

Adult patients with *de novo* acute myeloid leukemia show a functional deregulation of redox balance at diagnosis which is correlated with molecular subtypes and overall survival

The balance between the production of reactive oxygen species (ROS) and antioxidant systems, which defines the redox status (a state of oxidative stress if ROS are in excess), is a regulator of normal and malignant hematopoiesis. ROS status, sometimes linked with

molecular abnormalities, has pleiomorphic effects on the initiation and progression of acute myeloid leukemia (AML), but also on sensitivity to chemotherapy.¹⁻⁵ For example, variability in the levels of nicotinamide adenine dinucleotide phosphate oxidase (NOX), a ROS producer, influences the efficacy of AML induction therapy, improving complete remission when more ROS are generated.⁶ The two major sources of ROS production are the mitochondria and NOX activity, which itself contributes to increasing levels of ROS in more than 60% of AML.⁷ However, AML cells also have a singular mito-

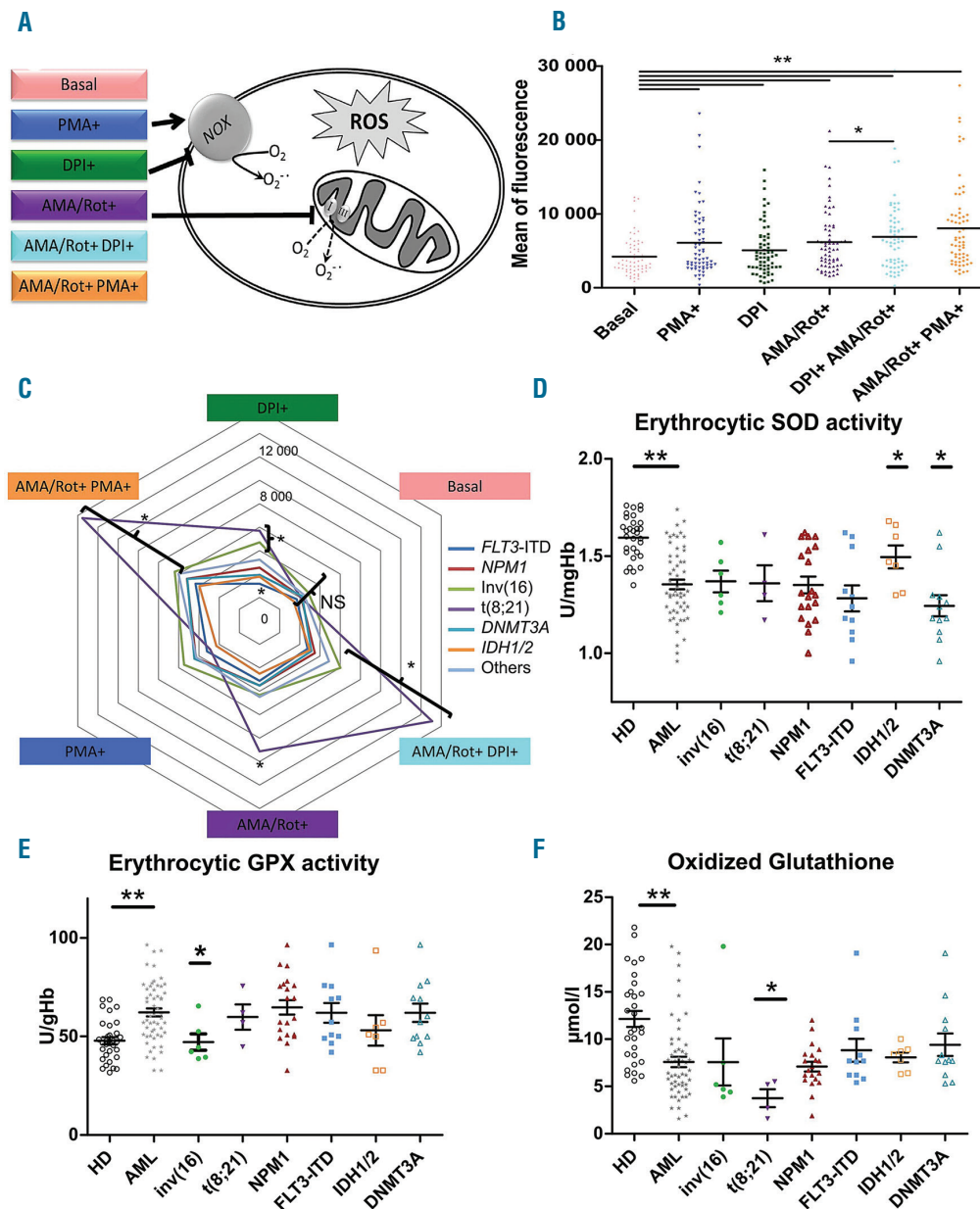


Figure 1. Reactive oxygen species profiles and antioxidant markers of leukemic cells. (A) Schema of reactive oxygen species (ROS) modulators stimulating or inhibiting NADPH oxidase and mitochondria used in the *ex vivo* ROS assays. (B) ROS profiles showing the effects of different ROS modulators in blast cells from bone marrow. Statistical analysis: Wilcoxon test for paired samples. (C) Spider chart of ROS profiles according to leukemic abnormalities. Colored lines represent leukemic abnormalities. Note that ROS profiles from t(8;21), inv(16) patients showed more variability in ROS production after the addition of modulators. "Others" refers to acute myeloid leukemia (AML) that did not express any of the listed abnormalities. (D) Distribution of erythrocytic superoxide dismutase activity at diagnosis in healthy donors (HD) and AML patients according to genetic abnormalities. (E) Distribution of erythrocytic glutathione peroxidase activity in HD and AML patients according to genetic abnormalities. (F) Distribution of oxidized glutathione in HD and AML patients according to genetic abnormalities. (B, D, E, F) * $P < 0.05$, ** $P < 0.01$. PMA: phorbol 12-myristate 13-acetate; DPI: diphenyleneiodonium; AMA: antimycin A; Rot: rotenone; NOX: NADPH oxidase; SOD: superoxide dismutase; GPX: glutathione peroxidase.

