, B, and C, Aurora kinases A and B are overexpressed in human tumors and represent potential targets for anticancer treatment . Aurora-A localizes to the duplicated centrosomes and to the spindle poles in mitosis. It has been implicated in several processes required for building a bipolar spindle apparatus, including centrosome maturation and separation. Aurora-A binds to, and its kinase activity is regulated by, a protein called TPX2, which is required for spindle assembly. Repression of Aurora-A expression by RNA interference (RNAi) delays mitotic entry in human cells, and overexpression of the wild-type kinase can compromise spindle-checkpoint function as well as inhibit cytokinesis. By activating translation of *Mos* mRNA, Aurora-A activates the ERK/MAPK signalling pathway, which in turn results in activation of maturation promoting factor (MPF, the CDK1-cyclin B1 complex). Aurora B functions in the attachment of the mitotic spindle to the centromere. Although Aurora-C has been shown to localize to spindle poles in late stages of mitosis, little is known about its functional role (1,2). Five members of the polo-like kinase family have been identified in humans (PLK1–5). Among these, PLK1, a serine/threonine kinase that plays an essential role in mitosis, stands out as a promising drug target in oncology, since its inhibition leads to a failure to complete mitosis, eventually resulting in cell death(3).

The first data to implicate this family of kinases in tumorigenesis came with the observation that Aurora-A and Aurora-B are overexpressed in primary breast and colon tumour samples. Many subsequent studies identified other tumour types, including breast, pancreatic, ovarian and gastric tumours, in which Aurora-A was amplified or otherwise overexpressed, as well as myeloid leukemias (4).

As Aurora kinases are overexpressed in many tumours, would they represent effective targets of anticancer drugs? In the hematological subset, large numbers of polo-like kinases and Aurora kinases are currently being evaluated. Information on the selectivity of these compounds *in vivo* is limited, but it is likely that off-target effects within the same kinase families will have an impact on efficacy and toxicity profile. Clinical results have been recently reported, in AML and CML patients respectively, on safety and efficacy of Barasertib (AZD1152), and Danusertib (PHA739358), showing promising anti-tumour effects. In a phase I/II Trial, AZD1152 was administered at doses of 50-1600 mg/d for 7 days every 21, by continuous intravenous (CIV) infusion to 32 patients with advanced AML. Toxicity was manageable and treatment resulted in an overall hematologic response rate of 25% (5). In Philadelphia positive leukemias preclinical models, Danusertib, an Aurora kinase A, B and C and FLT3 inhibitor, demonstrated activity against CML cells with wild-type BCR-ABL1 and cells possessing the ABL1 T315I mutation (6). Similarly, preliminary results of a Phase I clinical trials in 23 patients with advanced stage CML and Ph+ ALL, refractory or intolerant to TKI therapy (15 of which had the T315I mutation), have been recently reported. Five patients have achieved a haematological response and three have demonstrated cytogenetic responses (one complete, one partial, one minimum) (7). While results of these early phase clinical trials suggest a potential therapeutic role for Aurora kinase inhibitors in Ph+ ALL and CML, it remains uncertain whether efficacy is simply related to inhibition of BCR-ABL1 signalling or to the combined inhibition of Aurora and BCR-ABL1. Inhibitors of PLK1 elicit a typical “Polo” phenotype: cells arrest in mitosis with a monopolar or disorganized spindle and most eventually die from this mitotic arrest. There is a certain degree of overlap in the cellular phenotypes that are induced by the inhibition of PLK1 and Aurora kinases, suggesting that these kinases might co-regulate several processes in mitosis.

Taken together, inhibitors of polo-like kinases and Aurora kinases could prove to be effective anticancer drugs, but general antiproliferative effects will limit their widespread application. Therefore, in order to make optimal use of this interesting class of molecules, an exact understanding of the functions of these kinases in cell division is mandatory, along with a detailed comprehension of effects of combination therapy with classical antineoplastic drugs.

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Mutations of *NOTCH1* in chronic lymphocytic leukemia and richter syndrome

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Chronic lymphocytic leukemia (CLL) is the most common leukemia in adults. The clinical course of CLL ranges from very indolent, with a nearly normal life expectancy, to rapidly progressive leading to death and occasionally undergoing transformation to aggressive lymphoma, known as Richter syndrome (RS).

At presentation, several clinical and biological features may help to predict, at least in part, the clinical course of CLL. Of the biological prognosticators that have been developed, current guidelines for clinical practice recommend screening only for *TP53* disruption by mutation and/or deletion of the locus, that identifies a fraction of high risk CLL destined to experience a very short survival. High risk CLL, however, cannot be fully recapitulated by *TP53* disruption, since 40-50% high risk CLL are devoid of *TP53* abnormalities. Thus, it is conceivable that other genetic lesions may drive CLL aggressiveness and/or may cause the chemorefractory phenotype of the disease.

Genome-wide methods aimed at the characterization of the entire spectrum of genetic lesions present in the CLL genome may be useful to provide further insights into CLL pathogenesis, and might contribute to elucidate the molecular basis of CLL clinical evolution, including RS transformation and development of chemorefractoriness. On these grounds, we have exploited an integrated approach based on

next generation whole-exome sequencing (WES) to investigate the CLL coding genome. This approach has revealed two novel genes, namely *NOTCH1* and *SF3B1*, whose mutations predict poor outcome and preferentially associate with chemorefractory CLL.

