

## Molecular basis for the diagnosis and treatment of acute promyelocytic leukemia

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*Acute promyelocytic leukemia is characterized by gene rearrangements that always involve the retinoic acid receptor alpha on chromosome 15. In the majority of patients t(15;17) is detected, which generates the promyelocytic leukemia gene/retinoic acid receptor alpha rearrangement. This rearrangement interacts with several proteins, including the native promyelocytic leukemia gene, thus causing its delocalization from the nuclear bodies, impairing its function. The immunofluorescence staining technique using the anti-PML antibody may be used to provide a rapid diagnosis and to immediately start therapy using all-trans retinoic acid. The experience of the International Consortium on Acute Promyelocytic Leukemia has demonstrated that early mortality was significantly reduced by adopting the immunofluorescence technique. All-trans retinoic acid combined with chemotherapy is the standard therapy; this promotes complete remission rates greater than 90% and cure rates of nearly 80%. However, early mortality is still an important limitation and hematologists must be aware of the importance of treating newly diagnosed acute promyelocytic leukemia as a medical emergency.*

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### Introduction

The clinical features of acute promyelocytic leukemia (APL) were first described in 1949 by Croizat & Favre-Gilly.<sup>(1)</sup> Only in 1957, Hillestad recognized APL as a specific subtype of acute myelocytic leukemia (AML), highlighting the peculiar clinical features and laboratorial aspects of this entity.<sup>(2)</sup> Furthermore, in 1969, Rand speculated that leukemic cells were probably responsible for the activation of the fibrinolytic system.<sup>(3)</sup> But it was only in 1977 that Rowley described the characteristic translocation of APL<sup>(4)</sup> and, finally in 1999 the genes involved in the t(15;17) were cloned and characterized.<sup>(5)</sup> The introduction of all-trans retinoic acid (ATRA) in 1988 in the therapy of APL has revolutionized the management and outcome of this disease and, provided the first paradigm of molecular targeted treatment.<sup>(6)</sup> As a result, APL is today the most curable acute leukemia.

### Molecular and cytogenetic bases

The characteristic feature of APL is the break and fusion of the promyelocytic leukemia (PML) gene located in chromosome 15 and retinoic acid receptor alpha (RARA) located in chromosome 17, resulting in a t(15;17). This translocation is detected in more than 90% of APL cases<sup>(4)</sup> and produces two genes that encode the PML-RARA and RARA-PML hybrid proteins (the last detected in only 70% of cases).<sup>(5,7)</sup>

The PML gene is essential to many apoptotic pathways and is implicated in the control of genomic stability. Its inactivation in animal models caused resistance to apoptosis induced by several stimuli, increased cell growth and deregulated cell senescence.<sup>(8,9)</sup> In the nucleus, the PML protein is detected in nuclear structures as nuclear bodies (NBs), interacting with other proteins (p53, pRB, Daxx, CBP).<sup>(10)</sup> In t(15;17), the breakpoint in the PML gene occurs in three different sites: intron 6 (bcr1-breakpoint cluster region 1), exon 6 (bcr-2 - breakpoint cluster region 2), and intron 3 (bcr-3 - breakpoint cluster region 3).<sup>(11)</sup> Independently of the bcr subtype, most of the functional domains are conserved in the PML/RARA fusion protein.

The RARA nuclear receptor is distributed according to specific standards in each tissue and is particularly important for granulopoiesis.<sup>(12)</sup> Structurally, RARA has six functional domains five of which are preserved in the protein of the PML/RARA fusion.<sup>(5)</sup>

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In the presence of physiological concentrations of ATRA, the RARA protein is capable of linking to the receptor and migrating from the cytoplasm to the nucleus consequently dimerizing with a second class of retinoid receptors, the retinoic X receptor (RXR). This complex recognizes specific sequences of oligonucleotides present in the promoter regions of many genes. By self-linking to the nuclear co-repressors (N-Cor) Sin3a and Sin3b and, subsequently, to histone deacetylases (HDAC), it deacetylates histones, with consequent compaction of chromatin and repression of gene transcription. DNA methyltransferases (DNMT) are also part of the transcriptional repressing complex and are responsible for DNA methylation.<sup>(13)</sup> Hence, the expression of the PML/RARA oncoprotein is a deregulation of the epigenetic control in terms of both aberrant histone modification and DNA methylation in critical gene chromatin domains.

