OXFORD

BIOLOGY Methods & Protocols

Biology Methods and Protocols, 2024, bpad027

https://doi.org/10.1093/biomethods/bpad027

Advance Access Publication Date: 1 November 2023

Methods Article

Serum metallomics reveals insights into the associations of elements with the progression of preleukemic diseases toward acute leukemia†

Amna Jabbar Siddiqui[1], Noman Khan[2], Kauser Fatima[2], Sabiha Farooq[2], Muhammad Ramzan[2], Hesham R. El-Seedi[3,4,5,6], Jalal Uddin[7], Abdullatif Bin Muhsinah[8] and Syed Ghulam Musharraf [1,2,*]

[1]Dr. Panjwani Center for Molecular Medicine and Drug Research, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

[2]H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan

[3]Pharmacognosy Group, Department of Pharmaceutical Biosciences, Uppsala University, Biomedical Centre, Uppsala 751 24, Sweden

[4]Department of Chemistry, Faculty of Science, Menoufia University, Shebin El-Kom 32512, Egypt

[5]International Research Center for Food Nutrition and Safety, Jiangsu University, Zhenjiang 212013, China

[6]International Joint Research Laboratory of Intelligent Agriculture and Agri-products Processing (Jiangsu Education Department), Zhenjiang 212013, China

[7]Department of Pharmaceutical Chemistry, College of Pharmacy, King Khalid University, Asir 61421, Saudi Arabia

[8]Department of Pharmacognosy, College of Pharmacy, King Khalid University, Asir 61421, Saudi Arabia

*Correspondence address. H.E.J. Research Institute of Chemistry, International Center for Chemical and Biological Sciences, University of Karachi, Karachi 75270, Pakistan. E-mail: musharraf1977@yahoo.com

†This research work has been dedicated to Prof. Dr. Tahir Shamsi (late), National Institute of Blood Diseases, Karachi, Pakistan, for his countless efforts in the establishment of a bone-marrow transplant facility in Pakistan.

Abstract

Acute leukemia (AL) is a critical neoplasm of white blood cells with two main subtypes: acute lymphoblastic leukemia (ALL) and acute myeloid leukemia (AML). This study is focused on understanding the association of the preleukemic disease aplastic anemia (APA) with ALL and AML at metallomic level, using healthy subjects as a control. In this study, a validated and efficient inductively coupled plasma-mass spectrometry/MS-based workflow was employed to profile a total of 13 metallomic features. The study encompassed 41 patients with AML, 62 patients with ALL, 46 patients with APA, and 55 age-matched healthy controls. The metallomic features consisted of eight essential elements (Ca, Co, Cu, Fe, Mg, Mn, Se, and Zn) and five non-essential/toxic elements (Ag, Cd, Cr, Ni, and Pb). Six out of the 13 elements were found to be substantially different ($P < .05$) using absolute concentrations between serum samples of AL (ALL and AML) and preleukemia (APA) patients in comparison with healthy subjects. Elements including magnesium, calcium, iron, copper, and zinc were upregulated and only one element (chromium) was downregulated in serum samples of disease when compared with healthy subjects. Through the utilization of both univariate tests and multivariate classification modeling, it was determined that chromium exhibited a progressive behavior among the studied elements. Specifically, chromium displayed a sequential upregulation from healthy individuals to preleukemic disease (APA), and ultimately in patients diagnosed with ALL. Overall, metallomic-based biomarkers may have the utility to predict the association of APA with ALL.

Keywords: acute lymphoblastic leukemia; acute myeloid leukemia; aplastic anemia; inductive coupled plasma-mass spectrometry

Introduction

The normal level of elements is important in the human body because they play an essential role in the enzyme systems in many metabolic processes [1]. Zinc (Zn), Copper (Cu), and Manganese (Mn) are essential cofactors for a number of enzymes involved in DNA integrity [2]. They also play a role in membrane transport, nerve conduction, muscular contraction, and the function of sub-cellular systems such as mitochondria. These metals along with selenium (Se) also act as antioxidants [3]. These may affect immune responses and the production of free radicals [4].

Genetic hemachromatosis is caused by mutations in genes that lead to an increase in the level of iron to a harmful level [5]. Metal metabolism-related genetic diseases are caused by abnormalities in proteins and enzymes that are involved in nutrient metabolism and the production of energy. A lack or increase in iron (Fe), Cu, Mn, Zn, and Se can result from a variety of genetic diseases. [6]. The impact of various factors including trace elements and heavy metals in cancer etiology has been studied [7–9].

Acute leukemia (AL) is the malignant transformation of lymphoid or myeloid cells into primitive and undistinguishable cells [10]. Acute lymphoblastic leukemia (ALL) is the most common cancer in children, but acute myeloid leukemia (AML) instances in adults account for more than 80% of all cases [11]. Aplastic anemia (APA) is a blood disorder, and the molecular system responsible for the abnormality in the immune system and

Received: August 04, 2023. **Revised:** October 16, 2023. **Editorial decision:** October 17, 2023.

deficiencies in hematopoietic cells is genetic and involves alterations in telomere-repairing genes, and dysregulated pathways of T-cell stimulation [12]. Transformation of APA into ALL has been previously reported [13, 14].

The symptoms of ALL occur due to departure from regular hematopoietic processes, leading to the production of immature white blood cells (WBCs), which results in bicytopenia associated with leukocytosis. Patients with preliminary symptoms of pancytopenia and hypoplastic bone marrow are potential candidates for an APA diagnosis [15]. This aplasia is temporary, arises in about 2% of pediatric ALL cases, and is thought to be a preleukemic condition [16]. The etiology of the APA symptoms and the successive overt signs of ALL is unknown, and it is also uncertain whether the leukemic process occurs after the aplasia or if it is already present at the time of APA diagnosis. Although the exact initial event that causes ALL is unknown, some research suggests that ALL is caused by the acquisition of chromosomal aberrations, most commonly during fetal hematopoiesis, which results in a subclinical preleukemic clone of cells, and/or postnatal secondary genetic modifications [13]. Several studies have reported the level of serum trace elements in malignant disorders such as leukemia and lymphomas [17]. The current study utilizes inductively coupled plasma-mass spectrometry (ICP-MS) for the analysis of leukemia, pre-leukemia, and healthy serum samples. The aim is to establish the linear association of metal concentration with disease progression.

Material and methods

Auxological data of subjects

This study comprises samples including 41, 62, and 46 fasting sera of AML, ALL, and APA, respectively. The subtypes of AML, ALL, and APA are given in Supplementary Table S1. In this study, 55 healthy volunteers were also selected. Table 1 contained the biochemical parameters of AML, ALL, and APA. At the time of sampling, all healthy individuals included in the study exhibited normal signs, had no history of family members diagnosed with APA, AML, or ALL, and were free from any blood-related disorders or other medical conditions. Additionally, they were in good physical condition.

