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RESEARCH ARTICLE

A heavy metal baseline score predicts outcome in acute myeloid leukemia

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Abstract

Despite abundant epidemiological data linking metals to leukemia and other cancers, baseline values of toxic and essential metals in patients with leukemia and the clinical impact of these metals remain unknown. Thus, we sought to quantify metal values in untreated patients with acute myeloid leukemia (AML) and controls and determine the impact of metal values on AML patients' survival. Serum samples from patients with untreated AML and controls at Hospices Civils de Lyon were analyzed and compared for trace metals and copper isotopic abundance ratios with inductively coupled plasma mass spectrometry. Survival analysis was performed as a function of metal values, and a multi-metal score was developed for patients with AML. Serum samples were collected from 67 patients with untreated AML and 94 controls. Most patients had intermediate-risk cytogenetics (63.1%) without FLT3 internal tandem duplication mutations (75.6%) or *NPM1* mutations (68.1%). Most metal values differed significantly between AML and control groups. Patients with lower magnesium and higher cadmium values had the worst survival rates, with only 36% surviving at 6 months (P = .001). The adverse prognostic effect of this combination was maintained on multivariate analysis. Based on this, we developed a novel metal score, which accounts for multiple relative abnormalities in the values of five toxic and five essential metals. Patients with a higher metal score had significantly worse survival, which was maintained on multivariate analysis (P = .03). This baseline metal scoring system was also prognostic when we applied it to a separate population of front-line AML patients.

1 | INTRODUCTION

Toxic metals such as lead, cadmium, arsenic, nickel, and chromium enter the body as contaminants and often substitute for essential metals,[1-4] thereby competing for ligands, disrupting biochemical reactions,[3] and resulting in disease. Even brief exposure to toxic metals causes numerous gene expression and epigenetic changes that predispose cells to malignant transformation.[5] Acute myeloid leukemia (AML) is associated with various toxic metal exposures. For example, in a case-control study in Spain, an excess risk of childhood leukemia was observed near industrial and urban sites processing arsenic, cadmium, chromium, and nickel.[6] The participation of metals as effectors and modulators in cancer is not widely appreciated in clinical practice. Cadmium, lead, arsenic and other toxic metals are established carcinogens.[5] Relative deficiencies of the essential metals calcium, magnesium,[7] selenium, zinc,[8] and rubidium[9] are also associated with malignancies, as are elevations of the essential metals copper[6] and iron.[10]

Animal experiments have shown chronic exposure to cadmium has cytotoxic and genotoxic effects on peripheral blood and bone marrow cells.[11-15] Furthermore, exposure to cadmium and lead induces leukemia in mice[16,17] and accelerates leukemia cell proliferation in vitro.[18] Toxic metals are also immuno-toxic, impair innate and adaptive immunity,[19] and are associated with reduced natural killer (NK) cells.[20]

We hypothesized that there are imbalances of toxic and essential metals in patients with AML compared to healthy individuals, and that higher values of toxic and lower values of essential metals are associated with worse outcomes in AML patients. Thus, we compared serum metal values in patients with AML to those in healthy individuals and determined the extent to which metal values in AML patients correlated with clinical outcomes.

2 | METHODS

2.1 | Sample collection

For this case-control study, blood serum samples were collected from control participants with no hematologic malignancies, and patients with newly diagnosed AML presenting to Hospices Civils de Lyon, from January 2015 to April 2016. Samples were also collected at MD Anderson Cancer Center from patients with newly diagnosed AML from October 2005 to February 2017. Samples from patients with AML were collected prior to initiation of front-line chemotherapy. The diagnosis of AML was confirmed via analysis of bone marrow morphology. Control samples were also obtained from blood donors at Etablissement Français du Sang during the same time period. The study size was determined by the number of available serum samples. Participants in this study provided informed consent. The analysis of samples was approved by the Institutional Review Board of Hospices Civils de Lyon, Centre de Protection des Personnes du Centre Léon Bérard, ENS-Lyon, and the French Government Ministry of Health. The Institutional Review Board of The University of Texas MD Anderson Cancer Center also approved the sample collection and analysis. This research was conducted in accordance with the Declaration of Helsinki and is reported in accordance with the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement.

2.2 | Metal analysis

Serum samples were digested and mineralized in perfluoroalkoxy vessels at 130°C with HNO_3 and H_2O_2, and then evaporated in concentrated HCl at 120°C under a laminar flow hood. Stable dried specimens were analyzed for trace metal content using inductively coupled plasma mass spectroscopy (ICP-MS). Samples were also analyzed for the copper isotopic abundance ratio (dCu), the ratio of $^{65}Cu/^{63}Cu$, by using multicollector ICP-MS.

2.3 | Multi-metal score

A novel metal scoring system was utilized to risk-stratify the patients. We devised a scoring system based on whether a patient's values for 10 toxic and essential metals were above or below specified cutoff values. These cutoff values were determined based on a combination of clinical judgment and the serum metal value distributions in the control group. Patients were assigned one point for each metal that fell outside the "target range" (ie, a metal value above or below the predetermined limits) as shown in Figure 1. The overall score was a sum of the points. The rationale for constructing the score is also straight forward. Because relative deficiencies of calcium, magnesium,[7] selenium, zinc,[8] and rubidium[9] are associated with malignancies, patients received one point for any value lower than the specified values in Figure 1. Relative elevations of copper[6] and iron[10] also receive one point each because they have been associated with malignancies. Cadmium, lead, and arsenic[5] are established carcinogens,[5] and thus were also included in the scoring system, each receiving one point when exceeding the tabled limits in Figure 1. For seven patients, calcium levels by ICPMS were not available for the score and standard of care calcium values obtained from the clinical laboratory were used.

