Reference Intervals of and Relationships among Essential Trace Elements in Whole Blood of Children Aged 0–14 years

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Background: Essential trace elements are vital to human health. In this study, our aim was to establish reference intervals of and to evaluate relationships among Ca, Fe, Cu, Mg, and Zn for children. **Methods:** We collected blood samples of 3210 children aged 0–14 years from Lu'an, China, and concentrations of the above elements were determined by atomizer absorption spectrophotometer. A nonparametric method was used to establish the reference intervals. **Results:** Gender-related differences in concentrations were not statistically significant for the elements, except for Fe. There were strong positive and negative correlations between age and Fe ($R = 0.305$, $P < 0.001$), Zn ($R = 0.573$, $P < 0.001$); and age and Ca ($R = -0.372$, $P < 0.001$), Cu ($R = -0.127$, $P < 0.001$), respectively. Correlations between Ca-Mg

($r = 0.222$ – 0.384 , $P < 0.001$), Fe-Mg ($r = 0.495$ – 0.614 , $P < 0.001$), and Fe-Zn ($r = 0.239$ – 0.471 , $P < 0.001$) were the strongest compared with others. In multivariable linear regression, after adjusted for confounding factors, the associations between Zn-Fe and Mg-Fe were the strongest with per concentration quintile increase of Fe caused Zn and Mg increasing by 4.19% ($\beta = 0.041$; 95% CI: 0.037, 0.045; $P < 0.001$) and 3.87% ($\beta = 0.038$; 95% CI: 0.036, 0.040; $P < 0.001$), respectively. **Conclusions:** Gender- and age-based reference intervals of Ca, Fe, Cu, Mg, and Zn for children were established, and correlations between them were quite complex. More works are needed to illuminate these relationships and their impacts on children's health. J. Clin. Lab. Anal. 31:e22043, 2017. © 2016 Wiley Periodicals, Inc.

Key words: blood; children; reference interval; relationship; trace element

INTRODUCTION

Essential trace elements such as calcium (Ca), iron (Fe), copper (Cu), magnesium (Mg), and zinc (Zn) are involved as metal cofactors in the function of many enzymes and proteins, and they are essential for maintaining human health as they play important roles in human metabolism (1, 2). As key components of metalloenzymes, these essential elements are involved in vital biological function, such as oxygen transport, free radical scavenging, and hormonal activity (3). The animal and clinical human studies have suggested that Ca, Fe, Cu, Mg, and Zn are associated with hypochromia, febrile seizure, rickets, inflammatory processes, cardiovascular diseases, anemia, and other essential elements metabolism (1, 2).

In the special growth and development stage of life, digestive systems of infants and children are not well developed, and their dietary structures are gradually changed, along with improper feeding habits or

consuming overprocessed foods, which can usually cause malnutrition, so that they are more sensitive to deficiency or excess of essential trace elements. Unified reference intervals for infants, children, and adults may be inappropriate for assessment of nutritional status for infants and children and may result in misdiagnosis and incorrect classification of diseases. The International Federation of Clinical Chemistry (IFCC) had recommended that each laboratory should produce its own reference values. In recent years, a growing number of

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evidence suggests that reference values of those elements for children would be affected by gender and age (2, 4–9). Based on the above studies, established gender- and age-specific reference intervals for relatively healthy pediatric population in local region may help obtaining more accurate estimates of trace elements status. And, find out relationships among these essential trace elements may have important effects on their physiological functions and their associations with diseases.

The relationships among essential trace elements have been studied previously, especially in children (2, 3, 6, 7, 10–13). However, the results are not always consistent. Moreover, from these studies, we do not yet know whether the correlation between two essential elements was affected by age, gender, seasons of samples collection, and even other essential elements.

