

Minimal Residual Disease Detection in Promyelocytic Leukemia and its Correlation with Clinical OutcomeFarida Gad Alla MD¹, Thoraya Abdelhamid MD², Heba N. Raslan MD¹¹Clinical Pathology and ²Medical Oncology Departments, NCI, Cairo University
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Abstract: Background: Acute promyelocytic leukemia (APL) is a distinct subtype of acute leukemia characterized by a balanced reciprocal translocation between chromosome 15 and 17 resulting in the chimeric gene encoding PML-RARA protein. **Objectives:** Our aim was to study the PML-RARA fusion gene by real-time RT-PCR before and after therapy in a trial to assess its prognostic value and its impact on monitoring MRD to improve therapeutic strategies and decision making. **Patients and Method:** The study included 41 newly diagnosed APL patients. In addition to the standard work up, cytogenetic and FISH testing were performed at diagnosis and at CR. RT-PCR was done before treatment and after consolidation. We calculated the levels of PML-RARA by different methods: 1-The number of transcripts. 2-The ratio % which is calculated as: Copy number of PML-RARA /copy number of ABL. 3-The normalized copy number (NCN) which is calculated as: Copy number of PML-RARA/ copy number of ABL x 10000. To analyze the different prognostic factors, we correlated them to MRD cut-off values which were for the ratio% (1×10^{-3}), and (NCN=10) for NCN method. Accordingly patients were divided into: Low risk patients: those with ratio% $< 10^{-3}$ or NCN < 10 and high risk patients: those with ratio% $\geq 10^{-3}$ or NCN > 10 . Treatment plan: Induction and consolidation regimen included ATRA 45 mg/m² P.O. daily and Daunorubicin 60 mg/m² I.V. day 1-3 for three courses. Maintenance therapy was given with 6 mercaptopurine and methotrexate with ATRA intermittent courses for two years. **Results:** Median age was 36 years (18-66) with females to males ratio of 1.2:1. Only 30 patients achieved hematologic remission and were followed up for MRD detection while eight cases failed to achieve CR and three cases showed early death. The decrease in expression level of PML-RARA after treatment was highly significant. During the observation period of a median of 39.1 months (1-56), six cases showed relapse. The time elapsing from the detection of molecular relapse till the appearance of hematological relapse ranged from 1-3 months with most cases clustering in the 3rd month (4patients). The cumulative disease free survival (DFS) was 69%. The overall survival (OS) was 76.6% with a mean of 49.85 months with 95%confidence interval of (44.8-54.8). We found significant longer DFS and OS for the low versus high risk patients using the NCN cutoff at post consolidation analysis ($p < 0.001$ and 0.009 respectively). We found also high statistical significant correlation between two log reduction or more after consolidation and longer DFS but not OS. **Conclusions:** PML/RARA NCN method is simple and accurate; its use enables standardization and avoids differences among laboratories. Monitoring patients at short time points is valuable and essential for decision making and choice of proper therapy. [Farida Gad Alla , Thoraya Abdelhamid , Heba N. Raslan. **Minimal Residual Disease Detection in Promyelocytic Leukemia and its Correlation with Clinical Outcome.** *Nat Sci* 2014;12(1):12-19]. (ISSN: 1545-0740). <http://www.sciencepub.net/nature>. 3

Keywords: APL, PML/RARA, MRD, Quantitative real-time PCR.**1.Introduction**

Despite its rarity, acute promyelocytic leukemia (APL) is one of the most successful examples of translational research in medicine. In the past 20 years an extraordinary combination of laboratory and clinical research studies has in fact contributed to transform this once rapidly fatal disease into the most frequently curable adult leukemia. Moreover, APL has represented a model to unravel key mechanisms of leukemogenesis and a paradigm for innovative treatments including differentiation therapy and the use of chromatin remodeling agents and antibody-directed therapy [1].