We have assessed the prognostic role of *NOTCH1* mutations in CLL. Two series of previously untreated CLL were utilized as training (n=309, median follow-up 6 years) and validation (n=230, median follow-up 7 years) cohorts. *NOTCH1* mutations were analyzed by DNA Sanger sequencing in blind with respect to clinical data. In the training series, *NOTCH1* mutations occurred in 34/309 (11.0%) patients, being mostly represented (26/34, 76.5%) by a recurrent two bp frameshift deletion (c.7544_7545delCT). The remaining *NOTCH1* mutations (8/34, 23.5%) were frameshift deletions other than c.7544_7545delCT (n=7) and frameshift insertions (n=1). All mutations were predicted to disrupt the *NOTCH1* PEST domain. CLL with *NOTCH1* mutations preferentially carried unmutated *IGHV* genes (76.5%, p<.001). Other characteristics at presentation associated with *NOTCH1* mutations were advanced Rai stage (26.5%, p=.006) and trisomy 12 (44.1%, p<.001). By univariate analysis, *NOTCH1* mutations associated with an increase in the hazard of death (HR: 3.77; 95% CI: 2.14-6.66) and a significant overall survival (OS) shortening (p<.001) (Fig. 1A). Multivariate analysis selected *NOTCH1* mutations as an independent risk factor of OS (HR: 4.22; 95% CI: 2.15-8.28; p<.001), after adjusting for age (p<.001), Rai stage (p=.005), *IGHV* mutation status (p=.465), 11q22-q23 deletion (p=.128), trisomy 12 (p=.183) and *TP53* disruption (p<.001). The poor prognosis conferred by *NOTCH1* mutations was attributable, at least in part, to a shorter time to progression requiring treatment (p<.001), and a higher cumulative probability of RS development (p=.026). Although *NOTCH1* mutated patients were devoid of *TP53* disruption in 31/34 (91.2%) cases, the OS predicted by *NOTCH1* mutations was similar to that of *TP53* mutated/deleted CLL. Analysis of the validation series confirmed: *i)* the prevalence of *NOTCH1* mutations at CLL presentation (26/230, 11.3%); *ii)* the spectrum of *NOTCH1* mutations at CLL presentation (c.7544_7545delCT: 21/26, 80.7%; other mutations: 5/26, 19.3%); *iii)* the adverse prognostic impact of *NOTCH1* mutations in CLL both by univariate analysis and by

multivariate analysis (HR: 2.08; 95% CI: 1.10-3.93; p=.023); *iv)* the preferential mutually exclusive distribution of *NOTCH1* mutations with respect to *TP53* disruption (25/26, 96.2%); *v)* the similar poor OS of *NOTCH1* mutated and *TP53* disrupted CLL. Overall, our study on 539 CLL documents that *NOTCH1* mutations: *i)* represent one of the most frequent cancer gene mutations known to be involved at CLL presentation; *ii)* identify a subgroup of patients showing poor OS similar to that of *TP53* disrupted cases; *iii)* exert a prognostic role independent of widely accepted clinical and genetic risk factors; *iv)* predict OS in series from different institutions, as documented by the training-validation approach chosen for the design of our study. Different mechanisms might explain, at least in part, the poor prognosis associated with *NOTCH1* mutations in CLL. First, *NOTCH1* mutations lead to the acquisition of a progressive clinical phenotype that mandates treatment shortly after initial presentation, as documented by a median TFS of ~2 years for mutated cases versus ~9 years for *NOTCH1* germline patients. Second, our actuarial analysis indicates that *NOTCH1* mutated patients display a higher risk of developing RS, a condition that is frequently lethal and recurrently harbors *NOTCH1* mutations which, importantly, are present already at the time of CLL presentation in a significant fraction of RS patients.

A potential association of *NOTCH1* mutations with chemorefractoriness may explain the poor outcome associated with *NOTCH1* alterations. Although the relationship between *NOTCH1* mutations and response to treatment needs to be formally tested within clinical trials, indirect evidence for this hypothesis comes from the observation that *NOTCH1* mutations are enriched among chemorefractory CLL patients, and that *NOTCH1* activation *in vitro* confers resistance to apoptosis through NF-κB pathway activation. Given the availability of *NOTCH1* inhibitors that are under clinical development, mutations of *NOTCH1* in CLL may also provide a novel molecular therapeutic target.

Targeting leukemia stem cell: Anti-Hedeghog pathways strategies against LSC

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Hedgehog (Hh) pathway is one of the major factors regulating cell fate. Several reports have suggested that Hh signaling is critical for HSC survival and hematopoietic progenitor differentiation. Studies conducted in zebrafish revealed that embryos mutant for different components of the Hh pathway displayed defects in HSC pool formation indicating that Hh is required for definitive hematopoiesis. In mammals, there are three Hh proteins: Sonic Hh, Indian Hh and Desert Hh. Hh pathway activation is initiated when the Hh ligand binds to Patched receptor and the latter moves away from a second transmembrane protein called Smoothened (Smo), which is in turn able to signal. Smo to a cytoplasmatic complex that releases transcription factors of the Gli family to translocate to the nucleus where it activates target genes. Aberrant activation of the Hh pathway in cancer is caused either by mutations in the pathway or by Hh overexpression. Leukemic cells are thought to rely on autocrine signaling, whereby Hh ligand, produced by leukemic cells, acts on neighboring leukemic cells to stimulate their growth or survival. This model is supported by *in vitro* evidence that proliferation of tumor cell lines is accelerated by the addition of Hh ligand and is inhibited by the addition of an Hh neutralizing antibody or by cyclopamine, a Hh pathway antagonist. Moreover, Hh also acts through a paracrine mechanism; Hh ligand secreted by neoplastic cells signals to the tumor microenvironment and the stromal cell compartment signals back to malignant cells. The clear association between the Hh pathway and human leukemias led to research aimed at identifying of small molecules that block the pathway. In addition to cyclopamine, a natural Hh antagonist isolated from corn lilies that targets Smo, and different classes of small-molecule Hh antagonists have been identified through cell-based screens using an Hh reporter assay.

Among the molecules identified, a novel series of Hh pathway inhibitors, the 1-amino-4-benzylphthalazines, has been confirmed to act via the antagonism of the Smo receptor. An important issue that remains to be addressed is the potential toxicity of blocking a critical developmental pathway such as Hh. Thus far, treatment with Hh antagonists seems to be rather well tolerated.

Epigenetic check of normal and leukemic hematopoiesis: new insight

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Epigenetic changes play a major role during normal hematopoietic development and in the pathogenesis of leukemia. It is becoming clear that DNA methylation strongly interacts with other components of the epigenetic machinery such as histone modifications, and a high number of "epigenetic enzymes" to control for normal hematopoietic stem cell maintainance and differentiation towards the different cell lineages.
On the other hand, aberrant DNA methylation still is a crucial hallmark of cancer by itself. In Acute Myeloid Leukemia (AML), methylation changes are very frequent and include hypermethylation of tumor suppressor genes and hypomethylation of other regions of the genome, including oncogenes. Specific patterns of DNA methylation characterize AML, relate to karyotype abnormalities, but play an independent negative prognostic role. The contribution of epigenetic dysregulation to leukemogenesis in AML is currently unclear. However, interactions between recurrent translocations or mutations and epigenetic networks have been shown to be at least partially responsible for leukemic transformation, like in acute promyelocytic leukemia (APL) and t(8;21) leukemias. Also, mutations in the epigenetic master regulators EZH2, DNMT3A, IDH1 and IDH2 were recently identified in AML and in myelodysplastic (MDS) and myeloproliferative diseases, leading to secondary leukemia.
In this line, epigenetic therapies including hypomethylating agents show significant clinical activity in MDS and in a proportion of AML. While benefit is observed in many patients, DNA hypomethylating therapy by itself is not curative, and continuous treatment is required to maintain response. Furthermore, the kinetics of response and in vitro data suggest that cytotoxicity is also part of the mechanisms of action of these drugs.

