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Postmortem Blood Metal Levels: Establishing Updated Reference Ranges Using ICP-OES

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ABSTRACT: Postmortem testing for metals is crucial in forensic toxicology to determine whether metal exposure contributed to an individual's death. However, current reference ranges for metal concentrations, primarily based on living individuals, fail to account for postmortem physiological changes. This study addresses this gap by analyzing thelevels of zinc and iron postmortem blood levels over various postmortem intervals (PMI) using Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES). Fifty samples were analyzed, revealing a significant increase in metal concentrations over time, with zinc levels rising from 181 μg/dL to 24,935 μg/dL and iron levels from 155 μg/dL to 11,421 μg/dL across a 40-month PMI. These changes are attributed to the redistribution of metals from tissues into the bloodstream and decomposition processes. The study proposes postmortem-specific reference ranges, emphasizing the need for forensic pathologists and toxicologists to consider PMI in their assessments to avoid misinterpretation and inaccurate cause-of-death determinations. This research underscores the necessity of updating reference ranges for postmortem analysis, ultimately improving the accuracy of forensic toxicology reports and contributing to more reliable determinations of cause of death.

Keywords: Postmortem toxicology; Metal concentrations; Zinc; Iron; Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES); Postmortem interval (PMI); Forensic toxicology

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1. Introduction

Postmortem testing for metals is a vital aspect of forensic toxicology, as it can reveal whether exposure to certain metals played a role in an individual's death. In particular, the accurate interpretation of these data is essential, as it influences the determination of the cause of death, potentially impacting legal outcomes and public health responses. To ensure accurate interpretation, the analytical methods, sampling procedures, and reference ranges used must be reliable and suitable for postmortem conditions. Evaluation techniques such as atomic absorption spectrometry (AAS), inductively coupled plasma mass spectrometry (ICP-MS), and inductively coupled plasma optical emission spectrometry (ICP-OES) are commonly used to measure metal concentrations in biological samples like blood, urine, and tissues. These techniques offer high sensitivity and accuracy for detecting trace metals and are routinely applied to diagnose metal poisoning in living individuals. In particular, Inductively coupled plasma optical emission spectroscopy is widely recognized for its accuracy, sensitivity, and capability to analyze multiple elements simultaneously, making it a powerful tool in both clinical and forensic settings [1]. However, current reference ranges for metal concentrations are predominantly based on data from living individuals. These ranges do not account for the physiological and biochemical changes that occur after death, such as the redistribution of metals from tissues to the bloodstream, resulting in the misattribution of the cause of death, potentially triggering unnecessary legal investigations and incurring

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additional costs for families and judicial systems. As a result, the application of ICP-OES in postmortem scenarios is limited by the absence of validated reference ranges. This study is aimed to address this criticism by measuring postmortem whole-blood levels of zinc and iron and evaluating how these levels vary over time. These elements are susceptible to postmortem processes. The liver, spleen, and muscles, which are rich in zinc and iron, release these metals into the bloodstream as cells deteriorate. Furthermore, after death, the cessation of enzymatic activity can cause metals like zinc and iron, which are usually bound to proteins, to become unbound, resulting in increased concentrations in the blood. By analyzing samples from individuals who had died, this study aims to observe the natural progression of metal concentration changes as the postmortem interval increased. The ultimate goal is to establish reference ranges specifically suited for postmortem analysis, thereby improving the accuracy of forensic toxicology reports and ensuring that conclusions drawn from these reports are based on more relevant and reliable data.

2. Materials and Methods

2.1. Materials

Nitric acid 65% (CAS 7697-37-2) was purchased from Sigma-Aldrich (Milan, Italy) and used as received. Certified metal standards (e.g., Zinc standard for ICP traceCERT© 1 g/L in nitric acid 18562-100ML-F; Iron standard for ICP traceCERT© 1 g/L in nitric acid 43149) were obtained from Supelco. Water produced from a Milli-Q® Direct Water Purification System was used in all the experiments. All other reagents used in the experiment were of analytical grade and, when not indicated, were purchased from Sigma-Aldrich (Milan, Italy).

2.2. Sample Collection and Preparation

The study involved the collection of 50 postmortem whole blood samples from individuals with no reported physiological symptoms of metal intoxication. These samples were drawn from femoral veins, a common site for postmortem blood collection due to its relative isolation from immediate postmortem changes like hemolysis and contamination from nearby tissues. The time since death (preservation time) ranged from a few hours to several weeks, providing a broad spectrum for analyzing the effect of postmortem interval (PMI) on metal concentrations (Table 1). Each blood sample was carefully stored in sterile, metal-free containers to avoid contamination. The samples were then refrigerated at a consistent temperature of 4 °C until analysis. Prior to analysis, samples were brought to room temperature and homogenized to ensure uniformity. For analysis, samples were prepared by diluting 500 µL of blood with 4.5 mL of 7% *v*/*v* nitric acid in Milli-Q water. After adding nitric acid, the blood was centrifuged at 10,000 rcf for five minutes at room temperature to remove lipidic and protein precipitates.