2.4 | Cytogenetic and molecular analyses

Bone marrow aspirates and biopsies were obtained from all AML patients. All AML cases were sub-classified using the World Health Organization's 2016 AML classification criteria.[21] To determine the immunophenotypic features of the samples, multicolor flow cytometry was performed as previously described.[22,23] Conventional cytogenetic analysis was performed on G-banded metaphases from bone marrow aspirates cultured without mitogen stimulation using standard techniques, and was reported using the International System for

Metal	Level (SI)	SI units	Points Given for Each Metal Value
Ca	<2.432	mmol/L	1
Mg	<1.069	mmol/L	1
Se	<1.603	umol/L	1
Zn	<14.898	umol/L	1
Rb	<3.182	umol/L	1
Cd	>0.75	nmol/L	1
Pb	>2.268	nmol/L	1
Cu	>16.366	umol/L	1
Fe	>29.009	umol/L	1
As	>14.148	nmol/L	1

FIGURE 1 Metal scoring system. One point is given for each metal value that falls outside the "target range" (ie, a metal value above or below the predetermined limits). The score is a sum of the points

Human Cytogenetic Nomenclature.[24] Molecular analysis was performed using a polymerase chain reaction-based assay,[25-28] and fluorescence in situ hybridization analysis was performed on bone marrow cultures using a dual-color break-apart probe as previously described.[29] Patients were risk-stratified according to karyotype, gene mutations and per ELN classification.[30]

2.5 | Statistical methods

Characteristics of participants were tabulated using descriptive statistics. Metal values were reported by disease status using medians, minimums, and maximums. The AML and control groups were compared using Wilcoxon rank sum or Kruskal-Wallis tests, as appropriate. Side-by-side box plots were constructed to visually compare the distribution of metal values between AML and control groups and among cytogenetic risk groups of AML patients.

Overall survival (OS) was defined as the time from the date of AML diagnosis until death. Patients who were alive at the last follow-up were censored at that time. Survival estimates were calculated and plotted using Kaplan-Meier methods.[31] Univariate Cox proportional hazards regression models were used to assess the association between survival and each metal value as a continuous variable.[32] In order to normalize the data, natural log-transformations were taken of the metal value plus 0.000000001 to avoid calculating the log of zero. Each metal was then included in a model that controlled for age and sex. Metal values were reported transformed back to their original units. Hazard ratios and their 95% confidence intervals (CIs) were determined, along with *P* values assessing whether there was a continuous effect on survival risk with increasing metal. Each metal was then included in a multivariate Cox model that controlled for age and sex.

Next, univariate and multivariate recursive partitioning analyses using classification and regression trees (CART)[33,34] were conducted to identify subgroups of patients with different risks of death for each detected metal. The minimum number of patients in any terminal subgroup was set to 20. A two-sided log-rank test[35] was applied to assess differences between subgroups in each split. The splitting was stopped if the *P* value was greater than .05. In that case, a two-sided log-rank test was applied in pairwise fashion to compare terminal subgroups. Two terminal subgroups were combined if the *P* value was greater than .05.

Grouping of patients according to the metal score was based on an intuitive division into three groups. A multivariate Cox regression was implemented to determine whether this score group remained a significant predictor of survival after accounting for the ELN risk groups and blasts. Statistical analyses were performed in Stata 14.2 (StataCorp, College Station, TX) and SAS 9.4 (SAS Institute Inc. Cary, NC).

3 | RESULTS

3.1 | Patient and AML characteristics

A total of 67 patients with newly diagnosed AML and 94 healthy volunteers underwent serum collection for trace metal analysis at Hospices Civils de Lyon (Table 1). The median age was 67 years (range 28-87 years) for the AML patients, and 58 years (range 20-89 years) for the control group. The two groups had similar distributions of males and females. Most patients with AML lacked *FLT3* internal tandem duplication (ITD) mutations (75.6%) or mutations in *NPM1* (68.1%) and were considered to have intermediate-risk cytogenetic profiles (63.1%).

3.2 | Comparison of metal values in AML and control samples

Comparisons of the values of toxic and essential metals in AML patients and healthy control participants are shown in Table 2. Significant differences between the groups were found for most metals. We also found significantly lower values of selenium, arsenic, gold, calcium, and dCu (the isotopic abundance ratio of copper, $^{65}Cu/^{63}Cu$) in AML patients than in the control group. The AML patients had higher values of lead, titanium, zinc, chromium, iron, barium, and sodium.

