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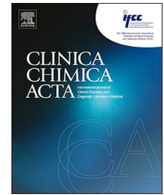

Comparison of two methods for the assessment of intra-erythrocyte magnesium and its determinants: Results from the LifeLines cohort study

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ABSTRACT

Background: Direct methods for the assessment of intra-erythrocyte magnesium (dIEM) require extensive sample preparation, making them labor intensive. An alternative, less labor intensive method is indirect calculation of intra-erythrocyte magnesium (iIEM). We compared dIEM and iIEM and studied determinants of dIEM and iIEM, plasma magnesium and 24-h urinary magnesium excretion in a large population-based cohort study.

Methods: dIEM and iIEM were measured using a validated inductively coupled plasma mass spectrometry (ICP-MS) method in 1669 individuals from the second screening from the LifeLines Cohort Study. We used linear regression analyses to study the determinants of IEM, plasma magnesium and 24-h urinary magnesium excretion.

Results: Mean dIEM and iIEM were 0.20 ± 0.04 mmol/10^{12} cells and 0.25 ± 0.04 mmol/10^{12} cells, respectively. We found a strong correlation between dIEM and iIEM (r = 0.75). Passing-Bablok regression analyses showed an intercept of 0.015 (95% CI: 0.005; 0.023) and a slope of 1.157 (95% CI: 1.109; 1.210). In linear regression analyses, plasma levels of total- and LDL -cholesterol, and triglycerides were positively associated dIEM, iIEM, and plasma magnesium, while glucose and HbA1c were inversely associated with plasma magnesium.

Conclusions: We observed a strong correlation between dIEM and iIEM, suggesting that iIEM is a reliable alternative for the labor intensive dIEM method.

1. Introduction

Magnesium is the second most abundant intracellular cation and is essential for regulation of many functions within the cell, including modulation of ion channels, protein synthesis and energy metabolism [1]. Of the total magnesium content in the human body, 55–65% is present in the skeleton, 34–44% in the intracellular space and only 0.3–1% in the circulation [2,3].

We previously showed that lower plasma magnesium concentrations were associated with increased risk of type 2 diabetes mellitus (T2DM) in women [4]. T2DM is characterized by disturbed glucose and lipid metabolism [5]. The effect of magnesium on insulin action could be one possible underlying mechanism by which magnesium affects diabetes risk, because the kinase function of the insulin receptors depends on the binding of two magnesium ions [6]. Thus, low intracellular magnesium concentrations may lead to insulin resistance and decreased cellular glucose utilization [7]. Unfortunately, plasma magnesium has been shown to be a poor predictor of intracellular magnesium [8].

Several laboratory tests to assess cellular magnesium concentrations in the human body have been evaluated, of which measurements in leukocytes generally correlate well with other magnesium pools, including skeleton, vascular smooth muscle and cardiac muscle [9]. However, concentrations of magnesium in leukocytes may vary according to pathological states and study population [10].

An alternative approach to assess intracellular magnesium concentrations is the assessment of intra-erythrocyte magnesium (IEM), which can be done with direct (dIEM) [11,12] and indirect (iIEM)

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methods [13]. Although the direct method has been suggested to be more accurate, this method requires labor-intensive sample preparation and high additional costs. Indirect methods, on the other hand, calculate IEM concentrations based on whole blood and plasma magnesium, while accounting for hematocrit. So far, only one study evaluated direct and indirect methods for the assessment of IEM, however, using samples of 10 subjects [14].

In order to obtain a valid method comparison between dIEM and iIEM, we compared dIEM and iIEM in LifeLines, a large population-based cohort study in the northern part of the Netherlands. Furthermore, we studied correlations between dIEM, iIEM, plasma magnesium and 24-h urinary magnesium excretion and additionally investigated the determinants of these magnesium parameters.

2. Material and methods

2.1. Study participants

Lifelines is a multi-disciplinary prospective population-based cohort study, examining in a unique three-generation design the health and health-related behaviors of 167,729 persons living in the North of The Netherlands. It employs a broad range of investigative procedures in assessing the biomedical, socio-demographic, behavioral, physical and psychological factors which contribute to the health and disease of the general population, with a special focus on multi-morbidity and complex genetics [15]. In brief, participants were invited to participate through their general practitioner unless they met any of the following criteria: severe psychiatric or physical illness, limited life expectancy (< 5 years), and/or insufficient knowledge of the Dutch language to complete a Dutch questionnaire.

