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**Origins of iAMP21 in children who later developed paediatric acute lymphoblastic leukemia: an investigation of neonatal blood spots.**

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**Running Head:** Prenatal Origins of iAMP21 in childhood ALL

**Disclosures:** The authors have no competing interests

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## Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding authors on reasonable request.

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## Authors contribution

- ***G. Leijonhufvud:** Conceptualization, data analysis, writing of the manuscript
- ***V. Ljungström:** Bioinformatics and WGS analysis

- **R.-M. Amini, I. Saft:** Facilitated retrieval of diagnostic material and ensured samples could be sent to London for analysis
- **B. Gustafsson#, AM. Ford#:** Conceptualization, supervision, NBS analysis, writing of the manuscript and review. [#] These authors contributed equally to this work

All authors have read and approved the final manuscript.

Certain genetic subtypes of paediatric acute lymphoblastic leukaemia (ALL) such as *KMT2A* rearrangements (*KMT2A-r*), *ETV6::RUNX1* fusion and high hyperdiploidy, can originate *in utero* (1-3). Recurrent chromosomal abnormalities correlate strongly with prognosis and therapeutic response and high-risk lesions such as *KMT2A-r, BCR::ABL1*, hypodiplody and intrachromosomal amplification of chromosome 21 (iAMP21) confer adverse outcomes and guide treatment intensification (1, 4-6). Interactions among inherited predisposition, early-life infectious exposures, and abnormal cytokine signalling have been proposed to contribute to the emergence and expansion of leukaemic clones (7-9). The developmental timing and cellular origins of iAMP21-ALL however remain incompletely defined (10). Clarifying whether these changes arise prenatally or postnatally is essential for understanding disease initiation and the subsequent clonal evolution of this subtype of paediatric ALL.

iAMP21 is observed in 2% of paediatric B-cell precursor (BCP) ALL cases. Clinically, iAMP21 is associated with older age at diagnosis (median 9 years) and low white blood cell counts (10, 11). Constitutional aberrations of chromosome 21 translocations, particularly the Robertsonian translocation, rob(15;21)(q10;q10)c, and ring chromosome 21, r(21)c, are associated with a markedly increased risk of developing iAMP21-ALL, often due to genomic instability and chromothripsis initiated at the dicentric Robertsonian or ring chromosome (12, 13). However, in a significant number of patients, the iAMP21 structure arises sporadically without a known underlying constitutional abnormality (10). Generation of the iAMP21 chromosome is an early clonal event (10), however the important question of how protracted the preclinical natural history can be in these older children remains unresolved.

In the present study, using patient-matched neonatal blood spots (NBS) collected at birth, we retrospectively investigated the presence of iAMP21 aberrations in two cases of paediatric ALL diagnosed at ages 8.3 and 14.4 years, respectively. At diagnosis, both cases presented

with >60% blasts and thrombocytopenia but a Robertsonian translocation or isodicentric chromosome was not detected (Supplementary Table 1). Initial treatment was administered according to the NOPHO intermediate-risk protocol. Patient 1 experienced a relapse four years after diagnosis and was subsequently treated under the IntReALL 2010 protocol. Following a second remission, the patient underwent hematopoietic cell transplantation (HCT) and remains alive 10 years after the initial diagnosis. In contrast, Patient 2 underwent HCT but died due to post-transplant complications (Supplementary Table 1). This study was approved by the Regional Ethical Review Board in Stockholm (DNR2020-00774). Clinical and genetic data at diagnosis (Supplementary Table 1) were obtained from the Swedish Childhood Cancer Registry, a nationwide quality registry.

In the first patient (iAMP21 patient 1), using whole-genome sequencing (WGS) followed by integrative genomics viewer analysis (IGV), we randomly identified several complex chromosome 21 aberrations at diagnosis, including a small deletion at chr21:40585321–40585440 (-119 bp) and a complex translocation detected at high-depth and predicted between chr21:16859181–17010117. Using PCR across the fusion regions, we were able to fully backtrack the small 119bp deletion detected at diagnosis to each NBS punch of the individual (Figure 1 A, B). Possible sequence microhomology at the breakpoints suggests involvement of microhomology-mediated end joining (MMEJ) in the repair process (14). The lack of an expected germline band for this region suggests either a germline homozygous deletion, patient polymorphism or a clonal event acquired prenatally. The breakpoint sequences of the diagnostic translocation (~141 kb apart) suggested an intergenic inversion or complex rearrangement, which was confirmed by PCR of the diagnostic DNA (Figure 1C, D). The variant allele fraction (~12–13%) further suggested either a subclonal population at diagnosis or a low-level mosaic germline event. Notably, only one of three NBS punches tested, produced a faint PCR product that had an identical DNA sequence to the diagnostic

rearrangement (Figure 1 E, F). This result is consistent with the existence of the rearrangement existing in only a subclonal population of cells prior to birth.