3.3 | Metal values and survival

We next examined how values of certain metals affected OS among AML patients. The median follow-up time among patients with AML

TABLE 1 Characteristics of the study cohort

Characteristic	AML patients*N* = 67	Healthy volunteers*N* = 94
Age, y, median (range)	67 (28–87)	58 (20–89)
Sex		
Female	24 (35.8%)	37 (39.4%)
Male	43 (64.2%)	57 (60.6%)
AML characteristics		
Hemoglobin concentration [g/dL], median (range)	8.5 (5.2-15.2)	
Absolute blast count [Giga/L], median (range)	40.97 (0.48-92)	
White blood cell count [$\times 10^6$/mL], median (range)	10.43 (1-1301)	
FLT3 ITD		
Positive	11 (24.4%)	
Negative	34 (75.6%)	
Unknown	22	
NPM1 mutation		
Positive	15 (31.9%)	
Negative	32 (68.1%)	
Unknown	20	
TP53 mutation		
Positive	1 (16.7%)	
Negative	5 (83.3%)	
Unknown	61	
ELN risk group		
Adverse	29 (46.8%)	
Intermediate	25 (40.3%)	
Favorable	8 (12.9%)	
Unknown	5	
Cytogenetic risk group		
Adverse	20 (30.8%)	
Intermediate	41 (63.1%)	
Favorable	4 (6.2%)	
Unknown	2	
Cytogenetic group by karyotype		
+8	2 (3%)	
−5/−7	3 (4.5%)	
Complex	15 (22%)	
Diploid	32 (48%)	
IM/ND	2 (3%)	
Misc	5 (7%)	
t(15;17)	3 (4.5%)	
t(3;3)/inv3	1 (1.5%)	
t(6;11)	3 (4.5%)	
t(8;21)	1 (1.5%)	
WHO category		
AML with *MLL* rearrangement	1 (1.5%)	
AML with myelodysplasia-related changes	23 (34.8%)	

(Continues)

TABLE 1 (Continued)

Characteristic	AML patients*N* = 67	Healthy volunteers*N* = 94
AML with mutated *NPM1*	15 (22.7%)	
AML with t(3;3)/inv (3)	2 (3%)	
AML with t(8;21)	1 (1.5%)	
AML with maturation	4 (6.1%)	
AML with minimal differentiation	2 (3%)	
AML without maturation	1 (1.5%)	
AML unclassified	1 (1.5%)	
AML with *BCR-ABL1*	1 (1.5%)	
Acute myelomonocytic leukemia	4 (6.1%)	
Acute monoblastic/monocytic leukemia	6 (9.1%)	
Acute promyelocytic leukemia	3 (4.5%)	
MDS with excess blasts-1	1 (1.5%)	
MDS with excess blasts-2	1 (1.5%)	
Unknown	1	

Abbreviations: AML, acute myeloid leukemia; ELN, European Leukemia Net; IM/ND, insufficient metaphases/not done; ITD, internal tandem duplication; MDS, myelodysplastic syndrome; WHO, World Health Organization.

TABLE 2 Comparison of metal levels in patients with AML and control participants

	AML		Control		
Metal (units)	Median	Range	Median	Range	*P*-value
Pb (nmol/L)	25.965	0.000-286.921	2.172	0.048-75.000	**<.001**
Cd (nmol/L)	0.000	0.000-5.160	0.089	0.089-6.316	.27
Se (μmol/L)	1.196	0.000-2.76	2.558	0.205-5.633	**<.001**
Ti (μmol/L)	0.311	0.000-1.049	0.067	0.000-4.030	**<.001**
Mg (mmol/L)	0.970	0.475-1.701	1.040	0.644-2.514	.06
Zn (μmol/L)	17.461	7.377-135.462	14.907	8.602-23.238	**.001**
Cr (μmol/L)	0.062	0.000-10.185	0.020	0.000-1.245	**.001**
Fe (μmol/L)	35.111	11.735-147.644	28.945	8.437-62.536	**<.001**
Co (μmol/L)	0.011	0.000-0.400	0.011	0.000-12.899	.75
Cu (μmol/L)	19.494	6.939-37.963	16.406	11.292-39.272	.16
As (μmol/L)	0.005	0.000-0.491	0.020	0.000-0.359	**.003**
Rb (μmol/L)	2.993	1.233-7.624	3.186	1.253-6.927	.64
Sr (μmol/L)	0.349	0.094-1.589	0.372	0.156-5.323	.58
Ba (μmol/L)	0.021	0.004-0.063	0.018	0.000-0.070	**.05**
Pt (nmol/L)	0.000	0.000-2.768	0.051	0.051-2.255	**<.001**
Au (nmol/L)	0.000	0.000-19.851	0.051	0.051-89.406	**<.001**
Ca (mmol/L)	2.437	2.417-3.118	2.463	2.416-3.116	**<.001**
Na (mmol/L)	134.737	89.571-196.658	129.759	99.119-181.679	**.03**
dCu ($^{65}Cu/^{63}Cu$)	−0.35	−1.15 to 0.02	−0.11	−0.83 to 0.18	**<.001**

was 51.6 weeks (range 0.2 to 129.9 weeks). Twenty-nine (43%) of the 67 AML patients had died, and 38 (57%) were alive at last follow-up. The median OS of the entire cohort of AML patients was 69.9 weeks (95% CI 29-not reached).

Univariate Cox proportional hazard regression models revealed that only magnesium values were individually associated with OS. With a hazard ratio of 0.05 (95% CI 0.01-0.31), on average, each increase of 1.0 in the natural log of the value of magnesium was associated with a 95% reduction in the risk of death (P = .001). We repeated this analysis including age and sex in a multivariate model for each metal. The results were similar: magnesium was the only metal with a significant survival effect, with a similar hazard ratio (P = .002).

