the actual variant would be performed. In the absence of P50 values, as with patients in this study, it is important to eliminate the possibility of Hb variants by sequencing the globin genes, which confirmed 6 positive cases on the registry.

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Pyruvate kinase M2 and prednisolone resistance in acute lymphoblastic leukemia

Treatment of childhood acute lymphoblastic leukemia (ALL) combines different classes of chemotherapeutic agents, such as *Vinca* alkaloids, anthracyclines and glucocorticoids (GCs). Although such therapy nowadays cures the majority of the patients, combination chemotherapy still fails in approximately 20%.[1] Most treatment failures can be explained by resistance to antileukemic agents,[2] and resistance to the glucocorticoids in particular has been shown to be related to an unfavorable event free survival.[3,4] Since the glucocorticoids prednisolone and dexamethasone play a crucial role in essentially all therapy protocols, the development of strategies to reverse resistance to these agents is important to improve ALL treatment efficacy. Recently, we have demonstrated that glucocorticoid resistance in pediatric ALL is associated with increased glucose metabolism, and that inhibition of glycolysis sensitizes prednisolone-resistant ALL cells to glucocorticoids.[5] Although it has been known for several decades that cancer cells shift their energy production from oxidative phosphorylation towards the less efficient glycolysis pathway (the so called Warburg effect)[6] it is still not clear how tumor cells establish this altered metabolic phenotype. Christofk *et al*. recently showed that altered expression of the glycolytic enzyme pyruvate kinase (PK), and more specifically the switch to the alternatively spliced isoform M2 (PKM2), is responsible for the increased rate of glycolysis observed in cancer cells.[7] Together, these findings suggest an upregulation of PKM2 in prednisolone resistant leukemia.

To investigate if pyruvate kinase M2 plays a role in glucocorticoid resistance in pediatric ALL, the expression of PKM2 was determined in leukemic cell samples of ALL patients and compared to PKM2 expression in normal peripheral blood and bone marrow samples. To distinguish between the different isoforms in real time quantitative PCR (RT Q-PCR), primers were designed that specifically amplify PKM2 or that recognize both isoform 1 and 2 of pyruvate kinase (PKM1/2, Figure 1A). The expression of PK isoforms was subsequently tested in different cell lines, confirming the specificity of the primer combinations (Figure 1B).

Next, the expression of the different PK isoforms was determined in normal peripheral blood lymphocytes, normal bone marrow and in leukemic samples from untreated children at initial diagnosis of ALL that were identified by an *in vitro* cytotoxicity assay (MTT)[8] as prednisolone-resistant (n=11, $LC_{50} \geq 150$ μg/mL), or sensitive to prednisolone (n=19, $LC_{50} \leq 0.1$ μg/mL).[6] In correspondence with the results of Christofk *et al*.,[7] a significant difference ($p<0.0001$) was found in the expression of PKM2 between normal and ALL cells (Figure 2A). However, PKM2 transcripts were found in all patient samples and no significant difference was observed between prednisolone-resistant or prednisolone–sensitive ALL cases