In this study, we assessed the concentrations of essential trace elements including Ca, Fe, Cu, Mg, and Zn in whole blood from 3210 children aged 0–14 years from Lu'an, China. The aim of this study is to establish reference intervals of these elements for infants and children, and evaluate the relationships among them, and the factors that have impacts on these relationships. And these may provide some useful information for better diagnosis and more reasonable micronutrient supplementation.

MATERIALS AND METHODS

Subjects and Blood Sampling

The population in this study comprised 3210 children, including 2150 boys and 1060 girls aged 0–14 years old. They all came for the routine health examination between January 2012 and December 2014 at the outpatient department of the Lu'an Affiliated Hospital of Anhui Medical University. Informed consent for the use of these children's detection results and personal information in this study was obtained from their parents or guardians. This study was approved by the Ethics committee of Lu'an People's Hospital. For all the children, after skin cleaning, about 2 ml of venous blood was drawn into vacuum tube (CHGD[®], Gaode, Wenzhou, China) with heparin sodium special for trace element analyses. Prior to element detection, all the blood samples were stored in 0–4°C for no more than 48 hr.

Elemental Analysis

Whole blood concentrations of Ca, Fe, Cu, Mg, and Zn were determined by flame atomic absorption spectrometry (BH5100, Bohui, Beijing, China) with hollow cathode lamps under 422.7, 248.3, 324.7, 285.2, and 213.9 nm, respectively. Quality controls were assured

by analyzing certified reference materials GBW(E) 080915, 080916, and 080917 from General Administration of Quality Supervision, Inspection and Quarantine of the People's Republic of China. Reference samples were analyzed every ten routine sample detection. If the values exceeded the uncertainty provide by the certificate of reference materials, the previous ten routine samples must be analyzed again.

Statistical Analysis

Data analysis was performed using SPSS version 19.0 (SPSS Inc., Chicago, IL) statistical software. Dixon's test was used to determine the outliers as recommended by National Committee for Clinical Laboratory Standards (NCCLS), if $D/R > 1/3$, the extreme value would be excluded as outlier, where D is the absolute difference between the largest or smallest value and the next largest or smallest value and R is the entire range of all values. For comparing variables between boys and girls, non-parametric Mann–Whitney test was used. Spearman rank correlation analysis was used for comparing essential trace element values with different age groups. Reference intervals were based on the central 95% of the data by using nonparametric method. The correlations at different gender, age, and season groups were conducted by partial correlation analysis. The associations of blood concentrations of one essential trace element with the increase of quintiles of other essential trace elements concentrations were determined by multivariable linear regression. A P -value < 0.05 (two-tailed) was considered as statistically significant.

RESULTS

The total number of children was 3,210, with age ranging from 2 months to 14 years. After analyzing by Dixon's test, no outliers were found in our study. Non-parametric reference intervals for the five elements are presented in Tables 1 and 2. The overall 95% reference intervals for blood levels of Ca, Fe, Cu, Mg, and Zn were 1.35–1.95 mmol/l, 6.72–9.92 mmol/l, 12.5–30.2 μ mol/l, 1.25–1.85 mmol/l, and 58.5–114.7 μ mol/l, respectively. Then, we examined the effects of gender and age on the concentrations of the five elements.

Effects of Gender on the Concentrations of the Five Trace Elements

As shown in Table 3, there were no significant differences in blood levels of Ca, Cu, Mg, and Zn ($P = 0.056$, 0.191, 0.577, and 0.078, respectively) between boys and girls. Gender-related difference was statistically significant only in Fe concentration ($P = 0.044$).