Sample collection and characterization methodology

In compliance with the ethical principles outlined in the Helsinki Declaration, sample collection was carried out at the National Institute of Blood Diseases and Bone Marrow Transplantation, Pakistan, with the informed consent of the individuals involved. The study received approval from the hospital's ethics committee, in accordance with the International Conference on Harmonization Good Clinical Practice criteria, as well as the Independent Ethics Committee (IEC) (-022-HB-2017). All experiments adhered to legal requirements and institutional guidelines. Each patient and healthy volunteer completed a comprehensive questionnaire, which was duly approved by the ethics committee. Disease samples were classified as per guidelines of the World Health Organization. Prior to sample collection, it was taken into account that the patients were neither in reduction stage nor had they received any therapy. The healthy individuals that were equivalent to their ages and genders were listed as negative controls to eradicate any effects of age and gender. Serum samples were collected from patients (after overnight fasting) in BD Vacutainer tubes (BD Franklin Lakes, NJ, USA, REF 367381), followed by centrifugation at 2000 rpm at 4°C for 10 min. Aliquots were then prepared and kept at −80°C until further analysis.

Reagents and standards

A water filtration and purification system (Thermo Scientific, MA, USA) produced deionized water (DI-water). Using the NanoPure Acid purification system (Nanonex, USA), analytical-grade reagent (AR, ACS), 70% HNO_3 (Cat # AR 1137, RCI Labscan Ltd, Bangkok, Thailand) was purified before being utilized for analysis. Thirty percent H_2O_2 was purchased from Merck KGaA Darmstadt, Germany (Cat # 107209). The tuning solution contains 2% HNO_3 and 1 g/l each of Mg, Li, Tl, Y, Co, and Ce. (Cat # 5185-5959), internal standard of 100 µg/ml (Sc, Bi, In, Lu, Ge, Rh, and Tb Cat # 5188-6525) and multi-element calibration solution 2A (Cat # 8500-6940) with the known concentration of 10 µg/ml of each element, were purchased from Agilent Analytical Technologies (Santa Clara, CA, USA). Tuning solution was used to optimize the ICP-MS parameters. Overnight, all glassware and bottles made of polypropylene were submerged in a 10% (v/v) HNO_3 reagent solution. All of the equipment was cleaned with DI water and dried in an Airstream® ESCO laminar-flow hood.

Preparation of standard solutions

Five percent HNO_3 aqueous solution was used as matrix solution for preparing standard calibrant for 13 elements (Ca, Co, Cu, Fe, Mg, Mn, Se, Zn, Ag, Cd, Cr, Ni, and Pb). Internal standards at a concentration of 100 µg/l were made in matrix solution. A calibration curve consisting of 16 points was made in the range from 0.0076 to 1000 µg/l of each metal using matrix solution. The same solution with no metals was used as a blank. Sensitivity was measured from the slope of the regression equation. Correlation coefficients, limit of detection (LOD), and limit of quantification (LOQ) were calculated to validate the study by using standard solution.

Preparation of the standard reference material

The methodology was authenticated through the measurement of both precision and accuracy from the known results of trace metal Seronorm™ serum L-1 (Sero, Billingstad, Norway, Cat # 201405). The manufacturer's protocol was followed for preparing the certified reference material (CRM). The contents of the vial were dissolved in 3 ml of deionized water and allowed to roll for 30 min to ensure thorough mixing. Subsequently, the mixture was transferred to a screw-cap plastic tube and further diluted with deionized water. The diluted CRM sample was analyzed using ICP-MS in triplicates for the analysis of each trace element.

Sample preparation for ICP-MS

A previously published method for sample preparation has been used in this study with slight modifications [18]. Microwave-assisted acid digestion of serum samples was performed using a microwave system equipped with multiwave ECO software version 1.51 and a 64MG5-T64 rotor (Anton Paar GmbH, Austria). Standard equipment, including a polytetrafluoroethylene (PTFE) lip seal tube and a single-use screw cap (Wheaton® 15 mm, cap 13-425), was utilized for this process. An aliquot of 50 µl of each serum sample was put into MG5 vials (Anton Paar, Hungary) together with 150 µl of 70% HNO_3 and 50 µl of 30% H_2O_2, which were then sealed with polytetrafluoroethylene (PTFE) lip and caps after being held in the fume hood for 15 min. By modifying the Anton Paar microwave system's settings, the samples were digested in two phases, with the first step's temperature set at 90°C and the second step's temperature set at 150°C. After the

Table 1. Experimental subject description of healthy and leukemia disease samples.

Patient characteristics	Healthy	ALL	AML	APA
Number of samples	55	62	41	46
Age in years min–max (median)	8–73 (26)	2–61 (22.5)	5–75 (30)	5–63 (24.3)
Gender (male/female)	44/11	43/19	25/16	34/12
Age male (years; $\mu \pm \sigma$)	31.3 ± 6.41	23.22 ± 10.51	33 ± 16	25 ± 14
Age female (years; $\mu \pm \sigma$)	30.18 ± 9.03	28.95 ± 17.44	37.12 ± 16.17	23.5 ± 16.64
Height (cm)	167.32 ± 7.95	–	161 ± 16	153 ± 15
Body weight (kg)	65.49 ± 6.99	–	55.42 ± 17.14	47.5 ± 16
BMI	23.59 ± 3.64	–	–	–
Fever	–	90% (56/62*100)	78.04% (32/41*100)	60.9% (28/46*100)
Weakness	–	73% (45/62*100)	58.53% (24/41*100)	63% (29/46*100)
Petechia/ecchymoses	–	–	–	39.1% (18/46*100)
Epistaxis/hematuria/gumbleed	–	–	–	41.3% (19/46*100)
Splenomegaly	–	26% (16/62*100)	14.6% (06/41*100)	8.7% (04/46*100)
Hepatomegaly	–	05% (3/62*100)	12.2% (05/41*100)	6.5% (03/46*100)
Lymphadenopathy	–	22% (14/62*100)	17.1% (07/41*100)	6.5% (03/46*100)
Packed red cell transfusion	–	–	29.3% (12/41*100)	71.7% (33/46*100)
Platelet transfusion	–	10% (6/62*100)	9.75% (04/41*100)	30.4% (14/46*100)
Hemoglobin 12–16 g/dl	–	9.43 ± 2.33	8.68 ± 1.97	8.49 ± 2.53
Total leukocyte count 4.5–11.0 × 10^9/l	–	39.13 ± 85.85	27.99 ± 40.86	3.09 ± 2.28
Neutrophils, %	–	30.75 ± 33.93	28.29 ± 26.13	22.72 ± 17.8
Absolute neutrophil count 1.8–7.8 × 10^9/l	–	2.36 ± 3.01	2.56 ± 3.74	0.66 ± 0.82
Platelet 150–450 × 10^9/l	–	83.92 ± 105.1	70.33 ± 95.5	20.02 ± 25.44
Bleed	–	48% (30/62*100)	29.3% (12/41*100)	–
Blasts, %	–	55.9 ± 28.2	57.5 ± 28.5	–
RBC transfusion	–	37% (23/62*100)	–	–
Lymphocytes, %	–	30 ± 23	–	–

MG5 vials were digested, they were all removed to cool at room temperature. The subsequent samples were taken in 15 ml of polypropylene tubes and diluted up to 3 ml by DI-water. Each sample was analyzed in triplicate followed by matrix correction using the solution of internal standard.