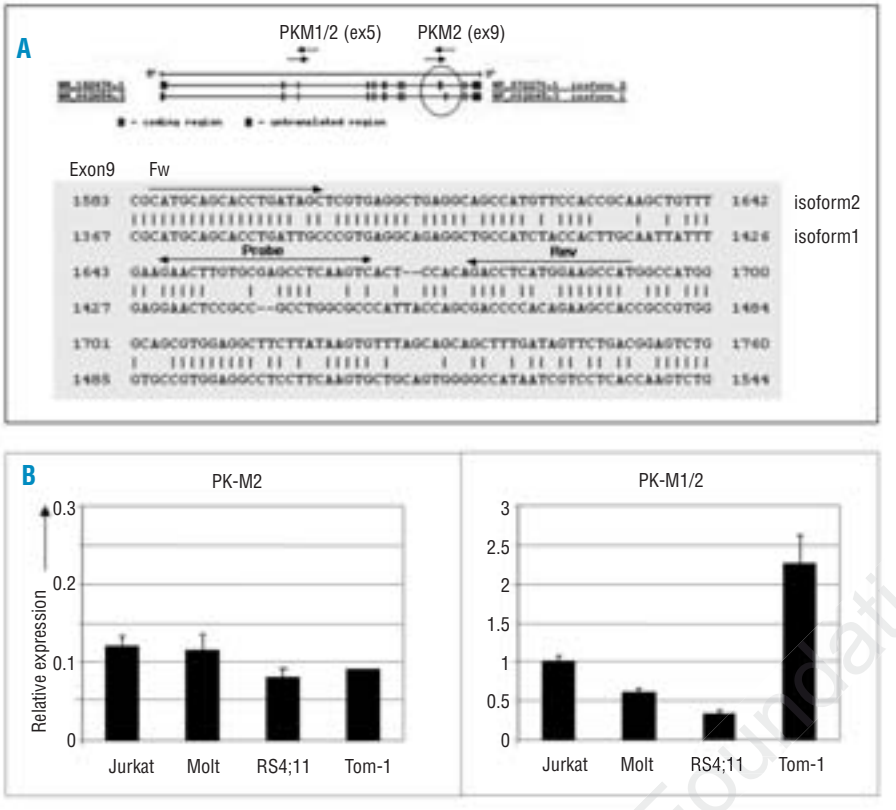

Figure 1. Verification of the real time PCR primer-probe specificity. (A) Graphic representation of primer design to distinguish pyruvate kinase isoforms. The primers located in exon 3 can not distinguish different PKM isoforms (PKM1/2). A primer-probe combination amplifying nt 1586-1692 (exon 9) was used to specifically amplify PKM2. Amplification efficiency was over 95% for all primer-probe combinations. Ribosomal protein S20 (RPS20) was used as a control gene for normalization. (B) Graphic representation of mRNA levels of pyruvate kinase isoform M2 (left panel) or isoform 1 and 2 (right panel) in different leukemic cell lines confirming primer-probe specificity. Expression of PK-M1/2 in Jurkat cells was set to be 100% and relative expression levels were calculated.

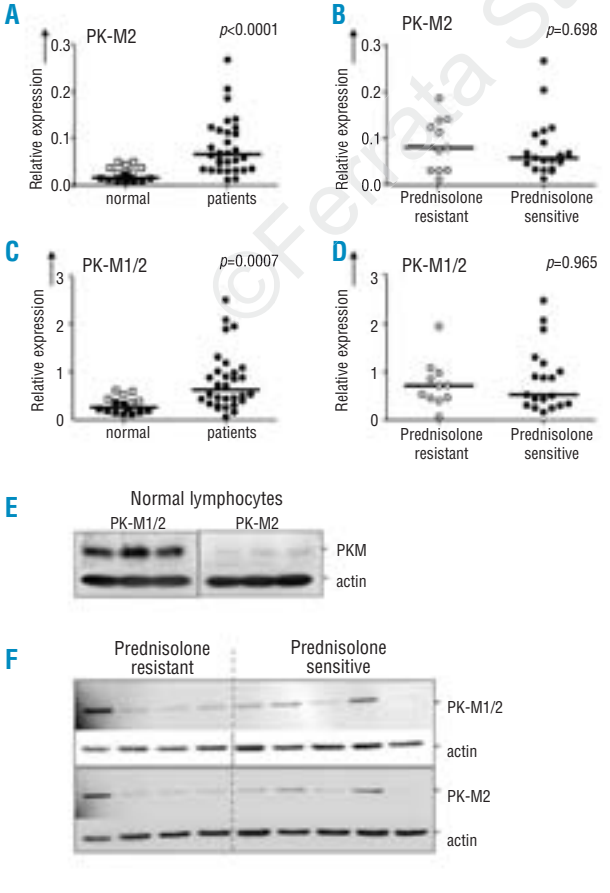

Figure 2. Expression of PKM isoforms in ALL patients. (A-D) Graphical representation of the expression of pyruvate kinase isoform M2 (panels a, b) or isoform M1 and M2 (panels c, d) as measured by Q-PCR. Expression of PK isoforms in normal lymphocytes versus ALL cells is depicted in panels a and c; expression of PK isoforms in prednisolone-resistant or prednisolone–sensitive patients in panels b and d. As normal samples peripheral blood (■) or bone marrow (□) was used. Medians are indicated as horizontal lines. (E-F) Western blots representing the expression of pyruvate kinase isoforms in normal lymphocytes (panel e) or in prednisolone-resistant or prednisolone–sensitive patients (panel f). 20 µg of protein was loaded and membranes were incubated with 1:1000 diluted antibody directed against PKM1/2 or PKM2 (Cell Signaling Technology Inc., Danvers USA).