TABLE 1. 95% Reference Intervals for Whole Blood Levels of Ca, Fe, and Cu in Lu'an Children Aged 0–14 years^a

		Ca (mmol/l)		Fe (mmol/l)		Cu (μmol/l)	
Age (years)	<i>n</i>	RI	Median [minimum, maximum]	RI	Median [minimum, maximum]	RI	Median [minimum, maximum]
Boys							
<1	247	1.44–2.01	1.73 [1.37, 2.16]	6.36–9.26	7.57 [6.14, 10.09]	12.5–32.1	20.8 [11.9, 38.0]
1~	350	1.43–2.02	1.65 [1.26, 2.12]	6.46–9.56	7.82 [5.72, 10.96]	13.0–32.5	21.3 [12.1, 35.9]
2~3	386	1.35–1.92	1.61 [1.21, 2.02]	6.82–9.81	8.05 [6.48, 11.78]	12.3–29.9	20.2 [11.8, 35.8]
4~6	509	1.34–1.90	1.59 [1.24, 2.05]	6.95–10.23	8.18 [6.21, 11.39]	12.9–30.3	19.9 [12.1, 33.4]
7~9	375	1.32–1.88	1.57 [1.21, 2.07]	6.88–10.05	8.16 [6.09, 10.73]	12.5–30.1	19.6 [11.8, 37.1]
10~14	283	1.31–1.91	1.55 [1.11, 2.05]	6.99–10.24	8.46 [6.61, 11.28]	12.3–28.3	18.8 [12.0, 35.6]
Girls							
<1	150	1.50–2.07	1.73 [1.33, 2.11]	6.47–9.16	7.60 [6.16, 11.20]	12.1–30.2	20.2 [11.9, 35.8]
1~	205	1.36–1.99	1.65 [1.31, 2.09]	6.72–9.82	7.92 [6.43, 10.24]	12.6–30.4	21.0 [12.2, 35.1]
2~3	220	1.37–1.91	1.61 [1.31, 2.08]	6.74–9.74	8.02 [6.01, 10.53]	12.2–31.1	20.2 [12.0, 35.2]
4~6	234	1.31–1.92	1.59 [1.20, 2.02]	6.81–10.23	8.08 [6.47, 10.94]	12.9–30.3	19.8 [12.0, 38.7]
7~9	144	1.33–1.85	1.58 [1.30, 2.01]	6.90–10.04	8.26 [6.61, 10.51]	12.6–28.2	19.6 [12.1, 32.3]
10~14	107	1.28–1.78	1.54 [1.20, 1.91]	6.70–10.33	8.31 [6.30, 11.35]	12.5–28.2	17.8 [11.9, 33.9]
Total <1~14	3210	1.35–1.95	1.61 [1.11, 2.16]	6.72–9.92	8.05 [5.72, 11.78]	12.5–30.2	20.1 [11.8, 38.7]

n = number of subjects.

^aIFCC criteria (nonparametric method) were used.

RI is the 95% nonparametric reference interval.

TABLE 2. 95% Reference Intervals for Whole Blood Levels of Mg and Zn in Lu'an Children Aged 0–14 years^a

Age (years)	<i>n</i>	Mg (mmol/l)		Zn (μmol/l)	
		RI	Median [minimum, maximum]	RI	Median [minimum, maximum]
Boys					
<1	247	1.23–1.84	1.50 [1.17, 2.04]	52.6–88.2	66.4 [51.4, 98.2]
1~	350	1.22–1.81	1.50 [1.14, 1.96]	57.7–97.3	71.2 [52.3, 106.1]
2~3	386	1.23–1.85	1.50 [1.14, 2.03]	62.1–108.9	77.7 [52.9, 127.5]
4~6	509	1.26–1.87	1.52 [1.16, 2.01]	66.1–118.4	85.3 [53.8, 149.7]
7~9	375	1.25–1.85	1.51 [1.14, 2.01]	66.0–120.7	86.2 [52.3, 146.8]
10~14	283	1.27–1.90	1.52 [1.13, 2.03]	65.0–119.1	89.4 [55.9, 130.8]
Girls					
<1	150	1.24–1.76	1.47 [1.16, 1.81]	53.7–94.9	65.6 [52.5, 99.1]
1~	205	1.24–1.89	1.53 [1.18, 2.02]	58.0–101.1	71.4 [52.2, 105.8]
2~3	220	1.23–1.86	1.50 [1.12, 1.99]	63.8–104.7	79.1 [54.0, 121.9]
4~6	234	1.27–1.83	1.49 [1.17, 1.94]	68.1–114.8	85.1 [53.2, 124.9]
7~9	144	1.24–1.81	1.52 [1.21, 1.89]	63.2–124.7	88.9 [58.5, 127.1]
10~14	107	1.30–1.86	1.49 [1.25, 1.88]	67.2–139.3	90.2 [61.5, 155.8]
Total					
<1~14	3210	1.25–1.85	1.50 [1.12, 2.04]	58.5–114.7	80.9 [51.4, 155.8]