ICP-MS analysis

The Agilent 7700x ICP-MS system (Santa Clara, CA, USA) was utilized for quantifying the selected elements. To eliminate contamination between runs, a washing solution consisting of 0.1% HCl and 2% HNO_3 in deionized water was employed. ICP-MS parameters and data were controlled by using workstation Mass Hunter software (Agilent Technologies, Santa Clara, CA, USA). Parameters for ICP-MS are presented in the Supplementary Table S2.

ICP-MS data pre-processing and statistical analysis

Using Agilent Technologies' Mass Profiler Professional software, chemometric analysis was carried out in a number of phases. Ten thousand counts of the total abundance were adjusted for the filtering procedure. In order to standardize the values for each sample using an external scalar for scaling up and scaling down, the data were normalized. In this article, variables that exhibited $P < 0.05$ and fold changes greater than 1.5 were considered significant variables.

Statistical comparison and analysis were carried out between healthy and disease samples and characterization through APA, AML, and ALL. Unpaired *t*-test and ANOVA were used for two groups and more than two group analyses, respectively. For multiple test corrections and *P*-value calculations, the asymptotic computation method along with the Benjamini–Hochberg false discovery rate (FDR) procedure was employed.

SIMCA MKS Umetrics AB (version 14.1) software was applied to processed data for the unsupervised principal component analysis (PCA), supervised partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA).

Method validation

In the concentration range of 0.0076–1000 g/l, a linear calibration curve was obtained for each element. To display the counts per second (cps) data against the measured concentrations, the least-square regression approach was utilized. Various criteria such as correlation coefficients (R^2), LOD, and LOQ were used to assess the validity of the quantification approach [19]. The calibration curve ($n = 3$) revealed an excellent linear relationship with R^2 values ranging from 0.993 to 1.000. LOD = 3.3/S and LOQ = 10/S were observed in the range of 0.002–5.490 g/l and 0.006–16.637 g/l (Supplementary Table S3).

To validate the developed method, Seronorm™ trace elements serum L-1, a reference standard material (CRM) for trace metal analysis in biological samples, was employed. The observed values obtained from this validation process were found to be in agreement with the certified values, displaying no significant differences between them. Supplementary Table S4 is presented for the percentage recoveries of each element with the observed variance.

The variance between the diluent used for sample preparation and the reference solution was utilized to monitor analyte detection. Additionally, blood samples with two concentration levels for each element were used in the spike recovery test to ensure that our procedure was reliable and precise (RSD, percent). The precision was mostly determined to be less than 10%. Precision was determined using the relationship (RSD, percent) = (standard deviation/CM) * 100. Supplementary Table S5 showed the coefficient of variation (percent RSD) for serum samples ranging from 0.376 to 7.169. As a result, our approach is sensitive and precise.

Results

Metallomic profiling of serum for pre- and acute-leukemia patients and healthy subjects

ICP-MS analysis was carried out for the screening of 13 elements in serum samples of leukemia and healthy subjects. Using absolute concentrations of elements, 6 out of 13 were found to be significantly different ($P < .05$), upregulated or downregulated between the serum of healthy people and leukemia patients (Table 2). In comparison to healthy individuals, leukemia patients exhibited upregulation of five elements, namely magnesium, calcium, iron, Cu, and Zn, while only chromium was found to be downregulated in their serum.

The normalized concentration of 13 elements was utilized to construct a PCA score plot, as depicted in Fig. 1A. This showed a separation between samples of healthy and leukemia patients. The x-axis component had a value of R2X of 0.447, while the second component had a value of 0.187. A 95% limit of confidence generated a few outliers from Hotelling's T2-test, removed prior to discriminant analyses.

Using six statistically significant metals, PLS-DA was used to validate that these elements can distinguish leukemia patients from healthy individuals (Fig. 1B). Between healthy controls and leukemia patients, the 2D-PLS-DA score plot showed a distinct separation.

The built model was analyzed for its sensitivity from the proportion of the disease samples, while specificity was determined from the proportion of control samples. According to the data in Supplementary Table S6, sensitivity was 98.2%, specificity was 75.53%, and the classification rate was 78.64%.

Adding a second orthogonal projection to the previously mentioned model (Fig. 1C) had no material effect on the results, showing a 76.21% classification rate, while sensitivity was reduced to 67.97% (Supplementary Table S6). The ROC plot (Supplementary Fig. S1) and permutation analysis (Supplementary Fig. S2) of the generated OPLS-DA model demonstrated a favorable area under the curve and a minimal disparity between the goodness of fit and predictive capacity.

The variable loading plot from a validated OPLS-DA model can be used for ranking all variables in terms of their discrimination performance. Figure 1D shows that group separation is mostly caused by magnesium, calcium, chromium, cobalt, nickel, Cu, Zn, silver, and lead, which have the highest variable importance in projection (VIP) value.

Table 2. List of elements in serum of leukemia patients (AML and ALL) and pre-leukemia (APA) that are significantly different from serum of normal healthy subjects

Element	P (corr)	Log FC (ALL versus HEALTHY)	Regulation (ALL versus HEALTHY)	Log FC (AML versus HEALTHY)	Regulation (AML versus HEALTHY)	Log FC (APA versus HEALTHY)	Regulation (APA versus HEALTHY)
24 Mg	6.09×10^{-40}	1.951	Up	1.730	Up	1.204	Up
40 Ca	1.52×10^{-15}	1.331	Up	1.081	Up	0.368	Up
52 Cr	7.20×10^{-35}	1.466	Up	−0.110	Down	1.409	Up
56 Fe	4.16×10^{-04}	0.738	Up	0.506	Up	0.621	Up
63 Cu	1.52×10^{-14}	1.312	Up	1.171	Up	1.070	Up
66 Zn	1.57×10^{-12}	1.122	Up	1.296	Up	0.860	Up