Table 1. Samples tipology.

Preservation Time (Months)	Samples
2	6
4	5
6	5
8	6
10	6
12	5
18	5
24	6
40	6

2.3. ICP-OES Analytical Procedure

The ICP-OES analysis was conducted using the instrument ICPE-9820 (Shimadzu Italy, Milan, Italy). In the optimized setup, radio frequency power was set at 1.20 kW, argon flow at 10.00 L/min, auxiliary gas was set at 0.60 L/min, and carrier gas flow was adjusted to 0.70 L/min. The exposure time for each sample was fixed at 30 s. Axial viewing configuration was employed due to its higher sensitivity, which is particularly important for detecting and quantifying low concentrations of metals such as zinc and iron. To ensure the precision and reliability of the ICP-OES measurements, the method was rigorously calibrated using certified reference materials (CRMs) for both zinc and iron. These CRMs provided known concentrations of the metals, allowing for the construction of accurate calibration curves. Multiple standards were used in the calibration process to verify the linearity of the instrument's response over a range of concentrations, ensuring that the measurements obtained were both accurate and reliable. This rigorous calibration

process is essential for validating the results and ensuring that the ICP-OES analysis delivers precise and reliable data for assessing postmortem metal concentrations.

2.4. Method Validation

2.4.1. Test Solutions and Calibration

A multi-element solution containing Al, Ba, Cu, Fe, Mn, Sr, and Zn was prepared for calibration in 7% (*v*/*v*) nitric acid. Additionally, four types of solutions were prepared to study spectral and matrix interferences, and determine detection limits for trace elements based on the average blood composition and the sample dissolution procedure. Solution 1 simulated the elemental composition of postmortem samples and contained 200 mg/L of Ca, 1200 mg/L of K, 140 mg/L of Mg, 500 mg/L of Na, 100 mg/L of P, and 1 mg/L of each trace element (Al, Ba, Cu, Fe, Mn, Sr, and Zn). Solution 2, which did not contain trace elements, was used as the matrix blank for solution 1. Solution 3 contained only the trace elements being analyzed. Solution 4 acted as the matrix blank for solution 3.

2.4.2. Interferences Studies and Selection of Analytical Lines

Spectral interferences were evaluated using Solutions 2 and 3, as described earlier in Section 2.4.1. A total of 41 spectral lines corresponding to 12 elements, recommended by the ICP-OES spectrometer library, were analyzed. Typically, more than two lines were examined for each element. Solutions 2 and 3 were nebulized consecutively, and the spectra were recorded within a ±0.8 nm range around the wavelength of maximum intensity for all emission lines. This allowed for detecting and eliminating inter-element overlapping spectral interferences by visually inspecting the registered spectra. Once interference-free conditions were confirmed, the signal-to-background ratio (R) and the matrix effect (ME) for 30 lines across seven trace elements were calculated using Equations (1) and (2), respectively:

$$R = I1/I2 \quad (1)$$

$$ME = [(I1 - I2)/(I3 - I4) - 1] \times 100 \quad (2)$$

Here, ME represents the matrix effect, and Ix (x = 1, 2, 3, and 4) corresponds to the net intensity measured for each line in the four solutions described in Section 2.4.1. Analytical lines were evaluated based on R and ME values, with higher R indicating better detection limits and lower ME values minimizing systematic errors (higher accuracy) due to macro-element presence. The final selection of trace element lines also considered the accuracy of determinations using certified reference materials (CRMs). For macro-elements, lines that were free from spectral interference and provided the highest accuracy were chosen. Interferences in both axial and radial view modes were also assessed.