chondrial organization with increased mitochondrial mass but a low spare reserve capacity that renders them susceptible to oxidative stressors.⁸ Finally, ROS concentrations can also be modulated by the microenvironment⁹ and by the antioxidant system.⁷

In this prospective study (registered as EudraCT: 2013 A00762-43), we determined the redox status of 84 adult patients with newly diagnosed AML through characterization of ROS production profiles at diagnosis in both the non-leukemic and leukemic compartments. We developed an original *ex vivo* ROS assay using mitochondrial and/or NOX modulators (Figure 1A). In order to construct ROS profiles, six different ROS conditions were studied after labeling with 10 μ M dihydroethidium (ThermoFisher Scientific): ROS production at basal state (phosphate-buffered saline vehicle) and after the addition of one or more ROS modulators [phorbol myristate acetate (PMA), antimycin A/rotenone (AMA/Rot), and/or diphenyleneiodonium (DPI)]. Candidate populations were targeted with CD45⁺ antibody (Becton Dickinson Biosciences), and side scatter (Online Supplementary Figure S1). Mean dihydroethidium fluorescence of each cell population was measured. The patients' characteristics are presented in Online Supplementary Table S1.

ROS production in leukemic cells from peripheral blood was well correlated with that in bone marrow blasts (Online Supplementary Table S2) and with leukocytosis ($r=0.508$, $P<0.001$). ROS production was increased by PMA, a NOX stimulator, and by the complex III and complex I inhibitors AMA/Rot ($P<0.001$ and $P<0.001$, respectively, compared to basal levels) (Figure 1B). Surprisingly, DPI, an inhibitor of NOX, stimulated ROS production in leukemic cells (basal vs. DPI, $P=0.002$ and AMA/Rot⁺DPI⁺ vs. AMA/Rot⁺, $P=0.012$). In fact, DPI has been shown to induce mitochondrial superoxide production through mitochondrial complex I.^{10,11}