n = number of subjects.

^aIFCC criteria (nonparametric method) were used.

RI is the 95% nonparametric reference interval.

Effects of Age on the Concentrations of the Five Trace Elements

As shown in Table 4, there was no correlation between Mg and age ($P = 0.161$). Ca and Cu were inversely correlated with age ($P < 0.001$ for each). However, Fe and Zn were positively correlated with age ($P < 0.001$ for each), especially Zn, which has the strongest correlation ($R = 0.573$) compared with

Ca, Fe, and Cu ($R = -0.372$, 0.305 , and -0.127 , respectively).

Correlations at Different Gender, Age, and Season Groups

The partial correlations among the five essential trace elements at different gender, age, and season groups are shown in Table 5.

TABLE 3. Effects of Gender on Whole Blood Levels of Ca, Fe, Cu, Mg, and Zn^a

	Boys (<i>n</i> = 2150)	Girls (<i>n</i> = 1060)	<i>Z</i>	<i>P</i>
Ca	1.60 [0.17]	1.61 [0.16]	−1.91	0.056
Fe	8.08 [1.00]	7.98 [0.95]	−2.015	0.044
Cu	20.20 [5.90]	19.95 [5.80]	−1.308	0.191
Mg	1.51 [0.20]	1.50 [0.17]	−0.558	0.577
Zn	81.30 [17.50]	80.20 [18.60]	−1.761	0.078

^aConcentrations of these elements were expressed as median [interquartile range].

TABLE 4. Correlation Relationships between Whole Blood Levels of Ca, Fe, Cu, Mg, and Zn

	Boys		Girls		Total	
	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>	<i>R</i>	<i>P</i>
Ca	−0.364	<0.001	−0.382	<0.001	−0.372	<0.001
Fe	0.307	<0.001	0.295	<0.001	0.305	<0.001
Cu	−0.135	<0.001	−0.116	<0.001	−0.127	<0.001
Mg	0.038	0.081	−0.003	0.928	0.025	0.161
Zn	0.562	<0.001	0.592	<0.001	0.573	<0.001

Overall, the most of these essential elements were positively correlated including Ca-Cu, Ca-Mg, Fe-Mg, Fe-Zn, Cu-Mg, and Cu-Zn; Ca-Fe and Fe-Cu were negatively correlated. Correlations between Ca-Mg, Fe-Mg, and Fe-Zn were the most stable and the strongest compared with others, and the correlations were not significantly affected by gender, age, and season. Fe-Cu, Cu-Mg, Cu-Zn, and Mg-Zn were mostly

affected by gender, age, and/or season, and only highly correlated at some of these subgroups. Ca-Zn was uncorrelated except for negative correlation at spring.

Associations of One Essential Trace Element with Concentration Quintiles of Other Ones

Associations of one of the five essential trace elements with concentration quintiles of other four ones were analyzed by multivariable linear regression after unadjusted (model 1), adjusted for age, gender, season and year (model 2), and adjusted for age, gender, season, year of samples collection, and the ln-transformed values of other three essential elements (model 3). The results are presented in Table 6.

