Prognostic value of Oncogenetic mutations in pediatric T Acute Lymphoblastic Leukemia:

A comparison of UKALL2003 and FRALLE2000T protocols.

Running Title: Prognostic value of oncogenetics in pediatric T-ALL

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Key points:

1. NOTCH1, FBXW7, RAS and PTEN mutations can predict response to treatment in T-ALL
2. Prognosis is protocol dependent and survival can be improved by better risk stratification and more effective treatment

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Abstract:

The mainstay of therapeutic stratification in pediatric T-ALL is end-of-induction Minimal Residual Disease (MRD), but this is increasingly challenged by oncogenetic status. A *NOTCH1*, *FBXW7*, *N/K-RAS*, *PTEN* classifier had marked prognostic impact in the FRALLE2000T protocol but not in the UKALL2003 protocol, whereas we now show the reverse following relapse. We undertook a replication analysis to determine the reasons for this discrepancy. This showed that oncogenetic low-risk (gLoR) combined to MRD<10^{-4} identified 33% of FRALLE2000T and 22% of UKALL2003 patients with a very low incidence of relapse (5y-CIR of 4% vs. 9%, respectively) whereas the UKALL2003 protocol led to better outcome for patients with MRD≥10^{-4} and gHiR patients with MRD<10^{-4}, mainly due to more widespread and effective intensification for patients with MRD≥10^{-4}. Increasing stringency for MRD negativity will not lead to detection of all gHiR patients, since 10% (6/60) were totally MRD negative. Instead, future studies should integrate oncogenetic status into therapeutic stratification and avoid treatment reduction in gHiR, MRD<10^{-4}/neg. patients. These data demonstrate the strength in comparing biomarker discrepancies between protocols as a means to identify optimal personalised therapy.

Introduction:

T-cell acute lymphoblastic leukaemia (T- ALL) accounts for 15% of pediatric ALL. Historically, T-ALLs respond less well than B-Cell precursor ALL (BCP-ALL) and outcome post relapse is very poor. While clinical, cytogenetic and response criteria exist for risk stratification in BCP-ALL, there are few day-one prognostic markers in T-ALL, so stratification is mainly based on early morphological or Minimal Residual Disease (MRD) response, including in the UKALL2003 (2003–2011) (Vora *et al*, 2013;Vora *et al*, 2014) and FRALLE2000T (2000-2010) (Petit *et al*, 2018)

Petit et al. retrospectively applied an oncogenetic N/F/R/P classifier based on *NOTCH1*, *FBXW7* and *N/K-RAS* mutations and *PTEN* mutation/deletion, whereby oncogenetic low risk (gLoR) patients demonstrated *NOTCH1* and/or *FBXW7* mutation (N/F^{mut}) but no *RAS* or *PTEN* abnormalities (R/P^{wt}) and oncogenetic high risk patients (gHiR) were N/F^{wt} and/or R/P^{mut}, to 213 patients achieving complete remission (CR) on the FRALLE2000T protocol. When combined with a d35 MRD cut-off of $0.01\%/10^{-4}$, this classifier identified a low risk group (gLoR and MRD$<10^{-4}$, 33% of patients) with a 5-year disease free survival DFS of 95% and a high-risk group (gHiR and MRD$>10^{-4}$, 23%) with a DFS of 51%.

While these results demonstrated that biomarkers could be useful for treatment stratification in T-ALL, the prognostic significance of N/F^{mut} in pediatric T-ALL is controversial. Several adult and pediatric groups have shown N/F^{mut} to be associated with improved outcome (Asnafi *et al*, 2009;Trinquand *et al*, 2013;Breit *et al*, 2006;Jenkinson *et al*, 2013), whereas other pediatric groups have not (Clappier *et al*, 2010;Zuurbier *et al*, 2010;Kox *et al*, 2010), indicating probable protocol dependence. Similarly, deregulation of the pro-proliferative Ras/Raf/MEK and PTEN/Akt/mTOR pathways have been associated with poor outcome in some studies (Gutierrez *et al*, 2009;Trinquand *et al*, 2013), but not in patients treated on UKALL2003 (Jenkinson *et al*, 2016). PTEN

deletions were found to have more prognostic impact than mutations (Gutierrez *et al*, 2009;Tesio *et al*, 2017).