Leukemia 20 – 30%: new entity for new therapy

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The historical FAB classification of Acute Myeloid Leukemia (AML) and Myelodisplastic Syndromes (MDSs), based on the morphological characteristics of blast cells and on the percentage of marrow infiltration by leukemic cells, has been overcome by the WHO classification since 2008. According to the WHO classification clinical, morphological, phenotypic and molecular data are considered together and very specific AML/MDS categories are identified. In particular, in the WHO classification, a percentage of blast cells in the bone marrow ≥ 20% suggest a diagnosis of AML, whereas a smaller leukemic mass is generally consistent with a diagnosis of MDS. However, there are some cases of relatively elderly patients, sometimes with bone marrow multilineage dysplasia and with a blast cell infiltration grater than 20%, but generally lower than 30 – 40%. These patients usually present with pancytopenia, and the clinical course may be relatively "indolent" compared to the AML with hyperleukocytosis, being similar to the old refractory anemia with excess of blast in transformation in the FAB era. The genetics of these cases is often similar to that observed in MDS, showing complex karyotypes, or abnormalities of chromosome 5 and 7, suggesting a possible origin of the leukemic clone from a myelodisplastic clone. Molecular studies of these AML cases is very important and should include at least the analysis of NPM mutations and Flt3-ITD. The prognosis of these patients is generally poor, with a lower rate of achieving CR than other AML types with conventional chemotherapy, and thus allogeneic stem cell transplantation should be considered as an option for younger and fit patients. New de-methylating agents (e.g Azacitidine) showed very promising results in this subset of patients.

A large and systematic biological study of the genes involved in the leukemogenesis and the disease progression using the Next Generation Sequencing technology is mandatory in order to better understand the pathogenesis of this group of diseases and to better address the issue of the most effective anti-leukemic therapy.

Gene modifier: is the microenvironment the therapeutic target?

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The specific niches within the bone marrow microenvironment may provide a sanctuary for subpopulations of leukemic cells to escape chemotherapy induced death and acquire drug resistance. Studies on the stroma/leukemia cross talk may result in the development of strategies against the acquisition of a chemoresistant phenotype and enhance the efficacy of therapies in leukemia. Novel therapeutic interventions have targeted the microenvironment/leukemia interactions focusing on SDF1/CXCR4,ILK/PI3/Akt,TGF beta and Notch signaling. On the other hand, gene transcriptional activity is regulated by chromatin modification and DNA methylation. Nuclear receptors such as RAR,RXR,PPAR gamma have histone acetyl transferase activity. The transcription of target genes is initiated following the ligation of these receptors, recruitment of coactivators and replacement of repressors. Histone deacetylase inhibitors interferen with these pathways acting as gene modifiers.
The interactions of drugs with DNMT3A mutations could be another possible pathway of gene modifications with possible clinical implications. Bioimmunomodifiers, such as lenalidomide and related compounds, have a possible role in modifying microenvironment and gene responses in leukemias; their role will be, too, focused during the meeting.

New biomarker for optimal use of Clofarabine

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Clofarabine (2-chloro-2'-fluoro-2'-deoxy-9-**β**-D-arabinofuranosyladenine) (CLO) is a second generation purine nucleoside analogue, thus acting as an antimetabolite. It has been developed to overcome the limitations given by the toxicity, especially dose-limiting neurotoxicity, known from the structurally related analogs cladribine and fludarabine, and to incorporate at the same time the best qualities of both congeners. Its growing use in pediatric and adult refractory and relapsed AML and ALL confirms that CLO is one of the most promising new molecules in this field of the last ten years.

Understanding genetic variants in the key candidate genes involved in mechanisms of action and pathway of CLO, as well as the pharmacodynamic targets of CLO, will provide an opportunity to identify patients at an increased risk of adverse reactions or decreased likelihood of response, based upon their genetic profile, which in future could help in dose optimization to reduce drug toxicity without compromising efficacy. To our knowledge, no studies of pharmacogenomics have been conducted until now on the different sensitivities of pediatric leukemia blast cells to Clofarabine.

We have assessed in vitro sensitivity to CLO in 17 acute lymphoblastic leukemia samples from children affected by T cell type ALL (T-ALL) and B cell type ALL (B-ALL). Study was performed on 10 T-ALL and 7 B-ALL samples. Median white cell count at diagnosis was $1.5x10^4$/µl and $15x10^4$/µl in B-ALL and T-ALL patients respectively. In vitro sensitivity to CLO was measured in terms of LC_{50}, drug concentration needed to kill 50% of leukemia cells (Fig.1). Median LC_{50} value of CLO in ALL cells was 0.057µM (range, 0.0095 µM - 289.4 µM). Median sensitivity to CLO was similar in B-ALL and T-ALL leukemic blast cells (B-ALL: 0.040 µM, range= 0.013 - 0.086µM, n=7; T-ALL: 0.240 µM**,** range= 0.009-289.40µM, n=10, *P*=0.315) (Fig.1). However, while B-ALL cells were similar in response to CLO being sensitive to low doses of the drug, in T-ALL samples we observed a variability in cell sensitivity with a spread distribution of LC_{50} values around the median concentration. T-ALL cells were then classified into two subgroups by their CLO sensitivity: T-ALL sensitive cells, T-ALLs (LC_{50} values lower than median value) and T-ALL resistant cells, T-ALLr (LC_{50} values higher than median). The difference in LC_{50} between the two groups is statistically significant, with T-ALLs cells having a median LC_{50} value of 0.018 µM and T-ALLr with a median LC_{50} value of 5.97 µM (*P* =0.0095).

