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A 3-Year Retrospective Analysis Comparing Flow Cytometry-Based DNA Ploidy (DNA Index) to Conventional Cytogenetics in Paediatric Patients Diagnosed with B-cell Acute Lymphoblastic Leukaemia

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A 3-YEAR RETROSPECTIVE ANALYSIS COMPARING FLOW CYTOMETRY-BASED DNA PLOIDY (DNA INDEX) TO CONVENTIONAL CYTOGENETICS IN PAEDIATRIC PATIENTS DIAGNOSED WITH B-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA

Dr Delwen Carla Henderson

A research report submitted, in the submissible article format, to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in partial fulfilment of the requirements for the degree of Master of Medicine in Haematological Pathology.

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Johannesburg, 2025

Declaration:

Student's contribution to article:

I, Delwen Carla Henderson, student number 2620016, declare that this Research Report is my own work and that I contributed adequately towards research findings in the article below. The article is planned for submission to *Cytometry: Part B* and is submitted for examination toward the Degree of Master of Medicine in Haematological Pathology at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University

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Agreement by co-authors:

By signing this declaration, the co-authors listed below agree to the use of the article below by the student as part of her Research Report.

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Abstract

Flow cytometric analysis is a valuable tool in the diagnostic work-up of patients with B-cell acute lymphoblastic leukaemia. Flow cytometry is commonly employed to assign the DNA index (an evaluation of leukaemic ploidy status) and the immunophenotypic profile of the blasts. This retrospective data analysis compared the DNA index to conventional cytogenetics to determine the clinical utility in accurately predicting ploidy states and examined the relationship between ploidy states and immunophenotypic profiles in the local patient population. The DNA index displayed poor sensitivity, specificity and predictive values and was deemed to not be a reliable indicator of the ploidy status as it is currently interpreted. Changes to definitions of the ploidy states improved the performance of the test and are a key factor in improving the utility of the assay. Brighter expression of CD34 and CD123 were noted in the cytogenetically hyperdiploid group, in keeping with the international literature. However, the decreased expression of CD45 and brighter expression of CD20 typically described were not appreciated. Further studies with larger numbers and more in-depth cytogenetic analysis are needed to clarify the role of the DNA index in the local patient population.

Keywords:

“DNA index”, “Ploidy analysis”, “Flow Cytometry”, “B-ALL”, “Cytogenetics”

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List of abbreviations

ALL: Acute lymphoblastic leukaemia

B-ALL: B-cell acute lymphoblastic leukaemia

CD: Cluster of differentiation

CMJAH: Charlotte Maxeke Johannesburg Academic Hospital

COVID-19: Coronavirus disease 2019

DNA: Deoxyribonucleic acid

DNAi: DNA Index

DNAi 1, 2, 3: Hypodiploid, diploid and hyerperdiploid classification by DNAi utilising the following limits:

	DNAi cut-offs		
	Hypodiploid	Diploid	Hyperdiploid
DNAi 1	<0.95	0.95 – 1.05	>1.05
DNAi 2	<0.95	0.95 – 1.10	>1.10
DNAi 3	<0.95	0.95 – 1.15	1.16 – 1.60

HIV: Human immunodeficiency virus

HLA-DR: Human leukocyte antigen - DR isotype

IQR: Interquartile range

LL: Lower limit

nTDT: Nuclear terminal deoxynucleotidyl transferase

UL: Upper limit

WCC: White cell count

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Introduction

Acute lymphoblastic leukaemia (ALL) is a potentially fatal haematological malignancy with rapid onset. It is defined by clonal proliferation of lymphoid progenitor cells and is associated with significant heterogeneity in clinical presentation, phenotype, genotype and response to therapy (Forero-Castro, Robledo et al. 2016, Pierzyna-Switala, Sedek et al. 2021, Qiu, Liao et al. 2021). ALL is sub-classified into two main entities, namely B- and T-cell ALL based on the cell of origin. B-cell acute lymphoblastic leukaemia (B-ALL) comprises approximately 85% of total ALL cases (Pierzyna-Switala, Sedek et al. 2021).

Risk stratification and prognostication of paediatric patients with B-ALL considers various genetic aberrations such as aneuploidy, which is a common finding. (Kumar, Bhatia et al. 2015, Swerdlow SH 2017, Bommannan, Arumugam et al. 2021). Conventional cytogenetics, despite being time-consuming, difficult to culture, and necessitating a high level of expertise, remains the gold standard for assessing the ploidy status (Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021). An alternative method of measuring ploidy is by using the deoxyribonucleic acid (DNA) index (DNAi) which is the ratio of the DNA content of the leukaemic cells to the DNA content of a healthy lymphocyte and is determined using flow cytometric analysis (Trueworthy 1992, Kumar, Bhatia et al. 2015, Gupta, Parihar et al. 2019, Alatawi, Bakhsh et al. 2023). Initial studies assessing the DNAi as a marker of prognosis demonstrated that patients with a DNAi ≥1.16, which corresponds with cytogenetic high hyperdiploidy, were 12 times less likely to fail treatment than those with a DNAi < 1.16 and that a DNAi between 1.1 and 1.2 served as a useful cut-off point for discriminating cases with a superior prognosis (Trueworthy 1992, Zaliova, Hovorkova et al. 2016, Qiu, Liao et al. 2021).

The DNAi, which can be done on residual flow cytometry sample, has a much quicker resulting time and can be used as an alternative or back-up (in the case of failed tests) to conventional cytogenetics. This is particularly useful in resource restricted settings such as sub-Saharan Africa where cytogenetics may not be readily available (Rachieru-Sourisseau, Baranger et al. 2010, Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021, Alatawi, Bakhsh et al. 2023). Within the public

sector in the South African health care system, seven laboratories have facilities for flow cytometry and five laboratories can process somatic cell cytogenetics, demonstrating that access to a DNAi is more feasible than access to conventional cytogenetics.

Sources differ as to the DNAi lower limit (LL) and upper limit (UL) for the different ploidy states (see table 1) (Arico, Valsecchi et al. 2008, Kumar, Bhatia et al. 2015, Noh, Park et al. 2017, Gupta, Parihar et al. 2019, Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021, Qiu, Liao et al. 2021). This lack of standardisation creates confusion when ploidy states are defined. Recently, there has been increasing interest in re-evaluating the DNA index as a prognostic factor in B-ALL, as well as the traditional cut-off values, particularly in the local patient population (Noh, Park et al. 2017, Qiu, Liao et al. 2021).

Table 1: Ploidy states by modal number of chromosomes and DNAi described in the literature

Ploidy status	**Modal number of chromosomes (karyotype)**	**DNAi (flow cytometry)**
Near-haploid	24 – 29	0.55 - 0.69
Low-hypodiploid	30 – 39	0.70 – 0.88
High-hypodiploid	40 – 45	0.89 – 0.95
Diploid	46	0.96 – 1.05 (LL 0.90 (Kumar, Bhatia et al. 2015))
Low-hyperdiploid	47 – 50	1.06 – 1.15 (UL 1.09 (Kumar, Bhatia et al. 2015))
High-hyperdiploid	51 – 65	1.16 – 1.39 (UL 1.41 (Pierzyna-Switala, Sedek et al. 2021) and 1.59 (Arico, Valsecchi et al. 2008); 1.10 – 1.60 (Kumar, Bhatia et al. 2015))
Near-triploid	66 – 80	1.40 – 1.79
Near-tetraploid	81 – 102	1.80 – 2.28

Adapted from Gupta et al (Gupta, Parihar et al. 2019). LL: lower limit, UL: upper limit.

Certain immunophenotypic profiles, white cell count (WCC) and size of blast cells have been demonstrated to be associated with different ploidy statuses (Arico, Valsecchi et al. 2008, Pierzyna-Switala, Sedek et al. 2021). The Beckman Coulter Kaluza C (Beckman Coulter Life Sciences, Indiana, United States of America) software for ploidy analysis and immunophenotypic analysis is widely used in South Africa, however, literature confirming the above findings in the local patient demographic is absent. This study aims to evaluate the DNAi in paediatric B-ALL samples using flow cytometry with reference to the gold standard conventional cytogenetic findings in the local patient population.

Materials and Methods

Samples and test principles

B-ALL samples received for flow cytometric analysis at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) (Johannesburg, South Africa) leukaemia immunophenotyping unit between March 2020 and September 2023 were analysed on the Navios flow cytometer (Beckman Coulter Inc, Indianapolis, United States of America) and interpreted using Kaluza C analysis software. Standard panels of fluorescently labelled monoclonal antibodies, which bind to cell surface receptors, were utilised which allowed identification of the immunophenotype of the leukaemic clone. Propidium iodide, a fluorescent dye which binds to DNA, was used to determine the DNAi by comparing the patient sample to a sex-matched control sample. Cytogenetics samples received at the somatic cell genetics unit at CMJAH were cultured, harvested, arrested in mitosis, and stained to allow the banding patterns of chromosomes to be appreciated. Thereafter the slides were scanned digitally for analysis by trained staff members using CytoVision software (Leica Microsystems, Wetzlar, Germany).

103 diagnostic paediatric (<18 years old) B-ALL samples were run during the study period and after exclusion of cases with failed cytogenetics or DNAi, 76 were included.

Study design

The study was a retrospective diagnostic method data analysis.

Ethical considerations

Ethics approval was granted by the Human Research Ethics Council of the University of the Witwatersrand (M230417 MED23-04-130).

Data collection

Data were collected, anonymised and captured onto the Excel spreadsheet designed for this purpose. Due to a recent change in laboratory standard operating procedures and the variation in international literature, two DNAi cut-off points were used to define hyperdiploidy (see table 2). Definitions used in this study for true and false positivity and negativity are shown in table 3.

Table 2: Definitions of ploidy states utilised for this study derived from local laboratory practice (NHLS 2024) and international literature*

Ploidy	**Modal number**	**DNAi 1[#]**	**DNAi 2[#]**
Hypodiploid	<46	<0.95	<0.95
Diploid	46	0.95 – 1.05	0.95 – 1.10
Hyperdiploid	>46	>1.05	>1.10

DNAi 1: DNA index cutoff 1, DNAi 2: DNA index cut-off 2. *Refer to table 1. [#] (NHLS 2024)

Table 3: Definitions used in this study to interpret specificity, sensitivity, positive predicative value and negative predicative value

	Hyper/hypodiploid on cytogenetics (gold standard)	**Diploid on cytogenetics (gold standard)**
Hyper/hypodiploid on DNAi	True positive	False positive
Diploid on DNAi	False negative	True negative

DNAi: DNA index

Statistical analysis

Microsoft Excel (Microsoft Office, Redmond, USA, version 2406) and R statistical computing (Vienna, Austria, version 4.4.1) were used for data analysis. Normality was established using the Shapiro-Wilk test. Medians were compared using the Kruskal Wallis test and percentages were compared using the Chi squared test. The Cohen kappa co-efficient was used to calculate the agreement between cytogenetics and DNAi.

Results

Of the 76 included samples, 41 (54%) were female and 35 (46%) were male with an age that ranged from 0.25 – 17 years. There was no difference between median age of male and female patients (4.0 years). The median WCC at presentation was 9.45 x 10^9/L (Interquartile range {IQR}: 3.23 – 43.57 x 10^9/L); further breakdown is provided in table 4. The median modal number of chromosomes was 46 (IQR: 46-47), and the median DNAi was 1.07 (IQR 1.02-1.17).

Table 4: White cell count (WCC) compared between cytogenetically defined ploidy states

	Hyperdiploid (n=28)	**Diploid (n=38)**	**Hypodiploid (n=10)**
Median WCC (x 10^9/L)	5.46	21.23	4.21
IQR (x 10^9/L)	2.64 – 74.11	6.79 – 74.11	2.81 – 12.19

Median white cell counts differed significantly between cytogenetically defined hyperdiploid and diploid groups (p=0.007) and cytogenetically defined hypodiploid and diploid groups (p=0.04). There was no statistically significant difference between cytogenetically defined hyperdiploid and hypodiploid groups (p=0.12). WCC: white cell count, IQR: interquartile range.

Human immunodeficiency virus (HIV) testing was performed in 49 patients, only 1 of which was positive (n=1, 2%). The samples were analysed at the height of the coronavirus disease 2019 (COVID-19) pandemic, however, despite this, the majority (n=48, 63%) had no documented COVID-19 results at diagnosis. Of the remaining 28 samples, 27 (96%) were negative, and one (4%) was positive for COVID-19 at diagnosis.