Ca was highly positively associated with Cu and Mg in all three models, highly negatively associated with Fe in model 1 and 3 but only weakly associated with Fe in model 2, and also highly negatively associated with Zn in model 1 but not in model 2 and 3. Fe was highly negatively and positively associated with Ca, and Mg and Zn, respectively, in all three models, and highly negatively associated with Cu in model 1 and 3 but not in model 2. Cu was highly positively associated with Ca and Mg in all three models, highly negatively associated with Fe in model 1 and 3 but not in model 2, and highly positively associated with Zn in model 2 and 3 but not in model 1. Mg was highly associated with Ca, Fe, and Cu in all three models, and Zn in models 1 and 2, however, was weakly negatively associated with Zn in model 3. Zn was highly negatively and positively associated with Ca and Fe,

TABLE 5. The Partial Correlations between Different Essential Elements of Children Aged 0–14 years

Characteristics	Number	Ca-Fe	Ca-Cu	Ca-Mg	Ca-Zn	Fe-Cu	Fe-Mg	Fe-Zn	Cu-Mg	Cu-Zn	Mg-Zn
Gender^a											
Boy	2150	−0.168***	0.138***	0.283***	—	−0.052*	0.553***	0.329***	0.075**	0.083***	—
Girl	1060	−0.156***	0.158***	0.291***	—	−0.072*	0.546***	0.422***	—	0.108***	−0.094**
Age (years)^b											
0~	397	−0.213***	—	0.253***	—	—	0.574***	0.282***	0.124*	—	—
1~	555	−0.093*	0.195***	0.294***	—	—	0.529***	0.239***	—	0.167***	—
2~3	606	−0.144***	—	0.314***	—	−0.088*	0.550***	0.358***	—	0.121**	—
4~6	743	−0.174***	0.092*	0.272***	—	—	0.587***	0.347***	—	0.083*	—
7~9	519	−0.136**	0.275***	0.296***	—	—	0.504***	0.362***	—	—	—
10~14	390	−0.202***	0.252***	0.224***	—	—	0.520***	0.471***	—	—	—
Season^c											
Spring	779	−0.162***	0.117**	0.384***	−0.115**	−0.076*	0.536***	0.319***	0.085*	0.120**	0.075*
Summer	1080	−0.101**	0.160***	0.222***	—	—	0.495***	0.368***	—	—	—
Autumn	895	−0.158***	0.164***	0.280***	—	−0.094**	0.606***	0.400***	0.087*	0.153***	−0.078*
Winter	456	−0.332***	—	0.309***	—	—	0.614***	0.305***	—	—	—

^aControlling for age, season, year of samples collection, and other three elements not in the analysis.

^bControlling for gender, season, year of samples collection, and other three elements not in the analysis.

^cControlling for gender, age, year of samples collection, and other three elements not in the analysis.

****P* < 0.001; ***P* < 0.01; **P* < 0.05.

TABLE 6. The Relationships between Essential Elements by Multivariable Linear Regression