We then explored the possibility that CLO sensitive and resistant patients represented two clinically or biologically different entities by correlating drug sensitivity to clinical parameters used for prognostic patient's stratification as WBC count at diagnosis and glucocorticoid response. There is no statistical correlation between CLO sensitivity or resistance and WBC count at diagnosis, considering the cutoff for poor prognosis both at >100000/µl (*P* =0.307), and at >50000/µl (*P* =0.302). No correlation was observed between CLO sensitivity and Prednisone response in vivo (*P* =0.633), or with Dexamethasone sensitivity in vitro (Spearman's rho = 0.16; *P*=0.657) (Fig. 2).

Since the sensitivity of T-ALL blast cells to clofarabine is highly variable, and no clinical or immunophenotypic data correlated with drug response, we asked whether specific gene expression signatures were associated with the phenotype. We performed gene expression profiling with oligonucleotide microarrays in the same patient's leukemic samples at diagnosis, and selected the genes differentially expressed between sensitive and resistant patients. More than 300 genes were significantly different in the two groups (*P* <0.01), and pathway analysis on Kegg map database showed that "Notch signaling" was the most significantly enriched pathway by 4 different statistics (Table 1). Indeed many genes belonging to the pathway and other markers of Notch activation in T-ALL (Chadwick et al., 2009; Rao et al., 2009) are significantly differentially expressed in sensitive *vs* resistant patients. Two of the most significantly different genes were also confirmed by qPCR.

KEGG pathway	Pathway description	EASE score	LS permutation	Efron-Tibshirani's GSA test	Goeman's global test
hsa04330	**Notch signaling pathway**	**0.015**	**0.00001**	**< 0.005**	**0.0048**
hsa04320	Dorso-ventral axis formation	NS	**0.0004**	**0**	**0.0048**
hsa00980	Metabolism of xenobiotics by cytochrome P450	NS	**0.00001**	**0.02**	**0.0048**
hsa04070	Phosphatidylinositol signaling system	**0.02**	NS	NS	NS
hsa04115	p53 signaling pathway	**0.05**	NS	NS	NS

Table 1. Kegg pathway enrichment of genes differentially expressed between T-ALLr and T-ALLs patients. *p* values are shown for each statistics.

These results demonstrate that the NOTCH pathway activation plays a fundamental key role in the genetic sensitivity of leukemic blasts to Clofarabine. Based on this consideration it is possible to speculate on the possibility that blocking the NOTCH signaling pathway, for example with gamma-secretase inhibitors, could potentially restore the sensitivity of resistant leukemic blasts to CLO. We are moving in this direction with our research in order to optimize the target to CLO and provide a better use of this new drug especially in the field of pediatric refractory/relapsed leukemia.

Tosedostat: an Aminopeptidase inhibitor

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Tosedostat "CHR-2797" is a novel metalloenzyme inhibitor that is converted into a pharmacologically active acid product (CHR-79888) inside cells. CHR-79888 is a potent inhibitor of a number of intracellular aminopeptidases. Aminopeptidases are key enzymes in the protein recycling pathway within cells. Inhibition of aminopeptidases leads to an amino acid deprivation response "AADR". This depletes sensitive tumour cells of amino acids and leads to an antiproliferative effect.
Oral tosedostat has been studied in both solid and liquid tumours. A summary of the clinical activity is included:
CHR-2797-001 (Reid et al, Clin. Cancer Res., 2009), Phase I/II study in solid tumours, 40 patients: 1 durable PR and 7 confirmed SDs, MTD 320 mg, MAD 240 mg.
CHR-2797-002 (Lowenberg et al, JCO, 2010), Phase I/II study in hem-onc tumours, 51 AML patients: 14 (28%) response (14% CR), RR AML subset (N=35): 11 (31%) PR or better, MTD 180 mg, MAD 120 mg
CHR-2797-038 OPAL (Oral presentation during ASH 2011), Phase II – R/R AML, 73 patients: ORR of PR or better = 22%
Tosedostat has shown promising anti-leukemic activity in a difficult to treat patient population. It is generally well tolerated with the main TEAEs (≥ 30 % of patients on tosedostat monotherapy) being fatigue, oedema, diarrhoea, thrombocytopenia, dyspnoea, nausea and anaemia. The majority of these events are G1 and G2.
The OPAL study data indicates that patients with prior MDS or treated with a HMA as primary induction for AML did particularly well on Tosedostat. A Global Phase III study targeting this patient profile is currently being set up and will look to start recruitment in Q3 of 2012.

Nelarabine

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Nelarabine is a new purine analogue currently approved for the treatment of patients with T-cell acute lymphoblastic leukemia (T-ALL) and T-cell lymphoblastic lymphoma (T-LBL), who have not responded to or have relapsed following treatment with at least two chemotherapeutic regimens (1). Nelarabine is demethoxylated by adenosine deaminase to become biologically active as 9-beta-D-arabinosylguanine (ara-G); ara-G incorporates into DNA, thereby inhibiting DNA synthesis and inducing an S phase-dependent apoptosis of tumor cells. The recommended dose in adults is 1,500 mg/m2 intravenously over 2 hours on days 1, 3 and 5 repeated every 21 days, while in pediatric patients a reduced schedule of 650 mg/m2 intravenously over 1 hour daily for 5 consecutive days repeated every 21 days is used (2). The duration of therapy with nelarabine has not been established. However, treatment is usually continued until disease progression, intolerable toxicity, or the patient become a candidate for bone marrow transplantation. Two phase II trials, one conducted in pediatric patients (3) and the other in adult patients (4), leaded to the approval of the drug for the above indications. Patients were in their first or subsequent relapse and/or were refractory to first-line therapy. Study endpoints were the rates of complete response (CR) and CR with incomplete hematologic or bone marrow recovery (CRi). The pediatric population consisted of 39 patients who had relapsed after, or had been refractory to, two or more induction regimens. CR following nelarabine treatment was observed in five patients (13%) and CR+CRi was observed in nine patients (23%). The adult efficacy population consisted of 28 patients. CR was achieved in five patients (18%) and CR+CRi was recorded in six patients (21%). Neurologic toxicity was dose limiting for both pediatric and adult patients. Other severe toxicities included hematologic, hepatic, and metabolic laboratory abnormalities in pediatric patients and gastrointestinal and pulmonary toxicities in adults. More recently, a