Examples of flow cytometry plots showing DNAi analysis are shown in figures 1 and 2.

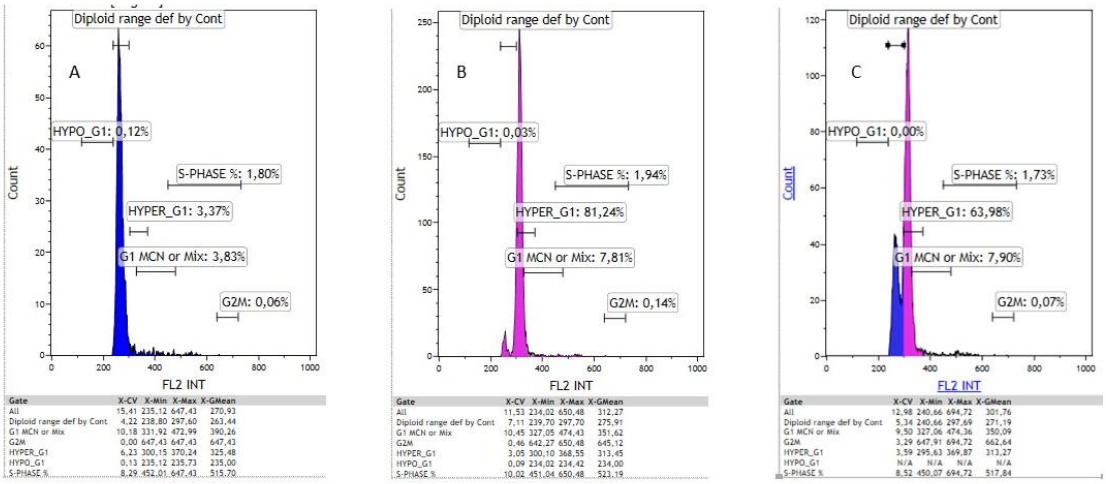


Figure 1: Example of DNA ploidy flow cytometry plots in a patient with hyperdiploidy as defined by the DNAi (DNAi = 1.19). A: control (blue), B: patient sample (pink), C: mix of patient and control demonstrating 2 distinct peaks (blue and pink).

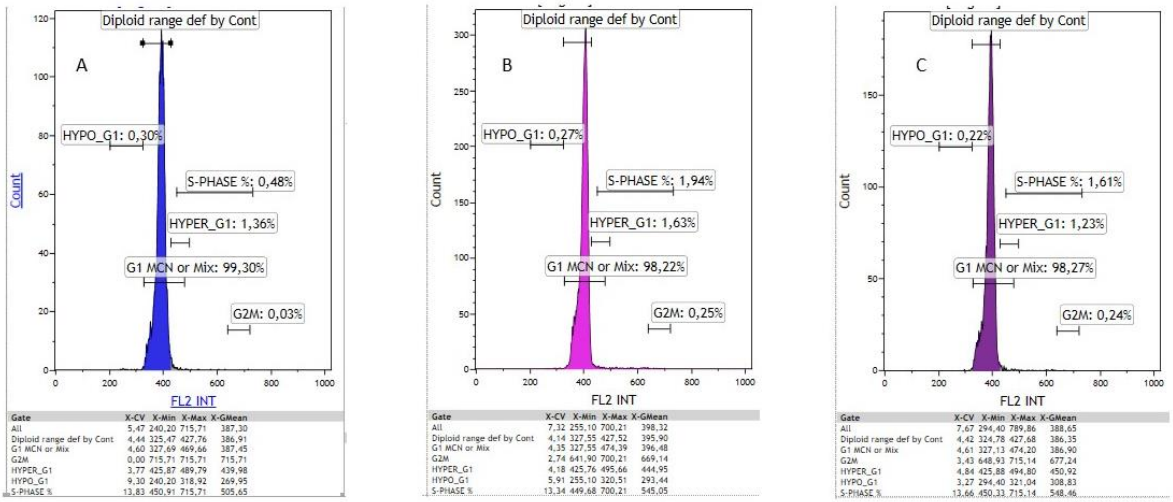


Figure 2: Example of DNA ploidy flow cytometry plots in a patient with diploidy as defined by DNAi (DNAi 1.02). A: control (blue), B: patient sample (pink), C: mix of patient and control demonstrating 1 peak (purple).

The number of samples assigned to each ploidy state by the different modalities is outlined in table 5. DNAi 1 and DNAi 2 overcalled hyperdiploidy and under called

hypodiploidy when compared to cytogenetics. Comparison of the DNAi to conventional cytogenetics (shown in table 6), did not demonstrate a statistically significant difference in results between the two ploidy states, DNAi 1 and DNAi 2 (p=1).

Table 5: Number of samples assigned to ploidy states by various modalities

	Ploidy	**Number of samples assigned**	**Percentage (%)**
Cytogenetics	**Hyperdiploid**	28	37
	Diploid	38	50
	Hypodiploid	10	13
DNAi 1	**Hyperdiploid**	42	55
	Diploid	32	42
	Hypodiploid	2	3
DNAi 2	**Hyperdiploid**	35	46
	Diploid	39	51
	Hypodiploid	2	3

DNAi 1: DNA index cutoff 1, DNAi 2: DNA index cutoff 2

Table 6: Comparison of DNA index to cytogenetics: sensitivity, specificity and predictive values

	DNAi 1	**DNAi 2**
Sensitivity	57.14%	51.43%
Specificity	44.74%	53.85%
Positive predictive value	48.78%	50.00%
Negative predictive value	53.13%	55.26%
Cohen Kappa co-efficient*	0.01	0.01

DNAi 1: DNA index cutoff 1, DNAi 2: DNA index cutoff 2

The specificity and sensitivity of the DNAi are poor using both DNAi 1 and DNAi 2. Whilst the specificity improves when DNAi 2 is used rather than DNAi 1, the assay, as it is currently interpreted, fails to pick up truly aneuploid or truly diploid cases.

In view of the poor performance of the DNAi 1 and DNAi 2 to predict the correct cytogenetically defined ploidy state, new DNAi and chromosomal modal number cut-offs (DNAi 3) were determined (as tabulated in Table 7) based on correlation with the gold standard as noted in the literature (Swerdlow SH 2017, Gupta, Parihar et al. 2019, Pierzyna-Switala, Sedek et al. 2021). Data were then reanalysed with an emphasis on the definition of high hyperdiploidy due to the prognostic significance of this particular entity. Cases classified as triploid or tetraploid (n=1) were excluded from this analysis.

Table 7: Definitions of ploidy states utilised for reanalysis according to numerical chromosomal definition of ploidy number derived from and international literature with specific emphasis on high hyperdiploidy

Ploidy	**Modal number of chromosomes***	**DNAi 3[#]**
Hypodiploid	<44	<0.95
Diploid	44-50	0.95 – 1.15
Hyperdiploid	51-65	1.16 – 1.60
Triploid/tetraploid	>65	>1.60

DNAi 3 : DNAi cut off 3. *Modal number of chromosomes: classification derived from WHO classification of Tumours of Haematopoietic and Lymphoid Tissue (Swerdlow SH 2017), [#] DNAi 3 cut-off values derived from Gupta et al's classification for high hyperdiploidy (Gupta, Parihar et al. 2019).

Upon reanalysis with the above parameters, 46 cases were classified as truly diploid (true negative), 12 cases as truly hyperdiploid (true positive) and a single case as triploid/tetraploid. Truly hypodiploid cases were absent. The sensitivity, specificity and negative predictive value improved to 75%, 78% and 92% respectively, however, the positive predictive value remained poor at 48%. The Cohen kappa co-efficient was 0.44 which indicates moderate agreement between the two methods using these parameters.

The antigen expression profile of true hyperdiploid samples (cytogenetically determined) is as follows: strong expression of CD19, CD10, CD34, CD38, CD123, HLA-DR, nuclear TdT, cytoplasmic CD79a and cytoplasmic CD22, weak expression of CD45 and negative/no expression of CD20, CD33, CD13 and CD7 in the majority

of samples. Hyperdiploid samples had stronger expression of CD123 and cCD22 compared with diploid and hypodiploid samples. CD34 had brighter expression in the hyper- and hypodiploid samples than in the diploid samples. Further breakdown of the immunophenotype of cytogenetically defined ploidy groups in provided in table 8.

Table 8: Immunophenotypic prolife of cytogenetically defined ploidy groups

Antigen	Hypodiploid cases (n=10)				Diploid cases (n=38)				Hyperdiploid cases (n=28)			
	N	W	P	NA	N	W	P	NA	N	W	P	NA
CD20	50%	40%	10%	0%	53%	24%	21%	3%	43%	25%	25%	7%
CD19	0%	20%	80%	0%	0%	0%	100%	0%	0%	7%	93%	0%
CD10	10%	0%	90%	0%	5%	3%	92%	0%	4%	11%	86%	0%
CD34	40%	10%	50%	0%	42%	21%	37%	0%	25%	11%	64%	0%
CD38	0%	30%	70%	0%	0%	13%	87%	0%	0%	11%	86%	4%
CD45	10%	90%	0%	0%	3%	89%	8%	0%	0%	100%	0%	0%
CD33	80%	0%	20%	0%	82%	16%	0%	3%	68%	29%	4%	0%
CD13	80%	10%	10%	0%	97%	0%	3%	0%	96%	4%	0%	0%
CD22	0%	0%	20%	80%	0%	5%	13%	82%	0%	0%	7%	93%
HLA-DR	0%	0%	90%	10%	3%	11%	76%	11%	4%	4%	93%	0%
CD7	100%	0%	0%	0%	100%	0%	0%	0%	100%	0%	0%	0%
nTDT	0%	10%	90%	0%	8%	11%	82%	0%	11%	4%	86%	0%
cCD79a	10%	10%	80%	0%	5%	8%	84%	3%	11%	0%	86%	4%
cCD22	50%	0%	20%	30%	53%	8%	24%	16%	29%	18%	43%	11%
CD123	50%	40%	10%	0%	50%	29%	21%	0%	29%	21%	46%	4%

The immunophenotypic antigen expression profile is shown by the number of cases in a cytogenetically defined ploidy group which express a particular antigen and the strength at which that antigen is expressed. N: negative, W: weak, P: positive, NA: not assessed, CD: cluster of differentiation, HLA-DR: human leukocyte antigen - DR isotype, nTDT: nuclear terminal deoxynucleotidyl transferase, cCD79a: cytoplasmic CD79a, cCD22: cytoplasmic CD22.

Discussion

Due to the prognostic implications of the ploidy status and the time sensitive nature of the disease, this study assessed the ability of the DNAi to correctly predict the cytogenetically defined ploidy states and thereby speed up risk stratification of patients. Antigen expression profiles were also documented according to ploidy subgroups with a comparison of local patient data with published literature. The

results show that the DNAi as currently analysed in our laboratory is not a reliable predictor of the ploidy status as defined by cytogenetics in the local patient demographic. The DNAi 1 and 2 missed approximately half of the patients who are truly hyper- or hypodiploid. These findings are contrary to those described in the international literature (Zaliova, Hovorkova et al. 2016, Gupta, Parihar et al. 2019, Bommannan, Arumugam et al. 2021, Alatawi, Bakhsh et al. 2023). In view of these discrepancies, the definitions of ploidy states were redefined as shown in table 7. Reassigning the definitions improved the sensitivity, specificity, negative predictive value and Cohen's kappa co-efficient of the DNAi suggesting that amendments to the laboratory's current SOP will lead to more reliable results.

Other possible reasons for the deviation from the international literature will be expanded upon below, with consideration given to the small sample size. Other studies have described a male preponderance in B-ALL (Arico, Valsecchi et al. 2008, Rachieru-Sourisseau, Baranger et al. 2010, Forero-Castro, Robledo et al. 2016, Bommannan, Arumugam et al. 2021, Qiu, Liao et al. 2021), however, this study as well as another study done in the South African context show a female preponderance (Vaughan, Bouwer et al. 2021). This may indicate an alternative disease profile locally and could explain the discrepancy noted. Despite the high prevalence of HIV in South Africa, the number of HIV positive patients in this study is negligible and therefore unlikely to explain this deviation of results from the literature. Due to the paucity of data in the international literature using the Kaluza C analysis software, analytical and post-analytical factors such as software limitations, human error in interpretation of results and failure of the controls cannot be excluded as possible reasons for the deviation of the results. In order to formally incorporate DNAi into the current laboratory workflow, validation of this system comparing DNAi to conventional cytogenetics (gold standard) should be performed to complement the previous method comparison with Paint-A-Gate platform (BD Biosciences, Franklin Lakes, New Jersey, United States of America). Delving into the specific cytogenetic abnormalities which result in a hyperdiploid DNAi may assist in discriminating a more suitable cut-off point for high hyperdiploidy. These measures would aid in determining a local population-specific reference.