Following IGV analysis of diagnostic DNA from patient 2, we again randomly identified a chromosome 21 translocation (chr21:26001836–26002391) predicted to represent a 540 bp inverted segment. PCR amplification and sequencing confirmed this inversion at diagnosis (Figure 2 A). All three NBS punches contained the same rearrangement, indicating that this region was broken, inverted and rejoined before birth. IGV data at diagnosis suggests that the translocation was present in all blood cells and could be a germline event (Supplementary Figure 1 A). Strikingly, a 69 bp duplication event also observed in patient 2 at diagnosis (chr21:21:43292455-43292524) had likely progressed postnatally from a small deletion present in the NBS before birth and at the same location (Figure 2 B), confirming clonal evolution. We similarly backtracked a small deletion at chr21:41540011–41540460 and confirmed its presence at birth in each NBS (Supplementary Figure 1 B). Sequence microhomology at the breakpoints of both these aberrations was again suggestive of MMEJ repair. Taken together, these results suggest that early genetic variants predispose to the acquisition of more dominant rearrangements and confirm that additional postnatal events and clonal evolution serve to drive overt iAMP21 leukaemia (Supplementary Figure 2).

Using WGS data and the MiXCR tool for immune repertoire analysis (15), we next assessed the clonality of *IGH* rearrangements in these two patients. Both cases showed clonal rearrangements at diagnosis involving one or both components of the IGH (VDJ) or IGL (VL) repertoires. In patient 1, after WGA of the NBS followed by patient-specific Q-PCR, we identified a matching, patient-specific *VDJ* rearrangement sequence identical to that observed at diagnosis, including the non-templated nucleotide regions inserted between the *VH* and *DH* and *DH* and *JH* segments respectively (Figure 3A). Backtracking of this clonotypic *IGH* rearrangement to the NBS further indicates the presence of a pre-leukaemic

clone at birth. In contrast, for patient 2 we did not detect the diagnostic patient-specific clonal *VDJ* rearrangement in any of the 3 NBS punches analysed (Figure 3B). Taken together, these findings demonstrate that selected iAMP21-associated chromosomal aberrations and clonal *IGH* rearrangement can be traced back to neonatal blood spots, providing evidence of a prenatal origin for at least some pre-leukaemic clones.

Backtracking of both iAMP21 patients, confirmed that some chromosome aberrations were detectable in both the diagnostic sample and the NBS, indicating either a germline origin or a somatic origin *in utero*, where blood cells are rapidly proliferating. These microdeletions and simple inversions on chromosome 21 can be germline only if they are small, simple, and do not involve essential 21q22 genes. We note that the small deletions and insertions we have analysed here, do occur in introns. Larger deletions, inverted duplications, and complex 21q rearrangements (such as iAMP21) are incompatible with germline events and must be somatic aberrations and, given the extensive latency to overt leukaemia, they are likely to be postnatal. In patient 1, the complex translocation identified at diagnosis was not present in all NBS punches examined, perhaps indicative of a secondary 'hit' rather than a germline event. An identical, patient-specific *VDJ* rearrangement to that observed at diagnosis was also detected in the NBS of this patient, providing more evidence for the presence of a pre-leukaemic clone at birth. In the second patient a small deletion detected in the NBS had progressed to a duplication event by diagnosis of iAMP21-ALL. These findings indicate that iAMP21 can arise through multiple developmental routes: either as a predisposing early embryonic (potentially germline) event, or as a clonally restricted prenatal lesion that later expands to clinical leukaemia.

A prenatal mutation in an early hematopoietic stem cell alone is insufficient to induce leukaemia. iAMP21 development is instead driven by chromosomal instability (10), often leading to amplification of the *RUNX1* region on chromosome 21, disruption of normal B-cell

maturation, and emergence of pre-B-cell leukaemic features. Accumulation of secondary mutations confers a proliferative advantage, resulting in aggressive clonal expansion and eventual dominance of the leukaemic clone in the bone marrow (10).

