doses).[11] This adds weight to the argument of starting chelation with deferasirox early in the course of MDS when lower doses may suffice and improve compliance.

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High-level induction of fetal haemoglobin by pomalidomide in β-thalassaemia/HbE erythroid progenitor cells

Studies have shown that increased expression of fetal haemoglobin (HbF; $\alpha_2\gamma_2$) can ameliorate red blood cell deficiencies in patients with β-thalassaemia and sickle cell disease (SCD).[1–3] Pharmacological induction of HbF expression in β-thalassaemia has been investigated using several classes of small molecules,[4] including 5-azacytidine,[5] decitabine,[6] hydroxyurea,[7] LSD1 inhibitors (tranylcypromine and RN-1),[8,9] and short chain fatty acid derivatives.[10,11] Among these molecules, hydroxyurea is the only U.S. Food and Drug Administration (FDA) currently approved drug for the treatment of SCD and/or β-thalassaemia. However, hydroxyurea has shown modest and variable responses with potential myelosuppression in β-thalassaemia patients. Therefore, more robust and safer HbF therapeutics are highly desired.

Pomalidomide, an FDA-approved immunomodulatory drug for the treatment of multiple myeloma,[12,13] stimulates γ-globin mRNA and HbF expression in erythroid progenitor cells by downregulating factors involved in γ-globin repression, including *BCL11A, SOX6, GATA1, KLF1* and *LSD1*.[14–16] In addition, treatment of a humanized mouse model of SCD with pomalidomide induced comparable HbF expression to hydroxyurea, but without myelosuppressive effects.[15]