The PML-RARA oncoprotein is able to interact with the parental proteins through its protein-protein interaction domains. In the case of PML, the expression of the PML/RARA oncoprotein leads to the delocalization of normal PML from its subcellular site, nuclear structures known as NBs. As a result, PML/RARA negatively acts on the function of the native PML protein.<sup>(10)</sup>

There are other chromosomal translocations associated with APL such as the t(11,17)(q23,q21), that generates the promyelocytic leukemia zinc finger (PLZF)/RARA hybrid gene with a frequency estimated at approximately 1%, and the t(11,17)(q13,q21) and interstitial duplication of 17q21-q23 generating, respectively, the nuclear matrix-mitotic apparatus (N $\mu$ MA)/RARA and signal transducer and activator of transcription (STAT5b)/RARA hybrid genes both with frequencies of less than 1%.<sup>(14)</sup> There are other translocations described such as the t(5,17)(q35,q21) generating the NPM (Nucleophosmin)/RARA hybrid genes, the t(4;17)(q12;q21) generating the FIP1L1/RARA hybrid genes and 17(17q24) generating PRKAR1A/RARA hybrid gene. These variant translocations lead to the formation of fusion proteins generally known as X/RARA, with differentiated sensitivity to retinoid acids. N $\mu$ MA/RARA, FIP1L1/RARA and NPM1/RARA are known for being ATRA-sensitive, whereas PLZF/RARA, PRKAR1A/RARA and STAT5b/RARA have different degrees of resistance to ATRA.<sup>(6,14-16)</sup>

## Epidemiology

APL predominantly affects adults between 20 and 59 years and there is no gender difference.<sup>(17)</sup> APL secondary to therapy is unusual, with a relative frequency close to 5%.<sup>(6,18,19)</sup> Around 10-15% of the AML diagnosed in adults are APL.<sup>(18,19)</sup> However, in some countries the proportion of APL cases among LMAs is higher; this includes Brazil (28.2%),<sup>(20)</sup> Mexico (20%),<sup>(21)</sup> Venezuela (27.8%)<sup>(22)</sup> and Peru (22%).<sup>(23)</sup> Whether there is a genetic cause underlying the differences in the geographic distribution of APL is controversial. A study involving 12 Brazilian institutions showed an elevated

frequency of the high relapse risk group as defined by the cooperative groups *Programa Español de Tratamientos en Hematología* (PETHEMA) and *Gruppo Italiano Malattie Ematologiche dell'Adulto* (GIMEMA) (36.9% versus 22.6%).<sup>(20,24,25)</sup>

## Clinical and laboratorial aspects

Constitutional symptoms resemble those of other AML, such as fever, fatigue, diminished appetite and weight loss. Characteristically, there is a tendency to bleed, disproportionate to thrombocytopenia. Bleeding events are present in about 60 to 80% of patients and this is the main reason for the high mortality rates during the early stages of treatment.<sup>(10,26-30)</sup>