We then calculated OS estimates as a function of metal values by using cutoffs determined with univariate and multivariate CART models in the cohort of AML patients (Table 3). Unlike the univariate

TABLE 3 Overall survival in AML patients by metal levels with cutoffs determined using classification and regression trees (CART)

Metal[a]	CART split[b]	No. of deaths/No. of patients	6-month OS (%)	SE	*P*-value
Univariate					
Ca	<2.43 mmol/L	12/20	31%	12%	**.005**
	≥2.43 mmol/L	11/40	73%	7%	
Cd	<0.750 nmol/L	13/39	63%	8%	**.02**
	≥0.750 nmol/L	16/28	46%	10%	
Mg	<1.07 mmol/L	24/44	46%	8%	**.02**
	≥1.07 mmol/L	5/23	77%	9%	
Se	<0.692 μmol/L	13/20	38%	11%	**.004**
	≥0.692 μmol/L	16/47	65%	7%	
Multivariate[c]					
	Mg ≥1.07 mmol/L and Cd < 0.729 nmol/L	14/45	66%	8%	**.001**
	Mg <1.07 mmol/L and Cd ≥0.729 nmol/L	15/22	36%	11%	
Metal Score[d]					
	1–3	2 / 11	81%	12%	**.01**
	4–6	15 / 38	60%	8%	
	>6	12 / 18	36%	12%	

Abbreviations: CART, classification and regression tree; OS, overall survival; SE, standard error.

[a]Metals not shown had no splits found. All metals were tested.

[b]Splits were identified in CART models using log-transformed data for all metals.

[c]The model included blasts, cytogenetic risk (advanced vs intermediate/favorable), and log values for all metals shown in Table 2 except Ca, which was excluded due to the number of patients with missing values.

[d]The metal score is based on 10 metals (5 toxic and 5 essential), with higher metal scores having less favorable metal status. Patients were assigned one point for each metal that fell outside the pre-determined limits shown in Figure 1. The overall score was a sum of the points. The patients' metal scores were segregated into these three groups by an intuitive approach.

Cox proportional hazard regression model described above, which identifies a consistent average change in risk with increasing metal values, the CART model can divide patients into meaningful risk subgroups. Significant risk subgroups were found in AML patients with respect to calcium (Figure 2), cadmium (Figure 3a), magnesium (Figure 3b), and selenium (Figure 3c). Patients with calcium, magnesium, or selenium values higher than the respective cutoffs or with cadmium values lower than the cutoff were more likely than others to have a longer median OS.

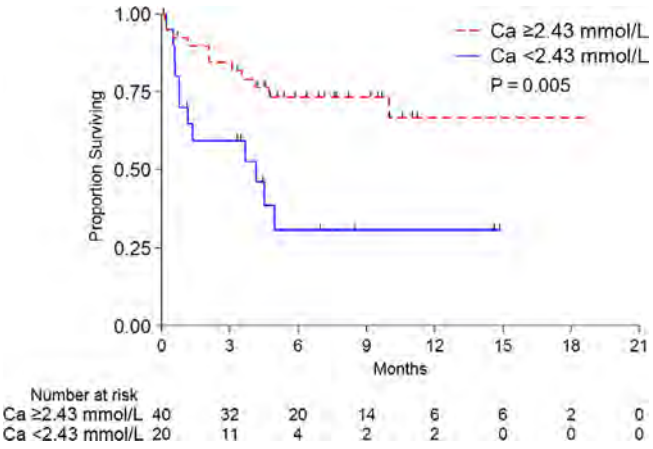

FIGURE 2 Kaplan-Meier curves comparing overall survival in AML patients with high (≥ 2.43 mmol/L) and low (< 2.43 mmol/L) serum calcium values [Color figure can be viewed at wileyonlinelibrary.com]

Furthermore, in an exploratory multivariate CART analysis, we found that magnesium and cadmium combined to form two risk groups (Figure 3d). Patients with magnesium values of 1.07 mmol/L or higher (Mg high) and cadmium values below 0.729 nmol/L (Cd low) had the longest survival, with a 6-month OS estimate of 66%. Patients with magnesium values below 1.07 mmol/L (Mg low) and cadmium values of 0.729 nmol/L or higher (Cd high) had the worst survival rates, with only 36% surviving at 6 months (P = .001). The adverse prognostic effect of higher cadmium and lower magnesium values was identified on multivariate analysis, including absolute blast count, cytogenetic risk group, and all other measured metals as covariates (except for calcium, which was excluded owing to a number of patients with missing values). We used cytogenetic risk rather than ELN for the exploratory model since more patients had complete cytogenetic risk data available. Age was omitted from the multivariate analysis to avoid collinearity because of the known close association between increasing age and both higher values of toxic metals[36-38] and adverse cytogenetics.[39-42] Values of other metals may be important for OS, but with the limited sample size, no further splits could be made. Therefore, in this analysis, cadmium and magnesium were the two metals whose values were most strongly associated with differences in survival.