The stronger expression of CD34 and CD123 in the hyperdiploid samples is in keeping with the literature which demonstrates an association between hyperdiploidy and overexpression of CD34 and CD123 (Borowitz, Shuster et al. 1990, Djokic, Bjorklund et al. 2009, Swerdlow SH 2017, Pierzyna-Switala, Sedek et al. 2021). Expression of surface CD22 (sCD22) is known to be associated with hyperdiploidy (Pierzyna-Switala, Sedek et al. 2021). There was no accessible data on sCD22 expression on the cases analysed, however, cCD22 expression was assessed and demonstrated no statistically significant variation in expression with regards to ploidy subtypes. The surface antigens, CD9, CD22, CD58, CD66c and CD86 have been described to be associated with hyperdiploidy (Pierzyna-Switala, Sedek et al. 2021, Kansal 2023), however, these were not tested in the majority of the study samples. Additionally, the literature describes dimmer CD45 expression and brighter CD20 expression in high-hyperdiploid cases (Pierzyna-Switala, Sedek et al. 2021, Kansal 2023), neither of which have been consistently demonstrated in this study.

A limitation of the study was that the sample size did not reach the required amount for statistical significance. Further studies with larger patient numbers, more detailed investigations into the type of cytogenetic aberrations, including unbalanced translocations, which specific chromosomes are duplicated or lost and assessment of FISH findings will be of value for more definitive and robust conclusions regarding the functionality of the DNAi in the South African context.

Conclusion

The results of this study do not support the ability of the DNAi as it is currently interpreted to correctly predict the ploidy status as defined by the gold-standard test, conventional cytogenetics, in the local patient population. When adjusting the definitions for ploidy states, taking into account the published literature, the performance of the DNAi improved suggesting that applying revised definitions may improve the utility of this test in our setting. Additional factors contributing to the unexpected results may include the limited study sample size and demographic differences in patients with B-ALL in South Africa. In keeping with the international literature, brighter expression of CD34 and CD123 was present in the hyperdiploid group, regardless of the method used to define hyperdiploidy. However, the

decreased expression of CD45 was not apparent. Further studies are needed in the South African context to better assess the functionality of the DNAi locally.

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Disclosures

The authors declare no conflicts of interest.

References

Alatawi, D., I. Bakhsh, S. Tashkandi, A. Alqarni, A. P. Shaik and M. Abudawood (2023). "Diagnostic Accuracy of Flow Cytometric DNA Index in Saudi Children with B Cell Acute Lymphoblastic Leukemia." Children (Basel) **10**(8).

Arico, M., M. G. Valsecchi, C. Rizzari, E. Barisone, A. Biondi, F. Casale, F. Locatelli, L. Lo Nigro, M. Luciani, C. Messina, C. Micalizzi, R. Parasole, A. Pession, N. Santoro, A. M. Testi, D. Silvestri, G. Basso, G. Masera and V. Conter (2008). "Long-term results of the AIEOP-ALL-95 Trial for Childhood Acute Lymphoblastic Leukemia: insight on the prognostic value of DNA index in the framework of Berlin-Frankfurt-Muenster based chemotherapy." J Clin Oncol **26**(2): 283-289.

Bommannan, K., J. R. Arumugam, T. Koshy, V. Radhakrishnan, T. G. Sagar and S. Sundersingh (2021). "Blast size-specific flowcytometric ploidy assessment using FxCycle(TM) Violet dye and its correlation with conventional cytogenetic ploidy in pediatric precursor B-lineage acute lymphoblastic leukemia patients." Int J Lab Hematol **43**(4): 760-770.

Borowitz, M. J., J. J. Shuster, C. I. Civin, A. J. Carroll, A. T. Look, F. G. Behm, V. J. Land, D. J. Pullen and W. M. Crist (1990). "Prognostic significance of CD34 expression in childhood B-precursor acute lymphocytic leukemia: a Pediatric Oncology Group study." J Clin Oncol **8**(8): 1389-1398.

Djokic, M., E. Bjorklund, E. Blennow, J. Mazur, S. Soderhall and A. Porwit (2009). "Overexpression of CD123 correlates with the hyperdiploid genotype in acute lymphoblastic leukemia." Haematologica **94**(7): 1016-1019.

Forero-Castro, M., C. Robledo, R. Benito, M. Abaigar, A. Africa Martin, M. Arefi, J. L. Fuster, N. de Las Heras, J. N. Rodriguez, J. Quintero, S. Riesco, L. Hermosin, I. de la Fuente, I. Recio, J. Ribera, J. Labrador, J. M. Alonso, C. Olivier, M. Sierra, M. Megido, L. A. Corchete-Sanchez, J. Ciudad Pizarro, J. L. Garcia, J. M. Ribera and J. M. Hernandez-Rivas (2016). "Genome-Wide DNA Copy Number Analysis of Acute Lymphoblastic Leukemia Identifies New Genetic Markers Associated with Clinical Outcome." PLoS One **11**(2): e0148972.

Gupta, N., M. Parihar, S. Banerjee, S. Brahma, R. Pawar, A. Rath, S. Shewale, M. Singh, A. Sasikumaran Nair Remani, S. Krishnan, A. Bhatacharyya, A. Das, J. Kumar, S. Bhave, V. Radhakrishnan, R. Nair, M. Chandy, D. Mishra and N. Arora (2019). "FxCycle Based Ploidy Correlates with Cytogenetic Ploidy in B-Cell Acute Lymphoblastic Leukemia and Is Able to Detect the Aneuploid Minimal Residual Disease Clone." Cytometry B Clin Cytom **96**(5): 359-367.

Kansal, R. (2023). "Diagnosis and Molecular Pathology of Lymphoblastic Leukemias and Lymphomas in the Era of Genomics and Precision Medicine: Historical Evolution and Current Concepts—Part 2: B-/T-Cell Acute Lymphoblastic Leukemias." Lymphatics **1**(2): 118-154.

Kumar, B. K., P. Bhatia, A. Trehan, A. P. Singh, D. Kaul and D. Bansal (2015). "DNA Ploidy and S-phase Fraction Analysis in Paediatric B-cell Acute Lymphoblastic Leukemia Cases: a Tertiary Care Centre Experience." Asian Pac J Cancer Prev **16**(17): 7917-7922.

NHLS (2024). Flow cytometric DNA Ploidy analysis, National Health Laboratory Service**:** 2-10.
Noh, O. K., S. J. Park, H. J. Park, H. Ju, S. H. Han, H. J. Jung and J. E. Park (2017). "Re-evaluation of DNA Index as a Prognostic Factor in Children with Precursor B Cell Acute Lymphoblastic Leukemia." Ann Clin Lab Sci **47**(5): 535-540.
Pierzyna-Switala, M., L. Sedek, J. Kulis, B. Mazur, K. Muszynska-Roslan, A. Koltan, M. Woszczyk, M. Niedzwiecki, A. Mizia-Malarz, G. Karolczyk, M. Lejman, J. Trelinska, W. Badowska, K. Derwich, T. Ociepa, I. Malinowska, B. Kazanowska, J. Kowalczyk and T. Szczepanski (2021). "Multicolor flow cytometry immunophenotyping and characterization of aneuploidy in pediatric B-cell precursor acute lymphoblastic leukemia." Cent Eur J Immunol **46**(3): 365-374.
Qiu, K. Y., X. Y. Liao, Z. W. He, R. H. Wu, Y. Li, L. H. Xu, D. H. Zhou and J. P. Fang (2021). "DNA index as prognostic factor in childhood acute lymphoblastic leukemia in the COG-TARGET database." BMC Cancer **21**(1): 813.
Rachieru-Sourisseau, P., L. Baranger, N. Dastugue, A. Robert, F. Genevieve, E. Kuhlein and A. Chassevent (2010). "DNA Index in childhood acute lymphoblastic leukaemia: a karyotypic method to validate the flow cytometric measurement." Int J Lab Hematol **32**(3): 288-298.
Swerdlow SH, C. E., Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Arber DA, Hasserjian RP, Le Beau MM, Orazi A, Siebert R (2017). WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue. Lyon, International Agency for Research on Cancer (IARC).
Trueworthy, R. S., J.; Look, T.; Crist, W.; Borowitz, M.; Carroll, A.; Frankel, L.; Harris, M.; Wagner, H.; Haggard, M.; Mosijczuk, A.; Pullen, J.; Steuber, P.; Land, v. (1992). "Ploidy of Lymphoblasts is the strongest predictor of treatment outcome in B-Progenitor Cell Acute Lympblastic Leukaemia of Childhood: A Pediatric Oncology Group Study." J Clin Oncol **10**(4): 606-613.
Vaughan, J., N. Bouwer, P. Willem, T. Wiggill and K. Hodkinson (2021). "The translocation t(1;19)(q23;p13) (TCF3/PBX1 fusion) is the most common recurrent genetic abnormality detected amongst patients with B-cell lymphoblastic leukaemia in Johannesburg, South Africa." 2021 **5**.
Zaliova, M., L. Hovorkova, M. Vaskova, O. Hrusak, J. Stary and J. Zuna (2016). "Slower early response to treatment and distinct expression profile of childhood high hyperdiploid acute lymphoblastic leukaemia with DNA index < 1.16." Genes Chromosomes Cancer **55**(9): 727-737.
Zhang, L., S. S. M. Habeebu and W. Li (2022). Prognostic and Predictive Biomarkers in Precursor B-cell Acute Lymphoblastic Leukemia. Leukemia. W. Li. Brisbane (AU)**:** 165–193.

Appendices

Appendix A: Copy of ethics clearance certificate

M230417 MED23-04-130

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Dr Delwen Henderson

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M230417 MED23-04-130

NAME: (Principal Investigator)	Dr Delwen Henderson
DEPARTMENT:	Haematology and Molecular Medicine Charlotte Maxeke Johannesburg Hospital Chris Hani Baragwanath Academic Hospital Helen Joseph Hospital Frere Hospital
PROJECT TITLE:	A 3-year retrospective analysis comparing flow cytometry-based DNA ploidy (DNA index) to conventional cytogenetics in paediatric patients diagnosed with B-cell acute lymphoblastic leukaemia a: Charlotte Maxeke Johannesburg Academic Hospital
DATE CONSIDERED:	05/05/2023
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Nikki Bouwer and Dr Anima Baiden
APPROVED BY:	Mrs M van Niekerk, Co-Chair, HREC (Medical)
DATE OF APPROVAL:	13/09/2023 **EXPIRY DATE:** 13/09/2028

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report** in January each year until study is closed. Failure to submit annual report will result in ethics clearance certificate suspension. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in April and will therefore be due in the month of April each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical). Email signed copy of this ethics clearance certificate prior to commencing with the study hrec-medical.researchoffice@wits.ac.za

Principal Investigator Signature

14/09/2023
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

05 December 2023

Dr Delwen Henderson
Haematology Laboratory
NHLS
Wits Medical School
Sent by email: delwenhenderson@gmail.com

Dear Dr Henderson,

Protocol no: M230417 MED23-04-130
Protocol Title: A 3-year retrospective analysis comparing flow cytometry-based DNA ploidy 9DNA index) to conventional cytogenetics in paediatric patients diagnosed with B-cell acute lymphoblastic leukaemia at Charlotte Maxeke Johannesburg Academic Hospital
Principal Investigator : Dr Delwen Henderson
Protocol Amendment:

Your letter dated 31 October 2023 refers.
This letter serves to confirm that the Chairman has approved your request to extend dates of eligible samples from March 2020-2023 to September 2023.