single-arm phase 2 study was conducted in adults (18-81 years of age) with relapsed/refractory T-ALL/LBL (5). After 1 or 2 cycles, 45 of 126 evaluable patients (36%) achieved CR, 12 partial remission (10%), and 66 (52%) were refractory. One treatment-related death was observed, and 2 patients were withdrawn before evaluation. A total of 80% of the CR patients were transferred to stem cell transplantation (SCT). Overall survival was 24% at 1 year (11% at 6 years). After subsequent SCT in CR, survival was 31% and relapse-free survival 37% at 3 years. Transplantation-related mortality was 11%. Neurologic toxicities of grade I-IV/grade III-IV were observed in 13%/4% of the cycles and 16%/7% of the patients. This large study with nelarabine in adults comfirmed impressive single-drug activity with acceptable toxicity profile in relapsed T-ALL/T-LBL, mainly as bridge to transplantation even in heavily pretreated patients. In one small study in pediatric patients, a combination of nelarabine with etoposide and cyclophosphamide was explored with evidence of response, but neurologic toxicity was remarkable and severe myelosuppression was recorded in all patients (6). Finally, in vitro data suggest that the combination of nelarabine with forodesine, another nucleoside analogues with promising anti-leukemic activity, would be pursued in pediatric patients with T-ALL, but also B-ALL and acute myeloid leukemia (7). The use of nelarabine as part of a combination regimen may show increased benefit, but more research is clearly needed in order to define more effective and safe regimens. Central and peripheral neurotoxicity has been the most identifiable toxicity associated with nelarabine administration (8). At the recommended adult dose of 1.5 g/m2, the incidence of motor and/or sensory peripheral neuropathy was around 20%. The incidence of reversible neurotoxicity occurs more rapidly in adult than pediatric patients, but the incidence of grade 3 and 4 neurotoxicity is more common in the pediatric population. More common central neurotoxicities include paresthesias, ataxia, tremor, neuropathy, amnesia, and sensory loss. Neurotoxicity is dose related, with larger doses of the drug, mainly in combination chemotherapy, resulting in neurologic dose limiting toxicity.

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mTOR inhibitors in AML

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The major thrust of novel therapeutics in AML is development of agents targeting critical cellular and molecular events that ultimately lead to leukemic transformation. One promising target for molecular therapy in AML is the PI3K/Akt signaling pathway, which is constitutively activated in AML samples and has been shown to be central to the proliferation and survival of the leukemic blasts. The serine-threonine kinase mTOR is downstream of PI3K/Akt and can be inhibited by selective inhibitors including rapamycin (sirolimus) and its first generation analogues (rapalogs). mTOR inhibitors have been shown to induce apoptosis of AML cells in vitro, to have in vivo activity in experimental models of AML, and to enhance the antileukemic activity of a variety of cytotoxic agents including etoposide, anthracyclines and cytarabine.
Clinical trials of rapalogs are ongoing in AML as single agents and in combination with chemotherapy. However, despite promising evidence of biological on target effects, rapalogs have proven only modestly successful. In part this reflects the fact that these compounds do not effectively inhibit the mTORC2 complex, and an incomplete understanding of the functions of mTOR and the complex feedback loops in its signaling network. In response to the limitations of the rapalogs, much effort is being made to develop a new generation of agents targeting the PI3K/Akt/mTOR network at multiple sites. Inhibitors targeting both mTORC1 and mTORC2 (ATP-competitive catalytic site inhibitors), as well as dual catalytic PI3K/mTOR inhibitors are currently undergoing early phase clinical testing. It is hoped that these agents will circumvent some of the shortcomings of the rapalogs and lead to more efficient targeting of this pathway for AML therapy.

Fluda associated regimens. New standard of care?

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Front-line induction chemotherapy of acute myeloid leukemia (AML) is conventionally based on regimens containing cytarabine (Ara-C) and one anthracycline, with or without the addition of a third drug. Complete remission (CR) can be obtained in about 70-80 % of younger patients with AML, but only 30% of cases survive longer than 10 years. The factors associated with poor response to standard therapy are secondary leukemia, advanced age, adverse karyotype, high leukemic burden, and over expression of multidrug resistance (MDR)-related proteins, like P-glycoprotein (PGP), that interfere with drug sensitivity. Several studies have confirmed the high efficacy and low toxicity of fludarabine-based regimens in AML, expecially in those with MDR over expression. The combination of fludarabine–cytarabine was initially studied in patients with relapsed AML; in this setting CR rates have been reported to range from 36 to 44%. Based on this activity, the combination of fludarabine–cytarabine was studied in newly diagnosed AML patients, with CR rates of 70-80%. The addition of granulocyte colony-stimulating factor before, during or after fludarabine–cytarabine treatment had no significant effect on the CR rates in this population. The FLAI (fludarabine, cytarabine, idarubicin) induction regimen has proved to be effective and mildly toxic when it was tested by our group in newly diagnosed AML patients aged <60 years, first in a pilot study (AML 97 study) and then in a multicentre randomised trial (AML 99 study). The rationale of FLAI combination is that fludarabine is a fluorinated purine analogue that is toxic to Multidrug Resistance (MDR) over expression cells and enhances Ara-C cytotoxicity by increasing cellular concentration of Ara-C 5-triphosphate, thus inhibiting DNA repair. Another schedule of combination chemotherapy consisting of fludarabine, ArA-C, and G-CSF, designated FLAG, has been suggested to provide effective antileukemic therapy without the toxic sequelae of high-dose ArA-C.

FLAG has become a quite popular schedule and is commonly employed for treating patients with high-risk MDS and AML. Promising results have been reported recently by different groups, using fludarabine containing combination therapy such as FLAG, FLAG plus mitoxantrone (FLANG), or FLAG plus Idarubicin (FLAG-Ida). However the randomized prospective trials are lacking and to date, fludarabine based regimens have not proven to be superior to Ara-C plus anthracycline in AML in induction phase. Despite the development of new drugs fludarabine based chemotherapy remain a valid option in clinical practice as induction or salvage treatment expecially in AML cases with MDR over expression.

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New Drug Development in United Kingdom Trials

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The UK National Cancer Research Institute (the successor to the MRC) runs large trails (n~1200 per annum) for both older and younger patients with AML and high risk MDS. With factorial design, several interventions are evaluated in a single trial. Currently the AML16 and AML17 trials asks 16 questions several of which include novel (unlicenced) agents. For younger patients novel agents in scrutiny are gemtuzumab ozogamicin (GO: Mylotarg) which is being asked in a dose question, following the clear benefit seen in the AML15 trial, in other than unfavourable disease, when added to conventional chemotherapy. The FLT3 inhibitor, lestautinib, is being tested in a placebo controlled randomisation in FLT3 mutated patients given as an adjunct to chemotherapy. mTOR inhibition is being similarly tested in other patients in combination, for those who are not high risk, and for high risk FLAG-Ida is being compared to Dauno/clofarabine prior to transplant. Since completion of recruitment to the GO randomisation the Dauno dose 60mg is being compared to 90mg in induction course 1.