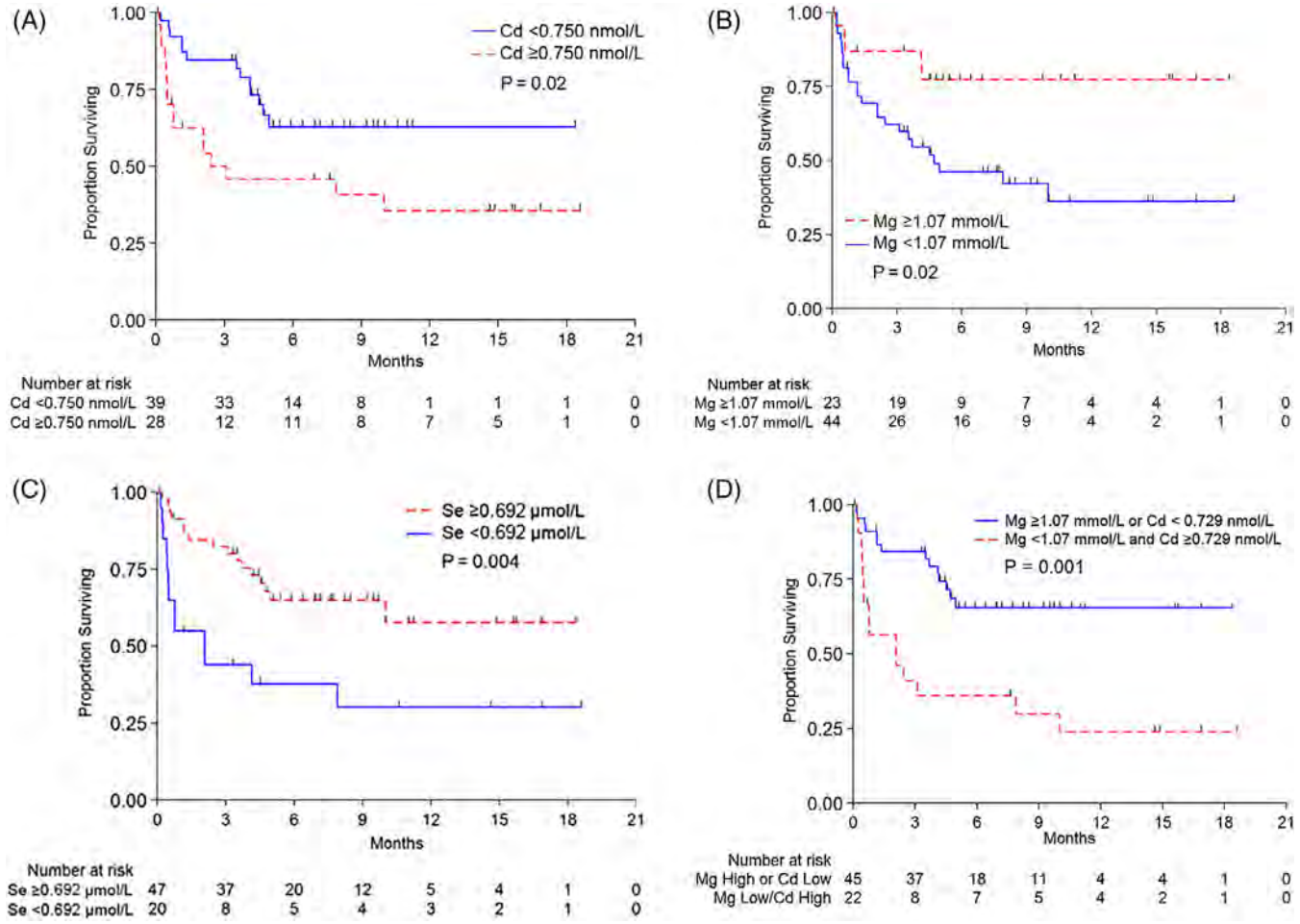

FIGURE 3 A, Kaplan-Meier curves comparing overall survival in AML patients with high (≥ 0.750 nmol/L) and low (< 0.750 nmol/L) serum cadmium values. B, Kaplan-Meier curves comparing overall survival in AML patients with high (≥ 1.07 mmol/L) and low (< 1.07 mmol/L) serum magnesium values. C, Kaplan-Meier curves comparing overall survival in AML patients with high (≥ 0.692 µmol/L) and low (< 0.692 µmol/L) serum selenium values. D, Kaplan-Meier curves comparing overall survival in risk groups identified by multivariate classification and regression trees. The Mg High and Mg Low groups are divided at 1.07 mmol/L, and the Cd High and Cd Low groups are divided at 0.729 nmol/L [Color figure can be viewed at wileyonlinelibrary.com]