The genetic hallmark of APL is the balanced reciprocal translocation (15; 17) (q22-24; q11-12) leading to a fusion of the promyelocytic (*PML*) gene on chromosome 15 and the retinoic acid receptor α

(*RARA*) gene on chromosome 17. Depending on the location of breakpoints within the *PML* site, the *PML/RARA* transcript subtypes *bcr1*, *bcr2* and *bcr3* may be formed. Of these, *bcr1* and *bcr2* are of similar size and together referred to as long (L) isoform, *bcr2* as variable (V) and *bcr3* as short (S) isoform. The *PML/RARA* fusion is detectable by fluorescence in situ hybridization (FISH) or reverse-transcriptase polymerase chain reaction (RT-PCR) in $> 95\%$ of morphologically defined APLs, while in the remaining cases several variant rearrangements have been described that constantly involve *RARA* and partner genes other than *PML*. These alternative fusions may involve at low frequency ($< 3\%$) the promyelocytic leukemia zinc finger (*PLZF*), or very rarely the nucleophosmin (*NPM*), nuclear mitotic apparatus (*NUMA*), and (*STAT5b*) partner genes. The

nature of the fusion partner has an important bearing on disease biology particularly with respect to all-trans retinoic acid (ATRA) sensitivity, with APL due to involvement of *PLZF* being characterized by retinoid resistance [2].

The PML-RARA protein functions as an aberrant retinoid receptor with altered DNA-binding properties as compared to wild type RARA. PML/RARA can hetero-dimerize with the RXR, with PML and with another PML-RARA chimeric protein forming a homodimer. Distinct from wild-type RARA, the repression induced by unliganded PML-RARA is releasable only by pharmacological doses of ATRA (10^{-6} M). This is explained by the fact that compared to wild-type RAR α , the PML-RARA hybrid binds the histone deacetylase (HDAC) recruiting co-repressor complex with higher affinity [3].

APL is a medical emergency frequently presenting with an abrupt onset. The high risk of early death (10-20%) and the potential for high cure rate (> 80%) account for the importance of immediate recognition and prompt initiation of specific treatment.

For the purpose of initial diagnosis, the morphologic appearance of hypergranular dysplastic promyelocytes allows typical cases to be identified and justifies immediate treatment initiation with RA, without waiting for diagnostic confirmation at the genetic level. Nevertheless, subsequent genetic diagnostic confirmation of APL is essential in all cases for several reasons: 1) it allows the clarification of cases with unusual morphology, such as the microgranular form, that are similarly responsive to RA and ATO 2) it permits the exclusion of the rare variant translocations not involving PML that are resistant to RA: and ; 3) it allows for the identification of the precise target for disease monitoring through the characterization of PML/RARA isoforms by RT-PCR.

The identification of the APL-specific genetic lesion in leukemic cells is feasible at the chromosome, DNA, RNA, and protein levels with the use of conventional karyotyping, FISH, RT-PCR, and anti-PML monoclonal antibodies, respectively. Both RT-PCR and FISH have the additional advantage that no dividing cells are required for analysis, and they allow results to be obtained in cases with few or poor-quality metaphases, or where the PML-RARA fusion gene is formed as a result of cryptic or complex rearrangements in the absence of the classical translocation [4].

Combined treatment with anthracycline-based chemotherapy and all trans-retinoic acid (ATRA) is highly successful in acute promyelocytic leukemia (APL), providing long-lasting remissions and

probable cures in up to 70% of newly diagnosed patients. Nevertheless, the persistence of resistant clones causing relapse and low survival still represents a problem in 15-25% of patients. Currently, detection of PML/ RARA transcripts by molecular techniques constitutes an important tool for monitoring minimal residual disease (MRD) and predicting evolution in APL patients. Conventional qualitative reverse transcriptase polymerase chain reaction (RT-PCR) has been widely used for genetic diagnosis and therapeutic monitoring of APL. Several reports have shown that RT-PCR positivity after consolidation treatment predicts hematologic relapse, whereas persistent RT-PCR negativity test is associated with long-term survival and a low relapse rate. However, this technique has several disadvantages such as the occurrence of false positive results due to cross-contamination and false negatives due to poor RNA quality or RT-PCR failures at different stages. In addition, the sensitivity of RT-PCR for measuring MRD is relatively low and the method is associated with significant inter-laboratory variability. Finally, qualitative RT-PCR requires significant post-PCR handling which is time and lab consuming and often leads to contamination of samples [5].