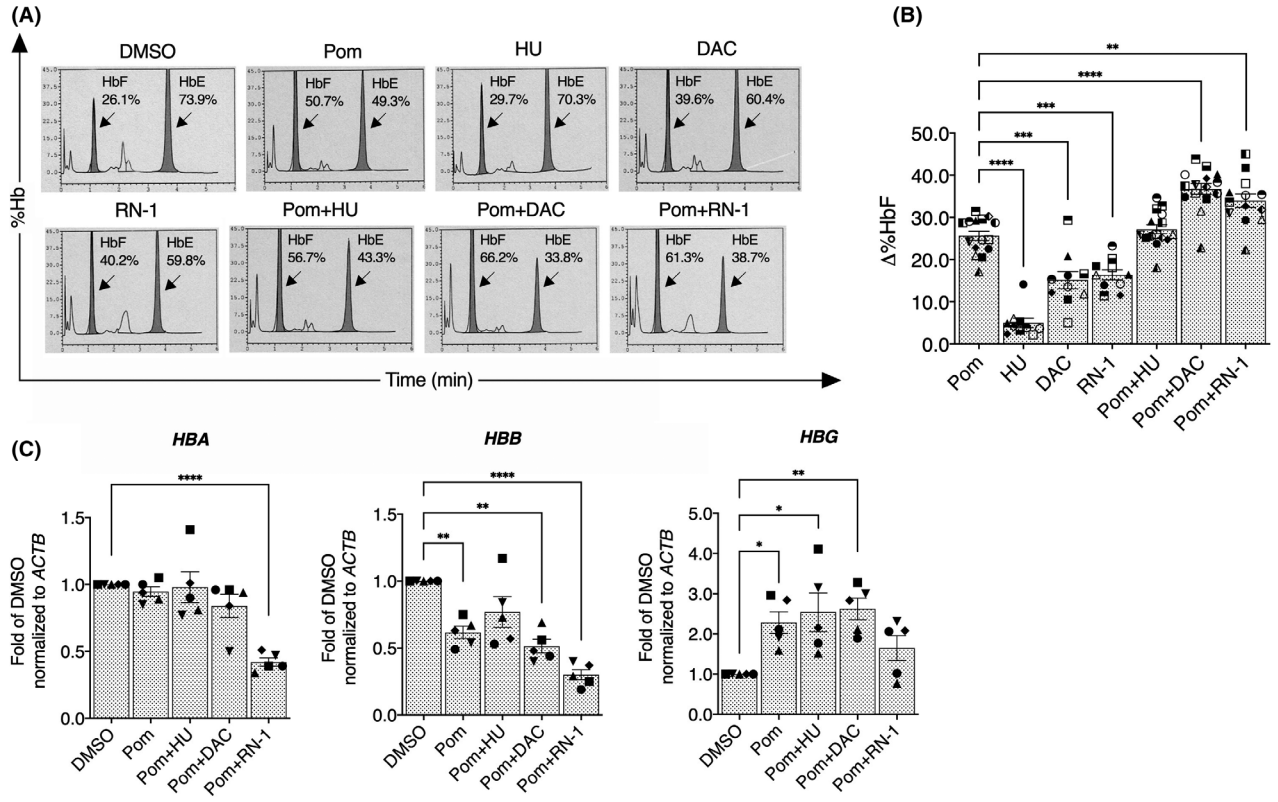

Fig 1. Robust HbF induction in β^0-thalassaemia/HbE erythroid cells by pomalidomide alone or in combination with other HbF inducers. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4–14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8–14), 0·02 μmol/l RN-1 only (from day 8–14), or in combination of Pom with HU, with DAC, or with RN-1. (A) Representative high-performance liquid chromatograms (HPLC) showing haemoglobin composition at day 14 of erythroid differentiation. (B) The percentage of HbF relative to total Hb (%HbF + %HbE) determined by HPLC at day 14 of erythroid differentiation. The increase in HbF percentage after treatment from the baseline level in dimethyl sulfoxide (DMSO) control was expressed as Δ%HbF (% HbF [compound treatment] − %HbF [DMSO control]). Mean ± standard error of the mean [SEM], n = 10 for HU, DAC and RN-1; n = 15 for Pom, Pom + HU and Pom + DAC; and n = 13 for Pom + RN-1. (C) Quantitative reverse transcription polymerase chain reaction analysis showing relative *HBA*, *HBB* and *HBG* mRNA expression levels normalised to β-actin (*ACTB*) at day 12 of erythroid differentiation. Data are presented as the mean (± SEM) of relative fold change of DMSO (n = 5) *P < 0·05; **P < 0·005; ***P < 0·0005; ****P < 0·0001.

Here, we investigated the therapeutic potential of pomalidomide and its combined effects with other HbF inducers, including hydroxyurea, decitabine and RN-1, in erythroid progenitor cells from compound heterozygous β^0-thalassaemia/HbE (*HBB*:c.79G>A) patients (Table SI) using a three-phase liquid culture system that supports terminal maturation of erythroid cells (Data S1 and Fig S1). Comparison of results using optimal conditions for each compound (Figs S2 and S3) revealed that pomalidomide was much more effective in inducing HbF expression than hydroxyurea, decitabine or RN-1 (Fig 1A,B). The greatest increase in HbF percentage from the baseline level was observed in pomalidomide-treated cells, achieving 25·6 ± 1·1% as determined by high-performance liquid chromatography (HPLC) (Fig 1A,B). β^0-thalassaemia/HbE precursors from patients of different β^0-thalassemic mutations (Table SI) showed similarly increased levels of HbF induction in response to pomalidomide treatment. This result suggested that deficient progenitors, regardless of specific β^0-thalassemic mutation or baseline HbF level, are all susceptible to strong induction with pomalidomide (Fig 1A,B and Tables SI and SII). The percentage of cells expressing HbF (F cells) increased from 49·8 ± 4·7% for dimethyl sulfoxide (DMSO) controls to 60·6 ± 2·5% after pomalidomide treatment (Fig S4). By quantitative reverse transcription polymerase chain reaction (RT-PCR), we found that pomalidomide significantly increased γ-globin (*HBG*) mRNA expression, achieving a 2·3 ± 0·3-fold increase over control cells, with coincidentally diminished β-globin (*HBB*) expression, without significant change in α-globin (*HBA*) expression (Fig 1C).