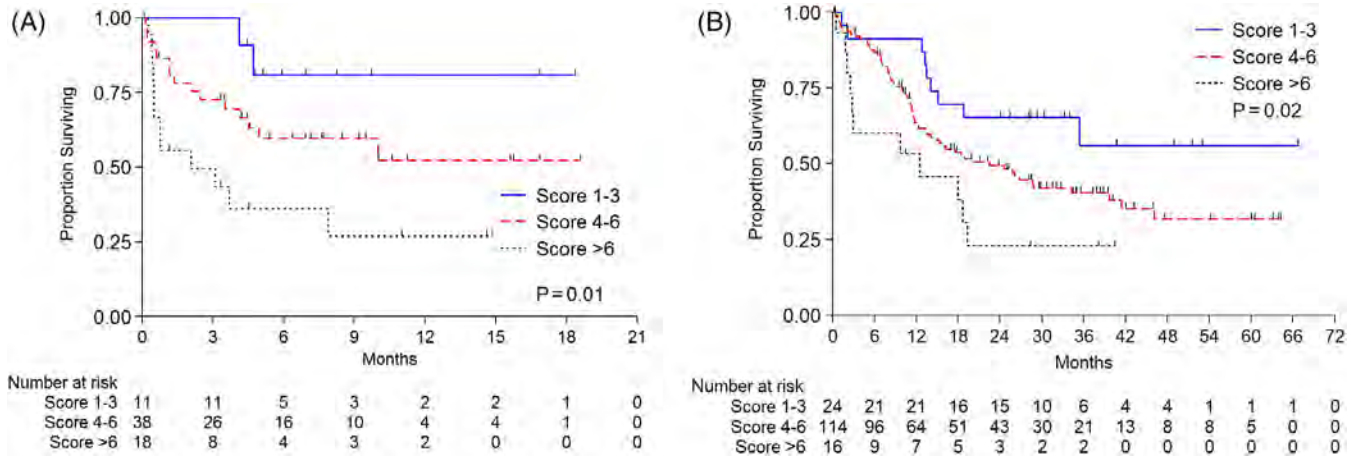

FIGURE 4 A, Overall survival by groupings of metal scores is shown for patients treated in Lyon. Survival was significantly worse in patients with higher metal scores. B, Overall survival by groupings of metal scores is shown for patients treated at MD Anderson. Survival was significantly worse in patients with higher metal scores [Color figure can be viewed at wileyonlinelibrary.com]

3.4 | Metal values and molecular/cytogenetic prognostic factors in AML

We also explored the association between metal values and three key prognostic factors in AML: *FLT3*-ITD mutations, *NPM1* mutations, and cytogenetic risk groups. Lower median sodium values were significantly associated with *FLT3*-ITD mutations; patients with *FLT3*-ITD mutations (n = 11) had a median sodium value of 127.8 mmol/L. However, patients without *FLT3*-ITD (n = 34) had a median sodium value of 136.5 mmol/L (P = .04). Higher copper values were significantly associated with mutated *NPM1*, with median copper values of 20.27 μmol/L in patients with *NPM1* mutations (n = 15), and 17.59 μmol/L in patients without this mutation (n = 32) (P = .05). We found no statistically significant associations between values of individual metals and cytogenetic risk groups.

3.5 | A Novel metal score predicts survival in AML

The findings of this study led us to explore the prognostic significance of combinations of various deficiencies and elevations of essential and toxic metals in the context of a metal scoring system (Figure 1). We hypothesized that patients with higher metal scores had significantly worse survival than patients with lower metal scores. We intuitively separated the patients according to three groups representing low (1–3), medium (4–6) and high metal scores (7–9); no patients in this cohort scored the maximum of 10 points. Specific 6-month survival estimates are presented in Table 3. Patients with higher metal scores did have significantly worse survival than patients with lower metal scores. For example, patients with metal scores 1–3 had a 6-month survival estimate of 81% vs 60% for scores 4–6 and 36% for scores 7–9 (P = .01) (Figure 4A). Table 4 further elaborates on the multivariate analysis of the metal score when controlling for ELN risk groups and blasts. The metal score remained significantly prognostic, even when accounting for ELN risk groups and blasts (P = .03). With only 29 events, models were limited to three variables.

In order to validate this observation, we next studied metals and metal scores in a separate population of patients with AML who presented to MD Anderson Cancer Center (clinical characteristics in Table S1). Similar statistically significant survival differences were observed between the three metal score groups. Patients with metal scores 1-3 had a 6-month survival estimate of 91% vs 87% for scores 4-6 and 60% for scores 7-9 (P = .02) (Figure 4B). On multivariate analysis of the metal score, the difference observed was nearly prognostically significant (P = .056) when applying the same model used for the Lyon cohort, controlling for ELN risk groups and absolute blast count.

3.6 | Metal score in relation to environmental health status

Because environmental health information was not available for the patients treated in Lyon, we next investigated whether higher metal scores were associated with other environmental and general health factors, including smoking and alcohol history, the presence of implanted metal medical devices, and other comorbidities. Smoking status was broken down into four groups: non-smokers (either never smoked or smoked <100 cigarettes in their lifetime), current smokers (smoked within the last month), ex-smokers (quit during the past 30 years up to >1 month ago), and remote smokers (quit more than 30 years ago).[43]

As shown in Table S2, the median metal score (IQR) for all patients across environmental and general health factors was 5 (4, 5). Most subgroups also had a median metal score (IQR) of 5 (4, 5) across environmental and general health factors with the following exceptions in subgroups of at least 10 patients: ex-smokers had a median score (IQR) of 5 (4,5.5) and remote smokers were lower with a median score (IQR) of 4 (3, 5); patients with and without diabetes mellitus had 5 (4, 6) and 4.5 (4, 5), respectively; patients with a history of other

TABLE 4 Multivariate analysis of metal score controlling for ELN Risk Group and blasts

Risk measure	Groups compared	Hazard ratio	(95% confidence interval)	*P*-value
ELN Risk Group				.29
	Adverse vs Favorable/Intermediate	1.55	(0.69, 3.45)	
Blasts				.43
	Each unit increase	1.01	(0.99, 1.02)	
Metal score				**.03**
	1-3 vs >6	0.08	(0.01, 0.65)	
	4–6 vs > 6	0.50	(0.23 1.10)	

Note: The metal score as an independent prognostic factor also remained significant when accounting for ELN risk groups and blasts.
Abbreviations: CART, classification and regression tree; OS, overall survival; SE, standard error.

cancers had 4 (4, 5); and patients with an implanted metal device had 5 (4, 5.5).