So, the advent of real-time PCR simplified the procedure and permitted clinical routine use. Real time PCR is a reliable and simple method to monitor APL patients with low risk of contamination, standardized by housekeeping genes and the short time it takes [6].

The aim of the present work was to study the leukemic specific chimeric PML-RARA fusion gene in cases of acute promyelocytic leukemia by real-time PCR to quantify the amount of the gene before and after ATRA therapy in a trial to assess its prognostic value by monitoring minimal residual disease to improve therapeutic strategies and decision making.

2. Patients and Methods:

Initially our study included 43 newly diagnosed patients with APL; however it was completed only on 30 patients due to failure of achievement of hematological remission in eight patients, presence of translocation other than t (15; 17) in two other patients, and death of three patients during induction. ALL patients were attendants of the Medical Oncology department, National Cancer Institute (NCI), Cairo University and they were 14 males and 16 females.

Patients were assessed by complete hemogram, bone marrow (B.M.) aspirate examination and Real Time RT-PCR for *PML-RARA* *PML/RARA* gene expression at diagnosis, and after consolidation. Immunophenotyping by flow cytometry,

cytochemical staining and cytogenetic investigation as well as FISH were done at diagnosis. For Real Time PCR, fresh sterile peripheral blood samples were collected on EDTA using sterile vacutainer tubes and samples were extracted using QIA Amp blood kit from Qiagen. The experimental procedure for real-time PCR with Taq-Man Technology was done using **PML-RARA Fusion *Quant*® Kit and Applied Biosystem 7000 apparatus.**

Total RNA was reverse-transcribed and the generated cDNA amplified by PCR using a pair of specific primers and a specific internal double-dye probe (FAM-TAMRA). An endogenous control (housekeeping gene; *ABL transcript*) is amplified from the sample as well as *PML-RARA bcr1* fusion transcript. Standard curves of known amounts of both the endogenous *ABL* control and the *PML-RARA* fusion cDNA allow the calculation of the ratio of *PML-RARA bcr1* fusion transcript signal to endogenous *ABL* signal in each sample. Specific primers and probe mixes and standard serial dilutions of control and fusion DNA are provided for the quantification of the *ABL* control and *PML-RARA bcr1* genes.

The number of PCR cycles necessary to detect a signal above the threshold is called the Cycle threshold (Ct) and is directly proportional to the amount of target present at the beginning of the reaction. Using standards with a known number of molecules, one can establish a standard curve and determine the precise amount of target present in the test sample. Results were relative to 100 ng of total RNA, corresponding to 5 μ l of cDNA. In our study, we expressed the PML-RARA /ABL ratio as follows: Copy number of PML-RARA /copy number of (ABL)

To determine the level of MRD, all patients had two samples to measure PML-RARA by RT-PCR, one at diagnosis and the second after consolidation. We calculated the levels of PML-RARA by more than a method: 1-the number of transcripts. 2-the ratio % which is calculated as follows: Copy number of PML-RARA /copy number of ABL and 3-the normalized copy number (NCN) which is calculated as follows: Copy number of PML-RARA/ copy number of ABL x10000. To analyze the different prognostic factors, we correlated them to the MRD cut-off values which were for the ratio% (1×10^{-3}), and (NCN=10) for NCN method. Accordingly patients were divided into two groups:

- **Low risk patients:** those with ratio% $< 10^{-3}$ or NCN<10.
- **High risk patients:** those with ratio% $\geq 10^{-3}$ or NCN>10.

