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Pharmacogenomics

Pharmacogenetics of 6-mercaptopurine in a black Zimbabwean cohort treated for acute lymphoblastic leukaemia

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Background: 6-mercaptopurine usage is associated with myelotoxicity and increased risk in patients carrying metabolism-related genetic variations. This study aimed to determine the frequency of candidate gene polymorphisms and their association with 6-mercaptopurine intolerance. **Methods:** A total of 41 patients on acute lymphoblastic leukaemia treatment were genotyped for *TPMT* and *NUDT15* (rs116855232) alleles, and their association with dose intensity was analyzed. **Results:** The defective *TPMT*3C* allele frequency was 9.8%. The median maintenance dose intensity for *TPMT*1/*3C* participants was considerably lower (47%) when compared with the *TPMT*1/*1* wild-type (77%), although not statistically significant. **Conclusion:** This is the first pharmacogenetics study carried out in a black Zimbabwean leukemia patient cohort. The high defective *TPMT*3C* (9.8%) allele frequency points to the potential utility of pharmacogenetics testing for safe usage of 6-mercaptopurine in this population.

Plain language summary: Acute lymphoblastic leukemia (ALL) is the most common malignancy affecting children in Zimbabwe and 6-mercaptopurine is frequently used as part of its treatment. However, 6-mercaptopurine is associated with side-effects such as severe neutropenia (a condition where you have a low number of white blood cells called neutrophils in your blood), with increased risk observed in patients carrying variants in genes involved in the metabolism of 6-mercaptopurine. Therefore, this study aimed to determine the frequency of polymorphisms in specific genes as well as their association with drug intolerance. A total of 41 patients on ALL treatment were studied. Review of treatment records was done to determine the cumulative 6-mercaptopurine dose and calculate dose intensity. Genotyping (to determine the versions of a gene a patient carries) for *TPMT* and *NUDT15* (rs116855232) was performed and results correlated with drug dose intensity. The most frequent genotype was *TPMT*1/*1*, occurring in 80% of the participants. The remaining 20% were carriers with two different copies of TPMT (*1/*3C). The defective *TPMT*3C* variation occurred at 9.8% and none had *TPMT*2*, **3A*, **3B* or *NUDT15* rs116855232 variants. Comparison analysis with dose intensity was done for 23 participants (56%) who had maintenance records available. The median dose intensity of 47% for *TPMT*1/*3C* participants was considerably low when compared to that of a normal *TPMT*1/*1* patient, which was 77%. However, no statistically significant difference was observed between *TPMT* genotype and dose intensity. This is the first study in a group of leukemic Zimbabweans to investigate the frequency of *TPMT* and *NUDT15* variants. With a high variation frequency of 9.8% for the defective *TPMT*3C*, pharmacogenetics testing for *TPMT* before treatment with 6-MP is recommended in the Zimbabwean population.

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Future Medicine

Acute lymphoblastic leukemia (ALL) is the most common pediatric blood cancer, accounting for around 25% of all cancers in children [1–3]. With advances in chemotherapeutics, molecular genetics and understanding of disease pathogenesis, the 5-year survival rate has increased to above 80% in favorable conditions [4]. Different treatment protocols exist that are generally grouped into remission induction, consolidation, reinduction and maintenance phases [5]. 6-mercaptopurine (6-MP), a thiopurine and an antileukemic drug, is the basis of most maintenance regimens required to foster complete remission in ALL patients. 6-MP is given on a daily basis for 2–3 years; however, significant inter-individual variability in its disposition and pharmacodynamics results in differences in tolerance and efficacy [6–8]. 6-MP side effects include severe life-threatening myelotoxicity, which may require dose adjustments to obtain a desirable degree of myelosuppression [9]. This necessitates population-specific studies, as individuals carrying defective alleles in *TPMT* and *NUDT15* exhibit intolerance to standard 6-MP doses, have interrupted treatment schedules, present with intense leukopenia, suffer severe infections, and may even die [3,10].

Considerable candidate gene and genome-wide association studies have been carried out to explain this inter-individual variability, extensively evaluating germline pharmacogenetics (PGx) markers that can possibly predict 6-MP toxicity and intolerance [11,12]. As a result, thiopurine-induced myelosuppression has been linked to genetic variations in thiopurine methyltransferase (*TPMT*) and nucleoside diphosphate-linked moiety X-type motif 15 (*NUDT15*), both of which are implicated in 6-MP disposal [13]. This has led to incorporation of *TPMT* and *NUDT15* variants in currently available clinical guidelines, recommendations for testing in clinical practice and adoption of PGx in most countries [14].