The dual patterns observed for iAMP21 raise the possibility that this lesion does not fit neatly into a single developmental category. Instead, iAMP21 may represent a biologically heterogeneous subtype, with prenatal initiation occurring in some patients and postnatal acquisition in others, but with clonal evolution occurring in both scenarios. The positive backtracking results for these older children with iAMP21 ALL, show that a very protracted covert natural history of iAMP21 ALL can occur prior to disease appearance and provides evidence that the postnatal latency in this subtype of ALL can be a decade or longer.
This study is limited by the small sample size, reflecting the rarity of both iAMP21 and the availability of suitable neonatal samples. While the sensitivity of our assays was high, extremely small pre-leukaemic clones may remain below the limit of detection. Larger population-based studies, ideally incorporating single-cell sequencing or ultra–deep targeted assays (10), family studies and sampling from different tissues will all be essential to determine the full spectrum of developmental origins for these lesions. Nevertheless, our data provide new and direct molecular evidence that contributes to the longstanding question of when specific paediatric leukaemia begins. By integrating neonatal sampling with modern genomic tools, we demonstrate that iAMP21 can initiate prenatally either as a germline or early clonal event or postnatally, so highlighting distinct developmental trajectories among ALL subtypes.

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# Figure Legends

## Figure 1

**Complex translocations and deletions within chromosome 21 of iAMP21 patient 1 at diagnosis backtrack to birth and show clonal evolution.**

A: A selected deletion at chr21:40585321–40585440 (-119bp) in iAMP patient 1 at diagnosis is present in the NBS at birth. Primers in grey blocks, prospective deleted sequences in yellow.

B: Identical fusion sequence to the deletion identified in patient 1 at diagnosis is observed in DNA extracted from the NBS punch. Boxed sequences show possible microhomology.

C: DNA sequence acquired from WGS of diagnostic DNA shows a complex translocation between (chr21:16859181-17010117). Primers in grey blocks.

D: DNA sequence confirms the expected translocation after PCR of diagnostic DNA from the patient.

E: Interrogation of 3 separate NBS punches suggests the presence of the translocation to be only in a subclone of cells within a single NBS. All samples (except diagnosis 1.dx) were isolated from NBS DNA. The *MLL* gene acts as a loading control.

F: Identical fusion sequence to the diagnostic translocation of patient 1 is observed as a subclone within a single NBS punch.

**Figure 2**

**Inversions and deletions within iAMP patient 2 at diagnosis backtrack to birth and show clonal evolution.**

A: The break and inversion of a translocation within chromosome 21 identified at diagnosis for patient 2 (chr21:26001836–26002391) is already present in the NBS at birth. Boxed sequences show possible microhomology.

B: The duplication event present in patient 2 at diagnosis (chr21:21:43292455-43292524) likely arises postnatally and subsequent to a small deletion already present in the NBS. Primer sequences in yellow blocks, potential duplicated region in red.

**Figure 3**

**IGH clonality by VDJ rearrangement analysis and patient-specific Q-PCR.**

A: Presence of a *IGH VDJ* pre-leukaemic clone in the NBS of patient 1 with DNA sequence identical to that identified at diagnosis (NBS3, including random base insertion, *N2*).

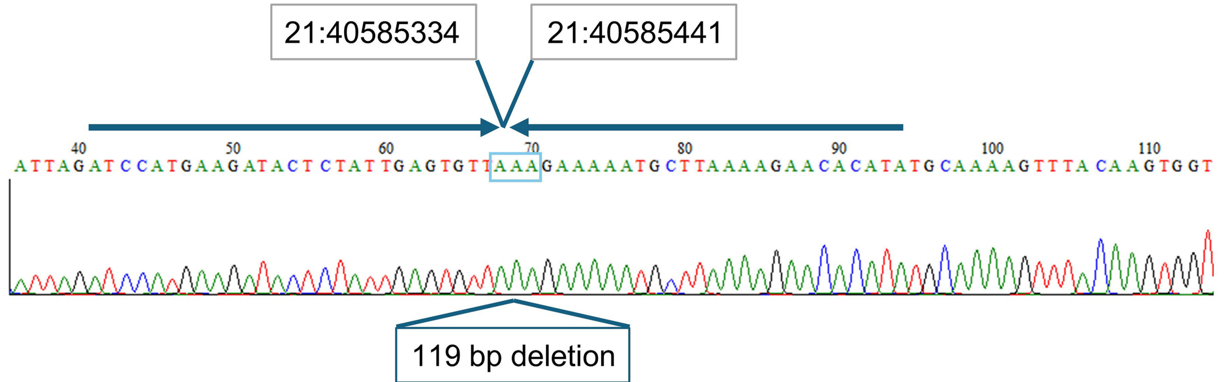
B: No clonal IGH sequences were found in the NBS of patient 2 at birth.

