# Heterozygous carriers of germline c.657_661del5 founder mutation in *NBN* gene are at risk of central nervous system relapse of B-cell precursor acute lymphoblastic leukemia

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## Supplementary Methods

### Patients

Patients were treated in 15 Polish Pediatric Oncology Centers between 2004 and 2015 according to the BFM backbone protocols (ALL-IC BFM 2002 n=214, 37.0% and ALL-IC BFM 2009 n=364, 63.0%). The median observation time was 3.28 years (range 0.11-9.97 years). The allocation of patients to risk groups was based on the criteria stated in ALL IC-BFM 2002/2009 protocols (see below - risk groups stratification). Minimal residual disease (MRD) monitoring is described in detail below. An isolated bone marrow (BM) relapse was defined by the presence of ≥25% lymphoblasts in the BM. An isolated CNS relapse was defined as the presence of MRI/cytologically proven CNS involvement in the absence of BM disease (M1 status with <5% blasts). Combined BM relapse was defined as ≥5% lymphoblasts in the BM in association with the involvement of one or more extramedullary sites[1]. The research protocol was approved by the Ethics Committee of Medical University of Lodz and informed consent was obtained from all participants and/or their parents.

### Comparison with the whole group enrolled to the trials in whole Poland

**ALL-IC 2002**

| Group | Sex (M/F) | Age [years] (median, 25-75%) | WBC count at diagnosis [x$10^3$] (median, 25-75%) | Poor steroid response (yes/no) | CNS involvement at onset (1/2/3) | Hypodiploidy (yes/no) | *ETV6-RUNX1* (yes/no) | *BCR/ABL* (yes/no) | *MLL/AF4* (yes/no) | Risk group (SR/IR/HR) |
|---|---|---|---|---|---|---|---|---|---|---|
| Whole ALL-IC 2002 cohort (n=1737) | 979 /758 | 5.32 (3.20-10.23) | 9.6 (4.0-34.08) | 201/1536 | 1583/83/71 | 41/1547 | 248/886 | 27/1699 | 56/1661 | 563/831/343 |
| Study group (ALL-IC BFM 2002, n=214) | 126/88 | 4.69 (2.66-9.73) | 12.6(4.85-41.2) | 18/184 | 184/13/10 | 5/150 | 25/113 | 5/209 | 12/202 | 60/101/41 |
| p value | 0.483 | 0.211 | 0.297 | 0.262 | 0.343 | 0.638 | 0.315 | 0.401 | 0.089 | 0.115 |

**ALL-IC 2009**

| Group | Sex (M/F) | Age [years] (median, 25-75%) | WBC count at diagnosis [x$10^3$] (median, 25-75%) | Poor steroid response (yes/no) | CNS involvement at onset (1/2/3) | Hypodiploidy (yes/no) | *ETV6-RUNX1* (yes/no) | *BCR/ABL* (yes/no) | *MLL/AF4* (yes/no) | Risk group (SR/IR/HR) |
|---|---|---|---|---|---|---|---|---|---|---|
| Whole ALL-IC BFM 2009 cohort (n=1055) | 564/491 | 4.95 (3.11-8.63) | 12.4 (5.1-42.3) | 104/814 | 833/76/49 | 28/705 | 136/434 | 15/996 | 24/983 | 143/577/217 |
| Study group (ALL-IC BFM 2009, n=364) | 182/182 | 4.49 (2.81-7.39) | 9.0 (4.0-30.1) | 27/389 | 301/15/14 | 12/270 | 51/234 | 5/358 | 17/352 | 63/196/63 |
| p value | 0.254 | 0.478 | 0.121 | 0.006 | 0.087 | 0.889 | 0.057 | 1.000 | 0.051 | 0.127 |

## Treatment

**ALL-IC BFM 2002 protocol**

| Treatment Element/Drug | Treatment Method | Single Dose | Per-Day Dose | Days of Administration |
|---|---|---|---|---|
| Induction | | | | |
| Protocol I' (SR BCP-ALL only) and protocol I (SR T-ALL, all IR and HR patients) | | | | |
| Phase I | | | | |
| Prednisone | PO | | 60 mg/m$^2$ | 1-28 |
| Vincristine | IV | 1.5 mg/m$^2$ (max. 2 mg) | | 8, 15, 22, 29 |
| Daunorubicin | PI over 1 hour | 30 mg/m$^2$ | | 8, 15, 22, 29 |
| L-asparaginase | PI over 1 hour | 5,000 IU/m$^2$ | | 12, 15, 18, 21, 24, 27, 30, 33 |
| Methotrexate | IT | 12 mgd | | 1, 12, 33 |
| Phase II | | | | |
| Cyclophosphamide | PI over 1 hour | 1,000 mg/m$^2$ | | 36, 64 |
| Cytarabine | IV | 75 mg/m$^2$ | | 38-41, 45-48, 52-55, 59-62 |
| 6-mercaptopurine | PO | | 60 mg/m$^2$ | 36-63 |
| Methotrexate | IT | 12 mgd | | 45, 59 |
| Consolidation | | | | |
| Protocol mM (only BCP-ALL, SR/IR) | | | | |
| 6-mercaptopurine | PO | | 25 mg/m$^2$ | 1-56 |
| Methotrexat | PI over 24 hours | 2,000 mg/m$^2$ | | 8, 22, 36, 50 |
| Methotrexate | IT | 12 mgd | | 8, 22, 36, 50 |
| Protocol M (only T-ALL, SR/IR) | | | | |
| 6-mercaptopurine | PO | | 25 mg/m$^2$ | 1-56 |
| Methotrexate | PI over 24 hours | 5,000 mg/m$^2$ | | 8, 22, 36, 50 |
| Methotrexate | IT | 12 mgd | | 8, 22, 36, 50 |
| Block HR-1' (all HR) | | | | |