The *TPMT* gene directly involved in 6-MP inactivation is notably polymorphic, with about 45 star alleles reported to the TPMT nomenclature committee [15]. *TPMT*2*, *TPMT*3A*, *TPMT*3B* and *TPMT*3C* alleles account for nearly 95% of people with lower TPMT activity and should be minimally included in all clinical PGx testing efforts [14]. Approximately 0.3% of Europeans or Africans have two alleles of the *TPMT* gene that results in loss of function, whereas 10% of patients have one loss-of-function allele, leading to no or intermediate *TPMT* activity, respectively [16,17].

The NUDT15 enzyme catalyses the transformation of active thioguanine triphosphates to less toxic 6-thioguanine monophosphates and 6-thioguanine diphosphate metabolites, preventing active metabolites from being incorporated into DNA and RNA [18]. Similarly, *NUDT15* is polymorphic, as the Pharmacogene Variation Consortium has currently catalogued approximately 21 variant alleles [15,19]. *NUDT15*2*, *NUDT15*3* and *NUDT15*9* are examples of loss-of-function allelic variants that have been identified [2]. Patients homozygous for *NUDT15* deleterious alleles are totally intolerant to 6-MP, in contrast to about 60 and 80% tolerance for those who are heterozygous and have wild-type alleles, respectively.

The drawbacks of these alleles, however, are brought on by the fact that they are relatively uncommon and occasionally exclusive to specific populations or races. The *NUDT15* variation, for instance, is uncommon among European individuals and more prevalent in Asian and Hispanic individuals [17,20]. *TPMT*3C* is frequently found in African (5–7%), and Southeast Asian (1–3%) people, whereas *TPMT*3A* is frequently seen among White people (8–10%) [16]. To the best of our knowledge, no studies have been carried out in the Zimbabwean population investigating the frequency of *TPMT* and *NUDT15* genotypes in ALL patients. This study therefore aims to determine the frequency of polymorphisms in candidate genes implicated in 6-MP metabolism in black Zimbabwean patients with ALL and evaluate whether these polymorphisms predict 6-MP intolerance during ALL therapy.

Methodology

An observational, cross-sectional study was conducted at the Parirenyatwa Group of Hospitals (PGH) in Zimbabwe which recruited 41 patients receiving standard-of-care treatment for ALL. Study participants were recruited from PGH pediatric and adult hemato-oncology wards and clinics, while specimen storage, processing and analysis were carried out at the African Institute of Biomedical Science and Technology (Harare, Zimbabwe). A sample size of 28 was calculated using Dobson's formula, and convenience sampling was used to enrol both children (<18 years old) and adults (≥18 years old). We retrospectively identified as potential participants all patients with a confirmed diagnosis of ALL from January 2015 up to October 2022. Participants who had a bone marrow transplant were excluded from the study as it affects the determination of the patient's genotype. ALL was diagnosed using a full blood count and a peripheral film, whereas a bone marrow aspiration and trephine biopsy examination were used

for confirmation. The clinical setting had limited access to advanced ALL diagnosis methods such as flow cytometry, cytogenetics, fluorescence *in situ* hybridization or molecular diagnosis.

A blood sample (3 ml) was collected from each enrolled participant for DNA extraction and genotyping. Participants' demographic information was gathered, and retrospective record reviews were carried out to look up baseline features and treatment data. All the adults (n = 4) were receiving induction therapy and none had started taking 6-MP; therefore, no 6-MP dosage information was available in their clinical records. The children were being treated using the Adapted Resource and Implementation Application (ARIA) guide protocol for ALL. The therapies given during the maintenance phase included weekly oral methotrexate (MTX), daily oral 6-MP, monthly intrathecal MTX, a monthly pulse of dexamethasone and monthly intravenous vincristine. For maintenance therapy, the starting dosages of 6-MP and MTX were 75 mg/m^2 per day and 20 mg/m^2 per week, respectively. At intervals of 4 weeks, a complete blood count was done, and 6-MP dosage was adjusted to keep the white blood cell level between 2.0 and 3.0 $\times$ 10^9/l. The 6-MP dose intensity (%) was defined as the ratio of the clinician-prescribed 6-MP dose to that of the protocol 6-MP dose during the maintenance stage of therapy to maintain a desirable absolute neutrophil count [18]. Based on the ARIA guide protocol, the 6-MP dose was adjusted according to toxicities and infections; therefore, the 6-MP dose intensity directly indicated drug sensitivity or tolerance. The dose intensity as a measure of tolerance to 6-MP was included in the comparison analysis with *TPMT* and *NUDT15* polymorphisms.