RFU is Relative Fluorescence Units.

**A**

ACAAAAAGGGTTGGAGAGATGGAAATATTGGAGCTCATTAGATCCATGAAGATACTCT
ATTGAGTGTTAAAGAAAAATGCTGCGTGAACCCGGGAGGCGGAGCTTGCAGTGAGCCG
AGATCCCGCCACTGCACTCCAGCCTGGGCGACAGAGCAAGTATGACTCCGTCTCAAAA
AAAAAAAAAAAAAAAAAAAAGAAAAATGCTTAAAAGAACACACAAAAGTTTACAAGTGGTA
ATAGATCTACAAGTCTTTTTTAAAGCACAGCAAAATACATGGGCAATTTCAGAGAGGATC
TATTTTTTCTTGTTTAATGCTGAATGAAGAATTATTCCACCCACCTCTCAGCAGTAGTGAC
TGACCAAATAAGAGGGTGTT

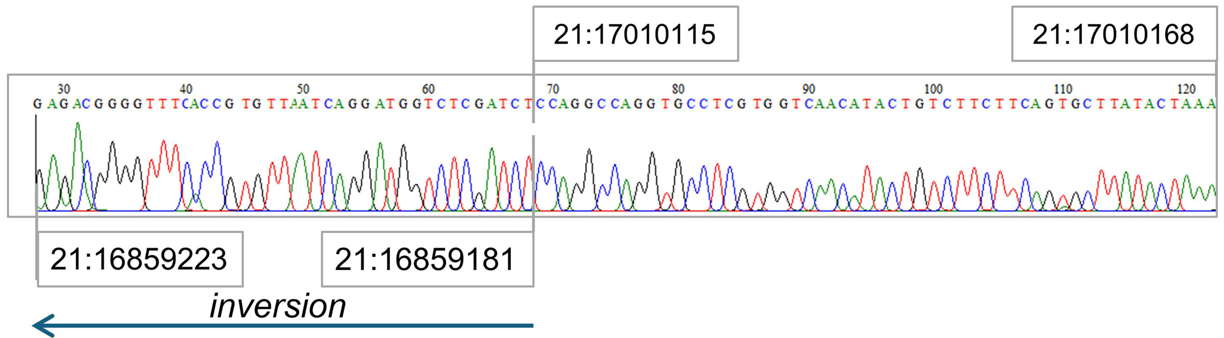
**B**



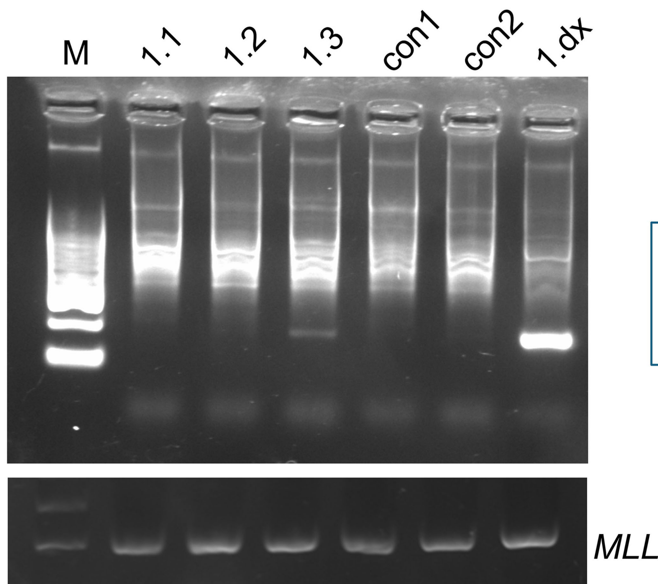

**C**

CTCCCAAGTAGCTGGGGTTACAGGTGCCCGCCACGACGCCCGGCTAGTTTTTGTATTTTCAGAGAG
ACGGGGTTTCACCGTGTTAATCAGGATGGTCTCGATCT//CCAGGCCAGGTGCCTCGTGGTCAACATAC
TGTCTTCTTCAGTGCTTATACTAAAGAATGGTCAGGGTGTTTTACAGCAG