The results were expressed using NCN method due simplicity and accuracy, according to Santamaria et al.[5].

Response criteria were used to define response to induction therapy according to Cheson et al. [7]. Molecular remission (MR) was defined a two consecutive Real time PCR results with NCN less than 10 after the end of consolidation therapy. Molecular relapse was defined as the reappearance of a positive molecular result (according to previous method) in two consecutive bone marrow samples at any time after consolidation therapy [8].

3. Results:

Initially our study included 43 newly diagnosed patients with APL; however it was completed only on 30 patients due to failure of achievement of hematological remission in eight patients, presence of translocation other than t (15; 17) in two other patients, and death of three patients during induction. The 30 studied patients included 14 (46.7%) males and 16 (53.3%) females (male: female ratio=0.875) with an age range of 18-66, mean of 37 ± 14.5 and median of 36 years. The 30 patients achieved complete hematological and cytological remission by FISH. Relapse was detected at an observation period of a median 39 months (range 1-56months). All patients achieved hematological remission following 1-3 courses of induction therapy; six patients (20%) relapsed. The time elapsing from the detection of molecular relapse till the appearance of hematological relapse ranged from 1-3 months with most cases clustering in the 3rd month (4patients), one patient in the 2nd month and one in the 1st month.

Results of monitoring of PML-RARA by real time PCR before and after therapy in APL patients:

Before therapy the PML-RARA expression as number of transcripts/5 μ l cDNA was 346.6 ± 1958 with a range of 1290-8480 and a median of 3236.5; after therapy the level was 81.5 ± 370 with a range of 0-2002 and a median of 2.6 ($P < 0.001$). Before therapy PML-RARA expression as NCN was 9305.9 ± 7232 with a range of 1040-26183 and a median of 6571.5; after therapy it was 42 ± 152 with a range of 0.8-785 and a median of 2.9 ($P < 0.001$).

Comparison between relapsed and non relapsed APL patients as regards hematological parameters and NCN PML-RARA level at presentation:

We compared between relapsed and non relapsed patients as regards age, hemoglobin (Hb) level, total leukocytic count (TLC), platelet count, and percent of blast equivalent in peripheral blood and bone marrow before therapy.

The age showed a statistically high significant difference between relapsed and non

relapsed (50.1 ± 16.2 Vs 31.3 ± 10.4 years with $p < 0.001$), Hb also showed statistical significance (6.11 ± 1.6 Vs 10.6 ± 12.9 mg/dl with $p = 0.03$), and platelets showed also statistically significant difference (41.52 ± 30.5 Vs $66.9 \pm 30.1 \times 10^9/L$ with $p = 0.04$), while TLC, blast equivalent % of peripheral blood and bone marrow showed no statistical significant difference. No statistically significant difference was detected between the two groups concerning the PML-RARA NCN level.

Comparison between relapsed and non relapsed APL patients as regards the level of PML-RARA after therapy:

When we compared relapsed and non relapsed APL patients as regards the level of PML/RARA (number transcript $5\mu L$ cDNA), relapsed patients had a mean \pm SD of 345.9 ± 811.4 and their median was 17.9 with range of 0.6-2002

,while non-relapsed had a mean \pm SD of 12.6 ± 32.8 and a median of 2.3 and a range (0-157). There was a statistically significant difference ($p = 0.053$) between the two groups. We followed up our patients for a period ranging from 1-56 with a median of 39.1 months. The cumulative disease free survival of all studied patients was 69.4%, with no median calculated as more than 50% of patients are still alive. We divided patients according to log reduction into two groups and calculated survival in both at 36 months (Fig 1):

1st group: with 1 log reduction (11 patients) showed cumulative survival of 30.3% at 36 months.

2nd group: with 2 or more log reduction (19 patients) showed cumulative survival at 36 months of 88.9%. This group showed significantly longer disease free survival ($p = 0.005$).