All the participants and their legal guardians provided written informed consent and assent for children aged 7–17 years, where appropriate. The study was approved by the Joint Research Ethics Committee (JREC) of Parirenyatwa Hospital, the University of Zimbabwe, and the Medical Research Council of Zimbabwe (MRCZ).

Genotyping of *TPMT* & *NUDT15* polymorphism

DNA were extracted from blood using the MagMax™ DNA Multi-Sample Ultra 2.0 Kit on an automated, high-throughput KingFisher Flex Magnetic Particle Processor system (Thermo Fisher Scientific, MA, USA). The extracted DNA was quantified using the Qubit 4 fluorometer (Thermo Fisher Scientific). TaqMan-based genotyping of *TPMT*2* (rs1800462), *TPMT*3A* (rs1800460, rs1142345), *TPMT*3B* (rs1800460), *TPMT*3C* (rs1142345), *TPMT*4* (rs1800584) and *NUDT15*2* (rs116855232) was done using the Genopharm® pharmacogenomics open array designed by Thermo Fisher Scientific [21]. The QuantStudio™ 12 K Flex real-time PCR system and TaqMan PCR reagents from Applied Biosystems (CA, USA) were used.

Statistical analysis

Quantitative participants' demographic data were presented as mean and standard deviation (SD) if normally distributed; otherwise, median and interquartile range (IQR) were used. Respective frequencies and percentages were shown for the qualitative data. Normality was assessed using histograms and the Shapiro-Wilks test. Statistical analysis was performed using Stata v15 and the PRISM software (GraphPad6, CA, USA) was used for visualizations. *TPMT* and *NUDT15* allele and genotype frequencies were computed from the obtained results. Hardy-Weinberg equilibrium (HWE) of the observed and expected genotype frequencies was tested using the χ^2 test, with a p-value $\leq$ 0.05 indicating deviation from HWE. The Mann-Whitney nonparametric test was used for comparing the differences of *TPMT* and *NUDT15* genotypes with 6-MP dose intensity. Associations between other variables such as gender and 6-MP dose intensity were performed using the Mann-Whitney U test. A p-value $<$ 0.05 was regarded as statistically significant for all analyses.

Results

General characteristics of study cohort

A total of 41 participants were enrolled in the study, of whom 37 were children (<18 years old) and 4 were adults (≥18 years old). Patients' baseline characteristics are shown in Table 1. For the children's group, the median age across ALL diagnoses was 4 years, with an IQR of 3–9 years. a total of 24 (65%) of the children were boys. The children's mean body surface area was 0.80 kg/m^2 with a SD of 0.18 kg/m^2. Four (11% of the 37 children) had B cells, four (11% of the 37 children) had T cells and 29 (78% of the 37 children) had a pre-B ALL diagnosis. Six children were in the induction phase, 32 had consolidation treatment records available, and 23 were in the maintenance phase. A total of four adults were enrolled in the study. Three (75%) were males and the median age was 29 years, with an IQR of 20–49 years old. All the adults (n = 4) were receiving induction therapy and none had started taking 6-MP; therefore, no 6-MP dosage information was available in their clinical records.

Table 1. General characteristics and demographics of the population (n = 41).

Characteristics	n (%)	Median (IQR)
Children (n = 37)		
Male	24 (65)	
Age (years)		6 (5–9)
Age at diagnosis (years)		4 (3–9)
BMI		15.9 (15.1–17.5)
BSA		0.8 (0.66–0.92)
ALL diagnosis		
B cell	4 (10.8)	
T cell	4 (10.8)	
Pre-B cell	29 (78.4)	
ALL risk classification		
Standard	18 (48.7)	
High risk	19 (51.30)	
Ethnicity		
Ndebele	3 (8.1)	
Shona	34 (91.9)	
Adults (n = 4)		
Male	3 (75)	
Age (years)		29 (20–49)
Age at diagnosis (years)		28.5 (19.5–49)
Ethnicity		
Shona	4 (100)	

ALL: Acute lymphoblastic leukemia; BMI: Body mass index; BSA: Body surface area.