2.4.3. Validation Studies

The validation of the method followed the ICH guideline of the European Medicines Agency (EMA) for assessing accuracy, precision, sensitivity, and linearity. To evaluate accuracy and precision, assays were validated using selected certified reference materials (CRMs). Method precision, defined as the closeness of agreement between independent test results, was expressed as the percentage variation coefficient (%CV). Precision was assessed in terms of both repeatability and reproducibility. Repeatability (intra-assay precision) was calculated as the %CV of results from six replicates of the CRM sample analyzed by a single analyst on the same day. Reproducibility (inter-assay precision), which refers to the precision of measurements under reproducible conditions, was determined by analyzing the same CRM in six separate runs—three by each of two analysts on different days. Accuracy was reported as the bias or percentage difference between the measured concentration and the assigned CRM value (%D), and it was evaluated under both repeatability (intra-assay accuracy) and reproducibility (inter-assay accuracy) conditions. The acceptance criteria for accuracy and precision were as follows: the mean %D and %CV should not exceed 10%; 75% of all samples must fall within 15% of the expected concentrations; and no more than one of the six samples at any concentration could exceed 10% of its expected value. The sensitivity of the method for each element was indicated by the slope of the linear regression equation (mg^{-1} mL), and linearity was assessed through the correlation coefficients of calibration curves. Linearity was considered acceptable if $r \geq 0.9995$.

Limits of detection (LD) and quantification (LQ) were calculated based on the mean blank signal ($\bar{x}_b$) and the standard deviation of blank responses (S_b) using the following equations:

$$LD = \bar{x}_b + 3S_b \quad (3)$$

$$LQ = \bar{x}_b + 10S_b \quad (4)$$

The values of $\bar{x}_b$ and S_b were determined from 10 blank measurements. Concentration values for the detection and quantification limits were derived from the corresponding signals (LD and LQ) using a calibration curve, y = f(c). Both limits were expressed in milligrams of each element per gram of dry sample weight.

2.5. Statistical Analysis

The relationship between metal concentrations and preservation time was analyzed using regression analysis. Higher R^2 values indicate a stronger correlation [2]. Proposed reference ranges were calculated using the Normal distribution method [2]. This method involves calculating the sample data's mean, variance, and standard deviation (SD). In particular, for a two-sided reference interval, the lower and upper limits of normality are calculated as:

$$\text{Lower limit} = \text{Mean} - 1.96 \times \text{SD}$$

$$\text{Upper limit} = \text{Mean} + 1.96 \times \text{SD}$$

while the 90% confidence interval for each limit is calculated using the formula:

$$\text{Limit} \pm 1.64 \times \text{Variance} \times \sqrt{(1/n + 2/(n-1))}$$

For data quality, values must follow a normal distribution, potentially after transformation, and a minimum sample size of 40 is recommended.

3. Results and Discussion

In the literature, toxic and lethal levels of zinc and iron vary depending on factors like exposure duration and individual health. For zinc, serum concentrations above 150 µg/dL are considered toxic, while lethal toxicity is rare but can occur from ingesting 4–5 g. Symptoms of zinc poisoning include nausea, vomiting, abdominal pain, diarrhea, headaches, dizziness, and in cases of inhalation exposure, respiratory distress and chest pain. Chronic exposure may lead to anemia, reduced immune function, and neurological issues like numbness. For iron, serum levels above 350 µg/dL are toxic, and ingestions of more than 60 mg/kg of elemental iron can be fatal, with concentrations over 1000 µg/dL linked to severe toxicity. Symptoms of iron poisoning include nausea, vomiting, abdominal pain, gastrointestinal bleeding, metabolic acidosis, shock, organ failure (especially liver failure), coma, and death. Chronic iron toxicity, such as from hemochromatosis, can cause liver cirrhosis, heart disease, diabetes, and skin discoloration. Understanding these toxic and lethal ranges is essential in forensic toxicology to determine if metal poisoning contributed to death. In this paper, we developed an analytical method based on ICP-OES spectroscopy to specifically detect Iron and Zinc (together with ten other metals) from postmortem blood samples. The matrix effect is critical in analytical methods, particularly when dealing with complex biological matrices like postmortem blood. In such cases, the presence of proteins, lipids, and other endogenous substances can interfere with the ionization efficiency of target analytes, leading to inaccurate quantification. In the developed method, the matrix effect was evaluated by comparing the responses of analytes spiked into the matrix with those spiked into a clean HNO_3 solution at a concentration of 1 µg/mL (Table 2). This method's accuracy and precision results are detailed in Tables 3 and 4. A comparison of element concentrations in the Certified Reference Materials (CRMs) with those from the intra-assay study (Table 3) shows that Al concentrations in our samples may have been underestimated by approximately 5.6%. In contrast, Ca, K, P, Ba, Cu and Sr concentrations were likely overestimated by 4–7%. The discrepancy between measured and expected concentrations (%D) was minimal for other elements. Inter-assay accuracy showed no significant variation (Table 4). As anticipated, the precision for reproducibility was slightly lower (coefficients of variation < 10%) compared to repeatability (coefficients of variation < 5%) across all elements. Nevertheless, both repeatability, reproducibility, and accuracy were within the acceptable criteria. The intensity of spectral lines increased linearly with concentration across the range used for each element, with correlation coefficients (r) equal to or greater than 0.9998, meeting the linearity acceptance criterion ($r \geq 0.9995$). Based on sensitivity, the studied elements are ranked as follows: Ba > Mn > Ca > Sr > Fe > Cu > Al > Mg > Zn > Na > K > P. Detection and quantification limits are shown in Table 5. The detection capabilities of our optimized