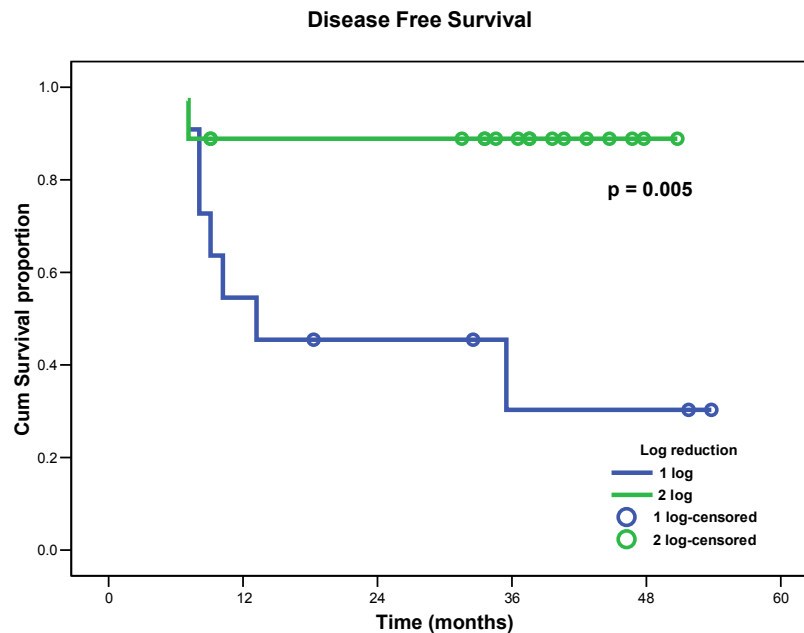


Fig (1): Relation between log reduction of PML/RARA expression (number transcript/ $5\mu L$ cDNA) and Disease Free Survival in 30 promyelocytic leukemia patients.

We compared disease free survival according to PML-RARA normalized copy number (NCN) at the post consolidation. We chose NCN=10 as a cutoff value according to Santamaria et al. [7] and we divided patients into 2 groups:

Group 1: included those who had NCN ≥ 10 (8 patients). This group had a cumulative survival of 15.5% with a mean of 20.5 month, a 95% confidence interval of 7.6-33.4 and a median of 10.2 month.

Group 2: who had < 10 NCN (22 patients). This group had a cumulative survival of 98.5% with 95% confidence interval of 41.1 -53.2. We found a high statistical significance between MRD level measured by NCN and DFS ($p < 0.001$). Those with NCN ≥ 10 showed a higher risk of relapse and shorter survival, while those with NCN < 10 had higher disease free survival (Fig 2).

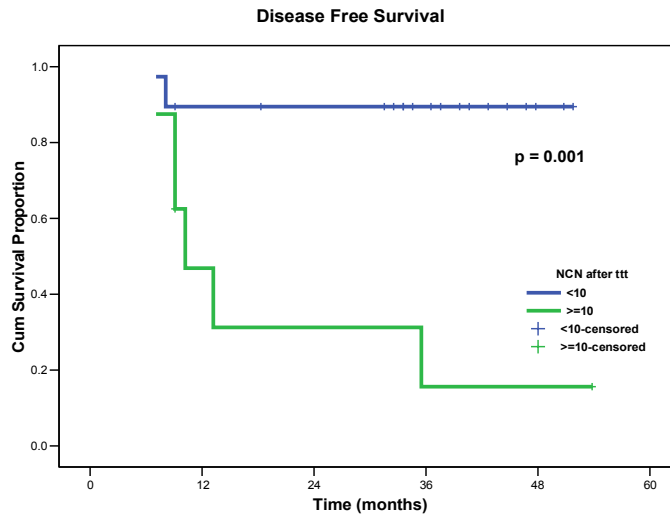


Fig (2): Relation between log reduction of *PML/RARA* expression (Normalized Copy Number) and Disease Free Survival in 30 promyelocytic leukemia patients.