| | | | | |
|---|---|---|---|---|
| Dexamethasone | PO/IV | | 20 mg/m$^2$ | 1-5 |
| Vincristine | IV | 1.5 mg/m$^2$(max. 2 mg) | | 1, 6 |
| Methotrexate | PI over 24 hours | 5,000 mg/m$^2$ | | 1 |
| Cyclophosphamide | PI over 1 hour | 200 mg/m$^2$ | | 2-4 (five doses, 12-hour intervals) |
| Cytarabine | PI over 3 hours | 2,000 mg/m$^2$ | | 5 (two doses, 12-hour interval) |
| l-asparaginase | PI over 2 hours | 25,000 IU/m$^2$ | | 6, 11 |
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | | 1 |
| Block HR-2’ (all HR) | | | | |
| Dexamethasone | PO/IV | | 20 mg/m$^2$ | 1-5 |
| Vindesine | IV | 3 mg/m$^2$ (max. 5 mg) | | 1, 6 |
| Methotrexate | PI over 24 hours | 5,000 mg/m$^2$ | | 1 |
| Ifosfamide | PI over 1 hour | 800 mg/m$^2$ | | 2-4 (five doses, 12-hour intervals) |
| Daunorubicin | PI over 24 hours | 30 mg/m$^2$ | | 5 |
| L-asparaginase | PI over 2 hours | 25,000 IU/m$^2$ | | 6, 11 |
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | | 1 |
| Block HR-3’ (all HR) | | | | |
| Dexamethasone | PO/IV | | 20 mg/m$^2$ | 1-5 |
| Cytarabine | PI over 3 hours | 2,000 mg/m$^2$ | | 1-2 (four doses, 12-hour intervals) |
| Etoposide | PI over 1 hour | 100 mg/m$^2$ | | 3-5 (five doses, 12-hour intervals) |
| L-asparaginase | PI over 2 hours | 25,000 IU/m$^2$ | | 6, 11 |
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | | 5 |
| Delayed intensification | | | | |
| Protocol II (for arms SR-1, IR-1, HR-2A, HR-2B) | | | | |
| Phase 1 | | | | |
| Dexamethasone | PO/IV | | 10 mg/m$^2$ | 1-21 |
| Vincristine | IV | 1.5 mg/m$^2$(max. 2 mg) | | 8, 15, 22, 29 |
| Doxorubicin | PI over 1 hour | 30 mg/m$^2$ | | 8, 15, 22, 29 |

| | | | | |
|---|---|---|---|---|
| l-asparaginase | PI over 1 hour | 10,000 IU/$m^2$ | | 8, 11, 15, 18 |
| Phase 2 | | | | |
| Cyclophosphamide | PI over 1 hour | 1,000 mg/$m^2$ | | 36 |
| Cytarabine | IV | 75 mg/$m^2$ | | 38-41, 45-48 |
| 6-thioguanine | PO | | 60 mg/$m^2$ | 36-49 |
| Methotrexate | IT | 12 mgd | | 38, 45 |
| Protocol III (for arms SR-2, IR-2, HR-1) | | | | |
| Phase 1 | | | | |
| Dexamethasone | PO/IV | | 10 mg/$m^2$ | 1-14 |
| Vincristine | IV | 1.5 mg/$m^2$ (max. 2 mg) | | 1, 8 |
| Doxorubicin | PI over 1 hour | 30 mg/$m^2$ | | 1, 8 |
| l-asparaginase | PI over 1 hour | 10,000 IU/$m^2$ | | 1, 4, 8, 11 |
| Phase 2 | | | | |
| Cyclophosphamide | PI over 1 hour | 500 mg/$m^2$ | | 15 |
| Cytarabine | IV | 75 mg/$m^2$ | | 17-20, 24-27 |
| 6-thioguanine | PO | | 60 mg/$m^2$ | 15-28 |
| Methotrexate | IT | 12 mgd | | 17, 24 |
| Block HR-1′, HR-2′, HR-3′ (for arm HR-2B only) as in HR consolidation | | | | |
| Interim maintenance therapy | | | | |
| Methotrexate | PO | 20 mg/$m^2$ | | Once per week |
| 6-mercaptopurine | PO | 50 mg/$m^2$ | | Once per day |
| Maintenance therapy | | | | |
| Methotrexate | PO | 20 mg/$m^2$ | | Once per week |
| 6-mercaptopurine | PO | 50 mg/$m^2$ | | Once per day |

Abbreviations: BCP-ALL, B-cell precursor acute lymphoblastic leukemia; HR, high risk; HR-1, high-risk experimental group; HR-1′, consolidation block HR-1′; HR-2′, consolidation block HR-2′; HR-3′, consolidation block HR-3′; HR-2A, HR control arm (Associazione Italiana Ematologia Oncologia Pediatrica option); HR-2B, HR control arm (Berlin-Frankfurt-Münster option); IR, intermediate risk; IR-1, intermediate-risk control group; IR-2, intermediate-risk experimental group; IT, intrathecal; IV, intravenous push; max., maximum; PI, intravenous infusion; PO, by mouth; T-ALL, T-cell acute lymphoblastic leukemia; SR, standard risk, SR-1, standard-risk control group; SR-2, standard risk experimental group.

**CNS-targeted Chemotherapy in ALL IC-BFM 2002 protocol**

| Risk Group | Systemic chemotherapy | | Locoregional chemotherapy | | |
|---|---|---|---|---|---|
| | MD/HD MTX | HD ARA-C | Intrathecal MTX | Triple intrathecal therapy (MTX/ARA-C/Pred) | Sum |
| BCP: SR-1<br>BCP: SR-2<br>BCP: IR-1 | 2g/m$^2$/24h x4 | | 15 + (4) | | 15 + (4) |
| T: SR-1<br>T: IR-1 | 5g/m$^2$/24h x4 | | 11 + (4) | | 11 + (4) |
| T: SR-2 | 5g/m$^2$/24h x4 | | 13 + (4) | | 13 + (4) |
| BCP: IR-2 | 5g/m$^2$/24h x4 | | 15 + (5) | | 15 + (5) |
| T: IR-2 | 5g/m$^2$/24h x4 | | 15 + (5) | | 15 + (5) |
| BCP/T: HR-1 | 5g/m$^2$/24h x2 | 2g/m$^2$ x6 | 11 + (5) | 3 + (1) | 14 + (6) |
| BCP/T: HR-2A | 5g/m$^2$/24h x2 | 2g/m$^2$ x6 | 9 + (6) | 3 + (1) | 12 + (7) |
| BCP/T: HR-2B | 5g/m$^2$/24h x4 | 2g/m$^2$ x12 | 7 + (4) | 6 + (2) | 13 + (6) |

Number in brackets are numbers of additional doses in initially CNS-positive patients (CNS status 3). These patients received/will receive tCRT at age-adjusted dosage.