ICP-OES method were sufficient to measure the concentrations of Al, Ba, Ca, Cu, Fe, K, Mg, Mn, Na, P, Sr, and Zn in postmortem samples.

Table 2. Matrix effect.

Element	Found Values in Matrix (µg/mL)	Found Values in Clean HNO_3 Solution (µg/mL)	Difference in %
Ca	1.04 ± 0.2	1.02 ± 0.2	1.92
K	1.03 ± 0.3	1.03 ± 0.3	0.00
Mg	1.02 ± 0.1	1.05 ± 0.1	−2.94
Na	1.04 ± 0.2	1.04 ± 0.2	0.00
P	1.07 ± 0.7	1.04 ± 0.7	2.80
Al	1.09 ± 0.8	1.05 ± 0.8	3.67
Ba	1.04 ± 0.4	1.09 ± 0.4	−4.81
Cu	1.03 ± 0.4	1.04 ± 0.4	−0.97
Fe	1.08 ± 0.4	1.11 ± 0.4	−2.78
Mn	0.98 ± 0.3	1.00 ± 0.3	−2.04
Sr	1.03 ± 0.4	1.06 ± 0.4	−2.91
Zn	1.05 ± 0.2	1.06 ± 0.2	−0.95

Table 3. Intra-assay accuracy and precision.

Element	Accuracy			Precision
	Certified Values	Found Values	D (%)	CV (%)
Ca	15.60 ± 0.2	16.60 ± 0.2	6.02	2.83
K	24.30 ± 0.3	25.60 ± 0.3	5.35	1.29
Mg	04.32 ± 0.1	04.45 ± 0.1	3.01	0.54
Na	24.00 ± 2.0	24.50 ± 2.0	2.08	1.37
P	01.37 ± 0.1	01.43 ± 0.1	4.19	4.07
Al	249.00 ± 8.0	235.00 ± 8.0	−5.62	1.88
Ba	124.00 ± 4.0	129.00 ± 4.0	4.03	1.99
Cu	13.00 ± 0.4	14.00 ± 0.4	7.69	2.72
Fe	218.00 ± 14.0	221.00 ± 14.0	1.38	2.05
Mn	98.00 ± 3.0	100.00 ± 3.0	2.04	3.33
Sr	53.00 ± 4.0	56.00 ± 4.0	5.66	7.41
Zn	55.00 ± 1.2	56.00 ± 1.2	1.82	0.74

Concentrations are expressed as (mean ± standard deviation for 6 replicates) are expressed in µg/g, except for Ca, K, Mg, Na and P, which are expressed in mg/g; Certified Reference Materials used: NIST1547.

Table 4. Inter-assay accuracy and precision.