Table 2. *TPMT* and *NUDT15* genotype frequencies.

Gene	Genotype	n	Frequency		HWE
			Observed	Expected	p-value
TPMT	**1/*1*	33	0.805	0.814	0.994
	**1/*3C*	8	0.196	0.177	
	**3C/*3C*	0	0	0.009	
NUDT15 (rs116855232)	CC	41	1	1	–

n: number of participants carrying the genotype; HWE: Hardy-Weinberg Equilibrium.

Frequency of *TPMT* & *NUDT15* polymorphisms

Genotyping of *TPMT* and *NUDT15* was carried out for the 41 participants. A total of 33 (80%) participants had the wild-type *TPMT*1/*1* genotype. The remaining 8 (20%) participants had a heterozygous *TPMT*1/*3C* genotype. The homozygous mutant *TPMT*3C/*3C* genotype was not observed among the study participants. None of the 41 participants carried the *NUDT15* rs116855232 CT or TT variant genotypes as shown in Table 2. The observed and expected *TPMT* genotypes conformed with the HWE. The allele frequency for *TPMT*1* was 90.2%, whereas it was 9.8% for *TPMT*3C*. Other *TPMT* alleles **2*, **3A* and **3B* were not detected in this study. The *NUDT15*1* allele was detected at a frequency of 100%.

Comparison between *TPMT* genotypes & 6-MP dose intensity

A total of 23 children had 6-MP maintenance records available (Table 1), whereas none of the adults had started taking 6-MP, so no 6-MP dosage information was available in their clinical records. The maintenance phase duration for the children ranged (IQR) from 6 to 16 months, and the median was 11 months. The median 6-MP dose intensity was 76%, ranging from 58 to 98%. The median 6-MP dose intensity was considerably low (47%) among *TPMT*1/*3C* individuals (n = 4) compared with *TPMT*1/*1* (77%) individuals (n = 19) (Figure 1). Similarly, the mean 6-MP dose intensity was lower (58%) for children with a *TPMT*1/*3C* genotype

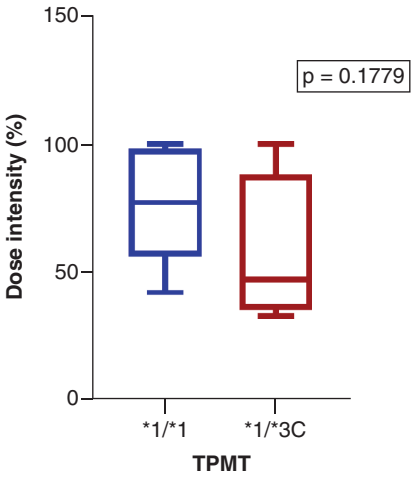

Figure 1. The comparison of 6-MP dose intensities among *TPMT1/*1 (wild type) and *TPMT**1/*3C (heterozygous) individuals.** P value was calculated using Mann-Whitney test.

Table 3. Comparison of allele frequencies from this study cohort with other populations.

			TPMT				
Population	***1***	***2***	***3A***	***3B***	***3C***	***4***	**p-value**
This study	0.902	0	0	0	0.097	0	–
African–American	0.923	0.005	0.008	0	0.024	0	0.997
South Asian	0.981	0.000	0.004	0.001	0.011	0.000	0.995
East Asian	0.980	0.000	0.000	0.000	0.016	0.000	0.996
European	0.953	0.002	0.034	0.002	0.005	0.000	0.994
Sub-Saharan Africa	0.922	0.000	0.002	0.000	0.053	0.000	0.999
			NUDT15				
rs116855232	**This study**	**African**	**Admixed American**	**East Asian**	**European**	**South Asian**	**p-value**
C	1	0.999	0.955	0.905	0.998	0.931	0.999
T	0	0.000	0.045	0.095	0.002	0.070	

Allele frequencies of other populations obtained were from the Clinical Pharmacogenetics Implementation Consortium *TPMT* and *NUDT15* allele frequencies table. A χ^2 test was used to calculate the p-values.

compared with *TPMT*1/*1* carriers (76%). However, comparison analysis using the Mann-Whitney test revealed no statistically significant difference (p = 0.1779) in dose intensity between *TPMT*1/*1* and *TPMT*1/*3C* genotypes. Because no participant carried a variant for *NUDT15*, no comparison analysis was performed with a 6-MP dose intensity.