For the present study, we used data from the second assessment that took place from 2014 until 2018. Within this second assessment, we collected blood and urine samples from August 2017 until December 2017 in which we measured IEM, plasma magnesium and 24-h urinary magnesium excretion. In total, we included 1669 participants aged > 18 years. All participants provided written informed consent. The Lifelines cohort study is conducted according to the principles of the Declaration of Helsinki and approved by the medical ethical committee of the University Medical Center Groningen, The Netherlands.

2.2. Measurements of clinical parameters

All participants visited the Lifelines research facility for a basic medical examination including blood pressure (ten times using a Dinamap automated blood pressure monitor), body height, weight (and body mass index (BMI)) and hip and waist circumference. Participants received oral and written instructions regarding the collection of 24-h urine [16]. Participants were instructed to postpone urine collection in case of urinary tract infection or menstruation, and to refrain as far as possible from heavy exercise during the collection period. On the day of collection, they were requested to discard the first morning urine, and to collect the subsequent urine for 24-h including the next morning's urine. Urine and blood samples were collected for storage and laboratory measurements. Plasma glucose was measured routinely on a Cobas 8000 platform (Roche, Mannheim, Germany) using a hexokinase UV test. Total cholesterol was measured using an enzymatic colorimetric assay, high-density lipoprotein cholesterol using a homogeneous enzymatic colorimetric assay, low-density lipoprotein cholesterol using a direct measurement, homogeneous enzymatic colorimetric assay, and triglycerides using a enzymatic colorimetric assay, not glycerol blanked, all measured routinely on a Cobas 8000 platform (Roche, Mannheim, Germany). Plasma sodium (indirect ISE) and potassium (indirect ISE) were measured routinely on a Cobas 8000 platform (Roche, Mannheim, Germany). Urine magnesium was measured on this platform as well, by means of the xylidyl blue colorimetric assay. Plasma and urine creatinine concentrations were determined using an Isotope Dilution Mass Spectrometry traceable enzymatic assay on a Roche Modular P analyzer [17]. Creatinine clearance was calculated using the following formula [18]: Creatinine clearance (ml/min) = $((U_{creatinine} * V)/P_{creatinine}) * (1000/1440)$ in which $U_{creatinine}$ represents urinary creatinine concentration in μmol/L, V represent the 24-hour urinary volume in liters and $P_{creatinine}$ represents plasma creatinine in μmol/L. Estimated glomerular filtration rate (eGFR) was estimated using the Chronic Kidney Disease Epidemiology Collaboration equation (CKD-EPI), based on plasma creatinine [19]. eGFR was categorized into 2 groups: ≥90 ml/min/1.73 m^2 and < 90 ml/min/1.73 m^2. HbA1c concentrations were measured on a Tosoh G8 (HPLC, Sysmex Corporation, Norderstedt, Germany). Hemoglobin, hematocrit, erythrocyte count, leukocytes, and mean corpuscular volume (MCV) were determined using a Sysmex XN20 automated hematology analyzer (Sysmex Corporation).

Data on alcohol consumption and smoking status was obtained from questionnaires. Non-drinkers were defined as not having consumed alcohol during the past month. Individuals were classified into four groups according to their daily alcohol intake: 0 drinks/day (non-drinker), ≤1 drink/day (light drinker), > 1–2 drinks/day (moderate drinker) and > 2 drinks/day (heavy drinker) [20]. Smoking status was defined as self-reported non-smoker, former smoker or current smoker.

2.3. Plasma magnesium assay

The plasma magnesium concentration was assessed using a colorimetric assay (Cobas 8000, Roche, Mannheim, Germany) at the University Medical Center Groningen. The colorimetric assay is based on the complex formation between magnesium ions and xylidyl blue in an alkaline solution (tris(hydroxymethyl)-aminomethane/6-aminocaproic acid buffer), forming a purple diazonium salt. The magnesium concentration is measured photometrically through the decrease in xylidyl blue absorbance. Ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA) is added to mask the calcium ions present in the sample. The intra-assay and inter-assay coefficients of variation are 1.1% and 1.3% at 0.59 mmol/L, 0.8% and 1.1% at 0.80 mmol/L and 0.7% and 0.9% at 1.35 mmol/L, respectively.

2.4. Sample preparation

EDTA whole blood samples were collected between August 2017 until December 2017. One aliquot sample was prepared for iIEM and one for dIEM. Erythrocytes were isolated from plasma according to a strict protocol to obtain washed erythrocytes for dIEM. First, plasma and buffy coat layer were removed after centrifugation of EDTA whole blood samples at 428*g* for 10 min at 4 °C. The erythrocytes were washed using phosphate buffered saline (PBS). Samples were centrifuged (428*g* for 10 min at 4 °C) to separate the PBS from the erythrocytes. Finally, the supernatant PBS was removed after centrifugation. These steps were then performed again, so that each sample was washed twice with PBS. After washing, a part of the packed red blood cells was used for an erythrocyte count. The rest of the packed red blood cells were stored at −80 °C until thawed for testing.