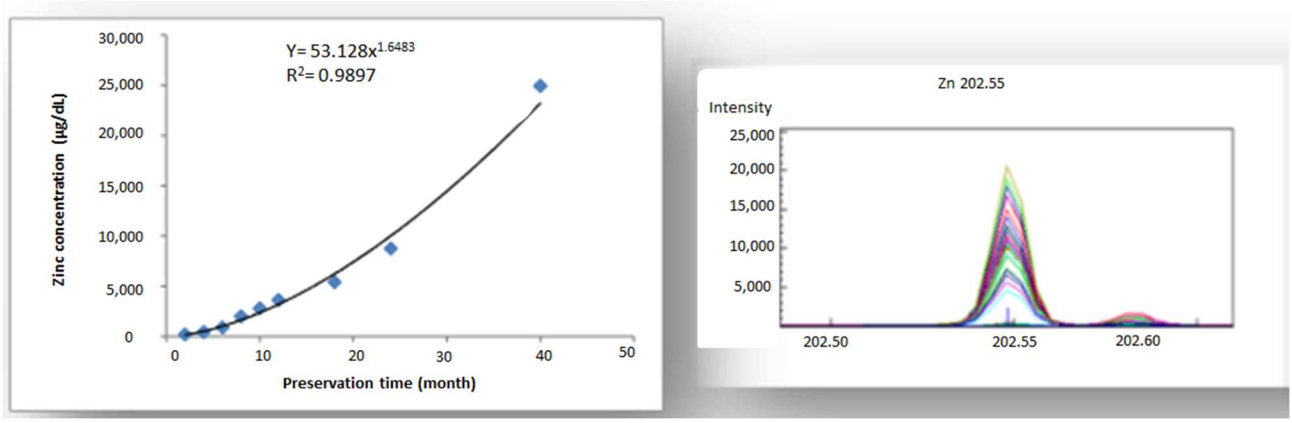
Element	Accuracy			Precision
	Certified Values	Found Values	D (%)	CV (%)
Ca	15.60 ± 0.2	15.80 ± 0.2	1.26	3.15
K	24.30 ± 0.3	24.40 ± 0.3	0.41	1.24
Mg	04.32 ± 0.1	04.41 ± 0.1	2.08	0.37
Na	24.00 ± 2.0	24.50 ± 2.0	2.08	2.10
P	01.37 ± 0.1	01.40 ± 0.1	2.14	5.21
Al	249.00 ± 8.0	254.00 ± 8.0	2.01	0.67
Ba	124.00 ± 4.0	125.00 ± 4.0	0.81	2.42
Cu	13.00 ± 0.4	13.50 ± 0.4	3.85	4.23
Fe	218.00 ± 14.0	223.00 ± 14.0	2.29	3.42
Mn	98.00 ± 3.0	100.00 ± 3.0	2.04	3.67
Sr	53.00 ± 4.0	55.00 ± 4.0	3.77	5.09
Zn	55.00 ± 1.2	55.50 ± 1.2	0.91	1.18

Concentrations are expressed as (mean ± standard deviation for 6 replicates) are expressed in µg/g, except for Ca, K, Mg, Na and P, which are expressed in mg/g; Certified Reference Materials used: NIST1547.

Table 5. Selected analyte lines with their detection and quantification limits.

Element	Wavelenght (nm)	Detection Limit (µg/g)	Quantification Limit (µg/g)
Ca	317.93	0.75	2.50
K	766.49	1.60	5.20
Mg	280.27	0.01	0.03
Na	589.59	1.40	4.60
P	213.62	1.20	3.90
Al	394.40	1.30	4.40
Ba	493.41	0.07	0.22
Cu	324.75	0.70	2.30
Fe	234.35	0.60	2.00
Mn	259.37	0.02	0.08
Sr	407.77	0.20	0.60
Zn	202.55	0.11	0.36

Although the validation covered a range of 12 metals, our work primarily focuses on the quantification of zinc and iron, which are particularly sensitive to postmortem variations. The study's findings demonstrated a significant and progressive increase in both zinc (Figure 1) and iron (Figure 2) concentrations in postmortem blood samples as the time since death lengthened.



(**A**) (**B**)

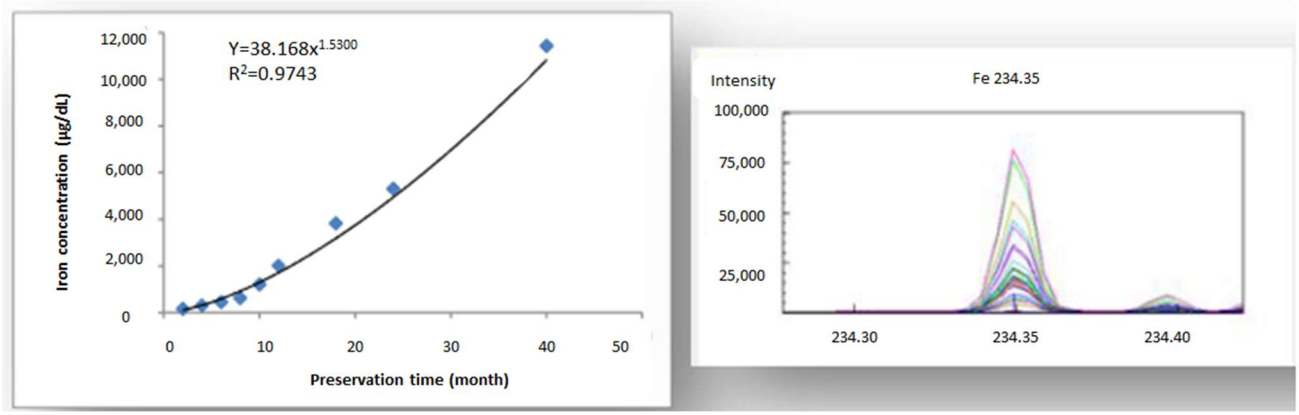
Figure 1. Zinc blood concentration in relation to preservation time. (**A**). Trend of zinc concentration increasing with preservation time. (**B**). ICP-OES spectra of zinc, where different colors represent varying preservation times. The peak area grows progressively as preservation time increases.