**Cranial radiotherapy in ALL IC-BFM 2002 protocol**

| Age by time of radiotherapy (years): risk group | pCRT (Gy) – CNS status 1/2 | tCRT (Gy) – CNS status 3 |
|---|---|---|
| **< 1 : all risk groups** | **0** | **0** |
| **≥ 1 < 2 : SR, IR** | **12 (T-ALL only)** | **12** |
| **≥ 2 : SR, IR** | **12 (T-ALL only)** | **18** |
| **≥ 1 < 2 : HR** | **12** | **12** |
| **≥ 2 : HR** | **18** | **18** |

Cases defined as CNS status 2 will receive extra IT MTX on day 18 and 27 but no tCRT. However, SR/IR T-ALL & all non-transplant HR patients with a CNS status 1/2 should receive pCRT.

**ALL-IC BFM 2009 protocol**

| Treatment Element/Drug | Treatment Method | Single Dose | Per-Day Dose | Days of Administration |
|---|---|---|---|---|
| Induction | | | | |
| Protocol I' (SR BCP-ALL only) and protocol I (SR T-ALL, all IR and HR patients) | | | | |
| Phase I | | | | |
| Prednisone | PO | | 60 mg/m$^2$ | 1-28 |
| Vincristine | IV | 1.5 mg/m$^2$ (max. 2 mg) | | 8, 15, 22, 29 |
| Daunorubicin | PI over 1 hour | 30 mg/m$^2$ | | 8, 15<br>(22 and 29 in IR and HR patients) |
| L-asparaginase | PI over 1 hour | 5,000 IU/m$^2$ | | 12, 15, 18, 21, 24, 27, 30, 33 |
| Methotrexate | IT | 12 mgd | | 1, 12, 33 |
| Phase Ib | | | | |
| Cyclophosphamide | PI over 1 hour | 1,000 mg/m$^2$ | | 36, 64 |
| Mesna | 1:1 cyclophosphamide | | | |
| Cytarabine | IV | 75 mg/m$^2$ | | 38-41, 45-48, 52-55, 59-62 |
| 6-mercaptopurine | PO | | 60 mg/m$^2$ | 36-63 |
| Methotrexate | IT | 12 mgd | | 45, 59 |
| Consolidation | | | | |
| Protocol mM (only BCP-ALL nad T SR/IR) | | | | |
| 6-mercaptopurine | PO | | 25 mg/m$^2$ | 1-56 |
| Methotrexate | PI over 24 hours | 2,000 mg/m$^2$ | | 8, 22, 36, 50 |
| Calcium folate | IV | 15mg/m$^2$ | | x3 at 42h, 48h and 54h after the start of MTX infusion |
| Methotrexate | IT | 12 mgd | age-adjusted | 8, 22, 36, 50<br>(1h after the start of MTX infusion) |
| Protocol M (only T-ALL, SR/IR) | | | | |
| 6-mercaptopurine | PO | | 25 mg/m2 | 1-56 |

| | | | | |
|---|---|---|---|---|
| Methotrexate | PI over 24 hours | 5,000 mg/m2 | | 8, 22, 36, 50 |
| Methotrexate | IT | 12 mgd | age-adjusted | 8, 22, 36, 50 |
| Block HR-1' (all HR) | | | | |
| Dexamethasone | PO/IV | | 20 mg/$m^2$ | 1-5 |
| Vincristine | IV | 1.5 mg/$m^2$ (max. 2 mg) | | 1, 6 |
| Methotrexate | PI over 24 hours | 5,000 mg/$m^2$ | | 1 |
| Calcium folate | IV | 15mg/$m^2$ | | x3 at 42h, 48h and 54h after the start of MTX infusion |
| Cyclophosphamide | PI over 1 hour | 200 mg/$m^2$ | | 2-4 (five doses, 12-hour intervals) |
| Cytarabine | PI over 3 hours | 2,000 mg/$m^2$ | | 5 (two doses, 12-hour interval) |
| L-asparaginase | PI over 2 hours | 25,000 IU/$m^2$ | | 6 |
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | age-adjusted | 1h after staring HD MTX infusion |
| Block HR-2' (all HR) | | | | |
| Dexamethasone | PO/IV | | 20 mg/$m^2$ | 1-5 |
| Vindesine | IV | 3 mg/$m^2$ (max. 5 mg) | | 1, 6 |
| Methotrexate | PI over 24 hours | 5,000 mg/$m^2$ | | 1 |
| Calcium folate | IV | 15mg/$m^2$ | | x3 at 42h, 48h and 54h after the start of MTX infusion |
| Ifosfamide | PI over 1 hour | 800 mg/$m^2$ | | 2-4 (five doses, 12-hour intervals) |
| Daunorubicin | PI over 24 hours | 30 mg/$m^2$ | | 5 |
| L-asparaginase | PI over 2 hours | 25,000 IU/$m^2$ | | 6 |
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | age-adjusted | 1h after staring HD MTX infusion |
| Block HR-3' (all HR) | | | | |
| Dexamethasone | PO/IV | | 20 mg/$m^2$ | 1-5 |
| Cytarabine | PI over 3 hours | 2,000 mg/$m^2$ | | 1-2 (four doses, 12-hour intervals) |
| Etoposide | PI over 1 hour | 100 mg/$m^2$ | | 3-5 (five doses, 12-hour intervals) |
| L-asparaginase | PI over 2 hours | 25,000 IU/$m^2$ | | 6 |

| | | | | |
|---|---|---|---|---|
| Methotrexate/cytarabine/prednisolone | IT | 12/30/10 mgd | age-adjusted | 5 |
| Delayed intensification | | | | |
| Protocol II (for arms SR-1, IR-1, HR-2A, HR-2B) | | | | |
| Phase 1 | | | | |
| Dexamethasone | PO/IV | | 10 mg/m$^2$ | 1-21 |
| Vincristine | IV | 1.5 mg/m$^2$ (max. 2 mg) | | 8, 15, 22, 29 |
| Doxorubicin | PI over 1 hour | 30 mg/m$^2$ | | 8, 15, 22, 29 |
| L-asparaginase | PI over 1 hour | 10,000 IU/m$^2$ | | 8, 11, 15, 18 |
| Methotrexate | IT | 12 mgd | In case of initial CNS involvement, age-adjusted | 1, 18 |
| Phase 2 | | | | |
| Cyclophosphamide | PI over 1 hour | 1,000 mg/m$^2$ | | 36 |
| Mesna | 1:1 cyclophosphamide | | | |
| Cytarabine | IV | 75 mg/m$^2$ | | 38-41, 45-48 |
| 6-thioguanine | PO | | 60 mg/m$^2$ | 36-49 |
| Methotrexate | IT | 12 mgd | age-adjusted | 38, 45 |
| Maintenance therapy | | | | |
| Methotrexate | PO | 20 mg/m$^2$ | | Once per week |
| 6-mercaptopurine | PO | 50 mg/m$^2$ | | Once per day |
| Methotrexate | IT | 12 mgd | B-ALL:SR;IR-3 x4; T-ALL with WBC < 100.000 and HR x6, age-adjusted | Every 4 weeks |