The aim of the present study was to compare the impact of the N/F/R/P classifier on UKALL2003 and FRALLE2000T T-ALLs in order to identify appropriate therapeutic regimens for gHiR patients, independently from, or within, an MRD-driven protocol. Whereas the latter is universally used for pediatric T-ALL management, this is not feasible for, closely related, T Lymphoblastic Lymphoma (T-LL), when MRD status is rarely available. N/F^{mut} has been shown to be associated with good prognosis in T-LL (Callens *et al*, 2012;Bonn *et al*, 2013) but the prognostic impact of the N/F/R/P oncogenetic classifier has not been published.

Methods:

The N/F/R/P mutational status in UKALL2003 was assessed as described (Jenkinson *et al*, 2013;Jenkinson *et al*, 2016), with denaturing HPLC screening for NOTCH1 exons 25-28 and 34 and FBXW7 exons 8-12. In the FRALLE2000 trial, NOTCH1 exons 25-28 and 34 and FBXW7 exons 9, 10 and 12 were screened directly by Sanger sequencing.

All UKALL2003 variables, patient endpoints and statistical tests were performed as described for FRALLE2000T (Petit *et al*, 2018). All analyses were done with Intercooled Stata, version 14, and R, version 3.4.3. MRD in both techniques was assessed by clone specific IG/TR quantitative PCR, using Euro-MRD group standardised techniques.

Results

Patient characteristics in UKALL 2003 and FRALLE 2000T

The UKALL2003/FRALLE2000T replication analysis and biomarker comparison was restricted to patients in complete remission (CR) at end of induction (EoI), so compared 156 UKALL2003 and 213

FRALLE2000T patients (Table 1, Fig. S1 consort). Demographic and clinical risk groups were similar overall, with the only significant difference being a higher incidence of MRD≥10^{-4} (p=0.017) and a trend for more frequent WBC≥200x10^9/L (p=0.12) in UKALL2003. The frequency of NOTCH1 and/or FBXW7 (64%/62%) and N/K-RAS (9.7%/7.5%) mutations was also very similar, whereas the frequency of PTEN alterations was higher in UKALL2003 (21%/13.1%; p=0.05), although this did not significantly alter the frequency of the oncogenetic classifier overall, with 55% of patients classified as genetic high risk (gHiR), compared to 49% in FRALLE2000T (p=0.3) (Table 1). The gHiR patients in UKALL2003 differed from their gLoR counterpart by more frequent leucocytosis (p=0.03) and MRD≥10-4 (p=0.0004), as in FRALLE2000T (p=0.0006).

Clinical outcome

UKALL2003 showed better overall results, with a DFS and OS of 86% and 90%, respectively, compared to 75% and 79% on FRALLE2000T, essentially due to improved outcome in gHiR and/or MRD≥10^{-4} patients (Table 1 and Fig. 1). On combining these parameters, the probability of relapse and DFS was similar in the gLoR/MRD<10^{-4} group, which corresponded to 34/122 (28%) of UKALL2003 and 71/191 (37%) of FRALLE2000T patients. In contrast, Cumulative Incidence of Relapse (CIR) and DFS were strikingly different in the gHiR/MRD<10^{-4} and MRD≥10^{-4} groups, suggesting that treatment on UKALL2003 was more effective for high-risk patients. This probably explains why the prognostic impact of the oncogenetic classifier was not seen in patients treated on UKALL2003 (p=0.78) unlike FRALLE2000T patients (p<0.0001; Table 1B). The different subgroups, their incidence and Subgroup analysis of UKALL2003 (Fig. S2) did not identify any subgroup for which the genetic classifier had significant impact.

The prognostic impact of MRD≥10^{-4} at EoI on DFS was also not statistically significant on UKALL2003 (p=0.22) but was on FRALLE 2000T (p=0.0006) (Table 1B). The incidence and therapeutic impact of

MRD≥10^{-4} was different in the 2 protocols. Among UKALL2003 patients, MRD≥10^{-4} was found in 28/62 (45%) of gLoR and 44/60 (73%) gHiR p=0.0004, compared to 28/99 (28%) and 49/92 (53%), respectively, of FRALLE patients (p=0.0006) (Table 1). Oncogenetic status in the FRALLE trial had prognostic significance in MRD<10^{-4} (p<0.0001) but not in MRD≥10^{-4} (p=0.15) patients (Petit *et al*, 2018).