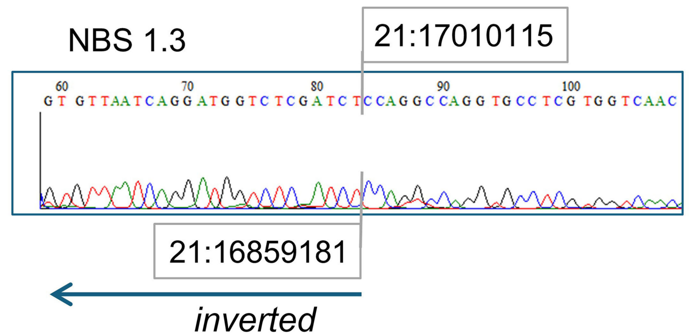
**D**




**E**




**F**



**A**

Control

21:26001843

21:26002391

TTGATTCTAATTAGCCAATCAAAGACAAGGATCCATCACATCTAGGCATGGC .......... AACACCTAGG

Pt2_NBS

21:26001843

21:26002391

inverted sequence

21:26001844

TTGATTCTAATTAGCCAATCAAAGACAAGGATCCACAATGTCAATACAAATGGGGACTAACTATTGTTTT

**B**

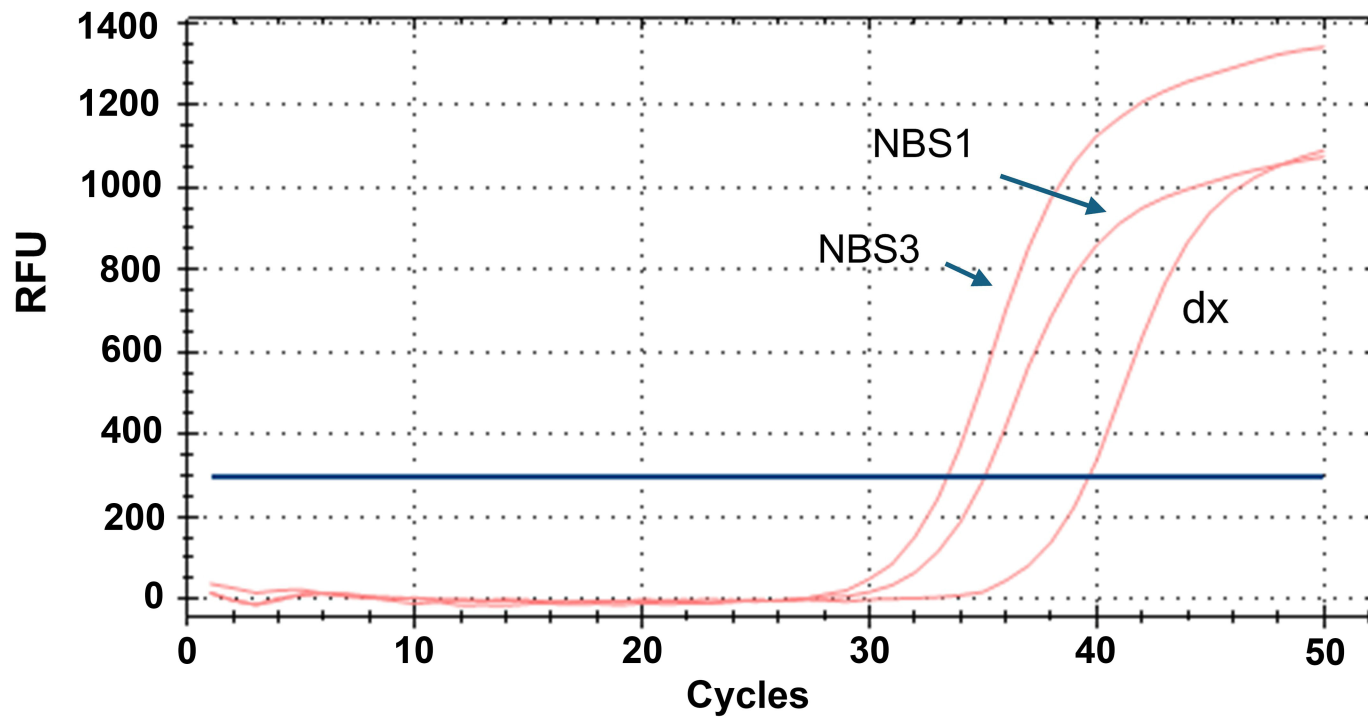
Pt2_NBS

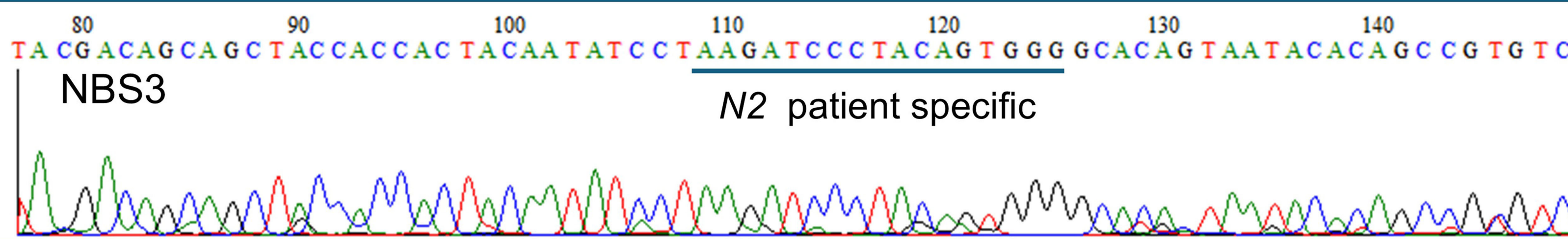
28bp deletion

Pt2_diagnosis

duplication

A




B

# Supplementary Material

**for**

**Origins of iAMP21 in children who later developed paediatric acute lymphoblastic leukaemia: An investigation of neonatal blood spots.**

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## Supplementary Table 1

| Patient ID | Year at Diagnosis | Age, years /Sex | Karyo-type | Leucocyte count ($x10^9/L$) | Platelet count ($x10^9/L$) | NOPHO protocol | Relapse? Yes=1, No=0 | Second Third Treat-ment | Alive =0 Dead =1 |
|---|---|---|---|---|---|---|---|---|---|