**CNS-targeted Chemotherapy in ALL IC-BFM 2009 protocol**

| Risk Group | Systemic chemotherapy | | Locoregional Chemotherapy | | |
|---|---|---|---|---|---|
| | MD/HD MTX | HD ARA-C | Intrathecal MTX | Triple intrathecal therapy (MTX/ARA-C/Pred) | Sum |
| BCP: SR<br>BCP: IR-3 | 2g/m$^2$/24h x4 | | 15 + (5) | | 15 + (5) |
| BCP: IR-4 | 5g/m$^2$/24h x4 | | 11 + (5) | | 11 + (5) |
| T<100000: IR | 5g/m$^2$/24h x4 | | 17 + (5) | | 17 + (5) |
| T>100000: IR | 5g/m$^2$/24h x4 | | 11 + (5) | | 11 + (5) |
| BCP/T HR | 5g/m$^2$/24h x4 | 2g/m$^2$ x12 | 7 + (5) | 6 + (6) | 13 + (11) |

Number in brackets are numbers of additional doses in initially CNS-positive patients (CNS status 3). These patients received/will receive tCRT at age-adjusted dosage.

Patients assigned to arm IR-2 and HR-2 receive 2 extra doses of IT MTX.

**Cranial radiotherapy in ALL IC-BFM 2009 protocol**

| Age by time of radiotherapy (years): risk group | pCRT (Gy) – CNS status 1/2 | tCRT (Gy) – CNS status 3 |
|---|---|---|
| < 1 : all risk groups | 0 | 0 |
| ≥ 1 < 2 : SR, IR | 0 | 12 |
| ≥ 2 : SR, IR | 0 | 18 |
| ≥ 1 :T-ALL + WBC>100000 | 12 | 12 |
| ≥ 2 :T-ALL + WBC>100000 | 12 | 18 |

| | | |
|---|---|---|
| **≥ 1 : HR (except BCP-ALL HR only x PPR)** | **12** | **18** |
| **≥ 2 : HR (except BCP-ALL HR only x PPR)** | **12** | **18** |

**Isolation of DNA**

Genomic DNA was extracted from bone marrow samples using the QIAamp DNA Blood mini kit (Qiagen,Hilden, Germany). The concentration and quality of isolates were determined by ultraviolet spectrophotometry (NanoDrop 8000, Thermo Scientific, Waltham, MA).

**Analysis of c.657_661del5 deletion in *NBN* gene**

The (c.657_661del5, pK219fsX19) mutation of *NBN* gene was identified using site-specific PCR with fluorescent-labeled primer (FAM). The primer sequences were as follows: Forward: 5'FAM-GATTAGATGCTTTTTGTCATTTTGTCCC-3'; Reverse: 5'-CACATTTTCACACTTCATCTTATTTTTTTGGG-3'.

The PCR reaction was carried out according to the manufacturer's protocol (HotStart Taq DNA Polymerase, Qiagen, Germany). The amplification products were quantified and identified by capillary electrophoresis on an ABI-PRISM3130 DNA analyzer (LifeTechnologies, USA). The results were analyzed using Gene Marker v.2.6.3 software (Softgenetics, State College, PA). Positive cases were subsequently confirmed using Sanger sequencing. The analysis of chromatograms were conducted using Sequencher DNA Sequence Analysis Software v. 5.2.0 (GeneCodes Corporation, USA).

**Multiplex ligation-dependent probe amplification (MLPA)**

Identification of the somatic copy number aberrations in leukemic cells was performed as described previously[2].

**Cell lines**

In total, n=16 Epstein-Barr Virus (EBV)-immortalized B cell lines representing wild-type *NBN* variant (n=2), heterozygous deletion 657del5 in *NBN* gene (n=8) and homozygous deletion 657del5 in *NBN* gene (n=6), were used as cell models for the measurement of spontaneous DNA double-strand breaks. The cell lines GM15817, GM15807, GM15819, GM15818, GM15809, GM15813, GM15812, GM15823, GM15790 and GM15842 were purchased from the Coriell Cell Repository. The remaining EBV-immortalized cell lines (MB, HK, AK, AS, ES, EwS) were generated according to Tosato et al.[3]. After thawing, all the EBV-immortalized cell lines were cultured in RPMI1640 medium with 20% FBS. After three passages, cells were suspended in the density 1 $x10^6$/ml in RPMI1640 medium with 20% inactivated FBS, 2mM/ml of glutamine, 200µg/ml gentamicin and ITS (insuline-5µg/ml, transferrine-5µg/ml, sodium selenate 5ng/ml) and incubated at 37°C and in 5% $CO_2$ in a humidified incubator for 72 hours.

**Measurement of DSBs by γH2AX cytometric assay**

After incubation, $1x10^6$ cells were washed in 1 mL of 0.1% BSA-PBS and pelleted (5min at 2000g), followed by fixation in Cytofix/Cytoperm solution (BD Biosciences, USA) for 20 minutes at $4^0$C. Subsequently, cells were washed and permeabilized using Perm/Wash solution (BD Biosciences, USA) for 15 minutes in RT. For intracellular staining of DNA double-stranded breaks, 5µl of mouse anti- γH2AX - Alexa Fluor 488 antibody (pS139) (Cat no. 560445, BD Biosciences, USA) was used at a final dilution 1:10 in Perm/Wash solution. Cells were stained in darkness for 40 minutes in RT, washed and then suspended in PBS with 1% of FBS. In order to perform cell cycle analysis, DNA stain - Hoechst 33342 (Life Technologies, USA) was added to the samples at a final concentration 1 µM, cells were incubated for 15 minutes in 37°C and immediately analyzed on a LSR II cytofluorometer (Becton Dickinson, USA). Cells were assessed for cell cycle phase based on the fluorescence of Hoechst 33342 (BD Biosciences, USA) and for γH2AX levels on a fluorescent-activated cell scanner. Each cell line was cytometrically assayed in two separate experiments.