The coagulopathy depends on three factors: activation of the coagulation cascade, increased fibrinolysis and proteolysis.<sup>(10)</sup> The promyelocytes of APL are able to activate the coagulation cascade by expressing tissue factor (TF)<sup>(31,32)</sup> and increase procoagulant activity in endothelial cells by expressing interleukin 1 $\beta$ 1 and tumor necrosis factor alpha.<sup>(33,34)</sup> In addition to these components, an isolated cysteine proteinase, a cancer procoagulant (CP) is released from blasts and directly activates factor X, maximizing the formation of thrombin and consequently fibrin.<sup>(35,36)</sup> The state of hyperfibrinolysis is evidenced by decreased fibrinogen, increased fibrin degradation products (D-dimer, fibrinopeptide A, prothrombin fragment 1+2 and thrombin-antithrombin complex), increased tPA (tissue plasminogen activator) and uPA (activator urokinase plasminogen),<sup>(35)</sup> and reduced levels of plasmin inhibitor  $\alpha$ 2 ( $\alpha$ 2PI),<sup>(35)</sup> plasminogen activator inhibitor-1 (PAI-1)<sup>(37)</sup> and 2-antiplasmin.<sup>(38)</sup> The coagulopathy of APL is usually described as similar to disseminated intravascular coagulation (DIC). However, both the average life of platelets and the levels of the natural anticoagulants, protein C and antithrombin, are normal in APL in contrast with DIC.<sup>(39,40)</sup> Furthermore the bleeding manifestations are disproportionate to the laboratory abnormalities observed.

Although the concentration of thrombin activated fibrinolysis inhibitor (TAFI) is normal, its activity is markedly reduced.<sup>(41)</sup> This change results in a framework compatible to an increased production of plasmin. There is also an increased expression of annexin II on leukemic cells of APL.<sup>(37)</sup> Other factors contribute to coagulation such as increased serum levels of elastase,<sup>(42)</sup> increased concentrations of von Willebrand factor and its fragments in circulation<sup>(43)</sup> and recently, microparticles containing TF, tPA, PAI-1 and annexin II.<sup>(44)</sup>

The complete blood counts usually show pancytopenia. Blasts with promyelocyte morphology are seen in peripheral blood. Most patients with APL have abnormal hemostasis tests, such as prolonged prothrombin time, prolonged activated partial thromboplastin time, prolonged thrombin time, hypofibrinogenemia, increased levels of fibrin degradation products and fibrin D-dimer.

The bone marrow aspirate normally shows an infiltration (commonly higher than 20% of nucleated cells) that is intensely positive to Sudan black and myeloperoxidase markers. Two morphological subtypes occur: the hypergranular and hypogranular (microgranular) patterns, the last corresponding to 25% of the cases.<sup>(16)</sup> The classic form is characterized by the presence of abnormal promyelocytes with abundant azurophilic granulation, irregular nuclei and Auer rods (frequently organized in bundles, characterizing cells known as Faggots).<sup>(16)</sup> In the hypogranular variant, usually associated with leukocytosis, a large number of blasts with reniform or bilobulated nuclei and discrete cytoplasmic granulation are observed.<sup>(16)</sup> There may be coexistence of a low number of typical hypergranular blasts. There is also a rare hyperbasophilic form associated with relapse.<sup>(16)</sup>

The immunophenotyping study of APL with t(15,17) (q22;q12) (hypergranular or "typical" variant) is characterized by blasts with high auto-fluorescence, expressing early myeloid markers such as CD117 (with low fluorescence intensity), CD13 and CD33 are also positive expressing heterogeneous and homogenous patterns of fluorescence intensity, respectively. The CD34 hematopoietic progenitor cell marker is negative or has a low expression, as does HLA-DR. The granulocytic differentiation markers CD15, CD11b and CD65 are negative or only weakly expressed and CD64 expression is common. In cases of microgranular morphology, which is frequently associated with the bcr3 subtype, blasts frequently co-express the T lineage-affiliated marker CD2 with myeloid markers CD13 and CD34, at least on a fraction of cells. For being a fast method, immunophenotyping is useful to investigate for APL, but it is not an appropriate method to confirm diagnosis.<sup>(6,16)</sup>