Discussion

In this study, we determined the frequency of candidate genes involved in the metabolism of 6-MP and their association with 6-MP intolerance and toxicity. We replicated the findings from the current body of knowledge that the defective allele *TPMT*3C* is the most frequent allele occurring in sub-Saharan Africans [22–24]. This allele was reported at a frequency of 9.8% in our study cohort of Zimbabwean ALL patients. Similar to what has been reported from studies in Africans, none of the participants carried the *NUDT15* c.415C>T variant associated with a significantly higher risk of 6-MP-associated leukopenia [25]. Comparison analysis showed that *TPMT*3C* individuals had a lower 6-MP dose intensity when compared with *TPMT* wild-types.

*TPMT*3C* was the only *TPMT* polymorphism reported in this study at a frequency of 9.8% (Table 3), which is considerably higher than the reported 5.3% for sub-Saharan Africans obtained from the PharmGKB database. However, it is comparable to the average global frequency of *TPMT* genetic variants, which is around 10% [7]. The comparison of allele frequencies from this study with those from other populations did not reveal any statistically significant difference. Of the fewer studies carried out in Africa so far, they have mostly looked at the frequency of *TPMT*2, *3A, *3B, *3C* and **4*, and barely *NUDT15* polymorphisms. Closer to our finding, an allele frequency of 7.6% for *TPMT*3C* was reported in 434 healthy Ghanaians [23]. In other studies in Africans, allele frequencies

of 5.3 and 5.6% were reported for *TPMT*3C* in healthy Nigerians (n = 360) and neurological patients of Black admixed South Africans (n = 184), respectively [26,27]. A study carried out in Kenya that recruited 398 ALL patients reported an allele frequency of 5.4% [28]. These differences in allele frequencies highlight the need to carry out ethnicity- or population-specific studies to guide the use of PGx in thiopurine dosing. All these findings support the uncontested claim that some ethnic groups exhibit considerably lower frequencies of these genetic variations than other groups, which warrants population-specific studies [7]. Based on the studies that have claimed considerable genetic diversity among African populations, it is somehow expected that these subpopulations have deviant allele frequencies [29,30]. In agreement with our study, these African studies did not report any *TPMT*2*, **3A*, **3B* or **4*, emphasising the importance of *TPMT*3C* in the PGx of thiopurines in Africans. Most importantly, none of the participants in our study had the *NUDT15* polymorphism, which agrees with previous studies [25].

The correlation of *TPMT* and *NUDT15* genotypes with 6-MP-induced toxic effects is usually performed during the maintenance phase of the therapy to exclude the influence of other drugs used in ALL treatment because it consists exclusively of 6-MP and low-dose MTX [31]. However, comparison analysis employing the Mann-Whitney test did not reach statistical significance (p = 0.1779) (Figure 1). Similar to this study, Correa-Jimenez *et al.* also failed to demonstrate statistically significant correlations between *TPMT* and *NUDT15* genetic variations and toxicity outcomes [32]. This lack of statistical significance is possibly due to the small sample size in our study [25]. Eight out of 23 children (35%) had low-maintenance dose intensities (range: 42–67%), and they did not carry any of the tested *TPMT* or *NUDT15* variants. These patients could have a low-maintenance dose because of poor adherence to their medication; however, we did not measure adherence in this cohort. Furthermore, other genes or genetic variants that affect the activity of the *TPMT* gene could be involved since we did not perform *TPMT* phenotyping in this study. This probably suggests the need for *TPMT* enzymatic activity testing or whole-exome sequencing, which might reveal other *TPMT* and/or *NUDT15* loss-of-function variants not tested in this study, which lead to 6-MP intolerance. Because no *NUDT15* polymorphism was found in this study, no analysis of the relationship between 6-MP dose intensity and hematological toxicity was performed. Sequencing could have revealed novel and rare *NUDT15* polymorphisms, as shown in the study by Moriyama *et al.*, who discovered three novel *NUDT15* variants only seen in African and European patients. One of the variants exhibited extremely low thermostability and had no catalytic activity at all, with one of the patients only tolerating 43.5 mg/m^2/day of 75 mg/m^2/day [17]. A genome-wide association study in children showed that patients with the homozygous *NUDT15* TT variant (*NUDT15* c.415C>T) could only tolerate 8.3% of their standard 6-MP dose, which was 75 mg/m^2/day [18]. Similarly, 6-MP dose reductions of 42 and 82% in patients with the CT and TT genotypes, respectively, were observed in Japanese children with ALL [33]. Another study of 124 ALL Uruguayan paediatrics discovered that the 6-MP dose was significantly lower in those with one or two *TPMT* and *NUDT15* risk alleles compared to those without risk alleles in all studied intervals [34].