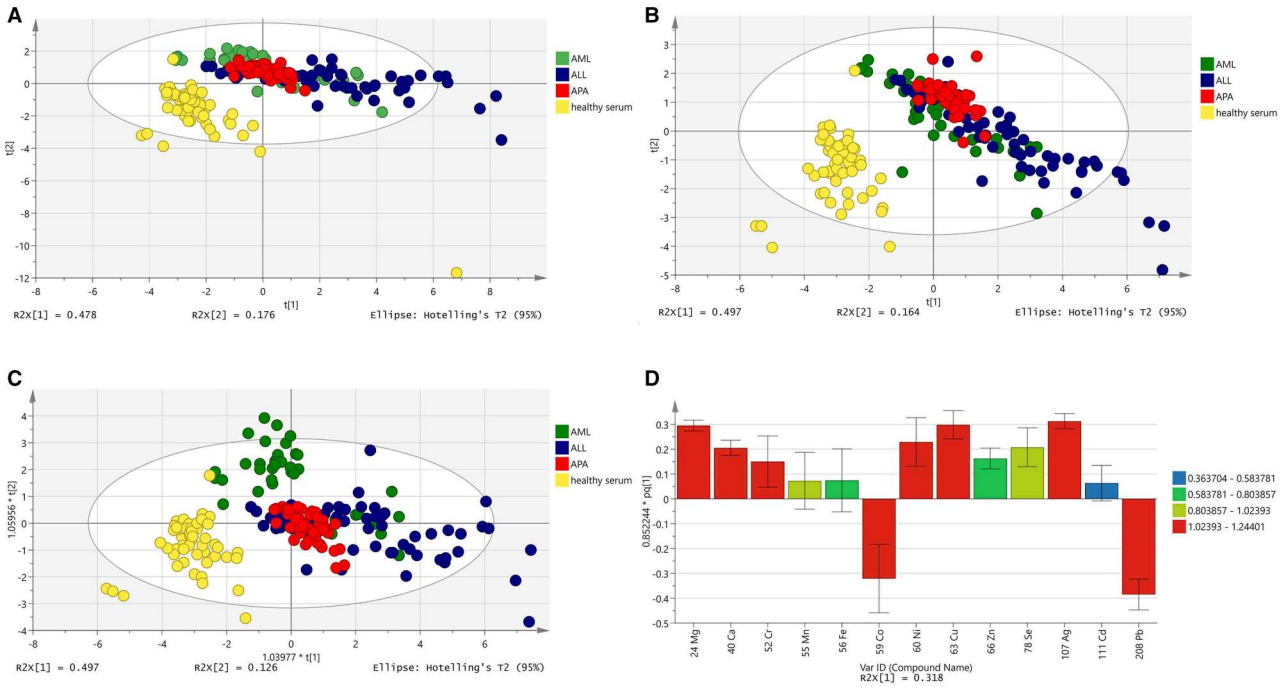

Figure 1. Metallomics showed differences among groups of AML, ALL, APA, and Healthy. (A–C) Score plots of PCA, PLS-DA, and OPLS-DA models showing the separation between the above mentioned groups, respectively. Each point represents a sample, green: AML patients, blue: ALL patients, red: APA patients, yellow: healthy controls. (D) Loading plot of OPLS-DA model colored as a function of VIP. Each bar represents the average of triplicate value against 13 elements.

Table 3. List of elements in serum of preleukemia (APA) with leukemia patients (AML) that are significantly different

Element	P (corr)	Log FC (APA versus AML)	Regulation (APA versus AML)
24 Mg	2.23×10^{-12}	−1.352	Down
40 Ca	1.14×10^{-12}	−1.370	Down
52 Cr	3.72×10^{-15}	1.483	Up
63 Cu	1.62×10^{-4}	−0.807	Down
66 Zn	4.00×10^{-13}	−1.397	Down

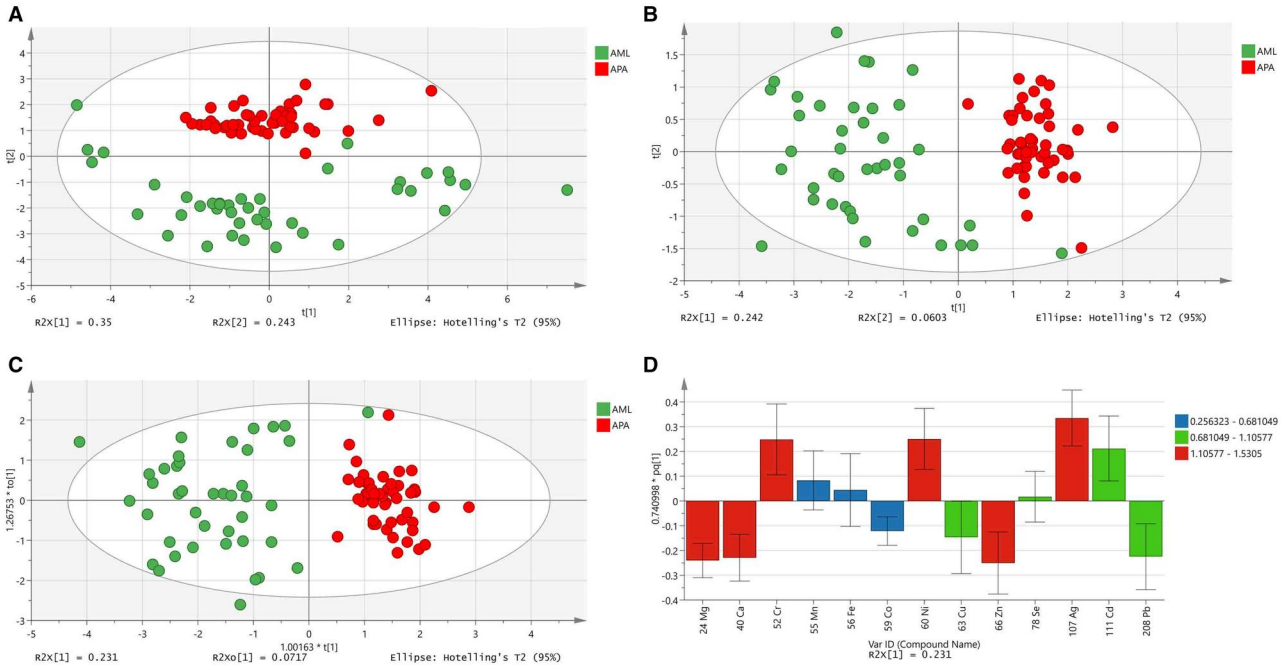

Figure 2. Metallomics showed differences among groups of AML and APA. (A–C) Score plots of PCA, PLS-DA, and OPLS-DA models showing the separation between the above mentioned groups, respectively. Each point represents a sample, green: AML patients, red: APA patients. (D) Loading plot of OPLS-DA model colored as a function of VIP. Each bar represents the average of triplicate value against 13 elements.

Chromium dysregulation in APA to AML patients

In the second phase of this study, a comparison was conducted between serum samples obtained from preleukemia patients (APA) and patients diagnosed with AML.

Five of the 13 elements were found to be significantly different ($P < .05$) when absolute concentrations were used (Table 3). Only chromium was shown to be upregulated in APA when compared to AML, while magnesium, calcium, Cu, and Zn were found to be downregulated.

The PCA score plot presented in Fig. 2A clearly demonstrates a noticeable differentiation between the APA and AML groups, based on the normalized concentration of elements. However, fewer outliers appeared at the 95% confidence level of Hotelling's T2-test. The first component on the x-axis, with an R2X value of 0.35, has higher variance than the second component, which has an R2X value of 0.243.