**Gating strategy**

The cells were acquired using the same sensitivity and compensation parameters in each experiment. From each sample, 100,000 cells were selected on two-dimensional SSC-FSC plots for further analysis. For the assessment of cell cycle phases, a Hoechst-A histogram was used. Apoptotic cells and dublets were eliminated by gating on Hoechst-W/ Hoechst-A graph. The percentage of cells positive for gamma-H2AX were assessed on a two-dimensional FL1- Alexa Fluor 488/ FL2-PE plot using a logarithmic scale and quadrant analysis. In each experiment, cells which were unstained for γH2AX, were used to cut off the signal from autofluorescent cells, and those with nonspecific fluorescence. Markers were placed in a position which allowed at least 98% of non-fluorescent cells to be obtained in the Q4(LL) quadrant. Results were analysed using FlowJo v10.3 software (FlowJo, LLC, USA).

**Statistical analyses**

Nominal variables were given as numbers with appropriate percentages, whereas continuous variables were presented as means with standard deviation. Chi-square tests were used to test associations between categorical variables. The normality of distribution was verified using the Shapiro-Wilk W-test. Normally distributed continuous variables were compared using the Student's t-test or one-way analysis of variance (ANOVA), otherwise Mann-Whitney test was used. *Post-hoc* comparisons were performed using Tukey's HSD test. For the outcome analyses, relapse-free survival (RFS) was defined as the time from diagnosis to relapse. Estimated RFS were presented using Kaplan–Meier survival curves compared using the log-rank test. A multivariate analysis of survival was performed using Cox's proportional hazard regression models. Statistica 12.5 (StatSoft, Tulsa, OK, USA) and VassarStats (www. vassarstats.net/) were used for statistical analyses. P-values $< 0.05$ were considered statistically significant in all tests applied.

Supplementary Table S1. Clinical characteristics of the ALL IC-BFM 2002 cohort

| No. | Age at diagnosis (years) | Sex | WBC count at diagnosis | Risk group[d] | MRD at day 15 (%) | MRD at day 15 >10% | MRD at day 33 (%) | CNS status at onset[d] | CNS relapse | Bone marrow relapse | Testicular relapse | Poor steroid response[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 6.51 | boy | 1.7 | IR | NA | NA | NA | 1 | yes | yes | 0 | 0 |
| 2 | 8.05 | boy | 1.7 | IR | NA | NA | NA | 1 | yes | yes | 0 | 0 |
| 3 | 0.26 | boy | 96.0 | HR | NA | NA | NA | 1 | 0 | 0 | 0 | 0 |
| 4 | 3.29 | boy | 80.5 | IR | 1.54 | (-) | 0.08 | 1 | 0 | 0 | 0 | 0 |
| 5 | 5.56 | boy | 3.3 | SR | <0.01 | (-) | NA | 1 | 0 | 0 | 0 | 0 |
| 6 | 0.46 | girl | 26.0 | IR | <0.01 | (-) | <0.01 | 1 | 0 | 0 | - | 0 |
| Group | Age at diagnosis (median, 25-75%) | Sex (M/F) | WBC at diagnosis (median, 25-75%) | Risk group (SR/IR/HR) | MRD at day 15 (median, 25-75%) | MRD at day 15 >10% (yes/no) | MRD at day 33 (median, 25-75%) | CNS status at onset (CNS 1/2/3) | CNS relapse (yes/no) | Bone marrow relapse (yes/no) | Testicular relapse (yes/no) | Poor steroid response[c] (yes/no) |
| 657del5 heterozygotes (n=6) | 4.42 (0.46-6.51) | 5/1 | 14.65 (1.7-80.5) | 1/4/1 | 0.00 (0.00-1.54) | 0/3 | 0.04 (0.00-0.08) | 6/0/0 | 2/4 | 2/4 | 0/5 | 0/6 |
| 657del wild-type (n=208) | 4.70 (2.66-9.74) | 121/87 | 12.6 (4.86-41.2) | 59/97/40 | 0.19 (0.02-1.20) | 11/122 | 0.00 (0.00-0.01) | 178/13/10 | 4/204 | 25/183 | 2/100 | 18/178 |
| p value | 0.2772[a] | 0.4044[b] | 0.5937[a] | 0.6848[b] | 0.2844[a] | 1.000[c] | 0.5594[a] | 0.6573[b] | 0.0094[b] | 0.1671[b] | 1.000[b] | 1.0000[b] |

[a] Mann–Whitney U test; [b] chi square test; [c] group allocation described in supplementary materials; NA - not available

Supplementary Table S2. Clinical characteristics of the ALL IC-BFM 2009 cohort

| No. | Age at diagnosis (years) | Sex | WBC count at diagnosis | Risk group[c] | MRD at day 15 (%) | MRD at day 15 >10% | MRD at day 33 (%) | CNS status at onset[c] | CNS relapse | Bone marrow relapse | Testicular relapse | Poor steroid response[c] |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 10.04 | boy | 18.0 | IR | NA | NA | 3.6 | 1 | 0 | yes | 0 | 0 |
| 2 | 4.00 | girl | 51.9 | IR | 2.2 | (-) | NA | 1 | 0 | 0 | - | 0 |
| 3 | 4.62 | girl | 6.3 | HR | 22.3 | yes | 1.53 | 1 | 0 | 0 | - | 0 |
| 4 | 6.68 | girl | 6.7 | IR | 3.3 | (-) | <0.01 | 1 | 0 | 0 | - | 0 |
| 5 | 4.35 | boy | 4.1 | IR | 0.11 | (-) | <0.01 | 1 | 0 | 0 | 0 | 0 |
| 6 | 4.09 | girl | 14.0 | IR | 3.94 | (-) | 0.024 | 1 | 0 | 0 | - | 0 |
| 7 | 2.76 | girl | 0.9 | SR | 0.03 | (-) | <0.01 | 1 | 0 | 0 | - | 0 |
| 8 | 4.28 | boy | 75.0 | IR | 0.5 | (-) | <0.01 | 1 | 0 | 0 | 0 | 0 |
| 9 | 0.20 | boy | 78.9 | IR | NA | NA | 0.408 | 1 | yes | yes | yes | yes |
| 10 | 1.99 | girl | 3.5 | IR | 2.6 | (-) | <0.01 | 1 | 0 | 0 | - | 0 |
| 11 | 5.26 | girl | 4.6 | SR | <0.01 | (-) | <0.01 | 1 | 0 | 0 | - | 0 |
| Group | Age at diagnosis (median, 25-75%) | Sex (M/F) | WBC at diagnosis (median, 25-75%) | Risk group (SR/IR/HR) | MRD at day 15 (median, 25-75%) | MRD at day 15 >10% (yes/no) | MRD at day 33 (median, 25-75%) | CNS status at onset (CNS 1/2/3) | CNS relapse (yes/no) | Bone marrow relapse (yes/no) | Testicular relapse (yes/no) | Poor steroid response[c] (yes/no) |
| 657del5 heterozygotes (n=11) | 4.28 (2.76-5.26) | 4/7 | 10.1 (4.1-51.9) | 2/7/1 | 2.20 (0.11-3.30) | 1/8 | 0.00 (0.00-0.41) | 11/0/0 | 1/10 | 2/9 | 1/3 | 1/10 |
| 657del wild-type (n=353) | 4.50 (2.82-7.39) | 178/175 | 8.8 (4.0-27.4) | 61/189/62 | 0.34 (0.05-3.23) | 50/300 | 0.00 (0.00-0.01) | 290/15/14 | 8/345 | 22/331 | 3/144 | 26/289 |
| p value | 0.4744[a] | 0.5425[b] | 0.9622[a] | 0.7618[b] | 0.5100[a] | 1.000[c] | 0.3905[a] | 0.5780[b] | 0.2437[b] | 0.1592[b] | 0.0774[b] | 0.6051[b] |