2.5. IEM assay

Magnesium can be accurately quantified using a variety of analytical techniques, including, but not limited to, atomic absorption spectrophotometry, particle-induced x-ray emission and inductively coupled plasma mass spectrometry (ICP-MS) [21]. An advantage of ICP-MS over other analytical techniques, such as atomic absorption spectrophotometry, is the lower limit of the detection [22]. In the present study, a validated ICP-MS method was used to determine magnesium in whole blood (for iIEM) and washed erythrocytes (for dIEM). The measurement of magnesium in serum and whole blood by an ICP-MS method has been previously validated [23]. However, the measurement

of IEM on an ICP-MS has not been validated before. Here, we describe the validation of magnesium in whole blood and washed erythrocytes on an ICP-MS in short that was performed at the University Medical Center Groningen. After mixing, 50 µl sample was diluted to 5 ml using a dilution reagent consisting of 0.1 mg/L Yttrium and 0.05% Triton-X100 and 0.05% EDTA in water. The diluted sample was measured with a Varian 820-ms ICP mass spectrometer. Six calibration standards, ranging from 5 to 50 mg/L were used and 3 quality control samples were measured with each run. The lower limit of quantitation was set to 5 mg/L. All measured samples were above the lower limit of quantitation. There were no matrix effects for whole blood or washed erythrocytes (equal slopes in different matrices) and calibration curve correlations were > 0.999. There were no significant interferences of other elements during the analyses. Accuracy was assessed at 4 concentration levels using Magnesium ICP-MS standard (VWR Chemicals, Radnor, Pennsylvania, US). For each concentration level, standards were measured 18 times over the course of 3 days. Bias was −3.2% at 5 mg/L, 2.4% at 7.5 mg/L, 3.0% at 25 mg/L and 3.4% at 45 mg/L. Within run and between run coefficient of variation were 0.5% and 2.0% for the LLOQ (5 mg/L), 0.6% and 2.7% for the low quality control sample (7.5 mg/L), 0.6% and 1.1% for the medium quality control sample (25.0 mg/L) and 0.4% and 0.8% for the high quality control sample (45 mg/L). iIEM was calculated according to the formula:

iIEM = whole blood magnesium − (plasma magnesium × (1 − hematocrit))/hematocrit [14].

2.6. Statistical analyses

Passing-Bablok regression analysis was used to calculate slopes and intercepts for the relationship between dIEM and iIEM (expressed in mmol/L and mmol/10^{12} cells). Bland-Altman plots were used to visualize and analyze bias between dIEM and iIEM. Data are presented as mean ± SD for normally distributed data and median [interquartile range (IQR)] for non-normally distributed data. Categorical data are presented as percentages. We used frequency distribution histograms and Q-Q plots to assess normality of our data. Data with a non-parametric distribution were $\log_{10}$ transformed. We tested correlations between dIEM, iIEM, plasma magnesium and 24-h urinary magnesium excretion with Pearson bivariate correlation coefficients. Linear regression analysis was performed to investigate determinants of dIEM, iIEM, plasma magnesium and 24-h urinary magnesium excretion. Regression coefficients were given as standardized beta values, referring to the number of standard deviations a dependent variable changes per standard deviation increase of the independent variable, thereby allowing for comparison of the strength of the associations of different variables. Clinical parameters included BMI, waist circumference, glucose concentrations, HbA1c, cholesterol and triglyceride concentrations, blood pressure, renal function (eGFR and creatinine clearance), plasma concentrations of sodium and potassium and hematology parameters (hemoglobin, hematocrit, and MCV). Potential confounders, including age, sex, BMI, eGFR (eGFR < 90 ml/min/1.73 m^2), plasma sodium and potassium and alcohol consumption and smoking status were taken into account. Missing data (present in data on alcohol consumption (30.9%) and smoking status (30.4%)) were handled with multiple imputations [24]. Results are reported for imputed data, except for the baseline characteristics. We evaluated potential effect modification in the associations of determinants with iIEM, dIEM, and plasma and urine magnesium by fitting models containing both main effects and their cross-product terms. Due to the shape of the cells, it is impossible to obtain only packed cells after washing and therefore, we indexed the intracellular magnesium concentrations to erythrocyte count and dIEM and iIEM were expressed as mmol/10^{12} cells. To study the robustness of the associations between determinants and dIEM and iIEM, we performed sensitivity analyses with dIEM and iIEM expressed as mmol per liter packed blood cells (Supplemental Table 3). A two-sided $p < 0.05$ was considered to be statistical significant. Data analyses were performed using SPSS 25.0 for Windows (SPSS Inc., Chicago, IL) and Rstudio version 1.1.383 (Vienna, Austria).