The following documents were received:

1. Motivation Letter
2. Revised research proposal
3. Latest copy of Clearance Certificate M230417



Yours sincerely,

Paul Ruff
Paul Ruff (Dec 5, 2023 20:04 GMT+2)

Prof Paul Ruff
Human Research Ethics Committee (Medical) Chair

Appendix B: *Cytometry Part B* Author guidelines

Available from:
https://onlinelibrary.wiley.com/page/journal/15524957/homepage/ForAuthors.html

Author Guidelines

Your main document file should include:

A short informative title containing the major key words. The title should not contain abbreviations;

The full names of the authors with institutional affiliations where the work was conducted, with a footnote for the author's present address if different from where the work was conducted;

Acknowledgments;

Abstract structured (intro/methods/results/conclusion) or unstructured;

Up to seven keywords;

Practitioner Points (optional) Authors will need to provide no more than 3 'key points', written with the practitioner in mind, that summarize the key messages of their paper to be published with their article;

Main body: formatted as introduction, materials & methods, results, discussion, conclusion;

References;

Tables (each table complete with title and footnotes);

Figure legends: At initial submission, figures can be included in the manuscript or can be submitted in separate files. Should your manuscript reach revision stage, figures and tables must be provided as separate files.

Manuscript Preparation

To ensure rapid and accurate publication, it is essential that the manuscript conform to the following instructions. Manuscripts that are not in accordance with these specifications may be returned to the author.

Manuscripts will appear under one of the following sections: Original Article, Review Article, Discussion Forum, Letter to the Editor, or Best Practice.

In all cases the manuscript should be consistent with style, spellings and use of abbreviations. We recommend that the manuscript be typed double-spaced with a 1 1/2" margin on all sides. Number the manuscript pages consecutively beginning with the title page. Hyphenation and justification should not be used. The manuscript should not exceed 8-12 double-spaced typewritten pages (including references, figures, and tables). If you wish to propose a manuscript size and page margin format outside of these requirements, you can do so by including a request in your cover letter which will then be considered by the Editor-in-Chief. Manuscripts should contain each of the following elements in sequence:

1. Title Page
2. Abstract
3. Text
4. Acknowledgments
5. References
6. Tables
7. Figure Legends

Define unusual abbreviations at the first mention in the text. Abbreviate measurements according to the Council of Biology Editors Style Manual (CBE Style Manual), fifth edition, available from the Council of Biology Editors, Inc., 11 South LaSalle Street, Chicago, IL 60603-1210. Enzyme names and units must follow the

recommendations of The Nomenclature Committee of the International Union of Biochemistry. Webster's Third International Dictionary is the accepted source for spelling and capitalization. The units for all measurements must conform to the International System of Units (SI). If a measurement is made in other units, then the SI equivalent should be given in parentheses. If authors refer to specific products of companies, they must identify the company by citing, in parentheses, its name, city, and state or country.

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Tables: Each table must have a title and should be self-explanatory. Avoid duplicating information in the text. Number tables with Arabic numerals in order of appearance in the text.

Figures: Images should be 300dpi or higher, with all labels and details large and clear enough to be legible to reviewers. Figures should be supplied in one of our preferred file formats: EPS, PS, JPEG, or TIFF. For additional guidance, read more at the Author Services page here.

References: References to published literature should be cited in the text by author name and year of publication and in alphabetical order in the reference list at the end of the article.

References must only be made to published papers and papers in press. Work in progress, unpublished experiments, and personal communications are specifically excluded from the reference list but may be acknowledged in parentheses in the text. References must be arranged alphabetically by the last name of the first author, by coauthors, and chronologically by the same author. Abbreviations of journal names

must conform to those used in Index Medicus . Authors are responsible for the accuracy of references. The following formats must be used for references:

Journals.

Brando B, Sommaruga E. Nationwide quality control trial on lymphocyte phenotyping and flow cytometry performance in Italy. Cytometry 1993;14:294-306.

Article in a book or comparable publication.

Gray JW, Cram LS. Flow karyotyping and chromosome sorting. In: Melamed MR, Lindmo T, Mendelsohn ML, editors. Flow cytometry and sorting. 2nd ed. New York: John Wiley & Sons, Inc.; 1990. p 503-529.

Books.

Givan AL. Flow cytometry: first principles. New York: John Wiley & Sons, Inc.; 1992. 1223 p.

Conflict of Interest Disclosure

Cytometry Part B: Clinical Cytometry requires that all authors disclose any potential sources of conflict of interest. Any interest or relationship, financial or otherwise, that might be perceived as influencing an author’s objectivity is considered a potential source of conflict of interest. These must be disclosed when directly relevant or indirectly related to the work that the authors describe in their manuscript. Potential sources of conflict of interest include but are not limited to patent or stock ownership, membership of a company board of directors, membership of an advisory board or committee for a company, and consultancy for or receipt of speaker’s fees from a company. The existence of a conflict of interest does not preclude publication in this journal.

If the authors have no conflict of interest to declare, they must also state this at submission. It is the responsibility of the corresponding author to review this policy with all authors and to collectively list in the cover letter to the Editor-in-Chief, in the manuscript (under the Acknowledgments section), and in the online submission system ALL pertinent commercial and other relationships.

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Manuscript Categories

Original Articles:

Manuscripts containing original work addressing the practice of clinical cytometry and the underlying methodology. Both flow or image cytometry are appropriate. This includes reports of clinical trials, diagnostic applications of flow and image cytometry and case series reports as well as new advances in instrumentation, methods, reagents, and data analysis techniques. Clinical research manuscripts including the

transition of new, and novel, cytometry techniques to clinical testing are welcome. Consensus and guideline documents relating to clinical cytometry are of high interest to the Journal and are welcome under this category.

Appendix C: Letter to the Editor

Medical School

University of the Witwatersrand

Johannesburg

South Africa

To: The Editor, *Cytometry: Part B*

RE: AMMENDMENTS TO THE DOCUMENT GUIDELINES FOR SUBMISSION FOR PUBLICATION

Dear Sir/Madam

This letter accompanies the attached article for consideration to be published in *Cytometry: Part B*. As noted in the author guidelines, a motivation may accompany a submission if any deviations from the guidelines are present. This article deviates from the guidelines with regard to the document length and is set with slightly wider margins and decreased line spacing than noted in the author guidelines.

The authors feel that if the proposed article was shortened, information which is crucial to full understanding of the subject at hand would have to be omitted and would thereby affect the quality of the research. The authors request that the Editor extends leniency with regard to the article length.

Sincerely

Dr DC Henderson

Appendix D: Turnitin report

D Henderson Mmed for turnitin-1.docx

by Delwen Henderson

Submission date: 11-Dec-2024 10:00AM (UTC+0200)
Submission ID: 2548890217
File name: D_Henderson_Mmed_for_turnitin.docx (116.57K)
Word count: 2649
Character count: 15153

A 3-YEAR RETROSPECTIVE ANALYSIS COMPARING FLOW CYTOMETRY-BASED DNA PLOIDY (DNA INDEX) TO CONVENTIONAL CYTOGENETICS IN PAEDIATRIC PATIENTS DIAGNOSED WITH B-CELL ACUTE LYMPHOBLASTIC LEUKAEMIA

Dr Delwen Carla Henderson

Supervisors/Co-Authors:

Dr Nikki Bouwer

Dr Anima Baiden

Johannesburg, 2024

Abstract

Flow cytometric analysis is a valuable tool in the diagnostic work-up of patients with B-cell Acute Lymphoblastic Leukaemia. Flow cytometry is commonly employed to assign the DNA index (an evaluation of leukaemic ploidy status) and the immunophenotypic profile of the blasts. This retrospective data analysis compared the DNA index to conventional cytogenetics to determine the clinical utility in accurately predicting ploidy states and examined the relationship between ploidy states and immunophenotypic profiles in the local patient population. The DNA index displayed poor sensitivity, specificity and predictive values and was deemed to not be a reliable indicator of the ploidy status. Brighter expression of CD34 and CD123 were noted in the cytogenetically hyperdiploid group, in keeping with the international literature. However, the decreased expression of CD45 and brighter expression of CD20 typically described were not appreciated. Further studies with larger numbers are needed to clarify the role of the DNA index in the local patient population.

Keywords:

"DNA index", "Ploidy analysis", "Flow Cytometry", "B-ALL", "Cytogenetics"

Introduction

Acute lymphoblastic leukaemia (ALL) is a potentially fatal haematological malignancy with rapid onset. It is defined by clonal proliferation of lymphoid progenitor cells and is associated with significant heterogeneity in clinical presentation, phenotype, genotype and response to therapy (Forero-Castro, Robledo et al. 2016, Pierzyna-Switala, Sedek et al. 2021, Qiu, Liao et al. 2021). ALL is sub-classified into two main entities, namely B- and T-cell ALL based on the cell of origin. B-cell acute lymphoblastic leukaemia (B-ALL) comprises approximately 85% of total ALL cases (Pierzyna-Switala, Sedek et al. 2021).

Risk stratification and prognostication of paediatric patients with B-ALL considers various genetic aberrations such as aneuploidy, which is a common finding. (Kumar, Bhatia et al. 2015, Swerdlow SH 2017, Bommannan, Arumugam et al. 2021). Conventional cytogenetics, despite being time-consuming, difficult to culture, and necessitating a high level of expertise, remains the gold standard for assessing the ploidy status (Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021). An alternative method of measuring ploidy is by using the DNA index (DNAi) which is the ratio of the DNA content of the leukaemic cells to the DNA content of a healthy lymphocyte and is determined using flow cytometric analysis (Trueworthy 1992, Kumar, Bhatia et al. 2015, Gupta, Parihar et al. 2019, Alatawi, Bakhsh et al. 2023). Initial studies assessing the DNAi as a marker of prognosis demonstrated that patients with a DNAi ≥1.16 were 12 times less likely to fail treatment than those with a DNAi < 1.16 and that a DNAi between 1.1 and 1.2 served as a useful cut-off point for discriminating cases with a superior prognosis (Trueworthy 1992, Qiu, Liao et al. 2021).

The DNAi, which can be done on residual flow cytometry sample, has a much quicker resulting time and can be used as an alternative or back-up (in the case of failed tests) to conventional cytogenetics. This is particularly useful in resource restricted settings such as sub-Saharan Africa where cytogenetics may not be readily available (Rachieru-Sourisseau, Baranger et al. 2010, Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021, Alatawi, Bakhsh et al. 2023). Within the public sector in the South African health care system, seven laboratories have facilities for

flow cytometry and five laboratories can process somatic cell cytogenetics, demonstrating that access to a DNAi is more feasible than access to conventional cytogenetics.

Sources differ as to the DNAi lower limit (LL) upper limit (UL) for the different ploidy states (see table 1) (Arico, Valsecchi et al. 2008, Kumar, Bhatia et al. 2015, Noh, Park et al. 2017, Gupta, Parihar et al. 2019, Bommannan, Arumugam et al. 2021, Pierzyna-Switala, Sedek et al. 2021, Qiu, Liao et al. 2021). This lack of standardisation creates confusion when ploidy states are defined. Recently, there has been increasing interest in re-evaluating the DNA index as a prognostic factor in B-ALL, as well as the traditional cut-off values, particularly in the local patient population (Noh, Park et al. 2017, Qiu, Liao et al. 2021).

Table 1: Ploidy states by modal number and DNAi described in the literature

Ploidy status	**Modal number (karyotype)**	**DNAi (flow cytometry)**
Near-haploid	24 – 29	0.55 - 0.69
Low-hypodiploid	30 – 39	0.70 – 0.88
High-hypodiploid	40 – 45	0.89 – 0.95
Diploid	46	0.96 – 1.05 (LL 0.90 (Kumar, Bhatia et al. 2015))
Low-hyperdiploid	47 – 50	1.06 – 1.15 (UL 1.09 (Kumar, Bhatia et al. 2015))
High-hyperdiploid	51 – 65	1.16 – 1.39 (UL 1.41 (Pierzyna-Switala, Sedek et al. 2021) and 1.59 (Arico, Valsecchi et al. 2008); 1.10 – 1.60 (Kumar, Bhatia et al. 2015))
Near-triploid	66 – 80	1.40 – 1.79
Near-tetraploid	81 – 102	1.80 – 2.28

Adapted from Gupta et al (Gupta, Parihar et al. 2019).

Certain immunophenotypic profiles, white cell count (WCC) and size of blast cells have been demonstrated to be associated with different ploidy statuses (Arico, Valsecchi et al. 2008, Pierzyna-Switala, Sedek et al. 2021), but literature confirming this in the local patient demographic using the Beckman Coulter Kaluza C (Beckman

Coulter Life Sciences, Indiana, United States of America) ploidy analysis and immunophenotyping platforms is limited. This study aims to evaluate the DNAi in paediatric B-ALL samples using flow cytometry with reference to conventional cytogenetic findings in the local patient population.