PLS-DA was used to perform class discrimination using five statistically significant metals (Fig. 2B). A strong separation drift between APA and AML patients has been seen in the 2D score plot. The sensitivity was 100%, and the specificity was 97.56%, with a classification rate of 98.89% (Supplementary Table S7). The addition of an extra orthogonal projection to the above model (Fig. 2C) had little effect on the model's sensitivity and specificity.

The variable loading plot from the verified OPLS-DA model was used to rank all variables in terms of their discrimination performance. Magnesium, calcium, chromium, nickel, Zn, and silver are mostly responsible for group separation, as seen in Fig. 2D.

Metallomic fingerprinting of serum for APA and ALL patients

The leukemia patients in this study were compared with patients with preleukemia, including APA. After conducting statistical significance analysis, five metals, namely magnesium, calcium, chromium, Cu, and Zn, were found to be significantly downregulated ($P < .05$) in the serum of APA compared to ALL, as indicated in Table 4.

The PCA analysis displayed a few outliers but did not demonstrate a clear differentiation between the APA and ALL groups, as shown in Fig. 3A. After orthogonal projection, the values of APA and ALL were not clearly differentiated from one another, according to the PLS-DA and OPLS-DA models (Fig. 3B and C, respectively). In contrast, the model's sensitivity and specificity are 81.63% and 85.48%, respectively, with 83.8% classification rate (Supplementary Table S7). The variable loading plot from the validated OPLS-DA model showed that magnesium, calcium, cobalt nickel, silver, and lead are largely responsible for group partition (Fig. 3D).

Metallomic profiling of serum for pre- and AL patients

Using absolute concentrations, 5 out of 13 elements showed significant differences ($P < .05$) (Table 5). Chromium was found for

Table 4. List of elements in serum of preleukemia (APA) with leukemia patients (ALL) that are significantly different

Element	P (corr)	Log FC (APA versus ALL)	Regulation (APA versus ALL)
24 Mg	2.74×10^{-7}	−0.989	Down
40 Ca	5.25×10^{-7}	−0.950	Down
52 Cr	4.52×10^{-5}	−0.784	Down
63 Cu	4.52×10^{-5}	−0.776	Down
66 Zn	8.90×10^{-5}	−0.740	Down

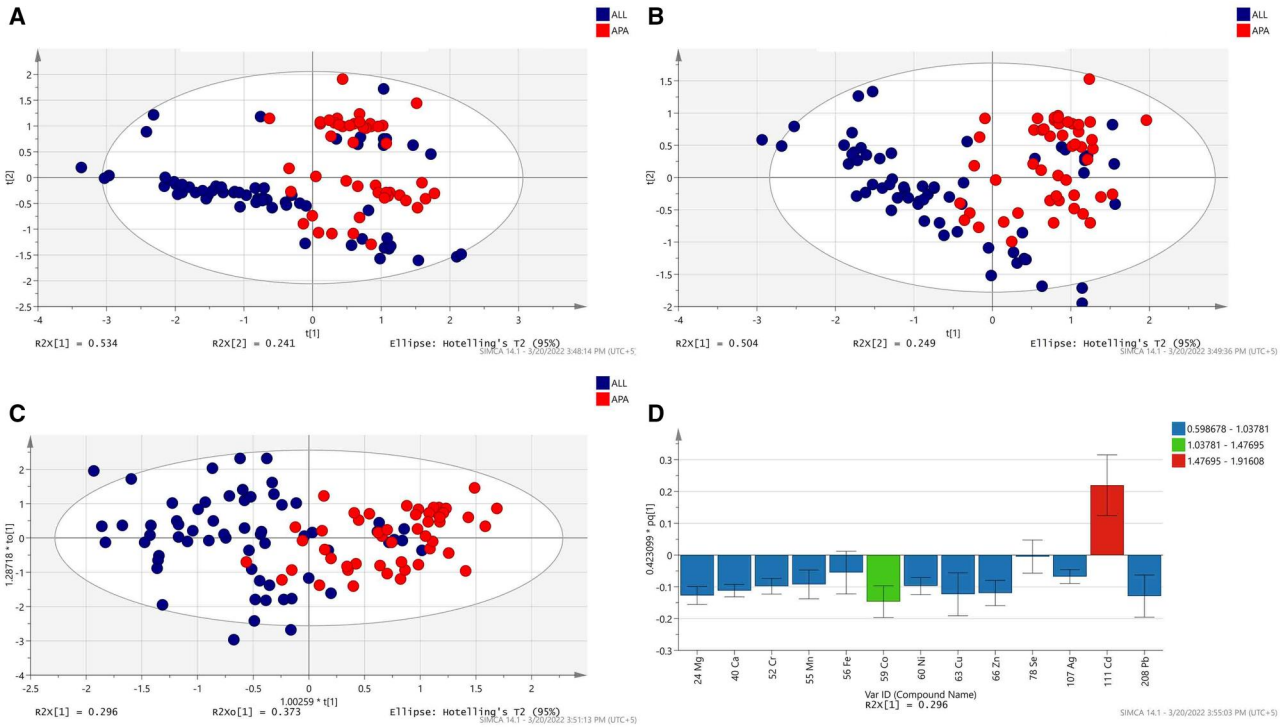

Figure 3. Metallomics showed differences among groups of ALL and APA. (A–C) Score plots of PCA, PLS-DA, and OPLS-DA models showing the separation between the above mentioned groups, respectively. Each point represents a sample, blue: ALL patients, red: APA patients. (D) Loading plot of OPLS-DA model colored as a function of VIP. Each bar represents the average of triplicate value against 13 elements.

Table 5. List of elements in serum of leukemia patients APA, ALL, and AML that are significantly different

Element	P (corr)	Log FC (ALL versus APA)	Regulation (ALL versus APA)	Log FC (AML versus APA)	Regulation (AML versus APA)
24 Mg	0.01	1.031	Up	0.726	Up
40 Ca	0.01	0.938	Up	0.695	Up
52 Cr	0.01	0.066	Up	−1.770	Down
63 Cu	0.01	0.780	Up	0.325	Up
66 Zn	0.01	0.667	Up	1.108	Up

downregulation only in AML as compared to APA while upregulated in ALL. Magnesium, calcium, Cu, and Zn were found to be upregulated in patients with ALL and AML as compared to APA.

PCA showed no separation between APA and ALL while clear separation can be seen between APA and AML patients (Fig. 4A). Substantial overlapping was also observed in the PLS-DA model with 70.01% sensitivity and 89.80% specificity with a classification rate of 75.66% (Fig. 4B; Supplementary Table S7). However, the OPLS-DA model (Fig. 4C) exhibited some group separation. Chromium, nickel, silver, and cadmium are key elements responsible for group differentiation in accordance to VIP values (Fig. 4D).