For older patients who are fit for an intensive approach Dauno/clofarabine has been compared to Dauno/ara-C as induction, and demethylation maintainance with azacytidine for 12 months is compared to no maintenance. Since the closure of the DA vs DClo randomisation, the role of the addition of ATRA to either DA or DA/etoposide is being tested.

For older patients who are not candidates for an intensive approach we have developed a "pick a winner" trial design to rapidly and efficiently, enable the assessment of several novel treatments simultaneously, against standard of care which continues to be Low Dose Ara-C (LDAC). The options tested so far in this design are LDAC + arsenic trioxide/ LDAC + GO/ LD Clofarabine/ LDAC + tipifarnib. Spacitibine/ Voseroxin/ LDAC+ Vosaroxin/ LDAC + AC220 and LDAC + Ganetespib (HSP90 inhibitor) are currently being introduced.

Pilot studies have been initiated to test the feasibility for the next trial in older patients which is to assess the use of the CXCR4 inhibitor (plerixafor) +/- G-CSF with each course of chemotherapy, and the combination of AC220 with standard chemotherapy.
The characteristics of the "pick a winner" Programme will be discussed and an update of these various interventions will be presented.

New FLT3 inhibitors: AC220

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FLT3 (Fms-like tyrosine kinase 3) is a member of the class III receptor tyrosine kinase family. The genetic alterations of FLT3 represent the most frequently identified mutations in cytogenetically normal (CN) Acute Myelogenous Leukemias (AML). These alterations lead to the constitutive activation of the receptor and to an abnormal signaling through pathways such as PI3 kinase / Akt, Ras / Raf / Mek or STAT5, resulting in proliferation and resistance to apoptosis (1). FLT3 alterations can also be secondary events in the leukemic process and some patients without mutations at diagnosis may relapse with a dominant mutated clone. Two categories of mutually exclusive alterations have been described: first, FLT3 Internal Tandem Duplications (ITDs) found in around 32% of CN AML and 38% of acute promyelocytic leukemias (APL) and second, tyrosine kinase domain mutation at the D835 position found in around 14% of CN AML, 20% of APL and 7% of CBF AML. The negative prognostic value of FLT3-ITDs is well established whereas the role of FLT3 D835 mutation is more controversial. FLT3-ITDs affect a partial sequence in exons 14 and 15 in a head-to-tail fashion that map to the juxtamembrane domain and consist of a sequence repetition of 3 to more than 400 base pairs that always preserves the reading frame. Interestingly, in approximately half of the cases, FLT3-ITDs are associated with a mutation in the NPM gene leading to the attenuation of the negative impact of FLT3-ITDs (2). In some studies, the size of the ITD and the proportion of mutated allele compared to wild-type allele have also been reported to be of prognostic importance.
Patients with CN AML associated with FLT3-ITDs are classified in the poor intermediate prognostic group (3). Once in Complete Remission, those patients may be offered an allogenic hematopoietic stem cell transplant (HSCT) depending on age and donor availability. In some studies, reinforced induction was reported to overcome the bad prognosis of FLT3-ITDs whereas in others this was not confirmed (4). Data from the German group have suggested that patients with

CN AML and unfavorable genetic markers such as FLT3-ITDs or no mutation of NPM-1 or CEBP□ may benefit from allogenic HSCT (2). Furthermore, FLT3-ITD MRD levels after induction chemotherapy are predictive of complete remission duration (5). In this context, inhibiting FLT3 tyrosine kinase may lead to clinical benefit for AML patients.

Recently developed FLT3 inhibitors have shown encouraging activity as monotherapy and in combination with conventional treatment. Among the more than 20 compounds studied, at least 8 molecules have been tested in clinical trial in the context of relapsed FLT3-ITD AML. The first generation FLT3 inhibitors (CEP-701, Lestaurtinib; PKC-412, Midostaurin for example) were more pan-kinase inhibitors rather than selective inhibitors for FLT3. *In vitro* IC50 were 2 and 6 nM respectively and *in vivo* IC50 as much as 700 and 1000 nM for CEP-701 and PKC-412 respectively. Duration of response was short (few weeks) for most of the patients whereas some patients experienced sustained responses. Sorafenib (BAY 43-9006) is a 1000 fold more selective inhibitor compared with the mentioned above. Treatment was prolonged and leads to long lasting responses especially in AML with high mutant to wt allelic ratios. Large international multicenter randomized studies are ongoing in newly diagnosed patients to test the efficacy of FLT3-TKI in combination with standard chemotherapy (6).

More recently, a second generation FLT3 inhibitor was evaluated in phase 1 and 2 studies. Quizartinib (AC220) was derived from a bis-aryl urea derivate with a high selectivity for FLT3 (6). 67 patients were included in a phase 1 dose-escalation study. AC220 was administered orally once daily with a maximum tolerated dose of 200 mg/d (dose limiting grade 3 QTc prolongation was reported at 300 mg/d). Overall response rate was 56% in the FLT3-ITD+ population compared to 20% in the FLT3 wild type population. The phase 2 study was recently reported at the ASH 2011 meeting. Patients with a relapsed or refractory FLT3-ITD AML were treated with AC220 at the starting daily dose of 90 mg in female and 135 mg in male. 62 patients were analyzed and the overall response rate was 40%

including CR (one patient), CRp (one patient) and CR with incomplete hematological recovery (all other patients). Interestingly, the duration of response was 12 weeks in median with a median survival of 25.4 weeks. Of note, ¼ of the patients were bridged to HSCT. Main adverse events were prolonged neutropenia (and febrile neutropenia) and QTc prolongation (7). Major mechanisms of secondary resistance to FLT3 inhibitors may involve resistance mutations in the ATP binding pocket of FLT3, FLT3 overexpression or the activation of alternative pathway.
In conclusion, the second generation of selective FLT3 inhibitors may represent a major step in the treatment of poor prognosis FLT3-ITDs AML (and perhaps also FLT3 wild type AML). The future challenges will be to safely integrate those new options in the management of first line patients.