Materials and Methods

Samples and test principles

B-ALL samples received for flow cytometric analysis at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) (Johannesburg, South Africa) leukaemia immunophenotyping unit between March 2020 and September 2023 were analysed on the Navios flow cytometer (Beckman Coulter Inc, Indianapolis, United States of America) and interpreted using Kaluza C analysis software. Standard panels of fluorescently labelled monoclonal antibodies, which bind to cell surface receptors, were utilised which allowed identification of the immunophenotype of the leukaemic clone. Propidium iodide, a fluorescent dye which binds to DNA, was used to determine the DNAi by comparing the patient sample to a sex-matched control sample. Cytogenetics samples received at the somatic cell genetics unit at CMJAH were cultured, harvested, arrested in mitosis, and stained to allow the banding patterns of chromosomes to be appreciated. Thereafter the slides were scanned digitally for analysis by trained staff members using CytoVision software (Leica Microsystems, Wetzlar, Germany).

103 diagnostic paediatric B-ALL samples were run during the study period and after exclusion of cases with failed cytogenetics or DNAi, 76 were included.

Study design

The study was a retrospective diagnostic method data analysis.

Ethical considerations

Ethics approval was granted by the Human Research Ethics Council of the University of the Witwatersrand (M230417 MED23-04-130).

Data collection

Data were collected, anonymised and captured onto the Excel spreadsheet designed for this purpose. Due to a recent change in laboratory standard operating procedures and the variation in international literature, two DNAi cut-off points were used to define hyperdiploidy (see table 2). Definitions used in this study for true and false positivity and negativity are shown in table 3.

Table 2: Definitions of ploidy states utilised for this study derived from local laboratory practice and international literature

Ploidy	**Modal number**	**DNAi 1**	**DNAi 2**
Hypodiploid	<46	<0.95	<0.95
Diploid	46	0.95 – 1.05	0.95 – 1.10
Hyperdiploid	>46	>1.05	>1.10

Table 3: Definitions used in this study to interpret specificity, sensitivity, positive predicative value and negative predicative value

	Hyper/hypodiploid on cytogenetics	**Diploid on cytogenetics**
Hyper/hypodiploid on DNAi	True positive	False positive
Diploid on DNAi	False negative	True negative

Statistical analysis

Microsoft Excel (Microsoft Office, Redmond, USA, version 2406) and R statistical computing (Vienna, Austria, version 4.4.1) were used for data analysis. Normality was established using the Shapiro-Wilk test. Medians were compared using the Kruskal Wallis test and percentages were compared using the Chi squared test. The Cohen kappa co-efficient was used to calculate the agreement between cytogenetics and DNAi.

Results

Of the 76 included samples, 41 (54%) were female and 35 (46%) were male with an age that ranged from 0.25 – 17 years. There was no difference between median age of male and female patients (4,0 years). The median WCC at presentation was 9,45 x 10^9/L (Interquartile range {IQR}: 3,23 – 43,57 x 10^9/L); further breakdown is provided in table 4. The median modal number was 46 (IQR: 46-47), and the median DNAi was 1.07 (IQR 1.02-1.17).

Table 4: WCC compared between cytogenetically defined ploidy states

	Hyperdiploid (n=28)	**Diploid (n=38)**	**Hypodiploid (n=10)**
Median WCC (x 10^9/L)	5.46	21.23	4.21
IQR (x 10^9/L)	2.64 – 74.11	6.79 – 74.11	2.81 – 12.19

Median white cell counts differed significantly (p=0.007) between cytogenetically defined ploidy states.

The majority of the samples were Human Immunodeficiency Virus (HIV) negative (n=48, 63%), however the HIV status was unknown in a large number (n=27, 36%) with a single sample documented to be HIV positive (n=1, 1,3%). The samples were analysed at the height of the Coronavirus Disease 2019 (COVID-19) pandemic, however, despite this, majority (n=48, 63%) had no documented COVID-19 results at diagnosis. Of the remaining 28 samples, 27 (36%) were negative, and one (1,3%) was positive for COVID-19 at diagnosis.

The number of samples assigned to each ploidy state by the different modalities is outlined in table 5. Comparison of the DNAi to conventional cytogenetics (shown in table 6), did not demonstrate a statistically significant difference in results between the two ploidy states, DNAi 1 and DNAi 2 (p=1).

Table 5: Number of samples assigned to ploidy states by various modalities

	Ploidy	**Number**	**Percentage (%)**
Cytogenetics	**Hyperdiploid**	28	37
	Diploid	38	50
	Hypodiploid	10	13

DNAi 1	**Hyperdiploid**	42	55
	Diploid	32	42
	Hypodiploid	2	3
DNAi 2	**Hyperdiploid**	35	46
	Diploid	39	51
	Hypodiploid	2	3

Table 6: Comparison of DNAi to cytogenetics: sensitivity, specificity and predictive values

	DNAi 1	**DNAi 2**
Sensitivity	57.14%	51.43%
Specificity	44.74%	53.85%
Positive predictive value	48.78%	50.00%
Negative predictive value	53.13%	55.26%
Cohen Kappa co-efficient*	0.01	0.01

There is no statistically significant difference between the two DNAi cutoffs (p=1). * Indicates no agreement between either DNAi cutoff and cytogenetic definitions of ploidy states.

The antigen expression profile of hyperdiploid samples was similar across the three different methods of hyperdiploid status assignation as shown in figure 1, namely: strong expression of CD19, CD10, CD34, CD38, CD123, HLA-Dr, nTdT, cytoplasmic CD79a (cCD79a) and cytoplasmic CD22 (cCD22), weak expression of CD45 and negative/no expression of CD20, CD33, CD13 and CD7 in the majority of samples. cCD22 expression showed minor variability with the 1.05 DNAi cut-off but this was not statistically significant (p=1).

On karyotype, two cases were noted to be near triploid (66-80 chromosomes). On sub-analysis, these cases were excluded from the expression profile analysis and when compared to the original analysis, no differences were noted.

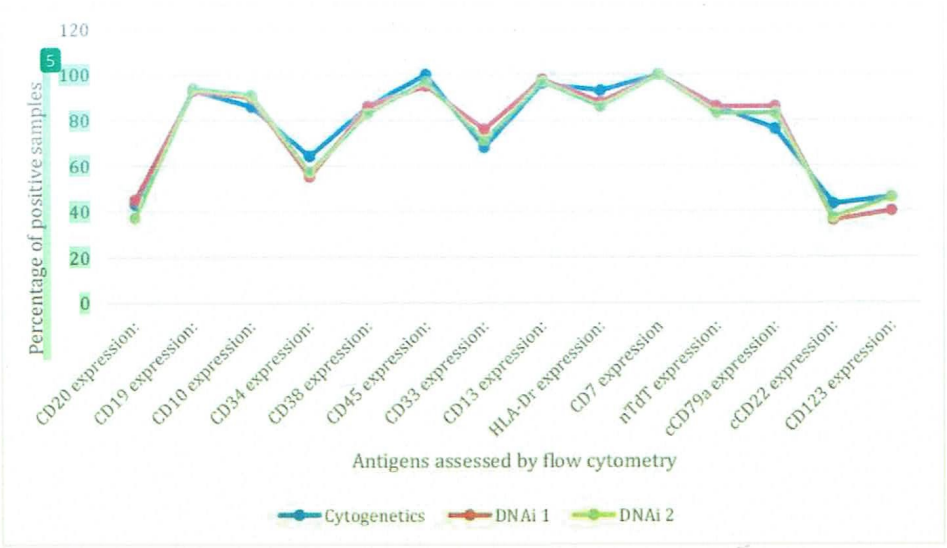


Figure 1: Hyperdiploid antigen expression profiles compared between cytogenetics, DNAi 1 and DNAi 2

Figure 2 shows the antigen expression profiles of the cytogenetically defined ploidy states and highlights the subtle differences therein.

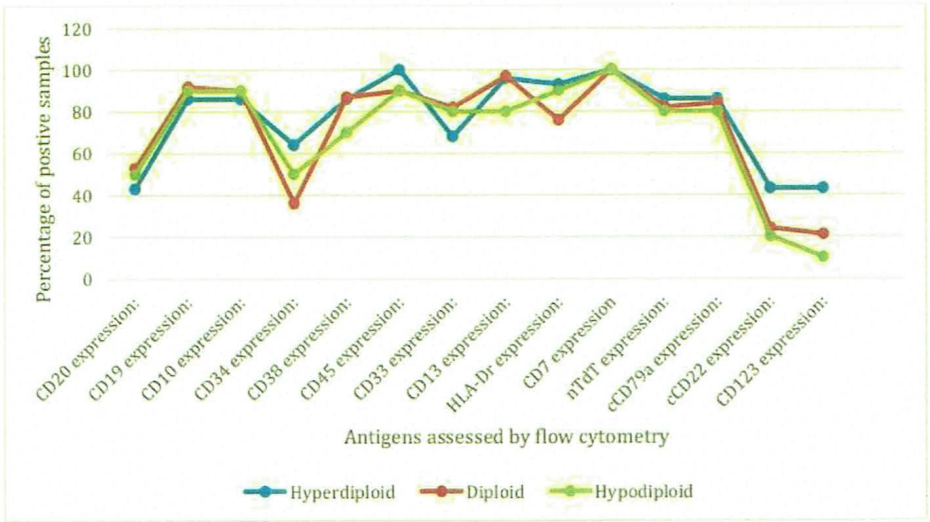


Figure 2: Antigen expression by cytogenetically defined ploidy states

Hyperdiploid samples had stronger expression of CD123 and cCD22 compared with diploid and hypodiploid samples. CD34 had brighter expression in the hyper- and hypodiploid samples than in the diploid samples.

Discussion

Due to the prognostic implications of the ploidy status and the time sensitive nature of the disease, this study assessed the ability of the DNAi to correctly predict the cytogenetically defined ploidy states, and thereby speed up risk stratification of patients. Antigen expression profiles were also documented according to ploidy subgroups with a comparison of local patient data with published literature. The results show that the DNAi is not a reliable predictor of the ploidy status as defined by cytogenetics in the local patient demographic. In this study, the DNAi missed approximately half of the patients who are truly hyper- or hypodiploid. These findings are contrary to those described in the international literature (Zaliova, Hovorkova et al. 2016, Gupta, Parihar et al. 2019, Bommannan, Arumugam et al. 2021, Alatawi, Bakhsh et al. 2023). Possible reasons for this will be expanded upon below, with consideration also given to the small sample size. The definitions of ploidy states used in this study may contribute to the unexpected findings, which highlights the impact of the lack of standardisation of DNAi values with regards to hyperdiploidy in international literature. Other studies have described a male preponderance in B-ALL (Arico, Valsecchi et al. 2008, Rachieru-Sourisseau, Baranger et al. 2010, Forero-Castro, Robledo et al. 2016, Bommannan, Arumugam et al. 2021, Qiu, Liao et al. 2021), however, this study as well as another study done in the South African context show a female preponderance (Vaughan, Bouwer et al. 2021). This may indicate an alternative disease profile locally and could explain the discrepancy noted. Despite the high prevalence of HIV in South Africa, the number of HIV positive patients in this study is negligible and therefore unlikely to explain this deviation of results from the literature. Due to the paucity of data in the international literature using the Kaluza C analysis software, post-analytical factors such as software limitations or human error in interpretation of results cannot be excluded as possible reasons for the deviation of the results. In order to formally incorporate DNAi into the current laboratory workflow, validation of this system comparing DNAi to conventional cytogenetics (gold standard) should be performed to complement the previous method comparison with Paint-A-Gate platform (BD Biosciences, Franklin Lakes, New

Jersey, United States of America). This would aid in determining a local population-specific reference.