No difference in basal ROS levels was observed according to molecular abnormalities (Figure 1C), although AML4 and AML5 expressed higher basal levels of ROS ($P=0.009$) (Online Supplementary Figure S2) as previously observed.¹² However, leukemic cells from patients with *FLT3*-internal tandem duplication (ITD) showed significantly lower ROS production when stimulated with DPI than did leukemic cells from patients without *FLT3*-ITD ($P<0.05$). In contrast, core binding factor (CBF)-AML cells comprising *inv(16)* and *t(8;21)* showed significantly higher levels of ROS production in several conditions (AMA/Rot⁺DPI⁺, AMA/Rot⁺PMA⁺, and DPI⁺ ($P=0.002$, $P=0.024$, and $P=0.028$, respectively). Hence, the CBF subgroup appeared highly sensitive to mitochondrial stress, possibly reflecting the poor stability of mitochondrial membrane permeability and a low mitochondrial threshold for apoptosis. In contrast, the weak response of the *FLT3*-ITD subtype to DPI suggests a 'mitochondrial resistance' to chemotherapy-induced apoptosis through oxidative stress. Levels of ROS production at baseline did not differ significantly between European LeukemiaNet prognostic groups (data not shown). Altogether, ROS production profiles based on the functional evaluation of NOX and mitochondria were more discriminative markers of AML heterogeneity than was basal ROS production.

We investigated whether non-tumoral cells from AML patients had ROS profiles equivalent to those from 65 healthy donors used as controls. ROS production at basal state was significantly higher in neutrophils from AML patients than in those from healthy donors ($P<0.001$) (Figure 2A). However, neutrophils from AML patients

exhibited a lower capacity to increase NOX-dependent ROS production in response to PMA ($P=0.001$) and AMA/Rot⁺PMA⁺ ($P<0.001$). Lymphocytes and monocytes from leukemic patients also displayed significantly lower ROS production than that of the corresponding cells from healthy donors whatever the ROS condition used ($P<0.001$) (Figure 2B,C). Thus, a dysregulated non-tumoral compartment might influence redox levels in the leukemic compartment.

Next, we investigated antioxidant and lipid peroxidation markers at diagnosis and their relationship with