3. Results

3.1. Baseline characteristics

Baseline characteristics are shown in Table 1. Overall, mean age was 51.0 ± 13.4 years, 57.5% were female, 36.0% were non-smoker and 11.9% non-drinker. Mean BMI was 25.8 ± 4.3 kg/m^2 and 32.2% of the participants had an eGFR < 90 ml/min/1.73 m^2. Finally, mean plasma magnesium was 0.85 ± 0.06 mmol/L and mean urinary magnesium excretion was 4.85 ± 1.76 mmol/24-h.

Table 1
Baseline characteristics of the LifeLines study.

Characteristics	Total (n = 1669)
Age, years	51.0 ± 13.4
Females, %	57.5
Anthropometry	
Body mass index, kg/m^2	25.8 ± 4.3
Waist circumference, cm	89.9 ± 12.7
Smoking status[a]	
Non-smoker, %	36.0
Former smoker, %	25.2
Current smoker, %	8.3
Alcohol consumption[b]	
Non-drinker, %	11.9
≤1 drink/day, %	38.3
1–2 drinks/day, %	14.1
≥2 drinks/day, %	4.7
Magnesium parameters	
dIEM, mmol/10^{12} cells	0.20 ± 0.04
iIEM, mmol/10^{12} cells	0.25 ± 0.04
Plasma magnesium, mmol/L	0.85 ± 0.06
Urinary magnesium excretion, mmol/24-h	4.85 ± 1.76
Glucose metabolism	
Glucose, mmol/L	5.00 (4.70–5.40)
HbA1c, mmol/mol	36.0 (34.0–38.0)
Lipids metabolism	
Total cholesterol, mmol/L	5.01 ± 0.98
LDL cholesterol, mmol/L	3.26 ± 0.90
HDL cholesterol, mmol/L	1.49 ± 0.42
Triglycerides, mmol/L	1.07 (0.77–1.51)
Blood pressure	
Systolic, mm Hg	127 ± 17
Diastolic, mm Hg	74 ± 9
Renal parameters	
Plasma creatinine, µmol/L	80.2 ± 16.9
Urinary creatinine excretion, mmol/24-h	13.3 ± 4.0
eGFR < 90 ml/min/1.73 m^2, %	32.2
Creatinine clearance, mL/min	116.5 ± 30.8
Plasma levels	
Sodium, mmol/L	140.9 ± 1.7
Potassium, mmol/L	4.01 ± 0.27
Hematology	
Hemoglobin, mmol/L	8.80 ± 0.79
Hematocrit, v/v	0.43 ± 0.03
MCV, fL	89.0 ± 4.14
Erythrocytes, 10^{12}/L	4.79 ± 0.45
Leukocytes, 10^{9}/L	6.08 ± 1.61

Data are presented as mean ± SD, median with interquartile ranges (IQR) or percentages. Abbreviations: dIEM, direct intra-erythrocyte magnesium; iIEM, indirect intra-erythrocyte magnesium; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c, MCV, mean corpuscular volume.
[a] Available in 1161 subjects.
[b] Available in 1153 subjects.