4 | DISCUSSION

In this hypothesis-generating study, we found that overall survival in patients with AML was significantly shorter when values of essential elements with antioxidant properties, such as selenium and calcium, were lower, and when values of toxic metals or essential metals that can become toxic above a narrow threshold, such as copper and iron, were higher. The significant survival impact of relatively high or low metal values was maintained on multivariate analysis by both the CART model when accounting for high Cd/low Mg, and by a comprehensive metal score that accounts for up to 10 metal values above or below predetermined limits. Given that individual patients can have a variety of different metal values, we devised the metal scoring system to provide a more complete approach for using multiple baseline metal values in prognostication. The metal score takes into account both the presence of toxic metals and relative deficiencies of certain essential metals.

It is noteworthy that we were able to verify that the same metal-score groups also significantly predicted survival in a separate and larger cohort of patients who were treated at MD Anderson. However, the proportion of patients within each metal score group differed between the two AML populations; a higher percentage of patients in the MD Anderson cohort than the Lyon cohort had scores in the "intermediate" group (metal scores 4-6). This difference in distribution may be due to environmental and lifestyle differences between the two groups. For example, the French Ministry of Health cited a survey showing that 31.9% of French people smoke,[44] whereas the US Centers for Disease Control estimated that 14% of Americans smoke.[45] Soil metal content also differs across countries and continents.[46] Despite these marked lifestyle and environmental differences, it is notable that the metal scoring system remained prognostic in two independent populations.

Although environmental health data were not available for the Lyon cohort, we did have environmental health data for the patients treated at MD Anderson and found that median and interquartile range (IQR) metal scores remained similar regardless of environmental health status, lifestyle, and comorbidities. In addition to occupational and environmental exposures,[47] multiple factors contribute to excess accumulation of toxic metals and deficiency of essential metals, including implanted metal devices,[48-50] genetic polymorphisms of metal transporters,[51] metal-metabolizing enzymes,[52] metal content in drinking water[53] and food,[54] as well as, gastrointestinal dysbiosis.[55] Importantly, several investigators have found increased rates of leukemia and lymphoma in patients with metal knee or hip prostheses.[48-50] This information and our findings suggest that acquired relative excesses of toxic metals and deficiencies of essential minerals can occur independently of specific lifestyles and occupations, and are thus an aspect of living in the industrial age.

A balance among various essential and nonessential metals is required for the proper function of enzymes and proteins; an excess of toxic metals leads to free-radical generation and DNA damage. Toxic metals can interfere metabolically with nutritionally essential metals such as copper, zinc,[56] iron, calcium, selenium,[3,4] and magnesium[57] by competing as ligands in enzymes and transport proteins in biological systems.[3] The resulting deficiencies of essential elements have been associated with cancer.[2]

Metals are also known to affect hematologic parameters in a variety of ways. For example, lead induces leukocytosis,[58] anemia, lymphocytosis and monocytosis in rabbits and humans,[54] and is associated with MDS and AML in humans.[59,60] Metals are stored long-term in the bone; for example, the half-life of lead and cadmium in the bone is up to 30 years.[61,62] Thus, when leukemia blasts are mobilized from the bone marrow to the peripheral blood, metals may be carried into the blood during leukemic infiltration and destruction of bone.[63] Therefore, higher peripheral blood blast percentages may contribute to higher metal values.

One interesting finding of our CART model was that the specific combination of high Cd/low Mg was an independent prognostic factor for survival in AML. This finding is notable because magnesium and cadmium antagonize each other.[57,64] While cadmium displaces and depletes magnesium, magnesium supplementation can reduce cadmium absorption and toxicity.[57,64] Patients in the higher magnesium group in our study had only mild hypermagnesemia (1.07-1.701 mmol/L), which is considered protective in a variety of diseases, including chronic kidney disease and vascular diseases.[65] Low magnesium values are associated with increased oxidative stress, inflammation, and immune impairment.[7] Moreover, magnesium is required to maintain genomic stability and is an essential cofactor in DNA replication and repair.[66] Human studies have shown that magnesium supplementation diminishes lymphocyte DNA oxidative damage[67]; thus, the potential benefit of magnesium supplementation in AML warrants more attention.