[a] Mann–Whitney U test; [b] chi square test; [c] group allocation described in supplementary materials; NA - not available

Supplementary Table S3. Molecular characteristics of the ALL IC-BFM 2002 cohort

| No. | HYPODIPLOIDY | HYPERDIPLOIDY | *MLL* | *ETV6-RUNX1* | *BCR/ABL* | *IKZF1* | *IGHD* | *CDKN2A* | *CDKN2B* | *PAX5* | *CRLF2* | *SHOX* | *CSF2RA* | *P2RY8* | *IL3RA* | *JAK2* | *BTG-1* | *EBF1* | *ETV6* | *RB1* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | NA | 0 | 0 | NA | NA | NA | NA | NA | NA | NA | NA | 0 | NA | NA | NA | NA | NA |
| 2 | 0 | yes | 0 | 0 | 0 | 0 | NA | NA | NA | NA | 0 | 0 | 0 | NA | NA | NA | 0 | 0 | 0 | 0 |
| 3 | 0 | 0 | yes | 0 | 0 | 0 | NA | 0 | 0 | 0 | 0 | 0 | 0 | NA | 0 | NA | 0 | 0 | 0 | del |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | yes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 6 | 0 | 0 | yes | 0 | 0 | 0 | 0 | 0 | 0 | del | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Group | (yes/no) | | | | | Deletion/no deletion | | | | | | | | | | | | | | |
| 657del5 heter. (n=6) | 0/6 | 2/6 | 2/4 | 0/5 | 0/6 | 0/6 | 0/3 | 0/4 | 0/4 | 1/3 | 0/5 | 0/5 | 0/5 | 0/3 | 0/5 | 0/3 | 0/5 | 0/5 | 0/5 | 1/4 |
| 657del wild-type (n=208) | 5/144 | 37/112 | 10/198 | 25/108 | 5/203 | 36/163 | 8/65 | 54/130 | 42/142 | 38/146 | 8/171 | 10/169 | 15/164 | 8/81 | 16/165 | 4/84 | 12/167 | 10/169 | 40/139 | 12/168 |
| p value[a] | 1.000 | 1.000 | 0.036 | 0.585 | 1.000 | 0.586 | 1.000 | 0.580 | 0.577 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 0.587 | 0.308 |

[a] Fisher's exact test (two-tailed), NA-not assessed, del – deletion

Supplementary Table S4. Molecular characteristics of the ALL IC-BFM 2009 cohort

| No. | HYPODIPLOIDY | HYPERDIPLOIDY | *MLL* | *ETV6-RUNX1* | *BCR/ABL* | *IKZF1* | *IGHD* | *CDKN2A* | *CDKN2B* | *PAX5* | *CRLF2* | *SHOX* | *CSF2RA* | *P2RY8* | *IL3RA* | *JAK2* | *BTG-1* | *EBF1* | *ETV6* | *RB1* |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | del | 0 | 0 | 0 | del | 0 | 0 | del | 0 | 0 |
| 2 | 0 | yes | 0 | 0 | 0 | 0 | NA | del | 0 | 0 | del | del | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 3 | 0 | yes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 4 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 5 | 0 | 0 | 0 | NA | 0 | 0 | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | del | 0 |
| 6 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 7 | 0 | yes | 0 | 0 | 0 | del | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 8 | 0 | 0 | 0 | 0 | 0 | del | del | del | del | del | 0 | 0 | del | del | del | del | 0 | 0 | 0 | 0 |
| 9 | 0 | 0 | yes | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 10 | 0 | yes | 0 | 0 | 0 | 0 | NA | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | del | 0 |
| 11 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | del | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Group | (yes/no) | | | | | Deletion/no deletion | | | | | | | | | | | | | | |
| 657del5 heter. (n=11) | 0/11 | 4/11 | 1/10 | 0/10 | 0/11 | 2/9 | 1/6 | 2/9 | 1/10 | 2/9 | 2/9 | 1/10 | 1/10 | 1/10 | 2/9 | 1/10 | 0/11 | 1/10 | 2/9 | 0/11 |
| 657del wild-type (n=353) | 12/270 | 78/210 | 16/342 | 51/234 | 5/347 | 52/290 | 32/243 | 99/246 | 87/258 | 71/269 | 26/311 | 26/311 | 22/315 | 31/294 | 28/309 | 21/303 | 34/303 | 20/317 | 86/251 | 25/312 |
| p value[a] | 1.000 | 1.000 | 0.409 | 0.219 | 1.000 | 0.676 | 0.586 | 0.735 | 0.305 | 1.000 | 0.219 | 0.594 | 0.534 | 1.000 | 0.243 | 0.532 | 0.610 | 0.501 | 0.737 | 1.000 |

Supplementary Table S5. Cox regression model for RFS.

| | Effect | p | Hazard ratio (95%CI) |
|---|---|---|---|
| *MLL* rearrangement | present | 0.000 | 6.94 (2.78-17.35) |
| MRD at day 15 (%) | | 0.009 | 1.04 (1.01-1.06) |
| Steroid resistance | present | 0.044 | 3.13 (1.03-9.49) |

Supplementary Table S6. Cox regression model for bone-marrow RFS.