According to Clinical Pharmacogenetics Implementation Consortium (CPIC) guidelines, individuals heterozygous for *TPMT* should start at 50% and receive 30–70% of the standard dose (75 mg/m^2/day) to avoid toxicity as they cannot tolerate their full dose [14]. From our study, 8 (20%) of 41 participants had a *TPMT*1/*3C* heterozygous genotype, and they could have benefited from PGx testing and dose adjustments. The **3C* allele occurred at a frequency of 9.8%, which is considerably high enough to consider using CPIC clinical guidelines in the Zimbabwean population. This implies that approximately 10 out of 100 individuals could benefit from *TPMT* screening. This would probably benefit both patients and the healthcare system by reducing the time needed to optimize the 6-MP dose by the clinicians, reducing unnecessary hospital visits due to frequent toxicity, and minimizing mortality and morbidity. Therefore, all patients diagnosed with ALL would need to be pre-emptively genotyped for *TPMT* before they are initiated on treatment. However, looking at resource-limited settings such as Zimbabwe, a reactive PGx approach could be considered. This means that only patients with severe, frequent toxicity and 6-MP intolerance would need to be genotyped for *TPMT* and *NUDT15*, and the CPIC guidelines recommendations would be applied based on the results.

Limitations for this study include a shorter study duration and the lack of complete clinical records for some participants. In the future, we recommend a large multicentre, prospective study with longer follow-up, documenting the toxicity and intolerance of 6-MP and its association with *TPMT* and *NUDT15* genotypes. A larger sample size would allow us to make better inferences about the association between toxicity outcomes and generalize the findings to a larger population of patients in Zimbabwe. Future studies should also take either the sequencing approach to uncover novel *TPMT* and *NUDT15* variations that may explain intolerance and toxicity with thiopurines or the genome wide association study (GWAS) approach to identify risk variants in other genes.

Summary points

- Use of 6-mercaptopurine (6-MP) in acute lymphoblastic leukemia patients is associated with myelotoxicity, and the risk is increased in patients carrying metabolism-related genetic variations.
- The goal of implementing *TPMT* and *NUDT15* pharmacogenetics (PGx) testing in clinical settings is to achieve safer and more effective personalized treatments for each patient.
- PGx information is lacking for African populations to guide thiopurine usage.
- This is the first study of 6-MP PGx in a black Zimbabwean leukemia patient cohort, and findings could guide implementation of clinical PGx guidelines, for example, the Clinical Pharmacogenetics Implementation Consortium.
- Genotyping for *TPMT*2*, **3A*, **3B*, **3C*, **4* and *NUDT15*2* (rs116855232) was performed, and results were associated with 6-MP drug dose intensity.
- The defective *TPMT*3C* allele occurred at an allele frequency of 9.8%.
- The highly defective *TPMT*3C* (9.8%) allele frequency points to the potential utility of PGx testing for the safe usage of 6-MP in this cohort.
- For future studies, we recommend large sample sizes for pharmacokinetics and pharmacogenetics studies in a Zimbabwean setting to guide the management of leukemias.

Author contributions

Pageneck Chikondowa contributed to research proposal development, conduct of the study, data analysis and writing of the paper. Derick Munkombwe contributed to development of the protocol, analysis of the results and writing of the paper. Zedias Chikwambi contributed to research proposal development, protocol development, data analysis and writing of the manuscript. Patience Kuona contributed to the protocol development, clinical supervision of the study, and writing of the manuscript. Collen Masimirembwa contributed to study conceptualization, research proposal development and writing of the manuscript.

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Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

Ethical conduct of research

This study was approved by the Joint Research Ethics Committee (JREC) of Parirenyatwa Hospital, University of Zimbabwe, and the Medical Research Council of Zimbabwe (MRCZ). All the participants and their legal guardians gave written informed consent and assent for children aged 7 to 17 years.

Data sharing statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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