The stronger expression of CD34 and CD123 in the hyperdiploid samples is in keeping with the literature which demonstrates an association between hyperdiploidy and overexpression of CD34 and CD123 (Borowitz, Shuster et al. 1990, Djokic, Bjorklund et al. 2009, Swerdlow SH 2017, Pierzyna-Switala, Sedek et al. 2021). Expression of surface CD22 (sCD22) is known to be associated with hyperdiploidy (Pierzyna-Switala, Sedek et al. 2021). There was no accessible data on sCD22 expression on the cases analysed, however, cCD22 expression was assessed and demonstrated no statistically significant variation in expression with regards to ploidy subtypes. The surface antigens, CD9, CD22, CD58, CD66c and CD86 have been described to be associated with hyperdiploidy (Pierzyna-Switala, Sedek et al. 2021, Kansal 2023), however, these were not tested in the majority of the study samples. Additionally, the literature describes dimmer CD45 expression and brighter CD20 expression in high-hyperdiploid cases (Pierzyna-Switala, Sedek et al. 2021, Kansal 2023), neither of which have been consistently demonstrated in this study. A sub-analysis was done which excluded 2 near-triploid cases from the antigen expression profile, however, the statistical outcomes remained unchanged.

This study did not discriminate between low hyperdiploidy and high hyperdiploidy, as this is not the current practice at CMJAH. The literature suggests a difference in prognosis between low hyperdiploidy (DNAi 1.10-1.16) and high hyperdiploidy (DNAi $\geq$1.16), the former being associated with an unfavourable prognosis and the latter associated with a favourable prognosis (Zhang, Habeebu et al. 2022). This lack of discrimination may be a contributing factor to the malalignment of the results with the international literature.

A limitation of the study was that the sample size did not reach the required amount for statistical significance. Further studies are required for definitive conclusions regarding the functionality of the DNAi in the South African context.

Conclusion

The results of this study do not support the ability of the DNAi to currently correctly predict the ploidy status as defined by the gold-standard test, conventional cytogenetics, in the local patient population. Possible reasons for this include the variation in the international literature on the definition of hyperdiploidy, the limited study sample size and demographic differences in patients with B-ALL in South Africa. In keeping with the international literature, brighter expression of CD34 and CD123 was present in the hyperdiploid group, regardless of the method used to define hyperdiploidy. However, the decreased expression of CD45 was not apparent. Further studies are needed in the South African context to better assess the functionality of the DNAi locally.

Funding

This study received no funding.

Disclosures

The authors declare no conflicts of interest.

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Appendix E: Copy of approved research protocol

Title

A 3-year retrospective analysis comparing flow cytometry-based DNA ploidy (DNA index) to conventional cytogenetics in paediatric patients diagnosed with B-cell acute lymphoblastic leukaemia.

Student: Dr Delwen Henderson

Student number: 2620016

Degree pursued: Master of Medicine in Haematological Pathology

Supervisor: Dr Nikki Bouwer

Qualifications: MBChB, MMed, FCPath Haem (SA)

Position: Senior Pathologist

Co-supervisor: Dr Anima Baiden

Qualifications: MBChB, MMed, FCPath Haem (SA)

Position: Pathologist

1. Introduction/Background

Acute lymphoblastic leukaemia (ALL) is a haematological malignancy which is defined by proliferation of clonal lymphoid progenitor cells. It is associated with significant heterogeneity in clinical presentation, phenotype, genotype and response to therapy. Most often onset is rapid, and the disease is usually fatal if left untreated (1, 2, 3). ALL is sub-classified into two main entities, namely B- and T-cell acute lymphoblastic leukaemia based on the cell of origin. B-cell acute lymphoblastic

leukaemia (B-ALL) comprises approximately 85% of total ALL cases, whilst T-cell acute lymphoblastic leukaemia (T-ALL) is in the minority, making up the remaining 15% (2).

Risk stratification and prognostication of paediatric patients with B-ALL takes into account various genetic lesions, the best described of which are detected using conventional cytogenetics (4). This ultimately dictates the treatment protocol prescribed and carries weight in the decision whether stem cell transplantation is needed or not (1, 2, 5, 6, 7). High-risk patients usually need more intensive therapy and are more likely to receive an allogeneic stem cell transplant. Certain toxic agents (e.g. anthracyclines) may be omitted or minimalised in patients with standard-risk disease which lessens long-term complications associated with these agents (8).

Aneuploidy is a common finding in B-ALL (6, 7). Ploidy status is conventionally defined by chromosomal modal number determined by cell culture and karyotyping (2, 6). Another method of measuring ploidy is by using the DNA index (DNAi) which is a representation of the DNA content of the leukaemic cells and is displayed as a ratio of leukaemic cell DNA content compared to DNA content in a healthy lymphocyte. The DNAi is determined using flow cytometric analysis (5, 6, 9).

Initial studies assessing the DNAi as a marker of prognosis demonstrated that patients with a DNAi ≥ 1.16 were 12 times less likely to fail treatment than those with a DNAi < 1.16 (9). The literature reveals that patients with a DNAi within the favourable range (high-hyperdiploid range) have a superior prognosis even if they

are classified as intermediate risk or high risk by other clinical criteria when treated with the BFM (Berlin-Frankfurt-Munich) protocol (which is the protocol used most commonly in our patient cohort) (8).

Additional chromosome gain, however, is not sporadic, and the chromosomes usually gained include 4, 6, 10, 14, 17, 18, 21 and X (2, 10). High-hyperdiploidy is associated with a better outcome whilst low-hypodiploidy and near-haploidy are associated with a poor outcome (2, 5, 6, 7, 9, 11). It has also been demonstrated that the favourable prognosis may be related to a DNAi ≥1.16 or the presence of specific trisomies (4, 6, 10, 17, 18, 22, double trisomy 4 and 10, double trisomy 10 and 17 or triple trisomy 4, 10 and 17) rather than absolute modal number (10). S-phase fraction analysis has not been shown to be useful in prognosticating these patients (2, 6, 12).

Conventional cytogenetics remains the gold standard for assessing the ploidy status. However, it necessitates a high level of expertise, is difficult to culture and is a time-consuming process (2, 7). Since the prognostication of B-ALL is important and will play a role in choice of treatment regimen, a quicker method of determining ploidy status is useful (7). Another method is also useful in cases where conventional cytogenetics has failed or is not available (such as in low resource settings) (2, 7, 11). DNAi assessment can be done on the same sample as the diagnostic flow cytometry immunophenotyping and can be available at the time of diagnosis, therefore speeding up the risk stratification of patients. The table below (Table 1) defines the different ploidy states by modal number and DNAi (2, 7). As seen below,

sources differ as to the DNAi lower limit (LL) upper limit (UL) for the different ploidy states, especially with regard to high-hyperdiploidy (2, 3, 5, 6, 8, 12). This lack of standardisation creates confusion when ploidy states are defined. In the past few years there has been increasing interest in re-evaluating the DNA index as a prognostic factor in B-ALL as well as the traditional cut-off values (3, 12).

Table 1

Ploidy status	Modal number (karyotype)	DNAi (flow cytometry)
Near-haploid	24 - 29	0.55 - 0.69
Low-hypodiploid	30 – 39	0.70 – 0.88
High-hypodiploid	40 – 45	0.89 – 0.95
Diploid	46	0.96 – 1.05 (LL 0.90 (6))
Low-hyperdiploid	47 – 50	1.06 – 1.15 (UL 1.09 (6))
High-hyperdiploid	51 – 65	1.16 – 1.39 (UL 1.41 (2) and 1.59 (8); 1.10 – 1.60 (6))
Near-triploid	66 – 80	1.40 – 1.79
Near-tetraploid	81 – 102	1.80 – 2.28

Adapted from Gupta et al (5).

It has been shown that hyperdiploidy on karyotyping is associated with an early response to treatment, but the DNAi has not shown the same association (3). Early response to treatment is measured by bone marrow response on day 7 or day 14 (<5%) blasts or by day 8 peripheral blood flow cytometric response (<1000 blasts/µL)(13).

Studies have shown certain immunophenotypic profiles, WCC and blast sizes to be associated with different ploidy statuses (2, 8), but literature confirming this in our patient demographic using the Beckman Coulter Kaluza ploidy analysis and immunophenotyping platforms was sparse at best.

Therefore, the aim of this study is to evaluate the DNA index using flow cytometry on the Navios flow cytometer and Kaluza C analysis software (Beckman Coulter Life Sciences, Indiana, US), which was introduced to our unit in March 2020, with reference to conventional cytogenetic findings in the South African patient population.

2. Study aims and objectives

Aim:

Evaluation of the DNA index functionality in paediatric patients with B-ALL.

Objectives:

i. To compare DNA index measured by flow cytometry on the Beckman Coulter Navios flow cytometer using Kaluza C analysis software and DNA ploidy as assessed by conventional cytogenetics (gold standard).
ii. To determine whether specific immunophenotypic profiles (see appendix B) in our population correspond with cytogenetically defined ploidy categories (hypodiploid/diploid/hyperdiploid).

3. Methods

Site of study

The study will take place at Charlotte Maxeke Johannesburg Academic Hospital (CMJAH) Leukaemia immunophenotyping unit and somatic cell genetics unit. Samples for leukaemia immunophenotyping are received from Chris Hani Baragwanath Academic Hospital, Helen Joseph Hospital, Frere hospital, Donald Gordon Medical Centre and Charlotte Maxeke Johannesburg Academic Hospital.

Participants/Samples

Inclusion criteria:

- Diagnostic paediatric B-ALL samples submitted for flow cytometry DNA ploidy analysis and conventional cytogenetics collected between March 2020 and September 2023.

Exclusion criteria:

- B-ALL cases run for flow cytometry DNA ploidy analysis with unsuccessful or unequivocal (low metaphase yield (<5), poor morphology, marker chromosomes) conventional cytogenetics result.

Sample size

All patients meeting the inclusion and exclusion criteria within the stipulated time frame will be included. Expected sample size is approximately 200 samples.

A sample size calculation using a prevalence of 23.4% (obtained from a local study in Johannesburg, South Africa) (14), confidence interval of 95% and a 5% margin for

error, equates to a sample size of 276. If the sample size calculation is done using 12.7% (obtained from an international study using the prevalence for southern Sub-Saharan Africa and corrected to exclude chronic leukaemias) (15), the sample size changes to 171.

Study design

The study will be a retrospective diagnostic method data analysis.

Data collection

Please see appendix A for data collection sheet. All data will be collected by the investigator using flow cytometry reports, DNA ploidy analysis from the Navios instrument, conventional cytogenetics reports and bone marrow aspirate and trephine reports traced on the laboratory information system (LIS). Data will be captured directly onto a password-protected google spreadsheet to minimize transcription errors and data loss. Data will be anonymised using study case numbers which will be stored in a separate document. No patient identifiers will be present on the data collection sheet. No patient names or dates of birth will be captured.

For this study, ploidy statuses derived from flow cytometry will be assigned using the table below (table 2), which has been derived from the current standard operating procedure (SOP) in use at CMJAH and current literature (see table 1). Ploidy status as defined by cytogenetics will be assigned using the ranges displayed in Table 2.

Table 2

Ploidy	**Modal number**	**DNAi**
Hypodiploid	<46	<0.95
Diploid	46	0.95 – 1.05
Hyperdiploid	>46	>1.05

Principles of tests used

Flow cytometry:

EDTA or heparin samples are suitable for processing. Samples are processed in the following steps:

1. Samples washed using phosphate buffered saline (PBS)
2. Addition of fluorochromes (fluorescent markers conjugated to monoclonal antibodies)
3. Incubation of fluorochromes and the patient sample in a dark chamber
4. Red cell lysis using isotonic ammonium chloride
5. Centrifugation and washing of the cells using PBS

The sampled are then loaded into the analyser when cells are directed into a single file and pass in front of a laser. The light from the laser is scattered by the cell allowing measurement of forward scatter (representation of the size of a cell) and side scatter (representation of the complexity of the cells). The laser also excites the fluorochromes which will then emit light at a specific wavelength. Light is captured by photo-multiplier tubes and every impulse generates an event which is represented

on a histogram. Using analysis software, the sample can then be interpreted to characterise the cells being analysed.

DNA ploidy assessment on flow cytometry involves staining of the DNA using Propidium Iodide which is a fluorescent dye. The fluorescence measured is directly proportional to the amount of DNA bound to the dye. The intensity of the fluorescence is compared to cells which contain a normal amount of DNA and in this manner DNA ploidy can be assessed. A sex matched peripheral blood sample is used as a control. The prepared sample used for flow cytometry as mentioned above is not used as pre-lysed cells can disintegrate during permeabilisation. 3 samples are prepared for DNA ploidy assessment: a control, the patient sample and a mix of the two. These cells are permeabilised and so-called DNA cocktail is added which contains the propidium iodide. This is done prior to loading the sample onto the analyser, after which cells pass in front of a laser and impulses are generated as described above.