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PKC412 as front line therapy

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A constitutively activated, mutated version of the class II receptor tyrosine kinase, FLT3 (*Fms*-Like Tyrosin kinase-3) is expressed in approximately 30% of patients with acute myeloid leukemia (AML). Two major classes of activating FLT3 mutations have been identified in AML patients: internal-tandem duplication (ITD) and tyrosin kinase domain (TKD) point mutation. ITD mutations occur in the juxtamembrane domain of FLT3 and are detected in 20-25% of AML patients whereas TKD mutations are described in 5-10% of AML cases. The expression of FLT3-ITD is significantly associated with increased white blood cell counts, high percentage of peripheral blood and bone marrow blasts and normal cytogenetics. The presence of FLT3-ITD correlates with an increased risk of relapse and poor long-term overall survival (OS). Some studies suggested the absence of wild-type allele as a predictor of poor outcome, thus underlining the prognostic role of the mutant/wild-type allele ratio. Although the expression of FLT3-TKD is also associated with high peripheral blood and bone marrow blast count and normal cytogenetics, its prognostic relevance is less clear. While some studies reported a very dismal outcome others did not; these conflicting results are likely due to the small numbers of patients observed and to the different chemotherapeutic regimens delivered. Based on this, aberrantly activated FLT3-kinase is regarded as an attractive therapeutic targets in AML. Midostaurin (PKC412) is one of several small molecules with FLT3 inhibitory activity that have been developed and used as single agents and/or in combination with chemotherapy for the treatment of FLT3 positive AML. Midostaurin has been used as single agent in 2 phase-II trials of 20 patients with relapsed/refractory FLT3-mutated AML or myelodysplastic syndrome (MDS), considered unfit for intensive chemotherapy. Fourteen patients experienced a greater than 50% decrease in peripheral blood blast count and 6 also achieved a greater than 50% reduction in bone marrow blasts infiltration. Of 10

patients in whom plasma inhibitor activity was determined, 8 showed a substantial inhibition of FLT3 tyrosine-phosphorylation. More recently, 95 patients with AML or MDS with either wild-type (n = 60) or mutated (n = 35) FLT3 were randomly assigned to receive oral midostaurin at 50 or 100 mg twice daily. A reduction in peripheral blood or bone marrow blasts greater than 50% was observed in 71% of patients with FLT3-mutant and 42% of those with FLT3 wild-type. These results suggest that midostaurin has hematologic activity in both patients with FLT3-mutant and wild-type and support the possibility to combine midostaurin with other agents such as chemotherapeutic ones. Actually, there are only few published data on this matter indicating that midostaurin can be safely associated with chemotherapy but whether or not such an approach translates in a longer OS and/or progression-free survival is still unclear. A large international multicenter randomized study (CALGB 10603/CTSU C10603), dealing with this issue, has been recently closed and results are eagerly awaited.

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JAK2 inhibitors: the COMFORT clinical trials

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Janus kinases (JAKs) are a family of four cytoplasmic tyrosine kinases (JAK1, JAK2, JAK3 and non-receptor tyrosine kinase 2 (TK2)) that mediate signals from the receptors for various cytokines and growth factors that play a key role in haematopoiesis and immune function. In 2005, several groups reported the presence of an activating mutation in the JAK2 gene, which results in a valine to phenyalanine substitution at codon 617 (JAK2V617F), in a substantial proportion of patients with chronic myeloproliferative neoplasm (CMPNs). Ectopic expression of JAK2V617F in mice results in MPN-like phenotypes, supporting an important role for this mutation, and JAK2 in general, in human CMPNs.

Ruxolitinib (formerly, INCB018424) is an orally available, ATP-competitive inhibitor, selective for tyrosine-kinases JAK1 and JAK2 and is the most advanced JAK1/JAK2 inhibitor in development for the treatment of CMPNs. The suggested mechanism of action of ruxolitinib is down-regulation of inflammatory cytokine activity via the inhibition of JAK1 and JAK2 (wild-type or mutated forms), resulting in antiproliferative and proapoptotic effects. In a mouse model of JAK2V617F-positive CMPNs, oral administration of ruxolitinib markedly reduced splenomegaly and levels of circulating inflammatory cytokines.

The efficacy and safety of ruxolitinib were studied in two Phase III trials in patients with myelofibrosis (primary, post-PV or post-ET myelofibrosis). The starting dose of ruxolitinib was based on platelet count; doses were individualized based on tolerability and efficacy, with a maximum dose in any group of 20 mg orally twice daily. In both studies, the primary efficacy end point was the proportion of patients achieving a ≥35% reduction from the baseline in spleen volume at week 24 as measured by MRI or CT. Secondary end points included the proportion of patients with a ≥50% reduction in total symptom score from the baseline to week 24, as measured by

the modified Myelofibrosis Symptom Assessment Form (MFSAF) v2.0 diary. This score captures the six major symptoms of myelofibrosis (abdominal discomfort, pain under left ribs, night sweats, itching, bone and/or muscle pain and early satiety).

COMFORT-I (Controlled MyeloFibrosis study with Oral JAK inhibitor Treatment) trial was a double-blind, randomized placebo-controlled trial involving 309 patients who were refractory to or not candidates for available therapy. Patients were randomized to ruxolitinib or placebo in a 1:1 ratio. The proportion of patients that achieved spleen volume reduction ≥ 35% from baseline to 24 weeks was 41.9% with ruxolitinib versus 0.7% with placebo ($P < 0.0001$). In addition, 46% of patients had a ≥50% reduction in total symptom score by week 24, compared with 5% of patients in the placebo group.

COMFORT-II trial was an open-label trial involving 219 patients who were randomized in a 2:1 ratio to ruxolitinib or the best available therapy (such as hydroxyurea and glucocorticoids), selected by the investigator on a patient-by-patient basis. Reductions in spleen volume ≥ 35% were observed in 31.9% of patients treated with ruxolitinib versus 0% with best available therapy at week 24, and 28.5% versus 0% at week 48 (both $P < 0.0001$).