Using Cox regression analysis, no correlation was encountered between disease free survival and *PML-RARA* transcript number ($p=0.912$). The overall survival of the studied patients was estimated as 76.6% with a mean of 49.85 months with 95% confidence interval of 44.8-54.8. We compared overall survival of patients according to log reduction, where the 1st group with 1 log reduction (11 patients) showed cumulative survival of 88.9% with a mean of 49.38 month and 95% confidence interval of 43.2-55.5, while the 2nd group with 2 or more log reduction (19 patients) showed cumulative survival of 93.7% with a mean of 48.35 months and 95% confidence interval of 43.78-52.93.

No statistically significant difference was found between the two groups ($p=0.19$).

We compared the overall survival of patients according to *PML-RARA* normalized copy number (NCN) at the post consolidation. Group 1, which included those who had $NCN \geq 10$ (8 patients) had a cumulative survival of 83.3% with a mean of 46.86 month with 95% confidence interval of 39.1-54.62, while group 2 which included patients who had <10 NCN (22 patients) showed cumulative survival of 100% and the median could not be calculated as more than 50% of patients are still alive. The mean was 47.1 month with 95% confidence interval of 41.1 - 53.2 ($p=0.009$) (Fig. 3).

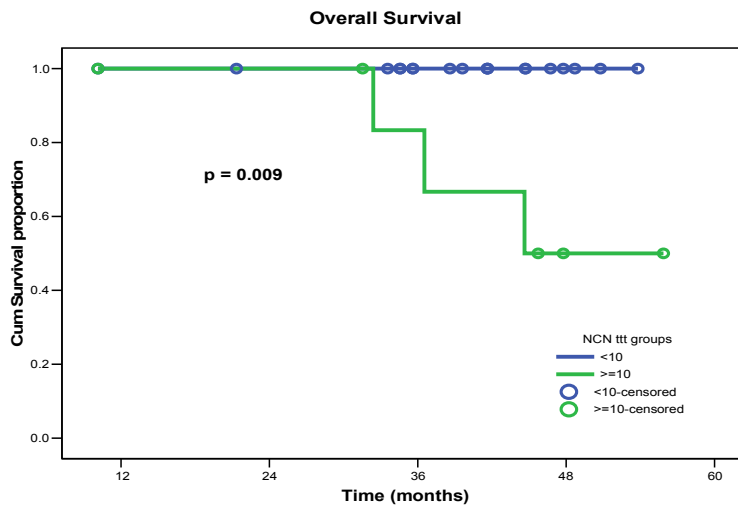


Figure (3): Relation between log reduction of *PML/RARA* expression (Normalized Copy Number) and Overall Survival in 30 promyelocytic leukemia patients.

PML-RARA expression level as number of transcripts showed no impact on overall survival ($P=0.34$).

Influence of clinical and hematological characteristics at diagnosis on disease free survival and overall survival:

Patients ≤ 30 years (11 patients) showed a longer disease free survival than those > 30 years (19 patients) ($p=0.02$).

Patients with $TLC \leq 10 \times 10^9 /L$ had longer disease free survival than those with $TLC > 10 \times 10^9 /L$ ($p=0.05$). Other parameters including sex, Hb level, percent of blasts in peripheral blood and bone marrow or number of platelets had no statistically significant impact on disease free survival or overall survival. So the older age and the higher initial TLC were the only parameters associated with a shorter DFS in our study.

4. Discussion:

Treatment of APL with conventional ATRA combined with chemotherapy has resulted in significant improvements in the clinical outcome [9]. Major causes of treatment failure include early mortality related to hemorrhagic diathesis and subsequent relapse [10]. Monitoring MRD by detection of the *PML-RARA* fusion transcript, in the peripheral blood or bone marrow, using conventional RT-PCR has been shown to be an effective strategy to predict relapse [11]. Early intervention at the time of molecular relapse is associated with a significantly superior survival advantage compared with initiation of treatment at the time of frank hematologic relapse [12].