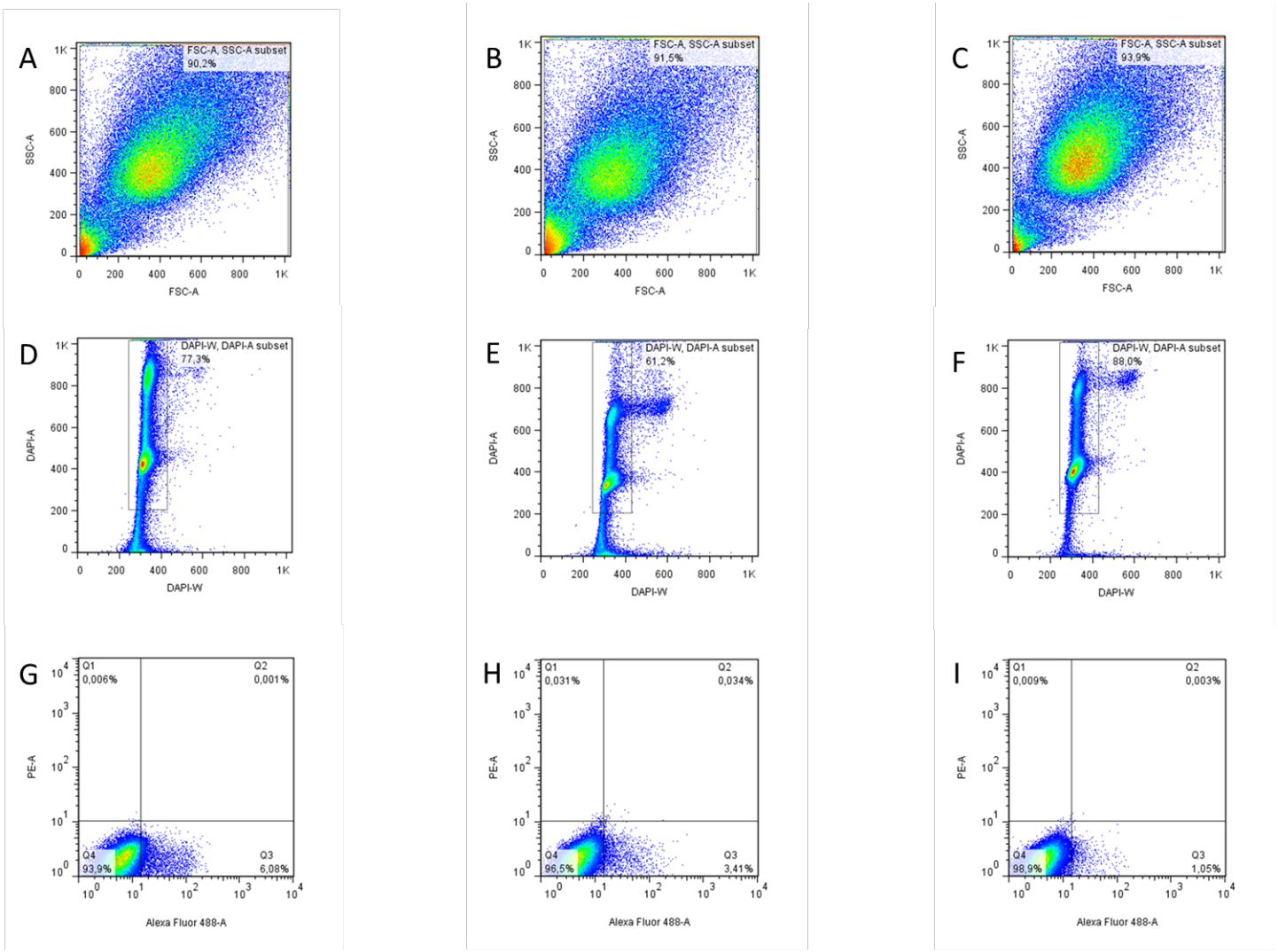
| | Effect | p | Hazard ratio (95%CI) |
|---|---|---|---|
| *MLL* rearrangement | present | 0.001 | 5.96 (2.11-16.80) |
| MRD at day 15 (%) | | <0.001 | 1.05 (1.02-1.07) |
| WBC at diagnosis ($x10^3$) | | 0.028 | 1.01 (1.00-1.02) |

Supplementary Table S7. Cox regression model for CNS RFS.

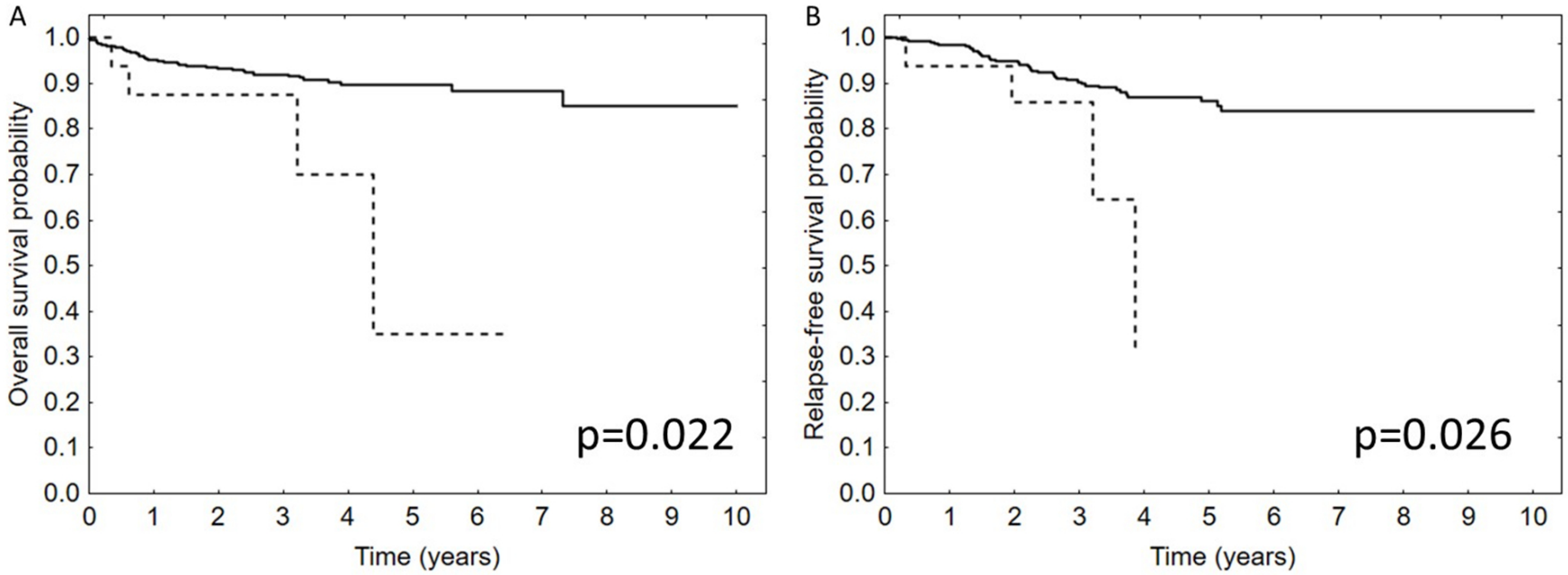
| | Effect | p | Hazard ratio (95%CI) |
|---|---|---|---|
| *MLL* rearrangement | present | <0.001 | 14.85 (4.21-52.26) |
| *NBN* c.657_661del5 status | heterozygous deletion | 0.004 | 7.98 (1.87-33.99) |