Dependent variables	Independent variables ^a	Model 1 ^b		Model 2 ^c		Model 3 ^d	
		β	95% CI	β	95% CI	β	95% CI
ln-Ca	Fe	-0.010***	-0.012, -0.008	-0.004**	-0.006, -0.002	-0.014***	-0.016, -0.011
	Cu	0.012***	0.010, 0.014	0.009***	0.007, 0.011	0.007***	0.005, 0.009
	Mg	0.011***	0.009, 0.013	0.011***	0.009, 0.013	0.016***	0.014, 0.018
	Zn	-0.012***	-0.015, -0.010	-0.002*	-0.005, -0.000	-0.002	-0.004, 0.001
ln-Fe	Ca	-0.013***	-0.015, -0.010	-0.005***	-0.008, -0.003	-0.010***	-0.012, -0.009
	Cu	-0.004***	-0.007, -0.002	-0.001	-0.003, 0.001	-0.004***	-0.006, -0.002
	Mg	0.031***	0.029, 0.033	0.035***	0.033, 0.037	0.033***	0.031, 0.034
	Zn	0.031***	0.029, 0.033	0.028***	0.026, 0.031	0.020***	0.018, 0.022
ln-Cu	Ca	0.032***	0.027, 0.038	0.025***	0.019, 0.031	0.023***	0.017, 0.029
	Fe	-0.008**	-0.013, -0.002	-0.001	-0.006, 0.005	-0.011**	-0.018, -0.004
	Mg	0.015***	0.010, 0.021	0.013***	0.008, 0.019	0.013***	0.007, 0.020
	Zn	0.001	-0.005, 0.006	0.016***	0.010, 0.022	0.020***	0.013, 0.027
ln-Mg	Ca	0.013***	0.011, 0.015	0.014***	0.011, 0.016	0.017***	0.014, 0.019
	Fe	0.032***	0.030, 0.034	0.037***	0.035, 0.040	0.038***	0.036, 0.040
	Cu	0.007***	0.004, 0.009	0.006***	0.003, 0.008	0.004***	0.002, 0.006
	Zn	0.011***	0.009, 0.014	0.014***	0.012, 0.017	-0.002*	-0.005, 0.000
ln-Zn	Ca	-0.029***	-0.033, -0.025	-0.008***	-0.012, -0.005	-0.006**	-0.010, -0.002
	Fe	0.056***	0.052, 0.060	0.042***	0.039, 0.046	0.041***	0.037, 0.045
	Cu	-0.001	-0.005, 0.003	0.008***	0.005, 0.012	0.010***	0.007, 0.013
	Mg	0.020***	0.016, 0.024	0.020***	0.017, 0.024	-0.004	-0.008, 0.000

^aIndependent variables were analyzed by a quantile increment.^bUnadjusted for covariates.^cAdjusted for age, gender, season, and year of samples collection.^dAdjusted for age, gender, season, year of samples collection, and the ln-transformed concentrations of other three essential elements.*** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$.

respectively, in all three models; nevertheless, it was not associated with Cu in model 1 but highly positively associated in models 2 and 3 and highly positively associated with Zn in models 1 and 2 but not in model 3.

In full-adjusted models (model 3), the associations between Zn-Fe and Mg-Fe were the strongest with per concentration quintile increase of Fe caused Zn and Mg increase by 4.19% and 3.87%, respectively, and per concentration quintile increase of Mg caused Fe increase by 3.36%. In some associations between two essential elements, their impacts on each other are different. For examples, per concentration quintile increase of Cu caused Ca only increase by 0.7%; however, per concentration quintile increase of Ca caused Cu increase by 2.3%.

DISCUSSION

Reliable and accurate reference intervals for infants and children are very important to medical practitioners and dietitians for clinical decision-making and nutritional assessment. It is difficult to obtain healthy children samples according to well-defined criteria. However, this problem can be overcome by gathering large population of hospital-based health examination

individuals (14, 15). This study establish the reference intervals of blood Ca, Fe, Cu, Mg, and Zn levels for infants and children in a large number of relatively healthy subjects, and make a more detailed classification considering the gender and age, which may be useful for decisive diagnoses of abnormality of the five essential trace elements and the related diseases.

The concentrations of blood Ca in this study were lower than previously reported in Shandong (6), Beijing (1), and Zhengzhou (16), while Cu and Zn levels were a little higher. It may turn out to be the reason of diverse diet habits in different regions of China. Shandong is located near the sea in the northeast of China, teeming with seafood such as shrimp and kelp characterized by high Ca content. In Beijing and Zhengzhou, people consume flour rich in Ca as staple food, which make them get adequate amounts of Ca. All those dietary habits are different from that in Lu'an. People here have a long-standing habit of drinking tea. Studies have shown that tea leaves contain Cu, Zn, Fe, and a great quantity of organic acid which could inhibit the Ca absorption (17, 18). Those may contribute to the elevated Cu and Zn levels and decreased Ca level in Lu'an. Nevertheless, there are no differences in Fe and Mg levels among the four places. To explore the causes, more works are needed to be