Initially, zinc levels were within the expected baseline range shortly after death, similar to those found in living individuals. However, over the course of several months, these concentrations surged dramatically, reaching levels as high as 30,000 µg/dL (Table 6).

Table 6. Zinc concentration in post-mortem samples.

Preservation Time (Months)	Average zinc Concentration (µg/dL)	Sample 1 (µg/dL)	Sample 2 (µg/dL)	Sample 3 (µg/dL)	Sample 4 (µg/dL)	Sample 5 (µg/dL)	Sample 6 (µg/dL)
2	181.00 ± 16.0	163	193	165	195	185	186
4	429.00 ± 32.0	428	395	409	458	456	
6	872.50 ± 45.0	829	924	905	834	870	
8	1974.00 ± 83.0	1904	2070	2033	1946	1914	1975
10	2793.40 ± 201.0	2659	2921	2991	2589	2935	2666
12	3606.76 ± 300.0	3417	3411	4001	3809	3401	
18	5394.05 ± 544.0	4921	5272	6010	5721	5045	
24	8735.12 ± 854.0	8617	9309	9730	8215	8518	8021
40	24,935.83± 3700.0	24,990	27,267	28,441	26,835	20,913	21,169

Similarly, iron concentrations, which initially fell within the normal range observed in living individuals, also increased substantially, exceeding 10,000 µg/dL after extended postmortem intervals (Table 7). The high R^2 values (0.9897 for zinc and 0.9743 for iron) are indicative of the quality of the analysis and suggest that preservation time is a significant factor influencing postmortem metal levels.



(**A**) (**B**)

Figure 2. Iron blood concentration in relation to preservation time; (**A**). Trend of Iron concentration increasing with preservation time. (**B**). ICP-OES spectra of Iron, where different colors represent varying preservation times. The peak area grows progressively as preservation time increases.

These trends indicate the complex physiological and biochemical changes that occur in the body after death, leading to significant alterations in the distribution and concentration of metals within the bloodstream. However, the variations follow the patterns shown in Figures 1A and 2A, allowing for a meaningful prediction of the increase in zinc and iron concentrations over extended periods.

Table 7. Iron concentration in postmortem samples.

Preservation Time (Months)	Average iron Concentration (µg/dL)	Sample 1 (µg/dL)	Sample 2 (µg/dL)	Sample 3 (µg/dL)	Sample 4 (µg/dL)	Sample 5 (µg/dL)	Sample 6 (µg/dL)
2	155.00 ± 9.0	149	159	145	156	160	163
4	302.00 ± 28.0	315	299	270	298	326	
6	450.00 ± 21.0	428	453	470	449	448	
8	621.00 ± 25.0	594	626	645	636	613	614
10	1202.00 ± 126.0	1104	1154	1356	1146	1304	1149
12	2001.00 ± 229.0	1898	1742	1990	2201	2175	
18	3821.00 ± 413.0	3585	3393	3880	4219	4029	
24	5302.00 ± 404.0	5020	5260	5829	5479	5332	4890
40	11,421.00 ± 2980.0	10,434	11,243	11,412	15,044	11,318	9077

Several postmortem physiological processes contribute to these observed increases in metal concentrations. One of the primary factors is redistribution, a process that occurs as cellular structures begin to break down after death [3]. In living organisms, metals like zinc and iron are stored in specific organs and tissues, such as the liver, muscles, and red blood cells. After death, the breakdown of cellular membranes and tissues allows these metals to diffuse more freely into the bloodstream [4]. For instance, zinc, which is heavily concentrated in the liver and muscles, leaks into the circulatory system as these tissues degrade [5]. Similarly, iron, primarily found in hemoglobin within red blood cells, is released into the bloodstream as red blood cells break down postmortem [6]. Another critical factor contributing to the rising metal concentrations is tissue autolysis and decomposition [7]. Autolysis is the process of self-digestion that occurs when enzymes within cells begin to break down the cell itself, leading to the deterioration of cellular structures. As autolysis progresses, the barriers that typically prevent metals from diffusing out of cells and tissues weaken, allowing more of these elements to enter the bloodstream [8]. Decomposition, driven by microbial activity, further accelerates this process, breaking down tissues and releasing stored metals into the circulatory system [8]. Environmental factors also play a significant role in influencing postmortem metal concentrations. The conditions under which a body is stored—such as temperature, humidity, and the type of storage container—can greatly affect the decomposition rate and, consequently, the redistribution of metals [9]. Higher temperatures, for example, can