| 1 | Pre-B-ALL 2016 | 8.4/ M | See below * | 14.6 | 36 | IR, high MRD day29 Block Therapy | 1, BM and CNS relapse | IntReALL 2010 protocol HCT | 0 |
| 2 | Pre-B-ALL 2009 | 14.4/ F | See below ** | 8.5 | 109 | IR, high MRD day 29 Block Therapy | 0 | HCT | 1 |

*NOPHO, Nordic Pediatric Hematology Oncology; IR, Intermediate Risk Protocol; MRD, Minimal Residual Disease; BM, bone marrow; CNS, Central nervous system;HCT, Hematopoietic cell transplantation*

**Patient 1**
*46,XY,del(7)(p12.1p14.2),del(7)(q32.1q32.3),del(7)(q33q34),del(8)(p21.2p21.3),del(12)(p13.2p13.2),del(13)(q14.2q14.2),del(17)(q11.2q12),del(21)(q22.3q22.3),qdp(21)(q11.2q22.3

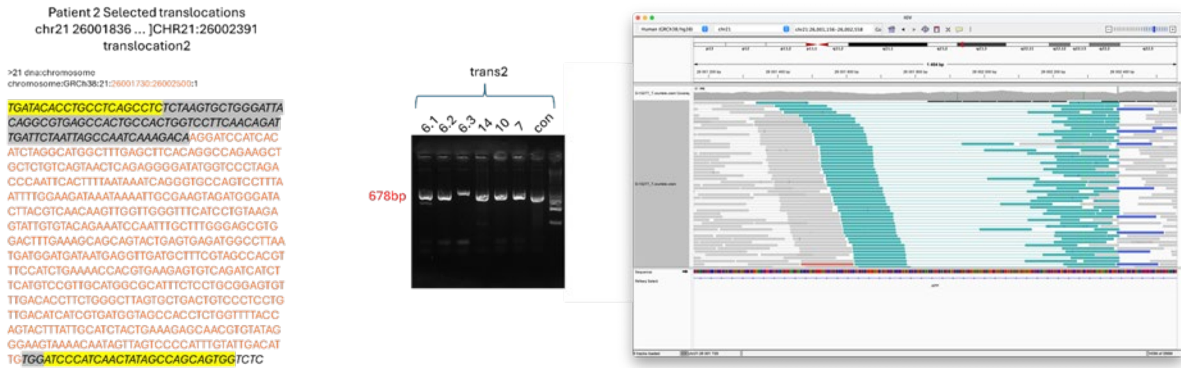
*FISH analysis did not show t(1;19), t(9;22), t(12;21), 11q23 (MLL) rearrangement, or del(9p21). There were also no signs of dic(9;20). Multiple extra RUNX1 signals were observed, consistent with iAMP21, and array analysis confirmed amplification of the long arm of chromosome 21. The result is thus consistent with iAMP21, indicating that the patient should be treated according to the intermediate-risk protocol.