(Figure 2B). Similar expression patterns were observed for PKM1/2 (Figure 2C and D), although the transcript levels of PKM1/2 were about ten-fold higher than the expression levels of PKM2. Together, these data indicate that not only the M2 isoform of pyruvate kinase was present in ALL patients but also PKM1, and that the mRNA expression levels of different PKM isoforms are no indication of glucocorticoid resistance in childhood leukemia.

Since it has been reported that tumor cells exclusively express the M2 isoform of pyruvate kinase[7,9-11] and not PKM1, we also determined the expression of the different PKM isoforms at the protein level. Western blotting was performed using antibodies that, like the primers in the Q-PCR experiments, specifically recognized PKM2 or both PKM1/2 isoforms. Blots incubated with these anti-

bodies showed a clear band for PKM1/2, but not PKM2, in normal lymphocytes (Figure 2E). In samples from ALL patients, however, equivalent expression levels of PKM1/2 and PKM2 were present (Figure 2F). Analogous to the Q-PCR results, no significant difference in protein levels were observed between prednisolone-resistant and prednisolone–sensitive cases, suggesting that pyruvate kinase isoform M2 is not responsible for glucocorticoid resistance in childhood leukemia. Whether the difference in expression between different isoforms of pyruvate kinase that was detected between normal bone marrow and leukemic cells reflects a difference in glycolytic rate is not known, since patient cells do not grow *in vitro* and we can not detect glucose consumption. Thus, although pyruvate kinase might play a role in the regulation of glycolysis in childhood ALL, glucocorticoid resistance is unlikely to be caused by selective expression of PKM2.

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Detection of twelve nucleotides insertion in the *BCR-ABL* kinase domain in an imatinib-resistant but dasatinib-sensitive patient with bi-phenotypic acute leukemia

Although targeted inhibition of BCR-ABL by imatinib (IM) is an effective therapy for patients with Philadelphia chromosome-positive leukemias, a minority of patients, most of them in advanced phase, acquire mutations in the BCR-ABL kinase domain (KD) leading to relapse.[1-5] These mutations consist, almost exclusively, in single nucleotide (nt) substitutions. Rare cases of splicing events inducing deletion or insertion of multiple nucleotides into ABL KD have been described. A deletion of 27 amino acids (aa) induced by the L248V mutation activating a cryptic splice site has been identified[6] as well as cases resulting from the insertion of 35 nt from intron 8 leading to a frameshift.[7,8] In the latter, this insertion could account for up to 62% of IM-resistant CML patients in chronic phase.[8] Here, we describe a novel mutation acquired at the moment of the IM resistance, consisting in an insertion of 12nt, and leading to the conservation of the open reading frame (ORF).

The 57-year old female patient was diagnosed in July 2006 with bi-phenotypic acute leukemia (hyperleukocytosis at 48 G/L with 47% of blasts exhibiting myeloid and lymphoid features: $CD13^+$, $CD33^+$, $CD19^+$, $CD10^+$ and $CD22^+$). The procedures followed were in accordance with the Helsinki Declaration as revised in 2008. A karyotypic analysis demonstrated a t(9;22)(q34;q11) as sole anomaly and molecular analysis detected *M*-BCR-ABL transcript. Hyper C-VAD and IM at 800 mg/day were used as induction regimen leading to complete remission. Consolidation therapy with alternating high-dose methotrexate plus cytarabine and Hyper C-VAD plus IM were given. She achieved a complete hematologic remission, a complete cytogenetic response and a major molecular response (*BCR-ABL/ABL* IS 0.06%). In April 2007, as she didn't have any HLA-matched donor she underwent high-dose therapy with cyclophosphamide plus total body irradiation (12Gy) conditioning regimen followed by the autologous transplantation of G-CSF collected PBSC. As the *BCR-ABL/ABL IS* rose to 0.09% IM was reintroduced at 600 mg/day leading to a sustained drop of the transcript level to 0.035% six months later (Figure 1). Then the transcript ratio rose rapidly within three months (0.7%; 2.4%; 8.1%). A mutation screen performed in April 2008 revealed an insertion of 12nt in 100% of the BCR-ABL transcripts and no other mutation. This mutation induced the insertion of 4 aa (A, F, G and S) between I293 and K294 (Figure 2). Retrospective analyses revealed that the mutation could be detected by a sensitive RQ-PCR on the cDNA five months before relapse, even while the patient experienced a molecular response (*BCR-ABL/ABL IS* at 0.035%). The proportion of the mutated clone in the setting of minimal residual disease assessed by nested PCR-RFLP analysis was 100% at this time (Figure 1). Comparison of this 12nt with