To enhance the level of HbF induction, we investigated the effects of combined treatment of pomalidomide either with or without other pharmacological HbF inducers. The combination of pomalidomide and decitabine had an additive effect on induction, as shown by the differential HbF level (Δ% HbF = 36·7 ± 1·3) when compared to treatment with any single agent (Fig 1A,B). Hydroxyurea did not generate any additional increase in HbF when combined with pomalidomide. The

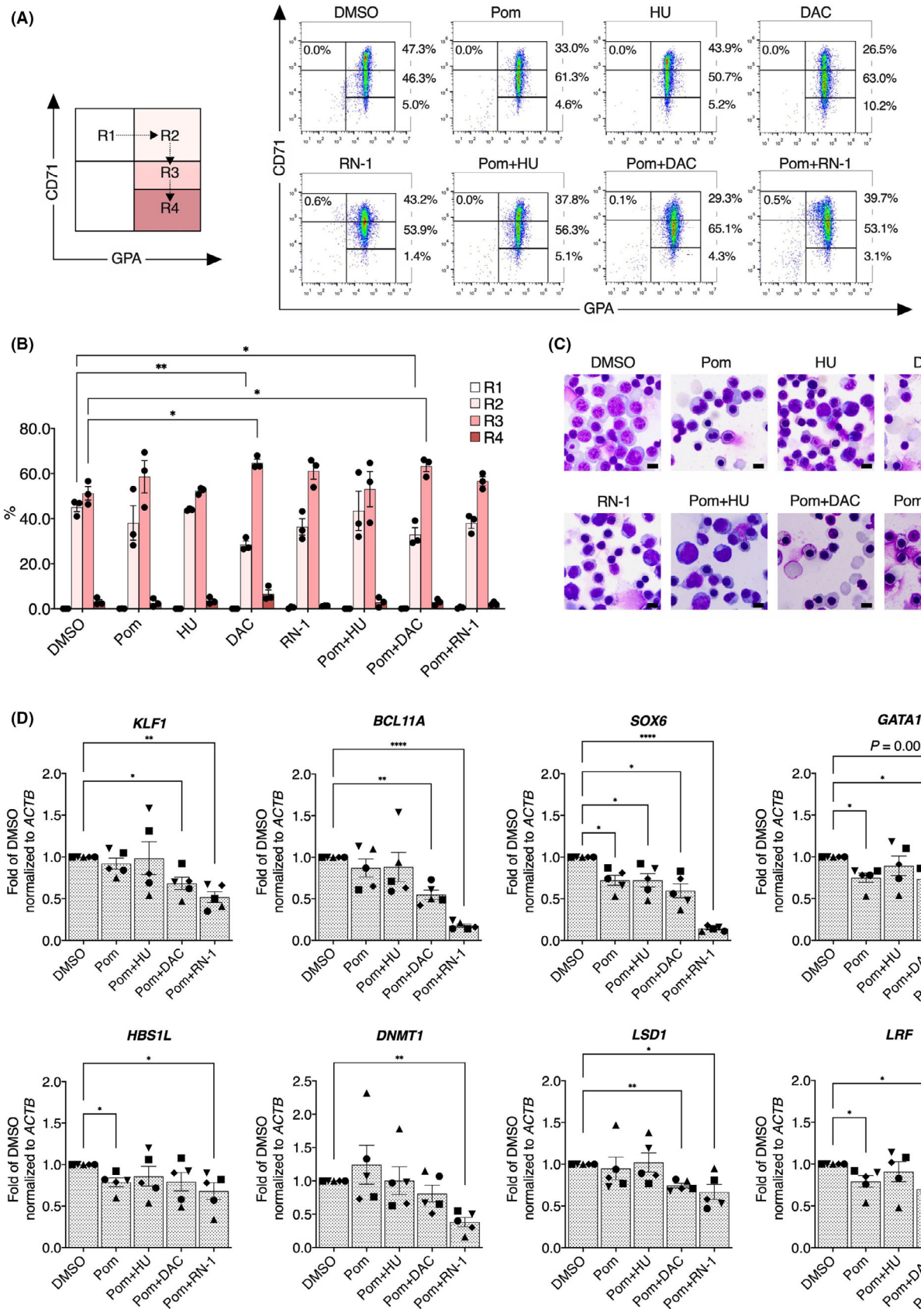

Fig 2. Effect of pomalidomide and its combinations on erythroid differentiation and mRNA expression of HbF regulators in cultured erythroid cells from β^0-thalassaemia/HbE patients. β^0-Thalassaemia/HbE erythroblasts were treated with 4·0 μmol/l pomalidomide only (Pom, from day 4 –14), 1·0 μmol/l hydroxyurea only (HU, from day 8–14), 0·1 μmol/l decitabine only (DAC, from day 8– 14), 0·02 μmol/l RN-1 only (from day 8–14), the combination of Pom with HU, with DAC, or with RN-1. (A) Representative flow cytometry dot plots for erythroid differentiation analysis on day 12 of culture. Erythroid cells were gated into R1 to R4 populations according to the expression levels of transferrin receptor (CD71) and glycophorin A (GPA/CD235a). (B) The histogram represents the quantitation of erythroid subpopulations analysed by flow cytometry. (mean ± SEM, $n = 3$). $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$, relative to dimethyl sulfoxide (DMSO) control. (C) Representative modified Giemsa-stained cytospins at day 12 of culture showing erythroid morphology after DMSO or compound treatments. Scale bar = 10 μm. (D) Relative mRNA abundance of known HbF regulators normalized to β-actin (*ACTB*) determined by quantitative reverse transcription polymerase chain reaction at day 12 of erythroid cell culture. Gene names are shown at the top of each histogram. Data are presented as the mean (± SEM) of relative fold change of DMSO . ($n = 5$) $^*P < 0{\cdot}05$; $^{**}P < 0{\cdot}005$; $^{****}P < 0{\cdot}0001$.