Univariate analysis of risk factors in the total cohorts identified only d28 MRD>5% in UKALL2003 but oncogenetics, MRD≥10^{-4}, d21 chemo-resistance (CHR), WBC≥200x10^9/L and male sex in the FRALLE2000T trial (Table 2). Multivariate analysis identified oncogenetics, MRD and WBC≥200 10^9/L as independent high-risk factors in FRALLE2000T (Petit et al.). This was the basis of a 3-parameter classifier, whose distribution and 5-year CIR for both FRALLE2000T and UKALL2003 is shown in Table 3. When regrouped into low, intermediate and high risk categories, there were more high-risk patients in UKALL2003 (40.2% vs. 32.5%) but with, as expected, a lower CIR. The low-risk category demonstrated higher prevalence in FRALLE2000T (30.3% vs. 23%) and a lower CIR (1.7% vs 10.7%).

Comparison of UKALL2003 and FRALLE2000T protocols:

Drug regimens and stratification criteria are detailed in Table S1. The main therapeutic differences were higher doses of anthracyclines, short acting L-Asparaginase and combined Prednisone/ Dexamethasone on FRALLE2000T, compared to Peg-Asparaginase and Dexamethasone only on UKALL2003 (Vora *et al*, 2014). Other differences include cytarabine scheduling, 6-Mercaptopurine loading doses, and CNS prophylaxis. Protocols for high-risk patients also differed. The high-risk Regimen C on UKALL2003 used a continuous moderate/high intensity regime that took patients through induction, consolidation, Capizzi and delayed intensification and maintenance without a break. In the high-risk T2 block of FRALLE 2000T (Petit *et al*, 2018), intensive blocks of treatment induction VEDA1, COPADM, VEDA2, delayed intensification and maintenance were used. The blocks

of treatment VEDA 1 and 2 and COPADM are more like the intensive blocks of treatment used in mature B cell non-Hodgkin's lymphoma, in which short intensive blocks of treatment are highly effective. So while both protocols are intensive, the main difference was that the T2 regimen gave intensive treatment upfront leading to gaps in treatment while waiting for WBC recovery, which may allow resistance to develop.

For risk stratification, UKALL2003 used age, WBC $50x10^9$/L and early morphological BM response to determine treatment intensity: Reg. A (age<10y and WBC<$50x10^9$/L); Reg. B (age>10y, and/or WBC>$50x10^9$/L) or Reg. C (slow early response at d15/8 respectively). Patients with d29 MRD neg. or pos.<10^{-4} and neg. at week 11 were randomised to have 1 or the standard 2 delayed Intensification blocks, with no difference in outcome (Vora *et al*, 2013). Those with d29 MRD≥10^{-4} were randomised between their original Regimen (A or B) vs. C, with improved outcome on the latter (Vora *et al*, 2014). On FRALLE2000T, initial response to prednisolone at d8, CHR at d21 and MRD≥10^{-2} at d35 were used to divide patients into standard risk T1 and high risk T2. The MRD 10^{-4} cut-off, identified retrospectively by Petit et al., was not used and patients with positive MRD 10^{-2}-10^{-4} were treated on the standard T1 arm.

Overall, 56/156 (36%) of UKALL2003 and 112/213 (53%) of FRALLE2000T patients were treated on Reg. C or T2 (Tables 1 and S1). Virtually all (92%) of Reg. C patients had MRD≥10^{-4} compared to only 55% of T2 FRALLE patients, which were mainly intensified based on d8 prednisone and or d21 morphological CHR. Only 19/191 (9%) patients overall, and 18/103 (17%) gHiR FRALLE2000T patients were MRD≥10^{-2} at d35 (Petit *et al*, 2018). Randomisation of MRD≥10^{-4} patients to Reg. C vs. A/B in UKALL2003 explains why the proportion of patients treated on Reg. C was lower than on T2 in FRALLE2000T, despite the higher incidence of MRD≥10-4 (59% vs. 40%). Taken together, intensification to high risk T2, based mainly on non-MRD criteria, was more frequent in