Confirmation of diagnosis must use techniques able to detect t(15;17) or the PML-RARA hybrid gene. Conventional cytogenetics, fluorescence in situ hybridization (FISH) and polymerase chain reaction by reverse transcriptase (RT-PCR) are the available options.<sup>(6)</sup>

Karyotyping on G-banded metaphases is usually performed by conventional methods on direct, 24-hour, and 48-hour cultures. It has the advantage of allowing the diagnosis of additional cytogenetic alterations, however it takes longer and is more expensive and it is not always possible to observe metaphases in the number and quality needed for analysis. In addition, secondary chromosomal abnormalities do not seem to have significant prognostic value in APL.<sup>(6)</sup>

RT-PCR is widely used for diagnosis. Standardized assays were established by the Biomed-1 Concerted Action group and allow the identification of the three main break points of PML (*bcr1*, *bcr2* and *bcr3*), fundamental for the research of minimal residual disease. The drawbacks of the method are the possibility of RNA deterioration, contamination and artifacts, which may lead to false results and a prolonged performing time.<sup>(6)</sup>

Another option available nowadays is immunofluorescence with anti-PML (PGM3). In patients with the PML-

RARA rearrangement, PML is found redistributed and it is possible to observe, by microscopy, a microparticle pattern. In the other LMA subtypes, the technique reveals only 8 to 10 large structures, which are NBs. Dimov et al. determined that the sensitivity and specificity of the anti-PML technique were 98.9% and 98.7%, respectively.<sup>(45)</sup> Immunofluorescence is useful for a faster diagnosis particularly where molecular methods are unavailable. However, this should not replace molecular confirmation.<sup>(6)</sup>

## Treatment

The combination of ATRA and anthracycline is currently considered the standard induction treatment in newly diagnosed patients<sup>(6)</sup> leading to a complete remission (CR) rate of 90 to 95%.<sup>(46)</sup> Two randomized trials<sup>(30,47)</sup> show that ATRA in association with other chemotherapy resulted in better outcomes mainly because of lower relapse rates.<sup>(46)</sup> However, which anthracycline is the best remains controversial.<sup>(6)</sup> The PETHEMA<sup>(48)</sup> and GIMEMA groups<sup>(49)</sup> used idarubicin and the International Consortium on Acute Promyelocytic Leukemia (IC-APL) used daunorubicin with similar results.<sup>(50)</sup>

Arsenic trioxide (ATO) is the most effective single drug against APL. It has been widely used as a single agent in induction in India and China with CR rates above 70%. A relatively good long-term remission can be obtained by using ATO as a single agent in newly diagnosed patients as has been evidenced with a 2-year disease-free survival rate (DFS) of 63.7% and a 3-year DFS of 87.2% in a large cohort of patients from India.<sup>(51)</sup>

Aggressive transfusion therapy is central in APL treatment, thus reducing cases of fatal bleeding.<sup>(6,18)</sup> Platelets are transfused to maintain the platelet count higher than from  $30 \times 10^3/\mu\text{L}$  to  $50 \times 10^3/\mu\text{L}$ , fresh frozen plasma is indicated to correct abnormal hemostasis and cryoprecipitate is administered to maintain the fibrinogen level higher than from 100 to 150 mg/dL. These tests should be performed at least once a day (more frequently if required).<sup>(18)</sup> Placement of a central venous catheter, lumbar puncture and other invasive procedures should be avoided until the coagulation abnormalities are resolved.<sup>(6)</sup>

Treatment with ATRA should be started immediately based only on clinical suspicion, even before the genetic confirmation. This measure itself leads to improvement of the coagulopathy and reduced risk of severe bleeding.<sup>(6)</sup> ATRA acts not only on cell cycle, but also leads to normalization of plasminogen,  $\alpha 2$ -plasminogen inhibitor, fibrinogen, D-dimers, the thrombin-antithrombin complex (TAT) and fibrinopeptides. It promotes a reduction in TF expression, resulting in an improvement of coagulation tests within 4-5 days. Nevertheless, it is paradoxically associated with thrombotic complications later in the course of the disease (usually 1-3 weeks after treatment introduction) because of an increase in tPA expression.<sup>(52)</sup>