Cytogenetics:

A heparin sample preferably from a bone marrow aspirate (containing at least 20% blasts) is suitable for cytogenetic analysis. Samples are added to growth medium (RPMI), foetal bovine serum (FBS) and an antibiotic and thereafter incubated at 37°C between 24 and 72 hours. Cells are harvested, firstly by centrifugation to remove the previously added media, after which colchicine is added to cause mitotic arrest. Hypotonic potassium chloride is added, followed by incubation for 25 min resulting in swelling of the cells to enable better visualisation of chromosomes.

Fixitive (methanol and acetic acid) is added and the sample is once again centrifuged. Once the supernatant has been removed, the sample is slow fixed with a higher volume of acetic acid and methanol. A last centrifugation and wash cycle is performed, after which the sample is stored in fridge. Slides are made the following day which are then baked and subsequently stained to allow the banding pattern of chromosomes to be appreciated.

Slides are scanned and uploaded digitally for analysis. Metaphase cells are analysed using specific software and a report is compiled. At least 20 metaphases should be analysed to be of good quality. All results are checked prior to authorisation of results.

4. Data analysis

Regarding patient demographics, categorical variables will be presented as the number (%) and continuous variables will be presented as mean values with standard deviation.

The relationship between conventional cytogenetics and DNAi will be assessed using the below definitions to determine specificity, sensitivity, positive predicative value and negative predicative value.

The following definitions will be used:

True positive: Hyperdiploid or hypodiploid on both DNAi and karyotype

False positive: Hyperdiploid or hypodiploid on DNAi but diploid on karyotype

True negative: Diploid on both DNAi and karyotype

False negative: Diploid on DNAi but hyperdiploid or hypodiploid on karyotype

Following on from this, agreement between the two tests (cytogenetic karyotype and DNAi) of the three classifications (hypodiploid/diploid/hyperdiploid) will be calculated using a kappa co-efficient.

The relationship between cytogenetically defined ploidy status and immunophenotypic profile will be presented descriptively. Rare ploidy states (near-tetraploid, near-triploid, near-haploid) will be excluded as outliers. The immunophenotypic profiles as well as the response to therapy will be compared across both the DNAi defined ploidy states and the cytogenetically defined ploidy states with known prognostic significance.

5. Ethics

Provisional ethics approval has been granted by the Human Research Ethics Council (M230417 MED23-04-130).

Confidentiality will be maintained by assigning non-identifiable consecutive study case numbers to subsequent cases (i.e. 1,2,3 etc.) with no patient names or dates of birth. Laboratory episode numbers corresponding to case numbers will be stored separately and will only be accessible to the investigators.

6. Timing

2023-2025	Feb - Apr	May-Jul	Aug-Oct	Nov-Jan	Feb-Apr	May-Jul	Aug-Oct	Nov-Jan	Feb - Apr
Literature review									
Protocol preparation									
Protocol assessment									
Ethics application									
Data collection									
Data analysis									
Write up									

7. Funding

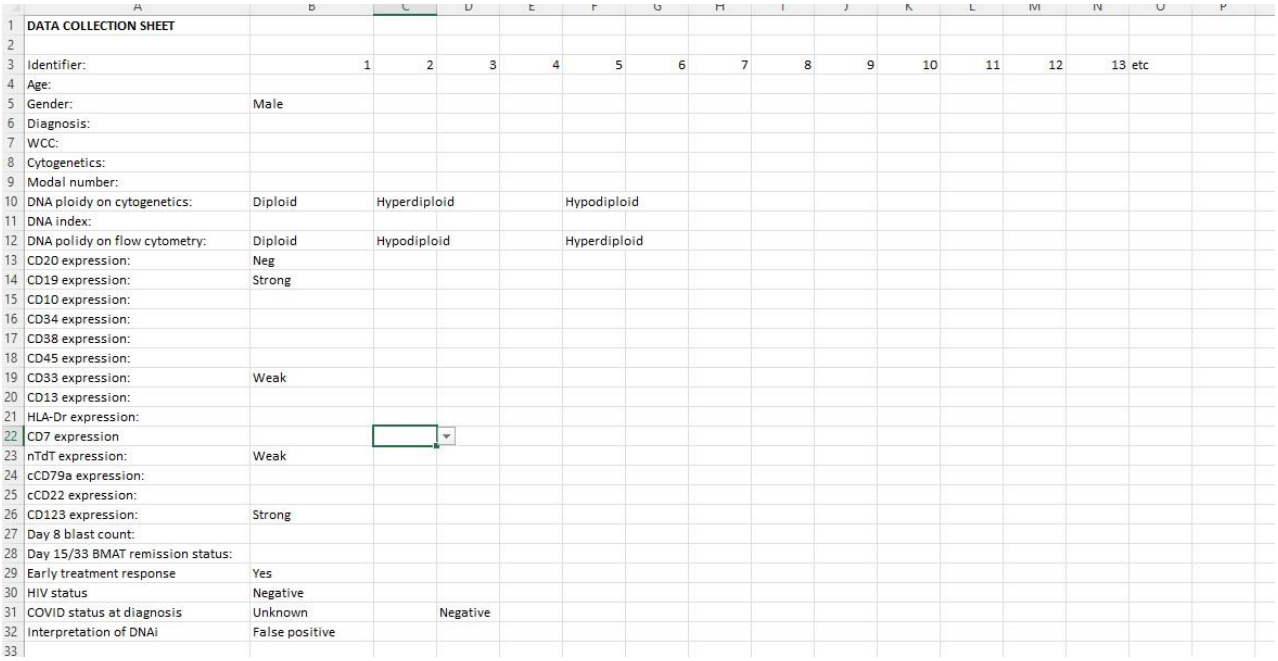
No funding is needed.

8. Limitations

The sample size may not reach the number required for statistical significance and this may become a limitation of this study.

9. Appendices

Appendix A: Data collection sheet



	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P
1	**DATA COLLECTION SHEET**															
2																
3	Identifier:	1	2	3	4	5	6	7	8	9	10	11	12	13	etc	
4	Age:															
5	Gender:	Male														
6	Diagnosis:															
7	WCC:															
8	Cytogenetics:															
9	Modal number:															
10	DNA ploidy on cytogenetics:	Diploid	Hyperdiploid			Hypodiploid										
11	DNA index:															
12	DNA polidy on flow cytometry:	Diploid	Hypodiploid			Hyperdiploid										
13	CD20 expression:	Neg														
14	CD19 expression:	Strong														
15	CD10 expression:															
16	CD34 expression:															
17	CD38 expression:															
18	CD45 expression:															
19	CD33 expression:	Weak														
20	CD13 expression:															
21	HLA-Dr expression:															
22	CD7 expression															
23	nTdT expression:	Weak														
24	cCD79a expression:															
25	cCD22 expression:															
26	CD123 expression:	Strong														
27	Day 8 blast count:															
28	Day 15/33 BMAT remission status:															
29	Early treatment response	Yes														
30	HIV status	Negative														
31	COVID status at diagnosis	Unknown		Negative												
32	Interpretation of DNAi	False positive														
33																

Appendix B: List of immunophenotypic markers investigated

The expression of the following antigens on the leukaemic blasts will be investigated. These antigens will be classified as one of the following three categories: negative, weak expression or strong expression.

- CD20
- CD19
- CD10
- CD34
- CD38
- CD45
- CD33
- CD13
- HLA-Dr
- CD7

- nTdT
- cCD79a
- cCD22
- CD123

10. References

1. Forero-Castro M, Robledo C, Benito R, Abaigar M, Africa Martin A, Arefi M, et al. Genome-Wide DNA Copy Number Analysis of Acute Lymphoblastic Leukemia Identifies New Genetic Markers Associated with Clinical Outcome. PLoS One. 2016;11(2):e0148972.
2. Pierzyna-Switala M, Sedek L, Kulis J, Mazur B, Muszynska-Roslan K, Koltan A, et al. Multicolor flow cytometry immunophenotyping and characterization of aneuploidy in pediatric B-cell precursor acute lymphoblastic leukemia. Cent Eur J Immunol. 2021;46(3):365-74.
3. Qiu KY, Liao XY, He ZW, Wu RH, Li Y, Xu LH, et al. DNA index as prognostic factor in childhood acute lymphoblastic leukemia in the COG-TARGET database. BMC Cancer. 2021;21(1):813.
4. Swerdlow SH CE, Harris NL, Jaffe ES, Pileri SA, Stein H, Thiele J, Arber DA, Hasserjian RP, Le Beau MM, Orazi A, Siebert R. WHO Classification of Tumours of Haematopoietic and Lymphoid Tissue. Revised 4th Edition ed. Lyon: International Agency for Research on Cancer (IARC); 2017.
5. Gupta N, Parihar M, Banerjee S, Brahma S, Pawar R, Rath A, et al. FxCycle Based Ploidy Correlates with Cytogenetic Ploidy in B-Cell Acute Lymphoblastic Leukemia and Is Able to Detect the Aneuploid Minimal Residual Disease Clone. Cytometry B Clin Cytom. 2019;96(5):359-67.
6. Kumar BK, Bhatia P, Trehan A, Singh AP, Kaul D, Bansal D. DNA Ploidy and S-phase Fraction Analysis in Paediatric B-cell Acute Lymphoblastic Leukemia Cases: a Tertiary Care Centre Experience. Asian Pac J Cancer Prev. 2015;16(17):7917-22.
7. Bommannan K, Arumugam JR, Koshy T, Radhakrishnan V, Sagar TG, Sundersingh S. Blast size-specific flowcytometric ploidy assessment using FxCycle(TM) Violet dye and its correlation with conventional cytogenetic ploidy in pediatric precursor B-lineage acute lymphoblastic leukemia patients. Int J Lab Hematol. 2021;43(4):760-70.
8. Arico M, Valsecchi MG, Rizzari C, Barisone E, Biondi A, Casale F, et al. Long-term results of the AIEOP-ALL-95 Trial for Childhood Acute Lymphoblastic Leukemia: insight on the prognostic value of DNA index in the framework of Berlin-Frankfurt-Muenster based chemotherapy. J Clin Oncol. 2008;26(2):283-9.
9. Trueworthy RS, J.; Look, T.; Crist, W.; Borowitz, M.; Carroll, A.; Frankel, L.; Harris, M.; Wagner, H.; Haggard, M.; Mosijczuk, A.; Pullen, J.; Steuber, P.; Land, v. Ploidy of Lymphoblasts is the strongest predictor of treatment outcome in B-Progenitor Cell Acute Lympblastic Leukaemia of Childhood: A Pediatric Oncology Group Study. J Clin Oncol. 1992;10(4):606-13.
10. Zaliova M, Hovorkova L, Vaskova M, Hrusak O, Stary J, Zuna J. Slower early response to treatment and distinct expression profile of childhood high hyperdiploid acute lymphoblastic leukaemia with DNA index < 1.16. Genes Chromosomes Cancer. 2016;55(9):727-37.
11. Rachieru-Sourisseau P, Baranger L, Dastugue N, Robert A, Genevieve F, Kuhlein E, et al. DNA Index in childhood acute lymphoblastic leukaemia: a karyotypic method to validate the flow cytometric measurement. Int J Lab Hematol. 2010;32(3):288-98.
12. Noh OK, Park SJ, Park HJ, Ju H, Han SH, Jung HJ, et al. Re-evaluation of DNA Index as a Prognostic Factor in Children with Precursor B Cell Acute Lymphoblastic Leukemia. Ann Clin Lab Sci. 2017;47(5):535-40.
13. Lauten M, Moricke A, Beier R, Zimmermann M, Stanulla M, Meissner B, et al. Prediction of outcome by early bone marrow response in childhood acute lymphoblastic leukemia treated in the

ALL-BFM 95 trial: differential effects in precursor B-cell and T-cell leukemia. Haematologica. 2012;97(7):1048-56.
14. Vaughan J, Bouwer N, Willem P, Wiggill T, Hodkinson K. The translocation t(1;19)(q23;p13) (TCF3/PBX1 fusion) is the most common recurrent genetic abnormality detected amongst patients with B-cell lymphoblastic leukaemia in Johannesburg, South Africa. 2021. 2021;5.
15. Dong Y, Shi O, Zeng Q, Lu X, Wang W, Li Y, et al. Leukemia incidence trends at the global, regional, and national level between 1990 and 2017. Exp Hematol Oncol. 2020;9:14.