**Patient 2**
**46,X,t(X;7)(p2?1;q2?1),der(21)i(21)(q10)invdup(21)(q11q22)[13].ish.iamp(21)(q22)/46,XX[6]

**Chromosome analysis from bone marrow showed a karyotype with additional material of unclear origin on the short arm of one chromosome 7. In addition, an isochromosome 21 with amplified material was identified (ic21amp). FISH analysis also demonstrated ic(21) amplification containing the RUNX1 gene. Initially classified as intermediate risk due to ic21 amplification.

# Supplementary Figure 1

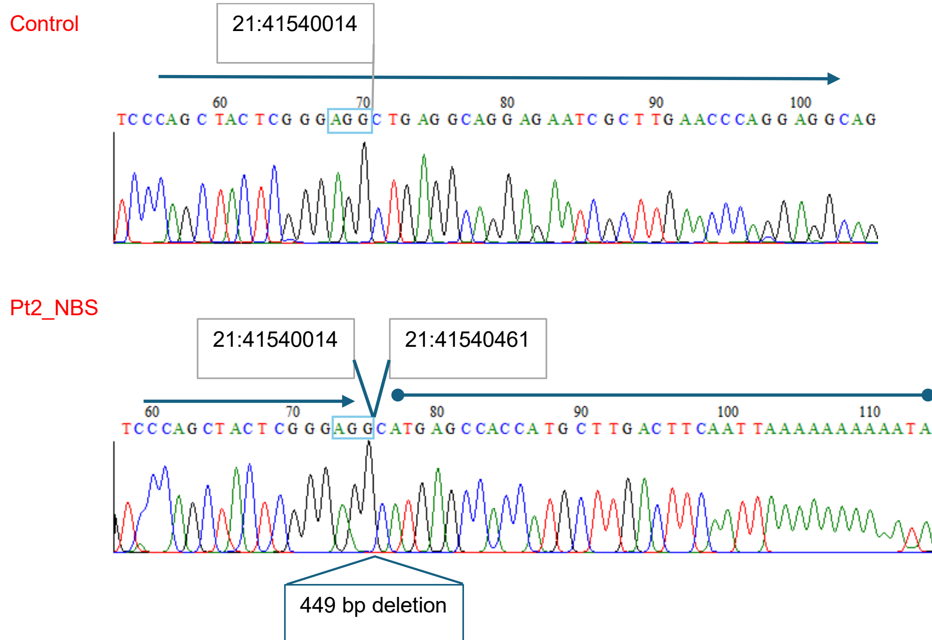
## A




**A selected translocation in iAMP-ALL patient 2 (inversion, see Figure 2A) is present at birth.**

Top: PCR primers are shown in yellow blocks, prospective inverted sequence is shown in brown, flanking regions in grey. Middle: gel picture after PCR. Lower panel: IGV of selected region at diagnosis of patient 2.

## B




**The small deletion within chromosome 21 identified at diagnosis for patient 2 (chr21:41540011–41540460) is already present in the NBS at birth.**

Control and NBS DNA sequence of PCR products. Boxed DNA sequences show possible microhomology