Regarding the consolidation therapy, the experience of the PETHEMA and GIMEMA groups suggests that therapy should be based on the relapse risk group (Table 1) with higher anthracycline doses in intermediate and high risk groups. Even so, patients classified as high risk still have relapse rates above 20%.<sup>(46)</sup>

Table 1 - Relapse risk in acute promyelocytic leukemia

Risk group	White blood cells (uL)	Platelets (uL)	5-year event-free survival
Low	≤ 10,000	> 40,000	100%
Intermediate	≤ 10,000	< 40,000	90%
High	> 10,000	-	75%

The relevance of maintenance therapy is controversial. Studies by the European APL group showed that the relapsed risk was increased in patients that did not receive maintenance.<sup>(53)</sup> Maintenance was adopted in the PETHEMA and GIMEMA trials, but no randomized comparisons were performed in order to establish whether maintenance is essential. Only a Japanese study compared no maintenance versus six courses of intensified maintenance chemotherapy without ATRA. This study showed no benefit from the use of chemotherapy alone in terms of reducing relapse rates with a significantly lower chance of survival in the maintenance arm. Despite the uncertainty of the benefit provided by maintenance therapy, the fact is that many patients who are PCR negative at the end of the consolidation will eventually relapse, especially those with high white blood cell (WBC) counts at diagnosis.<sup>(46)</sup>

The IC-APL adopted a treatment protocol based on the APL 2005 PETHEMA protocol, except that idaurubicin was replaced by daunorubicin. An interim analysis of 102 patients enrolled in Brazil, Mexico, Chile and Uruguay showed that the distribution of the relapse risk score at diagnosis according to PETHEMA-GIMEMA criteria was 14 low (14%), 54 intermediate (53%) and 34 high risk (33%). The incidence of low risk APL appeared lower than the values reported in developed countries.

Of 102 patients, 97 have toxicity and response data available; 12 (12.3%) experienced at least three symptoms/signs of differentiation syndrome (DS) and 77 (79%) patients achieved CR. Twenty-three deaths occurred with the causes of deaths including nine hemorrhages, eight infections and two DS. The 7- and 30-day mortality rates were 8% and 19.6%, respectively, the 1-year overall survival (OS) was 75% (95% confidence interval - 95% CI: 68%-84%). The median follow-up time among survivors was 14 months (range: 1.3-35 months). Of the 77 patients who achieved CR, the 1-year OS from the date of CR was 95% (95% CI: 89%-100%); only one patient relapsed. For patients surviving a minimum of 30 days, the outcome was similar to that reported by the PETHEMA-APL 2005 protocol in European patients. Prognostic factors for OS were examined using log-rank test as well as multivariate Cox models. Factors predicting OS were a high relapse risk score at

baseline (1-year OS: 59% for high, 87% for intermediate, 91% for low, p-value = 0.0007) and age. The 1-year OS was 85% for age < 25, 83% for 25-40, and 58% for age ≥ 40 (p-value = 0.008 for age ≥ 40 vs. < 40). In the Cox model, these two prognostic factors remain significantly associated with the OS [HR = 3.77, p-value = 0.004 for high relapse risk score; HR = 2.4, p-value = 0.047 for age ≥ 40]. In conclusion, the establishment of the IC-APL network resulted in a decrease of about 40% in early mortality and improvements in outcome to levels similar to those reported in developed countries.<sup>(50)</sup>