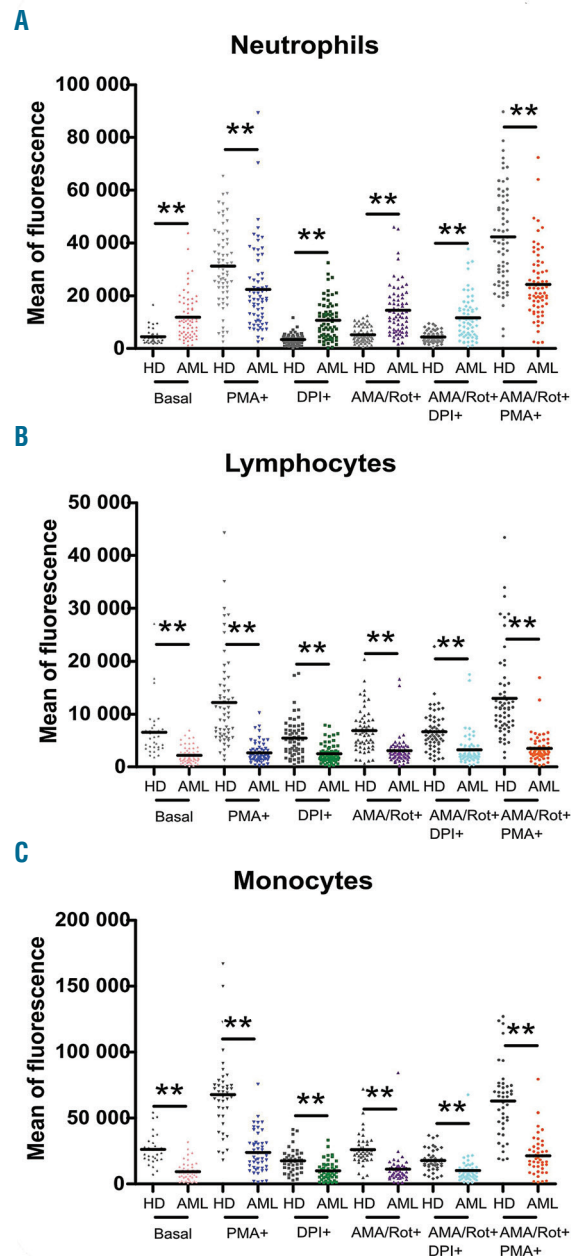


Figure 2. Reactive oxygen species profiles of non-leukemic cells from healthy donors and patients with acute myeloid leukemia. (A) At baseline, levels of reactive oxygen species (ROS) in neutrophils from patients with acute myeloid leukemia (AML) were higher than those in neutrophils from healthy donors. However, neutrophils from AML patients were less reactive to phorbol 12-myristate 13-acetate stimulation. In contrast, in lymphocytes (B) and monocytes (C) from AML patients, ROS production was lower whatever the ROS conditions. ** $P<0.01$. HD: healthy donors; PMA: phorbol 12-myristate 13-acetate; DPI: diphenyleneiodonium; AMA: antimycin A; Rot: rotenone.

molecular subtypes (for methodological details see the *Online Supplementary File*). The activity of erythrocytic superoxide dismutase was significantly lower in AML patients than in healthy donors ($P<0.001$) (Figure 1D). However, *IDH* and *DNMT3A* mutations were associated with, respectively, increased and decreased superoxide dismutase activity ($P<0.05$). Conversely, erythrocytic glutathione peroxidase activity was significantly increased in AML patients ($P<0.001$) (Figure 1E). Among molecular subtypes, patients with *inv(16)* presented significantly reduced erythrocytic glutathione peroxidase activity ($P<0.01$). Overall, total glutathione was decreased in AML ($P<0.001$), as were both oxidized glutathione ($P<0.001$) (Figure 1F) and reduced glutathione ($P<0.001$),

as previously described in $CD34^+$ AML cells.¹³ Interestingly, oxidized glutathione was significantly reduced in *t(8;21)* patients ($P<0.05$) leading to a higher glutathione redox ratio ($P<0.05$). The thiol ratio was also lower in AML patients than in healthy donors ($P<0.001$) and a significant decrease in total thiols was observed in patients with *FLT3-ITD* ($P<0.01$), *NPM1* mutation ($P<0.05$) and *DNMT3A* mutation ($P=0.059$) compared to patients without these mutations. Finally, we observed a significant reduction in lipid peroxidation, assessed by a malondialdehyde assay, in patients with *inv(16)* ($P<0.05$). However, antioxidant markers at diagnosis did not differ significantly between European LeukemiaNet prognostic classes (*data not shown*). Altogether, we showed that CBF-