combination of pomalidomide and RN-1 did increase the percentage of HbF (Fig 1A,B) and at the same time reduced *HBA*, *HBB* and *HBG* mRNA expression (Fig 1C), suggesting that this combination negatively affected total globin mRNA expression. Taken together, these results suggest that pomalidomide and decitabine act through independent pathways to induce, additively, high-level HbF expression, implying a cooperative therapeutic potential for the treatment of β-thalassaemia.

We next determined the cytotoxicity of treatments and found that pomalidomide did not significantly affect erythroid cell proliferation (Fig S5A) or viability (Fig S5B). However, pomalidomide plus decitabine showed a reduction in cell proliferation on day 12 of culture without affecting cell viability. Erythroid cell proliferation and viability were significantly reduced in cells exposed to pomalidomide plus RN-1 (Fig S5A,B), suggesting toxicity of the latter combination. Analysis of erythroid differentiation of cells treated with hydroxyurea or pomalidomide plus hydroxyurea was similar to that of DMSO-treated cells (Fig 2A,B), suggesting that these treatments did not affect erythroid terminal differentiation. We noted a trend towards increased differentiation of cells treated with pomalidomide, RN-1 and pomalidomide plus RN-1, compared with the controls. Interestingly, significantly accelerated erythroid differentiation was observed in decitabine alone and pomalidomide plus decitabine, as evidenced by elevated transferrin receptor $(CD71)^{medium}$/(glycophorin A $(GPA)^{high}$ population and decreased $CD71^{high}$/GPA^{high} cells (Fig 2A,B). Similarly, modified Giemsa-stained cytospins showed an increased number of late-stage erythroblasts in cells exposed to decitabine alone and pomalidomide plus decitabine when compared to control cells, indicating a shift towards normal erythroid cell maturation (Fig 2C and Fig S1). These results suggested that the differentiation of β^0-thalassaemia/HbE progenitor cells significantly improved after treatment with either decitabine alone or pomalidomide plus decitabine.