Appendix F: Raw data

Identifier	Age	Gender	Diagnosis	White cell count (x 10^9/L)	Cytogenetics	DNA index
3	6	Male	Pre B/Common ALL	72,42	46,XY[8]	1,13
4	4	Female	Pre B/Common ALL	4	46,XX[8]	0,99
6	3	Female	Pre B/Common ALL	132,14	46,XX,t(9;22)(q34;q11.2)[20]	1,03
7	4	Female	Pre B-ALL	3,01	45,X,-X,del(11)(q14)[16] /90,XX,del(11)x2[6]	0,96
9	1	Female	Pre B-ALL	1,91	46,XY,der(12)?inv(12)(p?13q?14)[8]/46,XY[12]	1,20
11	10	Male	Pre B-ALL	30,48	45,XY,dup(1)(q22q31),der(4;6)(6pter→6q13::4q35→4q31::4q13→4pter),i(7)(q10),t(9;20)(q12;p?12),-3,der(19)t(1;19)(q23;p13.3)[20]	1,16
12	2	Male	Pre B-ALL	4,09	45,XY,der(9;12)(q10;q10)[12]/46,XY[1]	1,05
13	3	Male	Pre B-ALL	86,62	46,XY[7]	0,93
14	13	Female	B-ALL	2,59	46,XX,add(7)(q?31)[8]/78<3n>,idem,+2,+4,+6,+10,+14,+17,+18,+18,+20,+21,+22[cp9]	1,52
15	9	Female	Pre B/Common ALL	368,93	46,XX,der(19)t(1;19)(q23;p13.3)[12]/46,XX,t(1;19)(q23;p13.3),i(9)(q10)[8]	1,13
16	1	Female	Pre B/Common ALL	1,76	46,XX,der(12)t(12;?)(p11.2;?)[3]/46,idem,add(19)(q13.4),+mar[cp5]/47,idem,+10[3]/46,XX[2]	1,23
18	6	Male	B-ALL	4,49	55,XY,+X,dup(1)(q?21q?25),+4,+der(6)t(1;6)(q?21;q?16),i(7)(q10),+9,+10,+14,+18,+21,+21[12]/55,XY,+X,dup(1)(q?31q?21),+4,+6,i(7)(q10),+9,+10,+14,+18,+21,+21[5]	1,13
20	14	Female	Pre B-ALL	3,86	46,XX[10]	1,19
21	2	Female	Pre B-ALL	20,77	46,XX[18]	1,12
23	5	Male	Pre B/Common ALL	17,39	54,XY,+X,+del(4)(q3?),+6,+10,+14,+17,+18,+21[15]	1,21
26	2	Male	Pre B-ALL	4,73	45,XY,del(1)(q?32q?42),der(7;12)(q10;q10),t(9;16)(q?13;q?24)[19]/46,XY[3]	1,06
30	10	Female	Pre B/Common ALL	6,12	46,XX,der(19)t(1;19)(q23;p13.3)[4]/46,idem,der(21)t(1;21)(q12;p?11.2)[17]	1,07
32	8	Female	Pre B-ALL	13,79	52~55,XX,dup(1)(q?21q?32),+4,+6,+8,+9,+10,+11,+14,+i(17)(q10),+21,+21[cp20]	1,15
33	8	Male	Pre B-ALL	66,6	46,XY[20]	1,07
34	2	Male	Pre B-ALL	21,68	46,XY,der(19)t(1;19)(q23;p13.3)[4]/46,idem,t(1;11)(p?31;q?21)[14]/46,XY[2]	1,14
37	17	Female	Pre B-ALL	18,9	46,XX,t(1;19)(q23;p13.3)[18]/46,XX[2]	1,04
38	4	Male	Pre-B/Common ALL	3,45	47,XY,del(11)(q23),+14[21]/46,XY[1]	1,16
39	3	Male	Pre B/Common ALL	4,91	~55,XY,+X,+5,+6,+10,+17,+18,+20,+21,+21[cp15]	1,18
41	7	Female	Pre B-ALL	6,4	47,XX,del(2)(p?22),+mar[3]/46,XX[1]	1,17
42	1	Female	Pre B-ALL	44,22	47,XX,+14[3]/46,XX[17]	0,97
43	9	Female	Pre B-ALL	4,33	45,X,-X?c,[18]/45,idem,t(1;6)(p?21;q27)[4]	1,11

44	13	Female	Pre B/Common ALL	1,76	50~52,XX,+X,+4,+9,+10,+18,+21[cp9]	1,18
45	12	Female	Pre B-ALL	10,16	46~47,XX,t(1;19)(q23;p13.3),del(3)(q?21q?27),add(5)(q?35),?inv(14)(q?q?),+mar,inc[cp17]	0,96
46	5	Male	Pre/Common B-ALL	3,72	45,XY,dic(2;12)(q?13;p?11.2),der(4)t(2;4)(q?13;p16)[20]/46,XY[1]	0,98
47	2	Female	Pre B-ALL	140,27	46,XX,der(19)t(1;19)(q23;p13.3)[20]	1,06
48	16	Female	Pre B/Common ALL	24,66	46,XX,t(17;19)(q22;p13.3)[18]/46,XX[2]	1,01
49	2	Male	Pre B-ALL	18,51	~64,XY,+X,+2,+4,+5,+6,+7,+8,+10,+11,+12,+14,+14,+15,+16,+17,+18,+21,+21inc[cp8]	1,35
51	9	Male	Pre B-ALL	0,64	48,XY,del(9)(p22),+mar1,+mar2[6]/46,XY[1]	1,05
52	3	Male	Pre B-ALL	49,75	47,XY,+22[6]/48,idem,+10[9]	1,04
53	2	Female	Pre B-ALL	131,14	55,XX,+X,+4,+6,+10,+14,+17,+18,+21,+21[6]	1,28
54	4	Female	Pre B/Common ALL	251	46~47,XX,i(7)(q10),add(9)(p?24),der(19)t(1;19)(q23;p13.3),+mar[cp15]/46,XX[5]	1,05
55	10	Female	Pre B/Common ALL	157,6	46,XX,der(19)t(1;19)(q23;p13.3)[8]/46,idem,del(9)(p?22)[4]/46,idem,t(1;6)(p?13;q?25.1)[8]	1,07
56	5	Female	Pre B-ALL	2,02	45,X,-X,t(4;12;15)(q?25;p?11.2;q?12)[17]/46,XX[1]	0,99
57	7	Male	Pre B-ALL	2,1	46,XY,t(2;12)(q?31;p?13),del(12)(p13)[21]	1,02
58	14	Female	Pre B-ALL	6,44	45~46,XX,t(2;6)(q?36;q?16),-19[9]/71~92,idemX2[cp11]	1,03
59	3	Female	Pre B/Common ALL	22,7	46,XX,der(19)t(1;19)(q23;p13.3)[5]/46,idem,t(13;16)(q?14;q?24)[11]/47,idem,+mar[3]/46,XX[1]	1,01
60	4	Female	Pre B/Common ALL	85,3	45,XX,del(6)(q?21q?25),der(8;9)(q10;q10),del(12)(p12),t(12;13)(q21;q?31), t(18;21)(p11.2;p13)[19]	1,03
61	12	Male	Pre B/Common ALL	2,8	47~48,XY,del(6)(q?13q?21),+21,+21[cp7]/46,XY[1]	1,01
62	10	Female	Pre B-ALL	41,6	46,XX[14]	0,96
63	8	Female	Pre B-ALL	171,3	46,XX,der(19)t(1;19)(q23;p13.3)[7]	1,13
65	5	Male	Pre B-ALL	18,35	46,XY,t(1;19)(q23;p13.3)[5]/46,idem,der(15)t(1;15)(q12;p11.2)[7]	1,1
67	4	Male	Pre B-ALL	49,2	46,XY[13]	1,15
68	9	Female	Pre B/Common ALL	8,73	47,XX,t(1;19)(q23;p13.3),+der(19)t(1;19)[15]	1,11
69	14	Male	Pre B-ALL	1,8	46,XY,-4,i(9)(q10),ins(15;?)(q?12;?),add(20)(q13),+mar1,inc[cp14]/46,XY[4]	1,18
72	2	Male	Pre B/Common ALL	7,46	46,XY[10]	1
74	2	Male	Pre B-ALL	11,44	46,XY,del(12)(p12)[9]/47,idem,+21[4]/46,XY[7]	1,17
76	1	Female	Pre B/Common ALL	28,39	46,XX[20]	1,22
77	1	Male	Pre B-ALL	79,16	46,XY,der(19)t(1;19)(q23;p13)[14]/46,XY[2]	1,01
78	4	Female	Pre B/Common ALL	2,95	56~59,XX,+X,dup(1)(q?31q?32),+3,+4,+5,+9,+10,+11,+14,+17,+18,+20,+21,+21,inc[cp8]	1,24
80	3	Male	Pre B-ALL	7,01	46,XY,der(7)?add(7)(p?)?inv(7)(p?q?),inv(14)(q?11.2q?32),inc[20]	1,04
81	3	Male	Pre B-ALL	25,9	58~62,XXY,del(1)(p?21),del(3)(q?21),-9,-12,-13,-16,-19,der(19)t(1;19)(q?21;q?13.4),+21,inc [cp7]	1,47
82	1	Female	Pre B/Common ALL	81	46,X,t(X;9)(q?13;p?13)[20]	1,01

83	1	Male	Pre B/Common ALL	10,8	46,XY,der(1)t(1;12)(q?42;q13),-12,+mar[20]	1,04
84	4	Female	Pre B/Common ALL	2,91	46,XX[3]	1,5
85	4	Male	Pre B/Common ALL	3,16	58~60,XY,+X,+X,+4,+6,+8,+9,+10,+del(12)(p13),+14,+17,+18,+21,+21,+21c,+mar1,+mar2[cp6]/46,XY[2]	1,46
86	4	Male	Pre B-ALL	12,26	52~56,XY,+X,dup(1)(q21q32),+4,+6,+10,+14,+17,+18,+18,+21,+21[4]/52~56,XY,+X,trp(1)(q21q32),+4, +6,+10,+14,+17,+18,+18,+21,+21[16]/46,XY[2]	1,26
87	3	Female	Pre B-ALL	2,23	52,XX,+4,+6,+14,+17,+21,+21[4]/53,idem,+X[13]	1,22
88	3	Female	Pre B-ALL	7,74	46,XX[20]	1,02
89	5	Male	Pre B-ALL	1,45	51~52,XY,+X,+9,+14,i(17)(q10),+18,+21,+21[cp10]	1,07
90	7	Female	Pre B/Common ALL	2,22	~45-46,X,+X,der(X;10)(p10;q10),inv(3)(p?25q?21),add(4)(p16),-11,del(12)(p?12p?11.2),-13,add(19)(p13),+mar[cp20]	1,05
92	5	Male	Pre B/Common ALL	2,01	47,XY,+21[5]	1,09
93	2	Male	B-ALL	2,21	48,XY,+i(1)(q10),t(2;9)(q?31;p?22),+21[2]/46,XY[6]	1,16
95	1	Female	Common B-ALL	6,09	45,XX,t(4;7)(q?13;q?31),del(14)(q22),t(9;16)(q10;q10),-16[5]	0,97
A1	6	Male	Pre B-ALL	6	46,XY,der(19)t(1;19)(q23;p13.3)[6]/54,idem,+5,+8,+18,+19,+20,+21,+21,+22[7]	1,03
A2	6	Male	Pre B/Common ALL	18	46,XY,der(19)t(1;19)(q23;p13.3)[20]	1,04
A6	6	Female	Pre B-ALL	3,44	54~55,XX,+X,+4,+add(6)(q?27),+9,+10,+14,+18,+21,+21,inc[cp3]	1,19
A8	7	Female	Pre B-ALL	1,08	54~57,XX,+X,+3,+4,+5,+6,+9,+9,+10,+14,+21,+21,inc[cp9]/46,XX[2]	1,19
A9	10	Male	Pre B-ALL	73,42	50,X,-Y,+X,+14,+14,+21,+21[8]/46,XY[3]	1,03
A10	0,25	Female	B-ALL (null phenotype)	17,37	46,XX[4]	0,98
A11	5	Male	Pre B/Common ALL	172,36	46,XY,inv(9)(p12q13)[6]	0,94
A12	4	Female	Pre B/Common ALL	58,11	46,XX,t(7;12)(q11.2;p13)[21]	1,02