Discussion

Numerous essential elements are required by various processes within the human body to function optimally. These elements serve as crucial cofactors for numerous enzymes and are integral for maintaining the integrity of DNA [20–22]. Furthermore, they play pivotal roles in muscle contraction, membrane transport, and nerve conduction, and are components of subcellular systems like mitochondria. Deviations from the optimal levels of these elements can have adverse effects on biological activities and have been linked to severe diseases such as cancer [23]. Many research studies have explored the concentration of trace elements in the serum of individuals with malignant diseases,

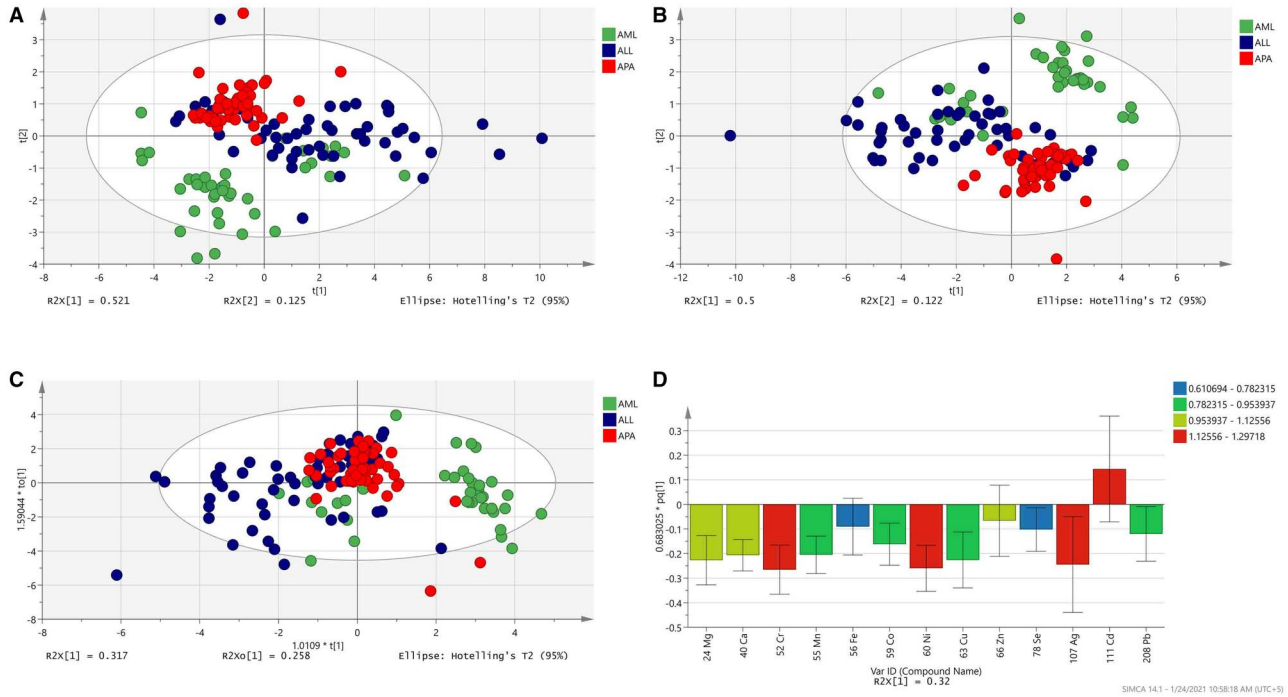

Figure 4. Metallomics showed differences among groups of AML, ALL, and APA. (A–C) Score plots of PCA, PLS-DA, and OPLS-DA models showing the separation between the above mentioned groups, respectively. Each point represents a sample, green: AML patients, blue: ALL patients, red: APA patients. (D) Loading plot of OPLS-DA model colored as a function of VIP. Each bar represents the average of triplicate value against 13 elements.

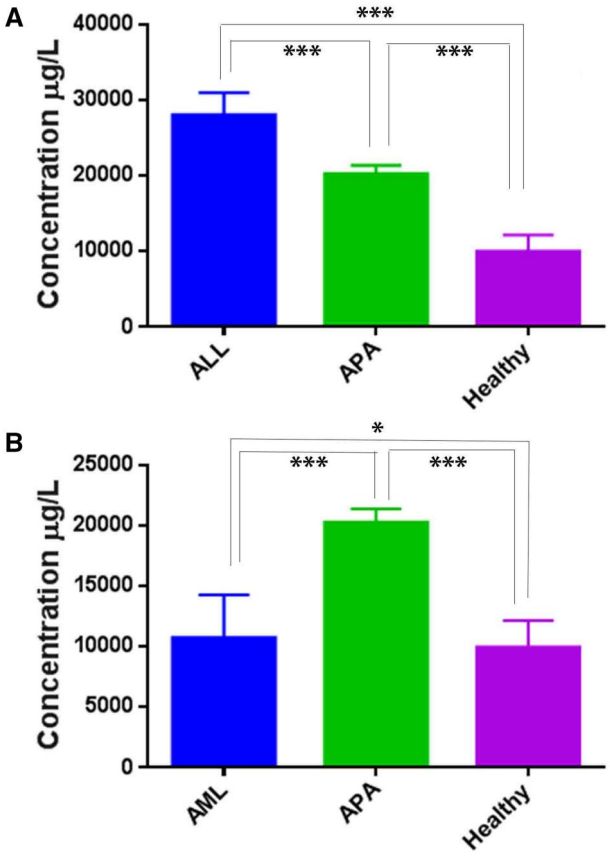

Figure 5. Absolute concentration of chromium element (µg/l) in serum of (A) ALL, APA, healthy control (B) AML, APA, and healthy control. All values are in triplicate. *P*-values *:>.05; **:<.05; ***:<.001.

including leukemia [24, 25]. However, there exist conflicting data in earlier research concerning the levels of trace elements in AL.

Metallomic analysis in our study has provided a snapshot of the trace elements that are differentially regulated in pre-leukemic and leukemia patients. Our findings initially compared the serum of participants with pre-leukemia, leukemia, and healthy individuals. Significance testing results indicated that among the elements examined in this study, six were found to be specific to the subjects. Notably, among these six elements, four essential metals exhibited a consistent pattern of increase in ALL, AML, and APA when compared to the healthy control group. Similarly, we observed elevated levels of all four elements in individuals with ALL/AML compared to those in the APA group. This suggests a consistent upward trend in their concentrations. While numerous studies have reported similar findings when comparing disease cases to controls [26–28], it is worth noting that a few studies have presented conflicting results [29, 30].