In the present study, we used quantitative RT-PCR technique to detect and monitor *PML-RARA* fusion gene in cases of APL at diagnosis and after consolidation. We designed to take 2nd RQ-PCR post-consolidation to determine MRD in agreement to Santamaria *et al.* [5] and Serna *et al.* [13], who concluded that there is correlation between RQ-PCR *PML/RARA* detection and a high risk of relapse. MRD assessment was not done after induction due to previous report of no correlation between PCR positivity after induction and outcome [14]. This is consistent with the concept that detection of *PML-RARA* transcript at this stage could very well relate to differentiating leukemic cells subject to subsequent apoptosis, which cannot be distinguished from residual APL blasts [15]. Furthermore, in contrast to other AML, induction treatment of APL can be associated with delayed leukemic cells clearance [2].

All of our samples were obtained as bone marrow samples, because they are more sensitive and precise [16]. We used t (15; 17) quantification kit to detect *PML-RARA* gene with BCR1 and BCR2 which are reported to be involved in 92%-95% cases [17].

We used ABL as a housekeeping gene, in consistence with previous studies [5, 17, 18, 19]. We expressed our results in three different ways, 1st as transcript number in 5 μ L cDNA according to Cassinat *et al.* [6] and Tobal *et al.* [17], 2nd as ratio% by dividing transcript number of *PML-RARA* by transcript number of housekeeping gene (*ABL*) according to Lee *et al.* [19] and Schnittger *et al.* [18] and 3rd as normalized copy number (NCN), derived by multiplying the *PML-RARA* copy number/ *ABL* copy number ratio by 10000, according to Santamaria *et al.* [5] and Fernandez *et al.* [20].

In our results, quantification of the mean of *PML/RARA* at diagnosis was 3.767 ± 1958 with a median of 3236.5 and a range of 1290-8480, as measured by number of transcripts. Our results are higher than those of Fernandez *et al.* [20] who had a *PML/RARA* transcript of a median of 3030 and a range of 826-9605 among their patients at diagnosis and lower than those of Tobal *et al.* [17] who had *PML/RARA* transcript of a median 2×10^6 and range of 0.7×10^6 - 3.5×10^6 . The difference between studies may be attributed to difference in RNA preparation, purification and storage which may affect quality of RNA. This explains the discrepancy in transcript and similarity of the ratio% and NCN as calculation methods between our study and that of Lee *et al.* [19] and Santmaria *et al.* [5]. Therefore, it is better to use ratio % or NCN methods to express the level of *PML/RARA*.

After treatment, the transcript number was significantly decreased to a median of 2.6×10^1 with a range of 0-2002, in agreement with Tobal *et al.* [17] who stated that after treatment the level of *PML-RARA* was significantly lower, but remained detectable in majority of samples even though BM was morphologically and karyotypically normal.

As ratio % and NCN methods are the same, for simplicity NCN was chosen over ratio% to represent MRD. By measuring *PML/RARA* as NCN we had a range of 1040-26183 and a median of 6571, in agreement with Santamaria *et al.* [5] who had a median of 5882 and a range of 1224-29750 and who stated that all samples at diagnosis showed >1000 NCN. All *PML-RARA* transcripts and NCN were significantly reduced after consolidation. This is in agreement with previous reports [20, 21].

In this study, 50% of our patients achieved hematological remission at 1 month after 1 course of induction therapy, while the rest achieved it after 3 courses; 20% of patients relapsed after consolidation. Both relapsed and non relapsed patients had comparable level of *PML-RARA* at diagnosis with no statistical significant difference. This coincides with Schnittger *et al.* [18] who stated that the initial expression of fusion gene had no impact on relapse.