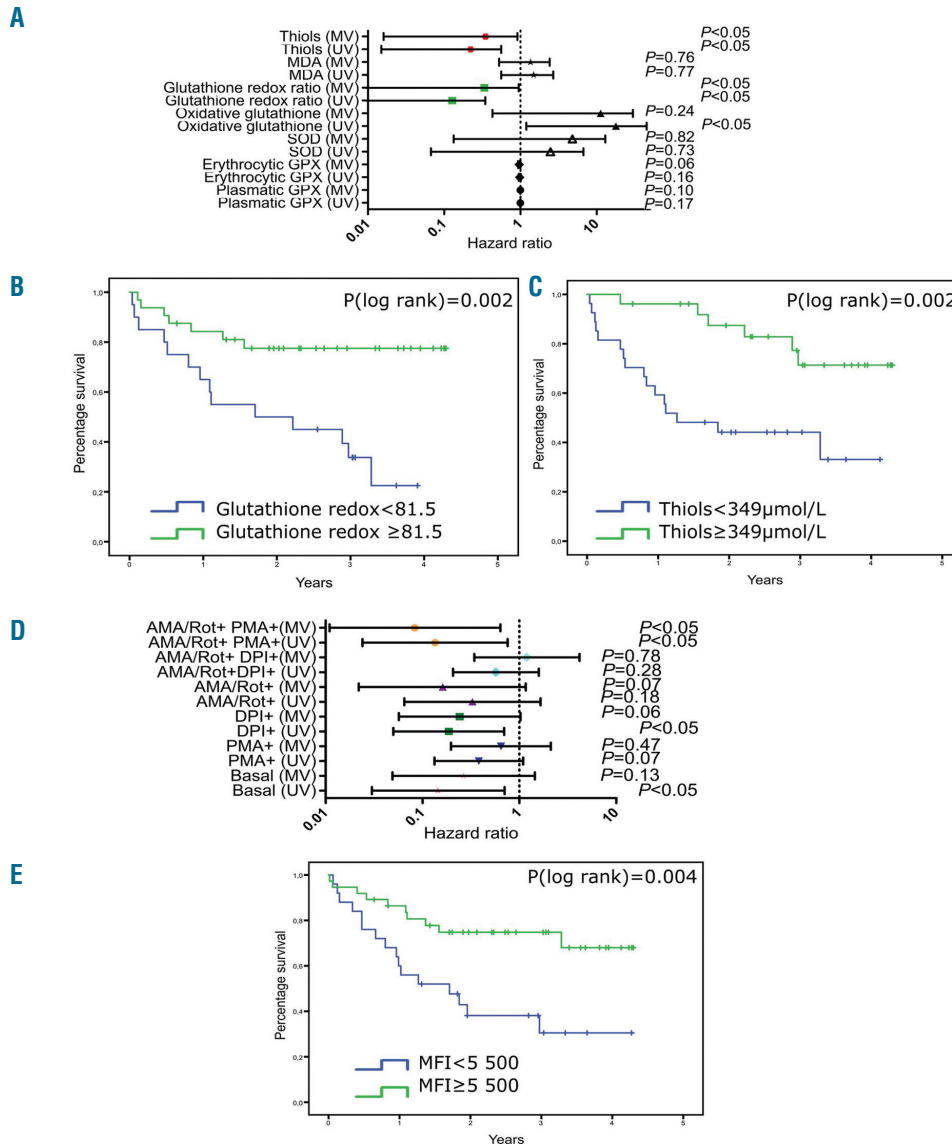


Figure 3. Prognostic value of antioxidant markers and leukemic reactive oxygen species profiles. (A) Forest plot representing the risk of death (with hazard ratios and 95% confidence intervals) according to antioxidant markers and malondialdehyde levels. (B) Kaplan-Meier survival curves of patients with acute myeloid leukemia (AML) receiving chemotherapy according to the cut-off for reduced/oxidative glutathione ratio (< vs. ≥ 81.5). (C) Kaplan-Meier survival curves of AML patients receiving chemotherapy according to the cut-off for thiol levels (< or $\geq 349 \mu\text{mol/L}$). (D) Forest plot representing the risk of death (with hazard ratios and 95% confidence intervals) according to reactive oxygen species (ROS) production by leukemic cells from bone marrow exposed to different ROS modulators (log-transformed variables). (E) Kaplan-Meier survival curves of AML patients receiving chemotherapy according to the cut-off for mean fluorescence intensity (MFI) in response to ROS modulators (< vs. $\geq 5,500$). High levels of MFI were predictive of survival ($P=0.004$). MV: multivariate analysis; UV: univariate analysis; MDA: malondialdehyde; SOD: superoxide dismutase; GPX: glutathione peroxidase; AMA: antimycin A; Rot: rotenone; PMA: phorbol 12-myristate 13-acetate; DPI: diphenyleneiodonium.

AML presented fewer markers of oxidative stress, with lower oxidized glutathione for t(8;21) AML and lower malondialdehyde levels for inv(16) AML, in contrast to the *FLT3*-ITD, *NPM1*, and *DNMT3A* subtypes of AML which showed lower levels of thiols. These data suggest that oncogenic mutations could also affect the antioxidant system and, together with alterations in ROS production, promote the proliferation of leukemic cells.

Next, we explored whether redox status at diagnosis influences survival. The statistical methods are described in the *Online Supplementary File*. We observed that the reduced/oxidized glutathione ratio at diagnosis was significantly higher in survivors than in non-survivors ($P=0.002$) and in non-relapsed patients versus relapsed patients ($P<0.05$). Furthermore, oxidized glutathione levels at diagnosis were lower in survivors than in non-survivors ($P=0.017$). Multivariate analysis including age, the 2017 European LeukemiaNet stratification and leukocyte counts confirmed that an increase in the reduced/oxidized glutathione ratio was an independent marker of longer survival [hazard ratio (HR)=0.055, 95% confidence interval (95% CI): 0.003-0.951, $P=0.04$] (Figure 3A). Moreover, an increase in thiol levels was also significantly associated with a lower risk of death and relapse (HR=0.12, 95% CI: 0.016-0.911, $P=0.04$ and HR=0.07, 95% CI: 0.007-0.753 $P=0.028$, respectively). Based on receiver operating characteristic curve analysis, we calculated a threshold for both the reduced/oxidized glutathione ratio and thiol levels. Patients whose glutathione redox ratio was ≥ 81.5 or patients whose thiol concentration was ≥ 349 $\mu\text{mol/L}$ at diagnosis had significantly higher survival rates ($P=0.002$ and $P=0.002$, respectively) (Figure 3B,C).