To investigate the effects of pomalidomide plus or minus these effectors on transcriptional regulation in β-thalassaemic erythroid progenitor cells, quantitative RT-PCR analyses revealed that one key γ-globin repressor mRNA, *BCL11A,* was only slightly reduced after treatment with pomalidomide or pomalidomide plus hydroxyurea. *BCL11A* was significantly downregulated (by 1·8- and 5·6-fold) after treatment with pomalidomide plus decitabine or pomalidomide plus RN-1 respectively (Fig 2D). Moreover, the expression of *SOX6*, *GATA1*, *HBS1L* and *LRF* were modestly but significantly downregulated by pomalidomide, whereas other erythroid regulators were unaffected (Fig 2D and Fig S6). In addition, combined pomalidomide and decitabine treatment, which showed additive effects on HbF induction, reduced the expression of *KLF1*, *LSD1* and *CHD4*. The combination of pomalidomide plus RN-1 significantly affected the expression of several key regulators, including *KLF1*, *SOX6*, *GATA1*, *HBS1L*, *DNMT1*, *LSD1*, *ID2*, *CHD4*, *FOXO3*, *NRF2* and *MYB* (Fig 2D and Fig S6), consistent with the fact that this same combination significantly reduced cell proliferation and viability (Fig S5). Taken together, these results indicate that the mechanisms of action of pomalidomide and several co-effectors in induction of HbF expression partly involve transcriptional regulation of key HbF repressors and/or co-repressors.

In summary, the present data show that pomalidomide is a potent HbF inducer and is more potent than hydroxyurea. The combination of pomalidomide and decitabine provide additive effects in inducing HbF expression in erythroid cells from β^0-thalassaemia/HbE patients. Despite these promising results, it must be emphasized that the potential risks associated with the use of pomalidomide include developmental defects (if taken during pregnancy), thrombosis and pancytopenia,[17] which are similar to the toxicities of the parental drugs, lenalidomide and thalidomide. Development of pomalidomide structural refinements or analogues with similar biological effects may lead to future, fully effective, reduced adverse effects and possible clinical application.

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Conflicts of interest

The authors declare no conflict of interest.

Author contributions

PK, TN, OS and NJ designed the research; PK, TN, PP and WK performed the experiments; PK, TN and NJ analysed data; DS, KP, SH and SF provided samples and resources; PK, JDE, and NJ wrote the manuscript; JDE, SH, SF, OS and NJ conceptualized the idea and supervised the project. All the authors read and approved the final manuscript.

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Supporting Information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Data S1. Supplemental methods.

Fig S1. Delayed erythroid differentiation is observed in β^0-thalassaemia/HbE comparing to normal erythroblast culture.

Fig S2. Time- and dose-dependent inducing effects of pomalidomide on HbF induction in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S3. HbF-inducing effects of hydroxyurea (HU), decitabine (DAC), and RN-1 in erythroid cells from β^0-thalassaemia/HbE patients.

Fig S4. Pomalidomide and its combinations increase erythroid cells expressing HbF (F-cells).

Fig S5. Effect of pomalidomide and its combinations on cell proliferation, viability of cultured erythroid cells from β^0-thalassaemia/HbE patients.

Fig S6. Treatment of pomalidomide and its combinations alter the expression of HbF regulators in β^0-thalassaemia/HbE erythroid cells.

Table SI Haematological parameters of β^0-thalassaemia/HbE patients participated in this study.

Table SII Induction of HbF in erythroid progenitor cells from β^0-thalassaemia/HbE after treatments with pomalidomide, hydroxyurea, decitabine, RN-1 and their combinations.

Table SIII Primers used in this study.