However, there was a significant difference in the transcript level between the two groups after treatment. This is in agreement with Tobal *et al.* [17] and Cassinat *et al.* [6] who reported that the *PML-RARA* levels increased significantly up to 4 months before the onset of clinical relapse and it reached up to 1.2×10^4

In our study the time elapsing from the detection of molecular relapse till the appearance of hematological relapse ranged from 1-3 months, similar to the results of Grimwade *et al.* [21]. We followed up patients for a period ranging from 1-56 with a median of 39.1 months. The cumulative DFS was 69.4% at 3 years which is lower than Lee *et al.* who determine DFS to be 81.5% , but similar to Asuo *et al.* [22] who reported 68.5% as cumulative disease free survival.

We calculated MRD of APL patients by different methods in order to evaluate which of them mostly correlates with clinical outcome. By using NCN at a cutoff value of 10, 8 patients with NCN level ≥ 10 had a cumulative survival of 15.5%, while the other 22 patients with NCN < 10 had a cumulative survival of 89.5%. This is in agreement with Santamaria *et al.* [5] who stated that patients with NCN < 10 had a favorable DFS as compared to patients with > 10 NCN who all finally relapsed.

The disappearance of malignant clone in APL is rare and using the term complete molecular remission is imprecise. In this context, we estimated the log reduction of *PML-RARA* ratio from the standardized base line which is the median value of *PML-RARA* at diagnosis. Patients with one log reduction showed significantly inferior DFS as compared to patients with ≥ 2 log reduction. This means that we can use the kinetics of tumor burden reduction to differentiate between patients who are at risk of relapse and those who remained in continuous complete remission. This is in agreement with a previous report [23].

The overall survival in our study was 76.6% at almost 3 years. This is lower than Asuo *et al.* [22] who calculated OS to be 83.9% at 6 years. This difference could be attributed to small number of patients in our study. We studied the impact of MRD upon overall survival. Low risk group with NCN < 10 showed cumulative survival of 100% and high risk group with NCN ≥ 10 had cumulative survival of 83.3%. This is coinciding with Xin *et al.* [24].

As regards the kinetics of tumor burden (log reduction), we could not detect any relation between log reduction and overall survival in contrast to Schnittger *et al.* [18] who stated that the decreased level of *PML-RARA* ≤ 2 log from diagnosis was found to be prognostically important. We also reported lack

of impact of the level of *PML-RARA* as a number of transcripts after treatment on OS.

Certain clinical and laboratory features are useful as prognostic indicators of risk of relapse. Patients' age at diagnosis appears of be an important clinical prognostic factor and in our study there was a significant relation between patients of age ≤ 30 years and longer disease free survival. In a large European trial, survival at 2 years was 67% for patient older than 65 years compared with 82% for younger patients [25] and in other multicentre study patients with age of < 30 showed longer remission [22]. Female gender has been shown in some trials to confer a favorable outcome compared to male gender [26] which was not encountered in our cohort. The results of our study also showed that patients who presented by WBC $\leq 10 \times 10^9/L$ had significantly better DFS ($P=0.02$). These results are supported by the joint study of the PETHEMA and GIMEMA cooperative group who reported the TLC count at presentation as the only prognostic factor with significant influence on remission duration

In conclusion: *PML/RARA* NCN method enables standardization and avoids differences among laboratories. The decrease in expression level of *PML-RARA* after treatment was highly statistically significant ($P<0.001$). The group of patients with ≥ 2 log reduction had higher DFS. Patients with NCN < 10 had higher DFS and OS. Patients with initial TLC $< 10 \times 10^9/L$ and age ≤ 30 years had a longer DFS. **Recommendations:** Appropriate treatment is to be given at the time of molecular relapse rather than waiting for frank hematological relapse. Confirmation of positive results should be done on a second sample to avoid problems of "false-positive" results due to PCR contamination, misidentification at any point between bone marrow aspiration and issuing of results.

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