FRALLE2000T than to Reg. C in UKALL 2003, both overall and in gHiR and gLoR oncogenetic groups, but this did not lead to overall therapeutic benefit. Although UKALL2003 patients with d29 MRD≥10^{-4} benefitted from Regimen C (Vora *et al*, 2014) there were too few gHiR patients (n=19) randomised to assess whether Reg. C was beneficial for this subgroup (CIR of 9.1% for 11 pts. vs. a CIR of 12.5% among the 8 who stayed on Reg. A/B, p=0.8).

Effect of oncogenetic markers at Relapse

Of the 156 UKALL2003 patients, 22 (14%) relapsed. In keeping with the lack of prognostic effect of the oncogenetic classifier, the ratio of gLoR:gHiR (45:55%) was the same among cases that did and did not relapse. However, the oncogenetic classifier was highly predictive of outcome after first relapse, when patients in the gHiR subgroup had a significantly inferior OS at 5 years after relapse: gHiR (n= 12), 17% (3-41%) vs. gLoR (n=10), 70% (33-89%), HR=4.14 (1.30-13.19), p=0.016. Of the 213 FRALLE2000T patients, 54 (25%) relapsed. The ratio of gLoR:gHiR was 28%:72%. After relapse, 5y-OS was 26%. It was 24.5% for gHiR (n=15) vs. 31% for gLoR (n=39), HR=1.035 (0.46-2.29), p=0.93. Treatment at relapse was variable, with 11/22 UKALL2003 patients treated on ALLR3 (Parker *et al*, 2010) and 11 off-protocol. At least 10/22 underwent ASCT. Treatment of FRALLE2000 relapsed patients was also variable; 24 were treated according to, ALLREZ BFM-like, COOPRALL 97 or 2007 (Domenech *et al*, 2008), 6 with a sequential BFM-inspired induction treatment, 10 with “myeloid inspired treatment” (mainly FLAG), 1 with nelarabine and 3 with a palliative chemotherapy. Among the 29 FRALLE2000T patients achieving second complete remission, 15 underwent ASCT, with 12 surviving patients.

Discussion:

We here identify a group of pediatric T-ALLs with gLoR and MRD<10^{-4} which correspond to 33% of FRALLE2000T and 22% of UKALL2003 patients that have a very low incidence of relapse, particularly on FRALLE2000T (5y-CIR of 4% vs. 9%) and merit consideration for treatment minimisation. 27 (79%) UKALL2003 patients in this category were treated on Reg. B (based on age and WBC), 5 on Reg. A and the remaining 2 on Reg. C.

We also show that the UKALL2003 protocol is preferable to FRALLE2000T for all MRD≥10^{-4} patients and gHiR patients with MRD<10^{-4}. The latter category in UKALL2003 was treated predominantly on Reg. A/B, and corresponded to 13% (16/122) of patients, which will not all be picked up by even more stringent MRD criteria, requiring total MRD negativity, since 6/16 were completely MRD negative and 10/16 MRD positive <10^{-4}. Our data suggest that intensification of these patients based on oncogenetic status merits consideration. Stratification of gLoR T-ALLs should be based on MRD status. A higher proportion of these patients were MRD≥10^{-4} in UKALL2003 than in FRALLE2000T (28/62, 45% vs. 28/99, 28%). This is similar to the variability in childhood ALL overall, when EoI MRD≥10^{-4} varied from approximately 25-50% (Enshaei *et al*, 2020) and may be explained by differences in induction therapy, timing of d29/35 EoI MRD, MRD technique and/or interpretation of low level MRD positivity. While all MRD laboratories for both protocols use the same qPCR techniques and participate in the same Euro-MRD quality assessments (van der Velden *et al*, 2007), minor differences in practise and interpretation have a disproportionate influence on very low level, borderline MRD positivity. Technological evolution towards MRD evaluation by next-generation flow cytometry, digital droplet PCR and next-generation sequencing will require integration of the most appropriate MRD cut-offs for multi-centre T-ALL trials.