Supplementary Table S8. Cox regression model for RFS in ALL-IC BFM 2002 protocol.

| | Effect | p | Hazard ratio (95%CI) |
|---|---|---|---|
| Age (years) | | 0.001 | 1.10 (1.04 - 1.16) |
| WBC at diagnosis ($x10^3$) | | 0.075 | 1.00 (1.00-1.01) |
| *NBN* c.657_661del5 status | heterozygous deletion | 0.017 | 4.32 (1.31-14.32) |




Supplementary Figure S1. Dot plots of a representative flow cytometry analysis of γ-H2AX expression performed on the EBV-immortalized cell lines harbouring wt-NBN (C, F, I), heterozygous 657del5 in *NBN* gene (B, E, H), homozygous 657del5 in *NBN* gene (A, D, G). Cells were first gated on SSC-FSC (A, B, C), than DAPI-A-DAPI-W (D, E, F) and PE-Alexa Fluor 488 (G, H, I).




Supplementary Figure S2. Kaplan-Meier curves for (A) overall survival and (B) relapse-free survival. Patients with 657del5 heterozygous deletion in *NBN* gene are represented by dashed line, wild-type patients are represented by black solid line.

**RISK GROUPS STRATIFICATION**

STANDARD-RISK GROUP (SR) - all criteria must be fulfilled

- At day 8: < 1,000 blasts/µL in the peripheral blood
- Age ≥ 1 year – < 6 years
- Initial WBC < 20,000/µL
- M1/ M2 marrow on day 15 (or FC MRD < 0,1%, if available - for ALL IC-BFM 2009 only)
- no M 2/3 marrow on day 33

INTERMEDIATE-RISK GROUP (IR)

- ALL IC-BFM 2002 – at day 8 < 1,000 blasts/µL in the peripheral blood and the following criteria:
    - Age < 1 year or ≥ 6 years and/or WBC ≥ 20,000/µL
    - M1 or M2 bone marrow on day 15
    - M1 bone marrow on day 33
    - M3 marrow on day 15 and M1 marrow on day 33

- ALL IC-BFM 2009 - All patients who are not stratified to SR or HR are intermediate risk patients.

HIGH-RISK GROUP (HR) – at least one of the following

- IR and M3 marrow on day 15 (or FC MRD >10%, if available - for ALL IC-BFM 2009 only)
- SR if available FC MRD >10% (for ALL IC-BFM 2009 only)
- at day 8 ≥ 1,000 blasts/µL in the peripheral blood
- M2 or M3 marrow on day 33
- Translocation t(9;22) [BCR/ABL] or t(4;11) [MLL/AF4]
- Hipodiploidy ≤ 44 (for ALL IC-BFM 2009 only)

**Minimal residual disease (MRD) monitoring**

Minimal residual disease (MRD) at day 15 and day 33 was measured by flow cytometry with EuroFlow eight-color antibody panels according to the protocols of the EuroFlow Consortium[4]. Samples were processed according to the lyse/wash protocol, with 1 FACS Lyse used for erythrocyte lysis (Becton Dickinson, San Jose, CA) and analysed with a FACS Canto II flow cytometer (Becton Dickinson, San Jose, CA). To ensure a sensitivity level of at least $10^4$, low-cellular samples were first subjected to erythrocyte lysis in 1x ammonium chloride solution (Pharm Lyse, Becton Dickinson, San Jose, CA) in a *bulk lysis protocol*. The reproducibility of the obtained results was ensured by complying with the standard operating procedures developed by EuroFlow based on daily quality assessment with fluorescent beads (Sphero Rainbow Calibration Particles, Spherotech, Lake Forest, IL)[4]. For data analysis, FACS Diva 6.1 software (Becton Dickinson, San Jose, CA) was used.

**POOR STEROID RESPONSE (**ALL IC-BFM 2002 and ALL IC-BFM 2009)

Patients with ≥ 1,000 blasts/µL in the peripheral blood) on day 8, after seven days of prednisone therapy, were considered as prednisone-poor responders

**CNS status (**ALL IC-BFM 2002 and ALL IC-BFM 2009)

CNS status 1

• No clinical evidence of CNS disease, including cranial nerve palsy, that would be unequivocally attributable to leukaemia.

• No imaging (CT/MRI) evidence of a CNS abnormality that would be unequivocally attributable to leukaemia.

• Normal fundoscopic finding.

• Blast-free CSF along with absence of any other evidence of CNS leukaemia.

CNS status 2

• Blasts unambiguously identified and RBC : WBC ≤ 100:1 on cytospin preparation of CSF with a cell count of ≤ 5/µL. With this RBC : WBC ratio, the LP is considered to be non-traumatic, and the CSF uncontaminated with blood

• Lymphoblasts identified and RBC : WBC > 100:1 on cytospin preparation of CSF. With this RBC : WBC ratio, the LP is considered to be traumatic, and the CSF contaminated with blood

• A traumatic LP (blood-contaminated CSF) is combined with an initial WBC of > 50,000/µL.

CNS status 3

• A mass lesion in the brain and/or meninges on CT/MRI.

• Cranial nerve palsy unrelated to other origin, even if the CSF is blast-free, or no circumscribed space-occupying lesion could be demonstrated within the neurocranium on MRI/CT scan

• Pure retinal involvement, i.e. with a blast-free CSF, and no mass on MRI/CT scan

Further details regarding stratification and treatment may be found in the full texts of ALL IC-BFM 2002 report[5] and ALL IC-BFM 2009 protocol available at (http://www.bialaczka.org/wp-content/uploads/2016/10/ALLIC_BFM_2009.pdf)

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