The major toxicities associated with JAK2 inhibition in the two COMFORT trials was a worsening of anaemia and thrombocytopenia. In COMFORT-I, 45.2% of patients in the ruxolitinib arm had Grade 3/4 anaemia at some point during treatment, compared with 19.2% of the patients in the placebo arm. Grade 3/4 thrombocytopenia was also more common in the ruxolitinib arm compared with the control arm (12.9% vs 1.3%). In COMFORT-II, the rates of low-grade anaemia remained similar (54% in the ruxolitinib arm, 63% in the best alternative treatment arm), while the rate of Grade 3/4 anaemia was substantially higher in the experimental arm (42% versus 28% in the control arm). Although low-grade thrombocytopenia was more common in the ruxolitinib arm in COMFORT-II than in the control arm (60% versus 22%), there was no difference in the proportion of patients who had Grade 3/4 thrombocytopenia (8% and 7%). Non-hematologic side effects include gastrointestinal disturbances, asymptomatic elevation of liver and pancreatic enzymes, peripheral neuropathy and hyperacute relapse of symptoms during treatment interruption.

Ruxolitinib is now approved by the US Food and Drug Administration (FDA) for the treatment of patients with intermediate or high-risk myelofibrosis, including primary, post-PV and post-ET myelofibrosis.

Early clinical development of drugs in myelofibrosis

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Myeloproliferative neoplasms (MPN)-associated myelofibrosis is the most disabling among the classical Philadelphia-negative MPNs. The conventional drug therapy for MPN-associated myelofibrosis is based on an array of drugs with only palliative benefits, none of which has been approved by the US Food and Drug Administration (FDA) or European Medicines Agency (EMA). With the advent of molecular studies and better insight into the pathogenesis of the disease, more mechanism-driven, targeted drugs are being sought aimed at improving survival in MPN-associated myelofibrosis. In this communication we first will discuss about indications and limitations of conventional therapies employed for treatment of MPN-associated myelofibrosis. Then we critically will review the information available for new therapies including the immunomodulatory and demethylating agents, histone deacethylase and mTOR and JAK2 inhibitors. From the results of literature review, three categories of drugs proved to have significant activity in MPN-associated myelofibrosis. Up to 40% response on anemia has been reported with the immunomodulator pomalidomide. m-TOR inhibitor RAD001, and different JAK2 inhibitors have documented profound effect on splenomegaly and constitutional symptoms, with some having activity also on anemia. These new drugs will give physicians more options to tailor therapeutic choice in this challenging disease. Future research emphasis will be on the use of combination of new drugs that could result in additive or synergistic efficacy

A selective JAK2 mutated inhibitors: we really need?

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The discovery of involvement of the JAK2/STAT signaling in patients with mieloproliferative neoplasms (MPNs), usually but not necessarily associated with the presence of JAK2 or MPL mutation[1], has represented the background for developing clinical trials with small molecules targeting JAK2[2]. INCB180424, now called Ruxolitinib, has already completed two phase III trials, showing clinically relevant activity against the enlarged spleen and the burden of constitutional symptoms that affect patients with myelofibrosis. The major end point of the studies was a reduction of spleen volume ≥35% by MRI, that equals to a ≥50% reduction in palpatory spleen length, at 24 and 48 weeks[3,4]. These results eventually led to the approval of the drug for patients with intermediate and high-risk MF by FDA in Nov 2011. Treatment was well tolerated, with few and modest non-hematologic effects; the DLT was represented by thrombocytopenia, while the commonest toxicity was anemia that required transfusion support in about a quarter of the subjects. Anemia has to be considered as an "on-target" side effect of the drug, since experimental data indicate that functional JAK2 is obligatory requirement for erythropoiesis. Ruxolitinib can be considered as the founding member of a larger family of small inhibitors that target JAK2, and include at least SAR302503, CYT387, Ly2784544, SB1518, to mention those with information on completed or ongoing clinical trials. All of these have reported a similar spectrum of activity on splenomegaly and constitutional symptoms, yet some difference begin to emerge. SAR302503 (previously TG10138) has been reported to produce more myelosuppression with effective control of leukocytosis and thrombocytosis, but more frequent gastrointestinal toxicity[5]. CYT387 appears to have an unique capacity to ameliorate hemoglobin level, at least in a sizable proportion of the patients with reported acquirement of transfusion independence, that appears as a paradox considering the expected consequences of JAK2 inhibition and suggests the role of

other unknown JAK2-independent regulatory pathways; pancreatic toxicity is the DLT[6].

Although it is not the time yet to draw firm conclusions about unique properties of one versus the other JAK inhibitors and more clinical experience is warranted, some considerations nevertheless apply. First, none of these inhibitors is specific for mutated JAK2 and all target the wild-type protein as well. This is a drawback, since one can hypothesize that availability of a selective inhibitor for mutated JAK2 could provide a key to targeted therapy (it will not easy to develop such an inhibitor, considering that the V617F mutation is located in the JH2 inhibitory domain rather than in a catalytically active domain); as a matter of fact, none of available inhibitors has yet been shown able to induce disappearance or marked reduction of the levels of V617F allele burden, at least in the short/medium term. Some evidence of a greater activity has been reported for SAR302503, but data are still scanty. Second, the lack of selectivity for the JAK2V617F mutated protein conversely represents an advantage: about 40% of patients with MF do not have the mutated allele yet they respond equally well to treatment with Ruxolitinib[7], supporting the evidence of an activated JAK/STAT pathway independent ok known JAK2/MPL mutations (and indirectly pointing to other unknown mutations insisting on the same signaling pathway). Toxicity seems to be generally acceptable for all of the JAK2 inhibitors, with some differences that likely point to other targets of the molecules, such as Flt3 that is probably associated with gastrointestinal manifestations. Also, most of these JAK2 inhibitors also target JAK1, and such a combined inhibition could represent one of the mechanisms contributing to the impressive reduction of the dysregulated cytokine level found in MF patients[8] and the ensuing clinical benefits as concerns constitutional symptoms[9].

As a whole, it is too early to state what is the role of JAK2 inhibitors in the management of myelofibrosis and the other MPNs. We know that none of these drugs produces molecular remissions (as reported, conversely, for pegylated interferon in polycythemia vera), but the question is: do we really need to obtain remission of the JAK2V617F mutated clone in these chronic disorders? Furthermore, we know that JAK2V617F is not the disease-driving mutations in MPNs, and as a matter of fact pegylated interferon did not lead to negativization of TET2-mutated clones in double mutated TET2 and JAK2V617F patients. We can be happy that JAK2 inhibitors have dramatically improved the life of patients with MF at an extent not obtained previously with conventional therapies; for many of the patients, they can indeed represent the current standard of care. Yet, we have to realize that the root to the cure of myelofibrosis, and other MPNs, is still far from the sight.

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