Oncogenetic gLoR status identified UKALL2003 relapsed patients who do well following achievement of second remission (70% 5y OS, vs. 31% for FRALLE2000T gLoR relapsed patients). Although the heterogeneous treatment modalities and small patient numbers limited detailed evaluation, this clearly needs to be evaluated prospectively in large scale trials, ideally with standardised management at relapse.

Extrapolation of these observations to management of patients with T-LL is difficult, since it is not yet clear whether addition of RAS/PTEN status improves the good prognostic value of N/F^{mut} status and virtually no MRD data exists. In the current European Intergroup for Childhood Non-Hodgkin's Lymphoma (EICNHL) LBL2018 trial (NCT-04043494/EudrCT2017-001691-39) patients with N/F^{wt} are randomised to more intensive treatment. The treatment regimen is similar to the leukaemia protocols used to treat T-ALL, with the intensive block more akin to UKALL2003 Reg. C. Although the two protocols differ in details, the intensity of treatment and the schema used is similar. Minimal Disseminated Disease (MDD) positivity ≥1% was reported to identify poor prognosis T-LL when treated on the Children's Oncology Group (COG) A5971 protocol, but this was not confirmed on the subsequent AALL0434 protocol, demonstrating that, as for T-ALL, prognostic markers are treatment dependent and that these differences can contribute to identification of optimal regimens.

In conclusion, the data presented here encourage inclusion of oncogenetic status in therapeutic stratification of immature T lymphoid malignancies in children, in combination with MRD in T-ALL. Recent description of a prognostic index based purely on WBC, MRD response and BCP-ALL cytogenetics, with relevance for T-ALL, would argue against this, presuming that treatment remains general/universal (Enshaei *et al*, 2020). It is not, however, adapted to personalised treatment, which will hopefully be increasingly used in future management of T-ALL.

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Authorship contributions:

1. Manuscript preparation – Taj M, Macintyre E, Moorman A, Petit A, Alby-Laurent F, Baruchel A, Vora A, Moppett J, Asnafi V.
2. Laboratory genetic studies, Mansour M, Gale R, Asnafi V
3. Statistics and analysis – Hamadeh L, Moorman A, Petit A, Chevret S.

(Schwab *et al*, 2010) from Table 1 to add to references

Coustan-Smith and Hayashi references to add

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Figure and Table Legends

Figure 1 – CIR and DFS of UKALL2003 and FRALLE 2000T according to genetic status and MRD

1A – gHiR vs gLoR

1B – gHiR/MRD<10^{-4}, gLoR/MRD≥10^{-4}, gHiR/MRD≥10^{-4}, gLoR/MRD≥10^{-4}

Table 1 Characteristics of the FRALLE2000T and UKALL2003 cohorts by genetic risk and treatment arm.

[1] Significant differences in overall cohort characteristics between FRALLE2000T and UKALL2003 are indicated (*p<0.05)

[2] Both protocols included cases with PTEN mutations and major, but not isolated minor, deletions. In UKALL2003 the 34 cases with PTEN alterations included 17 mutations, 12 deletions and 5 cases with both, compared to 16 mutations, 7 deletions and 5 with both in 28 FRALLE2000T patients.

Table 2: Univariate and Multivariate analysis of risk factors in FRALLE2000T and UKALL2003 trials

[1] Analysis is based on the 191 FRALLE2000T and 122 UKALL2003 cases with oncogenetic classifier and MRD data that achieved CR.

[2] Overall 8/49 UKALL2003 patients with WBC>200 relapsed but 6/8 patients had unknown MRD at end of induction

Table 3

Comparison of prevalence and 5 year Cumulative Incidence of Relapse (CIR) in the Oncogenetic, MRD and WBC based FRALLE200T classifier.

Supplementary data

Figure S1: CONSORT diagram for UKALL2003 and FRALLE2000T (in red) patients

Figure S2: Cumulative risk of relapse associated with the oncogenetic classifier in different patient subgroups among 156 UKALL2003 cases who achieved a complete remission.

Table S1: Differences in treatment between protocols.

[1] Only parameters relevant for T-ALL are cited here.