Cadmium affects a variety of epigenetic processes in cancer.[68] In the chronic myelogenous leukemia cell line K562, cadmium-induced global DNA hypomethylation led to increased cell proliferation.[69] Cadmium is also known to interfere with the efficacy of common chemotherapeutic agents.[70,71] For example, coadministration of cadmium negated the anticancer effects of 5-fluorouracil on breast cancer cells,[70] and exposure of lung cancer cell lines to cadmium increased metallothionein synthesis, resulting in increased resistance to etoposide.[71]

We also observed that lower selenium values in AML patients were associated with worse survival. Several studies suggest that selenium supplementation may be useful as an adjunct therapy for leukemia. Selenium supplementation activated apoptosis in leukemia stem cells and selectively eradicated leukemia stem cells in murine leukemia models and in leukemia cells from patients with AML.[72] In leukemia patients with hyperleukocytosis, selenium had an antiproliferative effect.[73] Whereas toxic metals have adverse effects on immunity and NK cells,[20] selenium enhances the cytotoxic potential of NK cells, CD8+ T cells, and a variety of other immune-stimulatory processes.[74]

The isotopic abundance ratio of copper, dCu ($^{65}Cu/^{63}Cu$), was significantly lower in the AML cohort compared to controls. This has been shown in other cancer populations, where it has been attributed to preferential deposition of the heavy isotope, ^{65}Cu, in the tumor tissue, thereby decreasing the dCu in the serum.[75] The dCu was not included in the current score, which was focused specifically on analyses that would be available in major leukemia treatment centers. Research is ongoing to elucidate the use of dCu as a predictive biomarker in AML.

While we found no significant associations between metal values and cytogenetic risk groups in our analysis of the Lyon cohort, we did determine that *NPM1* mutations were associated with higher copper values and that *FLT3*-ITD mutations were associated with lower sodium values. The association between *NPM1* mutations and copper values may be attributable to loss of NPM1 binding to the promoter of *SOD2*. This may compromise transcription of *SOD2*, leading to excess reactive oxygen species generation and higher copper values.[76-78] A link between excess copper, reactive oxygen species generation, and oxidative stress has been reported in hematologic malignancies.[77] To further explore the association between mutated *NPM1* and SOD2 expression, we queried the Oregon Health & Science University (OHSU) AML data base[79] using cBioPortal web-based software (http://www.cbioportal.org).[79-81] Of the 405 AML patients for whom messenger RNA (mRNA) data were available, 20% had *NPM1* mutations. The patients with *NPM1* mutations (n = 83) had significantly lower *SOD2* mRNA levels than the *NPM1* wild-type patients (n = 322) (Student *t* test derived $P < .001$) (Figure S1a), which further supports the association between *NPM1* mutated AML and compromised transcription of *SOD2*.

The significantly lower median serum sodium concentration observed in patients with *FLT3*-ITD mutations, as compared to those without *FLT3-ITD* mutations, was an unexpected finding. While the precise mechanism underlying this finding is unknown, some relationship to deregulation of *TESC*, associated with *FLT3*-ITD+ AML and sorafenib resistance,[82] might be inferred since the cellular Na+/H+ exchanger 1 (NHE1) (a potential determinant of interstitial sodium concentration) is modulated by TESC.[82] Additionally, in a large cohort of patients with AML (http://www.cbioportal.org),[79-81] *TESC* mRNA levels were significantly lower in *FLT3-ITD* mutation-positive patients (n = 123) than in *FLT3-ITD* mutation-negative patients (n = 282) (Student *t* test derived $P < .001$), further supporting an association between *FLT3-ITD* mutations and *TESC* deregulation (Figure S1b).

The observations from this study, including the lack of association between the metal scores and environmental health status, suggest that serum metal analysis by ICP-MS offers an accurate measurement of recent and ongoing metal exposure and a representation of the amount of metal in equilibrium with deep body compartments. This suggests that the metal score could perhaps be more clinically relevant than environmental health assessment, but more studies are needed. Note, ICP-MS is also a promising and cost-effective technology for incorporating metallomics into the field of hematology-oncology, as it is a proven technology for measuring metals and uses a high-throughput approach to rapidly obtain results from multiple samples. Thus, ICP-MS is more sensitive, more specific, and faster than atomic absorption, the technique used in most hospitals.

Our results suggest that further research into the role of heavy metals in AML is warranted, as our preliminary data suggest that metal status can affect clinical outcomes. Considering that toxic metals interfere with the efficacy of chemotherapy,[70] and administration of certain essential elements can improve responses to chemotherapy,[83,84] more research on modification of these parameters during cancer therapy is warranted. Although many previous studies have described associations between toxic metals, hematologic malignancies, and solid tumors,[6,59,85-91] none of them assessed the impact of baseline toxic metal values on survival outcomes. This study is first of its kind to analyze the associations between metals and survival in AML and to confirm a metal-scoring system that was significantly prognostic in two independent patient populations treated on separate continents. However, we recognize that further research in larger cohorts should follow to determine the most precise approach for risk stratification. We are currently studying larger populations of patients and controls, including collecting detailed environmental exposure histories and enhanced lifestyle characterizations, and will investigate the metal scoring system and its optimization through these efforts.

AUTHOR CONTRIBUTIONS

M.O. analyzed the data and wrote the manuscript. P.T. performed specimen analysis and wrote the manuscript. S.K. provided input on analysis and reviewed and approved the manuscript. F.A. performed specimen analysis and wrote the manuscript. P.R. helped analyze the publicly available cBioPortal data. R.S.T.T. performed statistical analysis and wrote the manuscript. A.P. made the pathologic diagnoses for the patients. R.K.-S. made the pathologic diagnoses for the patients. E.L.M. participated in sample collection and processing. J.C. provided input on analysis and reviewed and approved the manuscript. A.C. analyzed the data and wrote the manuscript. C.D. oversaw the data collection, analyzed the data, and wrote the manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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