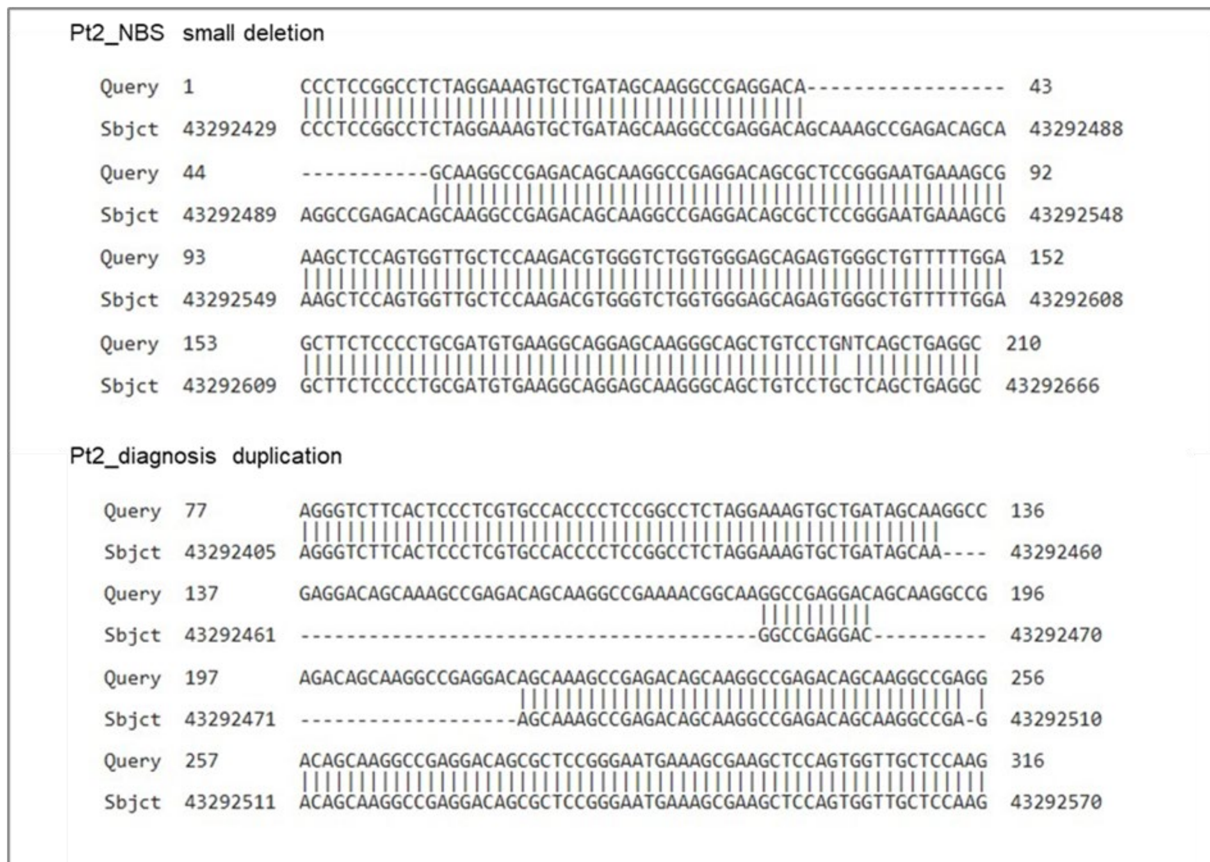
## Supplementary Figure 2

Patient 2 Selected duplication
chr21:43292455:43292524
+73 bp

GCTGGGGTAGAGCCTTTTAGCTATGGCCAGACCTGCTCTGCAGATTCCAACAAGGCCTCATGTC
CCAGCAACGGTGGCGTCCCTGCCAAAGGGGACGGGAGCTGAGCAAGGGTCTTCACTCCCTC
GTGCCACCCCTCCGGCCTCTAGGAAAGTGCTGATAGCAAGGCCGAGGAC***AGCAAAGCCGAGACA***
***GCAAGGCCGAAAACGGCAAGGCCGAGGACAGCAAGGCCGAGACAGCAAGGCCGAGGAC***AGCAAAGCCGAGAC
AGCAAGGCCGAGACAGCAAGGCCGAGACAGCAAGGCCGAGGACAGCGCTCCGGGAAGAA
AGCGAAGCTCCAGTGGTTGCTCCAAGACGTGGGTCTGGTGGGAGCAGAGTGGGCTGTTTTTGG
AGCTTCTCCCCTGCGATGTGAAGGCAGGAGCAAGGGCAGCTGTCCTGCTCAGCTGAGGCATG
GGATGGCCACAGCATCCCTGCAGAGCCCACCAGGATGCACTCAGAACTGACTTGT

>21 dna:chromosome: GRCh38:21:43292455-43292524

*GGCCGAGGACAGCAAAGCCGAGACAGCAAGGCCGAGACAGCAAGGCCGAGACAGCAAGGCCGAGGAC*




**A deletion in patient 2 at birth evolves to a postnatal duplication**

Top: Primer sequences in yellow blocks, potential duplicated region in red.

Bottom: Standard nucleotide BLAST comparison (Altschul SF et al., Basic local alignment search tool. J Mol Biol. 1990;215(3):403-10) between PCR amplicons derived around chr21:43292455-43292524 in patient 2. Sequence alignment shows that a small deletion present in the NBS had likely progressed to a duplication event at diagnosis of iAMP21-ALL.