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Clinical and biological correlates of the expression of select Polycomb complex genes in Brazilian children with acute promyelocytic leukaemia

De novo acute promyelocytic leukaemia (APL) is an aggressive subtype that accounts for 5–10% of all childhood acute myeloid leukaemia (AML). In some Latin American and European populations, APL is more frequent than in other geographic populations. Approximately 97% of patients with APL present t(15;17)(q22;q21.1)/promyelocytic leukaemia (PML)-retinoic acid receptor alpha (RARA) fusion protein and nearly all of the affected patients respond to therapy with all-*trans* retinoic acid (ATRA) combined with arsenic trioxide (ATO).[1] Additionally, studies in transgenic mice revealed that this transcript is necessary, but not sufficient, for APL development, suggesting that additional genetic or epigenetic changes are also required for the APL establishment.[2]

The FMS-like tyrosine kinase 3-internal tandem duplication (*FLT3*-ITD) has been frequently reported in patients with APL presenting a more aggressive disease course, with lower overall and disease-free survival rates. These mutations are known to co-operate with other initiating events to advance disease progression, but they do not initiate leukaemia independently.[3] Regarding paediatric APL, genomic studies reported that APL cells have fewer genetic alterations than those from other AML subtypes.[4] These data indicate that childhood APL development may require more than just genetic alterations to manifest the disease phenotype.

In this context, there are several reports on the epigenetic alterations implicated in AML development, and they are mainly presented in APL. For instance, patients with APL are characterised by a specific DNA methylation pattern, which may be due to relatively late events in APL leukaemogenesis, contributing to APL maintenance rather than leukaemia initiation.[5]

Besides, PML/RARA induces a multitude of alterations in chromatin architecture, including the recruitment of crucial epigenetic-modifying factors, such as histone deacetylase complexes and DNA methyltransferases. Moreover, pieces of evidence have revealed that the Polycomb repressor complex (PRC) could contribute to the alterations observed in the typical APL epigenetic landscape.[6]

Polycomb group (PcG) proteins are histone modifiers in two multiprotein complexes: Polycomb repressive complexes 1 and 2 (PRC1 and PRC2). Radulovic et al.[6] highlighted the emerging implications of these genes in haematopoietic neoplasms, including myeloid neoplasia. Therefore, changes in the expression profile of individual PcG genes might yield novel information about APL pathogenesis.

We evaluated the PcG gene levels, namely, enhancer of zeste homologue 2 (*EZH2*), Yin and Yang 1 protein (*YY1*), BMI1 proto-oncogene, Polycomb ring finger (*BMI1*) and suppressor of zeste 12 protein homologue (*SUZ12*), in a cohort of 25 Brazilian children with APL, with and without a *FLT3*-ITD mutation. In addition, we compared them to those found in patients with other AML subtypes, with and without the *FLT3* mutation, to verify whether this mutation status could be associated with the APL epigenetic landscape.

Amongst the 25 patients with APL, eight had additional chromosome abnormalities that mostly involved chromosomes 6, 8, 20 and 21. In the AML group, 39 patients had their karyotype evaluated and the abnormalities detected were as follows: 15 (38·5%) presented with lysine methyltransferase 2A (*KMT2A*) gene abnormalities; eight (20·5%) presented with non-recurrent chromosome abnormalities; four (10·3%) presented with RUNX1 translocation partner 1 (*RUNX1*)/*RUNXT1* fusion genes; four (10·3%) presented with core binding factor subunit beta (*CBFB*)/myosin heavy chain 11 (*MYH11*) fusion genes; four (10·3%) presented with normal karyotypes; three (7·7%) presented with abnormalities in chromosomes 5 and 7; and one (2·4%) presented with no mitosis. In relation to *FLT3* status, 21 (30·9%) patients had *FLT3*-ITD mutations, 13 (61·9%) were patients with APL and eight (38·1%) were patients with AML [AML-M5 (five patients), AML-M2 (two) and AML-M6 (one)] (Clinical data are in Table S1).