## Differentiation syndrome

Both ATRA and ATO, alone or in combination, can trigger DS. The incidence of DS is approximately one quarter of all APL patients receiving ATRA as induction therapy.<sup>(54)</sup> The combination of ATRA and ATO has an incidence of 16%.<sup>(55)</sup> DS usually starts between the third and fourteenth day of treatment with ATRA, but there are some reports after the first dose.<sup>(54)</sup> The clinical manifestations are quite varied and should be suspected in the presence of these signs and symptoms: shortness of breath with or without edema or pulmonary infiltrates and often mistaken for fluid overload with pleural effusions or pneumonia, fever, bone pain, weight gain, hypotension, edema, heart failure, kidney failure, cavity stroke and headache (which should be differentiated from the syndrome of pseudotumor cerebri). Sweet's syndrome and other findings are observed, usually preceded by an elevated WBC count.<sup>(6)</sup> The etiology of this syndrome is not fully understood. It is believed that there is an inflammatory process due to an excessive infiltration of different organs by myeloid cells with differential expression of adhesion molecules and cytokine production.<sup>(54,56)</sup> Early identification of symptoms and treatment are essential because retinoid syndrome leads to decreased EFS and OS. If the symptoms or signs are severe, ATRA (or ATO) should be discontinued and resumed at resolution of all signs and symptoms with the association of 10 mg dexamethasone twice daily<sup>(6)</sup> for at least three days until resolution of symptoms.<sup>(10)</sup>

## Monitoring

Cytogenetics and molecular testing are not needed at the end of induction because they have no prognostic value. In contrast, the detection of the PML/RARA fusion gene at the end of the third course of consolidation should be considered as resistance and the patient should receive alternative treatment. After maintenance therapy is completed, the frequency of monitoring is not clearly established. The good outcome for patients presenting with low risk disease is so favorable that frequent monitoring can be questioned. Patients with intermediate risk disease should be monitored every three to six months and those with high-risk disease every three months during and after the completion of maintenance therapy.<sup>(6,18)</sup>

## Relapse

Hematologic relapse follows the same criteria of diagnosis. Molecular relapse is considered in the presence of two consecutive positive RT-PCR results obtained with a minimum interval of two weeks. Initially salvage therapy was performed with anthracycline or mitoxantrone associated with intermediate or high doses of cytarabine, with or without etoposide, followed by transplantation. These regimens resulted in high rates of second CR (80% to 90%).<sup>(22)</sup> Since the availability of ATO, excellent results are obtained with this drug as a single agent in patients with relapse and refractory APL. The drug has three main mechanisms of action: 1) production of reactive oxygen species that induce the phosphorylation and activation of the Jun N-terminal kinase (JNK) pathway, triggering apoptosis; 2) phosphorylation of sumolization of PML/RARA, leading to its degradation; 3) inhibition of transcription of hTERT and consequent decrease in telomerase activity, leading to chromosome fusion and apoptosis.<sup>(10)</sup> The most significant adverse reactions are DS, gastrointestinal discomfort, elevated liver enzymes, neuropathy, hyperglycemia and cardiac arrhythmias (prolongation of the QT interval - careful monitoring is recommended to maintain the serum potassium above 4.0 mEq/L and serum magnesium above 1.8 mg/dL). ATO is administered intravenously at a dose of 0.15 mg/kg/d until hematologic remission or for a maximum of 60 days. There are reported rates of second CR of 80 to 90% and OS at one to three years of 50% to 70%. The results of molecular response to therapy after two cycles of ATO (one induction and one consolidation course) should then be taken into account for additional decisions on the continuation of therapy and, in particular, to identify patients at higher risk of additional relapse (i.e., those with persistent PCR positivity) who must be, when feasible, allocated to allogeneic hematopoietic stem cell transplantation. Therefore, the available options include continued treatment with ATO and/or chemotherapy plus ATRA followed by transplantation. So far there are no studies that compare autologous and allogeneic bone marrow transplantation. Four weekly courses of intrathecal methotrexate or alternating methotrexate and Ara-C should be given because of possible increases in the incidence in central nervous system relapse, especially for those patients with leukocytosis at presentation.<sup>(6,46)</sup>

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