done. Wang et al. (4) have reported that there were significant differences in blood Zn levels between female and male subjects, and no significant differences were found in Ca levels. However, some investigators (5) showed that mean serum Zn concentrations did not differ between boys and girls. There were also no significant gender-based differences in blood Ca, Fe, Cu, Mg, and Zn concentrations found in Zhao's work (2). However, in our study, no significant differences in Ca, Cu, Mg, and Zn concentrations between boys and girls were found, except that the concentrations of Fe were significantly lower in girls than in boys. Fe is an essential component of hemoglobin. It has been reported that female had lower hemoglobin than male starting at puberty (19). Some subjects enrolled in our study aged 10–14 may lead to the gender-based differences on Fe levels. In overall, those results are confusing. Possible reasons may be that age range or the divided age groups of children enrolled in these studies were different.

Many studies have indicated that the concentrations of essential trace elements were closely related to age (2, 4, 6–8). We found blood Zn and Fe concentrations are gradually increased with age, consistent with previous studies (2, 4, 6). While Ca and Cu levels are gradually decreased with age, similar to the results in Li (7) and Lin's study (8). No change in Mg concentrations with age was found in our study. The changes of children's diet structure along with age may contribute to the downtrend or uptrend of these elements concentrations. However, a limitation of our study is that the data of essential trace elements content from diet or drinks were not taken into consideration. Physical activities (20, 21) and even air pollution (22) may also affect the concentrations of essential trace elements. These influence factors would be monitored in our future studies.

For nutritional assessment, disease diagnosis, and therapy, appropriate reference intervals are important judgment principles. Nevertheless, this will not be enough. The correlations among these trace elements are also very important for more complete and precise interventions.

Correlations between essential trace elements have been studied in children previously (2, 3, 6, 7, 11–13). However, the results are not always consistent, possibly because of differences in the sample size, age group, or other characteristics of the study populations or discrepancies in the study designs and statistical methods. Our study has collected a huge number of subjects, and the statistical analysis methods applied are controlled for more related factors which might influence the correlations among the essential trace elements in whole blood of children.

In our study, correlations among Ca, Fe, Cu, Mg, and Zn in whole blood and the factors that have impacts on these correlations were analyzed. We found that Ca-Fe were negatively correlated (Tables 5 and 6), which was consistent with previous studies (3, 7), and this might be due to the significant inhibitory effect of Ca on Fe absorption (23–26). However, there were also studies showed that Ca-Fe were not correlated in whole blood (2, 13). Previous studies indicated that both Fe and Cu could inhibit the absorption of Zn in diet (27–32). Interestingly, in our study, Fe-Zn were highly positively correlated, and these correlation were stable across gender, age, and season (Table 5). There was a review indicated that Fe did not appear to have a negative effect on serum zinc concentrations (33). Several investigators reported that appropriate proportion of Fe-Zn (34, 35) (probably 1:1) and supplementation forms of Fe-Zn (34) (heme iron or oyster zinc) did not affect the absorption of the both, which might be a possible reason for the discrepancy of Fe-Zn correlation. Cu-Zn were also highly positively correlated in our study (Table 5). The situation similar to Fe-Zn might also exist between Cu and Zn. However, this does not exclude other mechanisms of the interaction. More studies are needed to verify the results and elucidate the mechanisms of these interactions.

As shown in Table 5, correlations of Cu-Mg and Mg-Zn were significantly affected by gender. Children aged 10–14 collected in our study are in the early period of pubertal stage. The changes of puberty-associated hormones or other related proteins are different between boys and girls (36, 37). Moreover, essential trace elements are reported to be closely associated with hormones metabolism, such as Zn and growth hormone (35, 38), Cu and gonadotropin-releasing hormone (39), and Mg and parathyroid hormone (40), which might be the explanations of gender-based correlations of Cu-Mg and Mg-Zn.