accelerate autolysis and microbial decomposition, leading to a faster and more pronounced increase in metal concentrations. Humidity levels can also affect tissue decomposition rate and metals' stability in blood samples.Inadequate storage conditions, such as fluctuating temperatures or exposure to moisture, can exacerbate these processes, resulting in higher than expected metal concentrations over time [10]. The significant increase in metal levels over time for postmortem samples presents substantial challenges to the current forensic and clinical practices that rely on reference ranges established for living individuals (In particular, normal results for blood iron are 70 to 175 mcg/dL for men, 50 to 170 mcg/dL for women, 50 to 120 mcg/dL for children while the estimated reference range of serum zinc level in sample population was 60–120 μg/dL [11,12]). These traditional reference ranges do not account for the dynamic changes that occur in the body after death and do not consider additional factors such as age, sex, and cause of death, which are all variables that can influence the found metal concentrations. In particular, age affects metal metabolism and distribution, with children, for example, being more susceptible to toxic effects from metals due to their smaller size and developing organs. Sex also plays a role, as hormonal differences between men and women can affect metal absorption, distribution, and excretion. For instance, women may have different iron levels due to menstrual blood loss, pregnancy, or hormonal influences, while men might accumulate more iron due to lower excretion rates. The cause of death is another important factor. Certain conditions, such as liver disease or renal failure, can result in abnormal metal concentrations, as these organs play a key role in the metabolism and excretion of metals. Traumatic deaths, infections, or chronic diseases can alter how metals are distributed and stored in the body. As a result, forensic pathologists and toxicologists are faced with the critical task of interpreting these elevated metal levels within the context of the postmortem interval. Failing to consider the PMI can lead to serious errors in determining the cause of death. For example, if elevated levels of zinc or iron are found in postmortem blood samples and are interpreted using reference ranges meant for living individuals, these levels might be mistakenly attributed to acute metal intoxication. This could lead to incorrect conclusions, suggesting that the individual was poisoned or exposed to dangerously high levels of these metals shortly before death when, in reality, the observed concentrations are a natural consequence of postmortem physiological processes. Such misinterpretations could have significant legal and medical implications, potentially resulting in wrongful accusations, unnecessary legal investigations, or misdirection in determining the actual cause of death. These implications also extend beyond forensic investigations. In clinical settings, where postmortem toxicology is used to understand underlying health conditions or exposure to toxic substances, inaccurate interpretations of metal levels could affect public health records and epidemiological studies. For instance, if a series of deaths is incorrectly attributed to metal poisoning based on postmortem data interpreted without considering the PMI, public health responses might be misinformed, leading to inappropriate health warnings or interventions. To address these challenges, forensic pathologists and toxicologists must incorporate postmortem-specific reference ranges and adjust their analyses according to the PMI. This approach requires the development of validated reference ranges that reflect the natural postmortem changes in metal concentrations over time to provide a more accurate framework for evaluating toxicological findings, helping to distinguish between genuine cases of metal intoxication and the normal postmortem elevation of metal levels. A reference interval, also known as a reference range or normal range, defines the interval that includes the central 95% of values from a population of healthy individuals [13]. In a two-sided reference interval, both low and high values are clinically significant, with the lower limit representing the value below which 2.5% of healthy individuals fall and the upper limit indicating the value above which 2.5% of healthy individuals fall. In cases where only low values are of concern, a left-sided reference interval is used, which includes only a lower limit (with 5% of healthy individuals falling below this value) and no upper limit.Conversely, when only high values are of concern, a right-sided reference interval is used, characterized by an upper limit (with 5% of healthy individuals above this value) and no lower limit [13]. The CLSI Guidelines C28-A3 recommend calculating a 90% confidence interval for both limits, with the confidence interval narrowing as the sample size increases, thereby increasing certainty in the reference limits [14]. Reference intervals can be calculated using three main methods: the normal distribution method, the percentile method, and the robust method [15]. The normal distribution method involves calculating the mean, variance, and standard deviation of the sample data, and it assumes the data follows a normal distribution, possibly after transformation, with a recommended minimum sample size of 40 units. The percentile method defines limits using specific percentiles and requires a minimum sample size of 120 units, with the confidence interval calculated following the method of Reed et al. [16]. The robust method, suitable for smaller sample sizes, employs an iterative process that refines central tendency and variability estimates, ultimately using bootstrapping to estimate the 90% confidence intervals. The choice of method depends on the data's characteristics and the sample size available [17]. On the basis of the sample

availability and the measured levels in this work, the reference range was calculated using the normal distribution method. The obtained data are described in Table 8 for zinc and Table 9 for iron.