Hypomagnesemia is a relatively common electrolyte abnormality in leukemic patients. Mg levels were significantly lower in serum with AL than healthy controls in a few studies [17, 31–33]. Guo *et al.* reported that hypomagnesemia resulted from the transfer of extracellular magnesium to the skeletal system during bone formation, which occurred prior to the initiation of ALL treatment [34]. It was investigated by Meyer, who found that Mg reduction could be due to many factors which control the Mg concentration status, such as reduced uptake and mobilization of bone Mg, intestinal hypo-absorption of Mg hyper-adrenoglucocorticism by decreased adaptability to stress, occasional urinary leakage, adrenergic hyporeceptivity, and insulin resistance [35]. In contrast to these findings, our study revealed higher magnesium levels in cases compared to controls. Therefore, it is essential to confirm the accuracy of the Mg measurements before formulating any hypotheses.

The other element which is upregulated in cases as compared to controls is Cu. A study reported that higher level of Cu in APA compared to healthy subjects [36] and no correlations were found with severity of disease, which is similar to our study. The higher level of Cu was reported in the AL patient compared to healthy individuals [17, 37, 38]. Serum Cu has been postulated as a possible biomarker for the degree of leukemia and malignant lymphoma, as well as a predictor of treatment response [24].

Along with the role of Cu, Zn has also been widely researched in health and disease. The reported studies showed that APA lowered the level of Zn in serum compared to healthy individuals [36] and correlated with severity of disease, which is in contrast to our study. It has also been reported that lower levels of Zn occur in AL compared to controls [17, 39]. It is worth noting that the redox-inactive effect of Zn in chelation processes allows Zn to work as a cell protector and a defender of the body against the oxidative stress. Therefore, systems in the body possess a multistep protection from the disturbance of this physiological concentration. In addition, the body has a branched system of Zn delivery to any organ, and the delivered concentration is controlled by many competing ligands. Therefore, overall results of these processes can induce the 'pendulum' effect which will enhance disorders of homeostasis.

Hazardous metals can contaminate the body and often substitute for essential metals, thereby competing for ligands, disrupt biochemical reactions, and result in disease [40]. Even short-term exposure to hazardous metals causes several gene expression and epigenetic variations that can drive cells to malignant transformation [41]. The toxicity of chromium (VI) involves mitochondrial and DNA damage of blood cells, which leads to cancer development [42]. Toxicity and carcinogenicity of Cr (VI) is the result of its harmful effects on individual gene expression and on entire biochemical pathways [43]. It is interesting that among all metals, only chromium was observed for group separation between APA and AML. In this study, sequential increase of the metal chromium has been observed in the progression from healthy to preleukemia, that isAPA and lastly ALL (Fig. 5A). While in the case of AML, it was observed that absolute concentration of the metal chromium has been decreased in AML in comparison to APA and almost equal to healthy subjects (Fig. 5B).

Conclusion

The findings of this study demonstrate that metallomic profiling of ALL, AML, APA, and the healthy control groups has revealed significant associations between specific elements and preleukemia, which play a crucial role in the development of cancer. The results indicate that calcium, magnesium, Zn, Cu, and chromium exhibit distinct disease-related patterns and can serve as diagnostic and prognostic markers. These elements could potentially be utilized as supplementary predictive markers in cases where there is a reported progression of preleukemic diseases toward AL. However, validation of these results is required with larger cohorts before the routine clinical use of such markers.

Acknowledgements

The authors are thankful to all individuals who provide us their samples (blood) on voluntarily basis. Authors are thankful to Mr. Arsalan Tahir and Junaid-ul-Haq for their technical assistance in ICP-MS analysis.

Author contributions

Amna Jabbar Siddiqui (Data curation [equal], Formal analysis [equal], Project administration [lead], Software [equal], Visualization [equal], Writing—review and editing [equal]); Noman Khan (Data curation [lead], Formal analysis [equal], Investigation [equal], Methodology [equal], Software [lead], Validation [equal], Visualization [equal], Writing—original draft [lead]); Kauser Fatima (Formal analysis [equal]); Sabiha Farooq (Formal analysis [supporting], Methodology [equal]); Muhammad Ramzan (Data curation [supporting], Software [supporting], Visualization [supporting], Writing—review and editing [equal]); Hesham R. El-Seedi (Conceptualization [supporting], Funding acquisition [supporting], Writing—review and editing [equal]); Jalal Uddin (Funding acquisition [supporting], Investigation [equal], Writing—review and editing [equal]); Abdullatif Bin Muhsinah (Funding acquisition [lead], Resources [equal], Writing—review and editing [equal]); Syed Ghulam Musharraf (Conceptualization [lead], Funding acquisition [lead], Project administration [equal], Resources [lead], Supervision [lead], Writing—review and editing [equal])

Supplementary data

Supplementary data is available at *Biology Methods and Protocols* online.

Conflict of interest statement. None declared.

Funding

S.G.M. acknowledged the financial support from the Higher Education Commission (HEC project I.D. 4493) for conducting this research. The authors (A.B.M. and J.U.) extend their appreciation to the Deanship of Scientific Research at King Khalid University for funding this work through the Large Groups Project under grant number (RGP2/381/44).

Institutional review board statement

The study was conducted in accordance with the Declaration of Helsinki, and approved by the Institutional Review Board (or Ethics Committee) of ICCBS (protocol code ICCBS/IEC-022-HB-2017/Protocol/1.0 and 25 January 2017).

Informed consent statement

Informed consent was obtained from all subjects involved in the study.

Data availability

Data will be provided upon request.

References

1. Gundogdu M, Kaya H, Gulcin I *et al.* Oxidase activity of ceruloplasmin and some acute phase reactant and trace element concentrations in serum of patients with chronic lymphocytic leukemia. *Scott Med J* 2007;**52**:24–7.
2. Leach Jr, R. Role of manganese in mucopolysaccharide metabolism. *Fed Proc* 1971;**30**:991–4.
3. Shenkin A. Micronutrients and outcome. *Nutrition* 1997;**13**:825–8.