Our results showed that age had significant impacts on correlations between Cu and other four essential elements. We also found that the correlations among these five essential trace elements in whole blood were affected by seasons, which were rarely mentioned in other studies, to our known. As shown in Table 5, seasons of sample collection also had apparent impacts on correlations between Cu and other four elements and correlations between Ca-Zn and Mg-Zn. However, the reasons are still unknown. People here have different diet habits in different seasons; meanwhile, children have different diet habits with age. Furthermore, these essential elements are mainly obtained from diets. We speculate that the impacts of age and season on the correlations among these essential elements might be partially caused by seasonal diet habits and

different diet pattern with age. Moreover, it is interesting that Cu-Fe, Cu-Mg, Cu-Zn, and Mg-Zn were either highly or weakly correlated in spring and autumn, but all uncorrelated in summer and winter. Lu'an (116°30'37"E, 31°44'40"N) is located in eastern China, and the average temperature here in both spring and autumn is about 17.5°C and in summer and winter is about 26.0 and 5.0°C, respectively. Does this suggest that the temperature also have some impacts on some of these correlations between the five essential elements? Or it might have something to do with seasonal diseases. For example, increased incidence of respiratory infection is linked with season alternate, especially in the beginning of spring and the end of autumn with big change of temperature here.

Coexistence of multiple-micronutrient deficiencies are common among children due to their rapid growth leading to high requirements, especially Ca, Fe, and Zn. Prophylactic supplementation with multiple micronutrients seems to be an appealing strategy and effective intervention for children's health. Concerns on pivotal role of trace elements is gradually increased among parents. Many children, who are suffering from infectious diseases or even healthy, without trace elements detection, are routinely supplemented with trace elements in form of micronutrient-fortified foods and single- or multiple-micronutrient preparations. Previous studies and our results all showed that Ca-Zn and Ca-Fe were negatively correlated. From birth to 2 years of age or even older, children in Lu'an are recommended to be supplemented with Ca and vitamin D, Fe, and Zn for prevention of rickets, anemia, and hyp immunity. However, these supplementations are conducted without the awareness of the relationships among these essential elements. Considering the negative effects of Ca on Fe and Zn in blood of children, is it reasonable to simultaneously intake these nutrients? Although we have found the strong positive correlation of Fe-Zn, as aforementioned, proper ratio of Fe-Zn may contribute to the increased absorption of the two elements and alleviate the adverse effects of Fe on Zn absorption. So we should pay some attention to the content of each essential trace element when purchasing commercial micronutrients products. It should be noted that although essential trace elements supplementation are benefit for mineral-deficient children, the effects of extra supplementation on healthy and well-fed children are somewhat disappointing. Zn- and Fe-combined supplementation has been reported to be inefficacious on immune function among nourished and healthy children (41) and has less positive effect on growth than Zn-only supplementation (42). It has been suggested that excessive Fe intake of infants may increase the risk of infection (35). Our findings raise

questions about the absorption efficiency of single essential element from multi-micronutrient preparations. Hence, in addition to the concentration measurements of these essential elements, a particular consideration should be drawn to the relationships among them before we implement the preventive supplementation of trace elements or assess the effects of single trace element on children's health.

In conclusion, reference intervals for whole blood levels of Ca, Fe, Cu, Mg, and Zn for pediatric subjects are present, which could be used as a tool for clinical diagnosis. And our results indicated that the relationships between Ca, Fe, Cu, Mg, and Zn in peripheral blood of children are more complex than what we thought. Their correlations were affected by many factors such as gender, age, season of samples collection, all of which should be considered as confounding factors in the future works to obtain a more accurate conclusion. It seems that a fine balance of essential trace elements in blood is very important to children's health. Our findings are important for better diagnosis and establishing recommendations for optimal trace element supplementation in infants and children healthy or suffering from diseases.

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