Table 8. Proposed Zinc post-mortem reference range.

Preservation Time (Months)	Zinc Lower Limit (µg/dL)	Zinc Upper Limit (µg/dL)
2	149.64	309.29
4	366.28	749.91
6	784.30	1582.23
8	1811.32	3633.19
10	2399.44	4903.90
12	3018.76	6216.77
18	4327.81	9026.50
24	7061.28	14,694.11
40	17,683.82	38,360.30

Table 9. Proposed Iron post-mortem reference range.

Preservation Time (Months)	Iron Lower Limit (µg/dL)	Iron Upper Limit (µg/dL)
2	137.36	172.64
4	247.12	356.88
6	408.84	491.16
8	572.00	670.00
10	955.04	1448.96
12	1552.16	2449.84
18	3011.52	4630.48
24	4510.16	6093.84
40	5580.20	17,261.80

Through the utilization of the proposed reference range for zinc and iron, this research enhances the ability to differentiate between ante-mortem exposure to toxic levels of metals and postmortem concentration changes, ultimately leading to more reliable cause-of-death determinations.

4. Conclusions

This study offers critical insights into the dynamic behavior of zinc and iron concentrations in postmortem blood, underscoring the significant limitations of applying reference ranges established for living individuals to postmortem cases. The data showed significant increases in metal levels over time after death, which are not reflected in the reference ranges commonly used in forensic toxicology.These findings highlight the urgent need for establishing specific reference ranges for postmortem blood metal levels, acknowledging that the current reliance on reference ranges developed for living individuals can lead to significant inaccuracies in forensic investigations. The proposed postmortem reference ranges were carefully stratified based on the postmortem interval (PMI) to provide more precise and contextually appropriate guidelines for interpreting toxicological results. By incorporating a broad spectrum of cases, this study developed more nuanced reference ranges that reflect the complex physiological changes that occur after death. The implications of this study are far-reaching, as they pave the way for the development of more accurate and appropriate postmortem reference ranges specifically tailored to account for the physiological changes that occur after death. These set postmortem-specific reference ranges provide forensic pathologists and toxicologists with a more reliable framework for interpreting metal concentrations in the context of a deceased individual, thereby improving the accuracy of forensic toxicology reports. This advancement is particularly crucial in cases where the cause of death is unclear or where there is suspicion of metal poisoning. By using reference ranges that reflect the postmortem interval and the natural progression of metal redistribution, forensic experts can make more informed and precise determinations regarding the role of metals in the cause of death. As research in this area continues to evolve, adopting further updated postmortem reference ranges will significantly enhance the reliability and validity of postmortem metal analysis. This progress will contribute not only to more accurate determinations of cause of death in forensic investigations but also to the broader field of forensic science by establishing a more scientific basis for toxicological evaluations. Future research must focus on expanding the sample size of studies, including a more diverse cohort of subjects. This expansion would enable the stratification of reference ranges according to additional factors such as age, sex, and cause of death, which are all variables that can influence metal concentrations in the body. A larger and more varied dataset would help ensure

that the reference ranges developed are more universally applicable and accurate. Moreover, while this particular study concentrated on zinc and iron, it is essential to broaden the scope of research to include other metals, such as lead, mercury, and cadmium. These elements are of significant forensic interest due to their toxicity and potential to be involved in poisoning cases. Establishing a comprehensive reference range for a wider array of metals would greatly enhance the forensic community's ability to accurately assess whether metal exposure contributed to a person's death. In addition to expanding the range of metals studied, future research should explore the impact of storage conditions on metal concentrations in postmortem samples. Factors such as temperature, humidity, and the type of container used for storing samples can significantly affect the stability and concentration of metals over time. Understanding how these conditions influence postmortem metal levels is crucial for developing more reliable storage protocols and ensuring that the reference ranges used are applicable across different storage scenarios.

Author Contributions

Conceptualization, R.C.; Methodology, S.R., A.C., R.F. and G.N.; Validation, R.C. and S.R.; Formal Analysis, S.R., A.C., R.F. and G.N.; Investigation, R.C., S.R., A.C., R.F. and G.N.; Resources, M.I.; Data Curation, R.C. and M.I.; Writing—Original Draft Preparation, R.C.; Writing—Review & Editing, R.C., S.R., A.C., R.F. and G.N.; Supervision, R.C.; Funding Acquisition, R.C. and M.I.

Ethics Statement

Not applicable.

Informed Consent Statement

Not applicable.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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