4. Wiernsperger N, Rapin J. Trace elements in glucometabolic disorders: an update. *Diabetol Metab Syndr* 2010;**2**:70–9.
5. Pietrangelo A. Hereditary hemochromatosis: pathogenesis, diagnosis, and treatment. *Gastroenterology* 2010;**139**:393–408.e2.
6. Umair M, Alfadhel M. Genetic disorders associated with metal metabolism. *Cells* 2019;**8**:1598.
7. Sohrabi M, Nikkhah M, Sohrabi M *et al.* Evaluating tissue levels of the eight trace elements and heavy metals among esophagus and gastric cancer patients: a comparison between cancerous and non-cancerous tissues. *J Trace Elem Med Biol* 2021;**68**:126761.
8. Singh V, Madeshiya AK, Ansari NG *et al.* CYP1A1 gene polymorphism and heavy metal analyses in benign prostatic hyperplasia and prostate cancer: an explorative case-control study. *Urol Oncol* 2023;**41**:355.e9–355.e17.
9. Nguyen HD. Prognostic biomarker prediction for glioma induced by heavy metals and their mixtures: an in-silico study. *Toxicol Appl Pharmacol* 2023;**459**:116356.
10. Jiang N, Kham SKY, Koh GS *et al.* Identification of prognostic protein biomarkers in childhood acute lymphoblastic leukemia (ALL). *J Proteomics* 2011;**74**:843–57.
11. Jemal A, Siegel R, Xu J *et al.* Cancer statistics, 2010. *CA Cancer J Clin* 2010;**60**:277–300.
12. Young NS, Calado RT, Scheinberg P. Current concepts in the pathophysiology and treatment of aplastic anemia. *Blood* 2006; **108**:2509–19.
13. Horsley SW, Colman S, McKinley M *et al.* Genetic lesions in a preleukemic aplasia phase in a child with acute lymphoblastic leukemia. *Genes Chromosomes Cancer* 2008;**47**:333–40.
14. Villarreal-Martínez L, Jaime-Pérez JC, Rodríguez-Martínez M *et al.* Acute lymphoblastic leukemia of childhood presenting as aplastic anemia: report of two cases. *Rev Bras Hematol Hemoter* 2012;**34**:165–7.
15. Melhorn DK, Gross S, Newman AJ. Acute childhood leukemia presenting as aplastic anemia: the response to corticosteroids. *J Pediatr* 1970;**77**:647–52.
16. Kelly K, Murphy P. Aplastic anaemia preceding acute lymphoblastic leukaemia in an adult with isolated deletion of chromosome 9q. *Leuk Res* 2008;**32**:1936–8.
17. Demir C, Demir H, Esen R, *et al.* Altered serum levels of elements in acute leukemia cases in Turkey. *Asian Pac J Cancer Prev* 2011; **12**:3471–4.
18. Hassan S, Mazhar W, Farooq S *et al.* Assessment of heavy metals in calcium carbide treated mangoes by inductively coupled plasma-mass spectrometry (ICP-MS). *Food Addit Contam Part A Chem Anal Control Expo Risk Assess* 2019;**36**:1769–76.
19. Tehreem S, Rahman S, Bhatti MS *et al.* A UPLC-DAD-based bioscreening assay for the evaluation of the angiotensin converting enzyme inhibitory potential of plant extracts and compounds: pyrroquinazoline alkaloids from Adhatoda vasica as a case study. *Molecules* 2021;**26**:6971.
20. Morales ME, Derbes RS, Ade CM *et al.* Heavy metal exposure influences double strand break DNA repair outcomes. *PLoS One* 2016;**11**:e0151367.
21. Kumar S, Budhwar R, Nigam A *et al.* Cytoprotection against Cr (6+)-induced DNA damage by alpha-lipoic acid: implications in reducing occupational cancer risk. *Mutagenesis* 2009; **24**:495–500.
22. Restrepo HG, Sicard D, Torres MM. DNA damage and repair in cells of lead exposed people. *Am J Ind Med* 2000;**38**:330–4.
23. Bi X, Pohl N, Dong H *et al.* Selenium and sulindac are synergistic to inhibit intestinal tumorigenesis in Apc/p21 mice. *J Hematol Oncol* 2013;**6**:8–7.
24. Tessmer CF, Hrgovcic M, Thomas FB *et al.* Long-term serum copper studies in acute leukemia in children. *Cancer* 1972; **30**:358–65.
25. Ilicin G. Serum copper and magnesium levels in leukaemia and malignant lymphoma. *Lancet* 1971;**2**:1036–7.
26. Akanni EO, Onuegbu AJ, Adebayo TO *et al.* Assessment of some selected trace metals in chronic myeloid leukemia patients in a tertiary health facility in South West Nigeria. *AJMS* 2013;**5**:71–5.
27. Lyle DB, Wang XH, Ayotte RD *et al.* Calcium uptake by leukemic and normal T-lymphocytes exposed to low frequency magnetic fields. *Bioelectromagnetics* 1991;**12**:145–56.
28. Trikannad Ashwini Kumar AK, Vellanki S, Krishnamoorthy G. Hypercalcemia as the sole initial presentation of precursor B-cell acute lymphoblastic leukemia. *Cureus* 2022;**14**:e22081.
29. Ohanian M, Telouk P, Kornblau S *et al. Am J Hematol* 2020; **95**:422–34.
30. Afridi HI, Kazi TG, Talpur FN. Correlation of calcium and magnesium levels in the biological samples of different types of acute leukemia children. *Biol Trace Elem Res* 2018;**186**:395–406.
31. Van Laecke S, Nagler E, Vanholder R. Magnesium and lymphoma: opportunities in translation. *Leukemia* 2014;**28**:729–31.
32. Birt D. Effects of the intake of selected vitamins and minerals on cancer prevention. *Magnesium* 1989;**8**:17–30.
33. Su Y, Chen LJ, He JR *et al.* Urinary rubidium in breast cancers. *Clin Chim Acta* 2011;**412**:2305–9.
34. Guo CY, Halton J, Barr R *et al.* Hypomagnesemia associated with chemotherapy in patients treated for acute lymphoblastic leukemia: possible mechanisms. *Oncol Rep* 2004;**11**:185–90.
35. Meyer KA, Kushi LH, Jacobs DR *et al.* Carbohydrates, dietary fiber, and incident type 2 diabetes in older women. *Am J Clin Nutr* 2000;**71**:921–30.
36. Gupta V, Kumar A, Asthana RK. Serum zinc and copper levels in aplastic anemia. *Indian Pediatr* 2012;**49**:493–4.
37. Carpentieri U, Myers J, Thorpe L, *et al.* Copper, zinc, and iron in normal and leukemic lymphocytes from children. *Cancer Res* 1986;**46**:981–4.
38. Akkuş İ, Gürbilek M, Çalişkan U *et al.* Blood and fingernail copper (Cu) and magnesium (Mg) levels of children with acute lymphoblastic leukemia. *Turkish J Med Sci* 1998;**28**:155–6.
39. Modaressi A, Hadjibabaie M, Shamshiri AR *et al.* Trace elements (Se, Zn, and Cu) levels in patients with newly diagnosed acute leukemia. *Int J Hematol-Oncol Stem Cell Res* 2012;**6**:5–10.
40. Abdulla M, Chmielnicka J. New aspects on the distribution and metabolism of essential trace elements after dietary exposure to toxic metals. *Biol Trace Elem Res* 1989; **23**: 25–53.
41. Alonso ML, Montaña FP, Miranda M *et al.* Interactions between toxic (As, Cd, Hg and Pb) and nutritional essential (Ca, Co, Cr, Cu, Fe, Mn, Mo, Ni, Se, Zn) elements in the tissues of cattle from NW Spain. *Biometals* 2004;**17**:389–97.
42. Ray RR. Adverse hematological effects of hexavalent chromium: an overview. *Interdiscip Toxicol* 2016;**9**:55–65.
43. Nigam A, Priya S, Bajpai P *et al.* Cytogenomics of hexavalent chromium (Cr6+) exposed cells: a comprehensive review. *Indian J Med Res* 2014;**139**:349.