Furthermore, we showed that ROS production at baseline was significantly correlated with overall survival in univariate analysis (from bone marrow samples, HR=0.14, 95% CI: 0.03-0.7, $P=0.02$) although this was not confirmed in multivariate analysis (from bone marrow and peripheral blood samples, HR=0.27, 95% CI: 0.05-1.45, $P=0.13$ and HR=0.35, 95% CI: 0.12-1.03, $P=0.06$, respectively) (Figure 3D). However, leukemic cell ROS measured after incubation with modulators could be an independent prognostic marker of survival. Indeed, the risk of death was significantly decreased in patients with the highest levels of DPI-induced ROS (in bone marrow and peripheral blood HR=0.169, 95% CI: 0.041-0.694, $P=0.014$ and HR=0.26, 95% CI: 0.075-0.90, $P=0.035$, respectively, in univariate analysis). An increase in DPI-dependant ROS in bone marrow samples was also associated with longer relapse-free survival and event-free survival in multivariate analysis (HR=0.1, 95% CI: 0.02-0.49, $P=0.005$, and HR=0.17, 95% CI: 0.04-0.66, $P=0.01$, respectively). Interestingly, increased ROS production by leukemic cells under AMA/Rot⁺PMA⁺ stimulation was significantly associated with longer survival in both univariate and multivariate analyses (HR=0.14, 95% CI: 0.02-0.76, $P=0.02$ and HR=0.08, 95% CI: 0.01-0.64, $P=0.02$, respectively). Moreover, higher ROS production under AMA/Rot⁺PMA⁺ stimulation was significantly associated with longer relapse-free survival and event-free survival in multivariate analysis (HR=0.028, 95% CI: 0.003-0.278, $P=0.002$ and HR=0.12, 95% CI: 0.023-0.73, $P=0.02$, respectively). These results were confirmed in peripheral blood (for overall survival and event-free survival: HR=0.28, 95% CI: 0.1-0.813, $P=0.019$ and HR=0.33, 95% CI: 0.13-0.84, $P=0.02$, respectively). Finally, we identified a threshold using receiver operating characteristic curves in which patients whose blasts from bone marrow or blood at diagnosis produced $\geq 5,500$ ROS

in response to AMA/Rot and PMA had a significantly higher survival rate ($P=0.004$) (Figure 3E).

Our approach underlines the importance of functional NOX and mitochondria in chemosensitivity and confirms that mitochondrial fitness is critical to prognosis. We suggest that blast cells resistant to mitochondria and NOX stress are more resistant to chemotherapy (or at least include subclones resistant to chemotherapy), probably because in these patients chemotherapy failed to induce efficient ROS-mediated apoptosis of leukemic cells. Farge *et al.* demonstrated that chemoresistant leukemic cells displayed a high OXPHO signature characterized by active polarized mitochondria with no loss of mitochondrial membrane potential after cytarabine-treatment.¹⁴ We hypothesize that our patients resistant to mitochondrial and NOX stress could be similar to those with high OXPHO and may be good candidates for ROS/mitochondria targeted therapy. For example, mitochondrial complex I inhibitors such as metformin can induce an energy shift from high OXPHOS to low OXPHOS (the so-called Pasteur effect) and improve the sensitivity of *FLT3*-ITD AML to cytarabine. Finally, the fact that both NOX and mitochondria-dependent ROS production were associated with better survival argues for cooperation between ROS producers. Indeed, superoxide generated from leukemic NOX2 drives mitochondrial transfer from stromal cells.⁹

In conclusion, our study showed that AML patients had a dysregulated redox balance linked to their molecular status, involving leukemic cells, non-tumoral cells and the antioxidant system, which play a major role in the prognosis of patients.

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