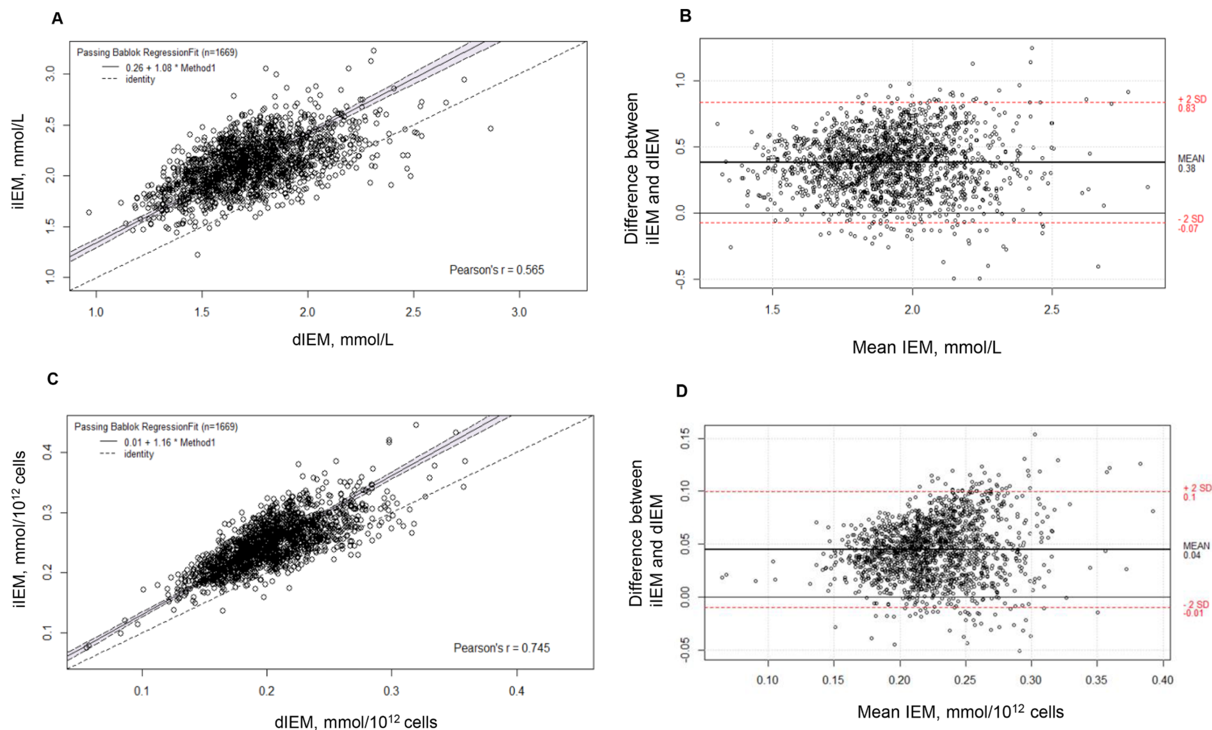

Fig. 1. Passing-Bablok regression analysis for the method comparison and Blant-Altman plots to visualize bias expressed in mmol/L (A, B, respectively) and expressed in mmol/10^{12} cells (C, D, respectively). dIEM; direct intra-erythrocyte magnesium; iIEM; indirect intra-erythrocyte magnesium.

3.2. Method comparison

We compared dIEM and iIEM in 1669 samples from the LifeLines study, both expressed as mmol per liter packed blood cells and mmol per 10^{12} red blood cells. Mean dIEM concentrations were 1.73 ± 0.24 mmol/L and 0.20 ± 0.04 mmol/10^{12} cells and mean iIEM concentrations were 2.11 ± 0.24 mmol/L and 0.25 ± 0.04 mmol/10^{12} cells. When expressed in magnesium per liter packed blood cell, we found a Pearson correlation coefficient of 0.57 between dIEM and iIEM. Ordinary linear regression analysis produced an intercept of 1.092 (1.019; 1.164) and a slope of 0.590 (95% CI: 0.549; 0.631) and Passing-Bablok regression produced an intercept of 0.263 (95% CI: −0.156; 0.359) and a slope of 1.077 (95% CI: 1.019; 1.142) (Fig. 1A). A Bland-Altman plot showed a systematic bias of 0.44 mmol/L with iIEM being slightly higher as compared to dIEM (Fig. 1B). When expressed as magnesium per 10^{12} cells, a higher correlation was observed between dIEM and iIEM (r = 0.75). Ordinary linear regression analysis produced an intercept of 0.078 (0.070; 0.085) and a slope of 0.836 (95% CI: 0.800; 0.872) and Passing-Bablok regression produced an intercept of 0.015 (95% CI: 0.005; 0.023) and a slope of 1.157 (95% CI: 1.109; 1.210) (Fig. 1C). A Bland-Altman plot showed a systematic bias of 0.04 mmol/10^{12} cells with iIEM being slightly higher as compared to dIEM (Fig. 1D).

3.3. Correlations between dIEM, iIEM, plasma magnesium and urinary magnesium excretion

We found a positive correlation between plasma magnesium and dIEM (r = 0.18, P < 0.001) (Fig. 2A). We observed no correlation between 24-h urinary magnesium excretion and dIEM (r = −0.002, P = 0.94) (Supplemental Fig. 2A). Similar correlations were found between plasma magnesium and iIEM (r = 0.14, P < 0.001) (Fig. 2B) and 24-h urinary magnesium excretion and iIEM (r = 0.00, P = 1.00) (Supplemental Fig. 2B). Finally, 24-h urinary magnesium excretion was not correlated with plasma magnesium (r = 0.05, P = 0.07) (Supplemental Fig. 2C).

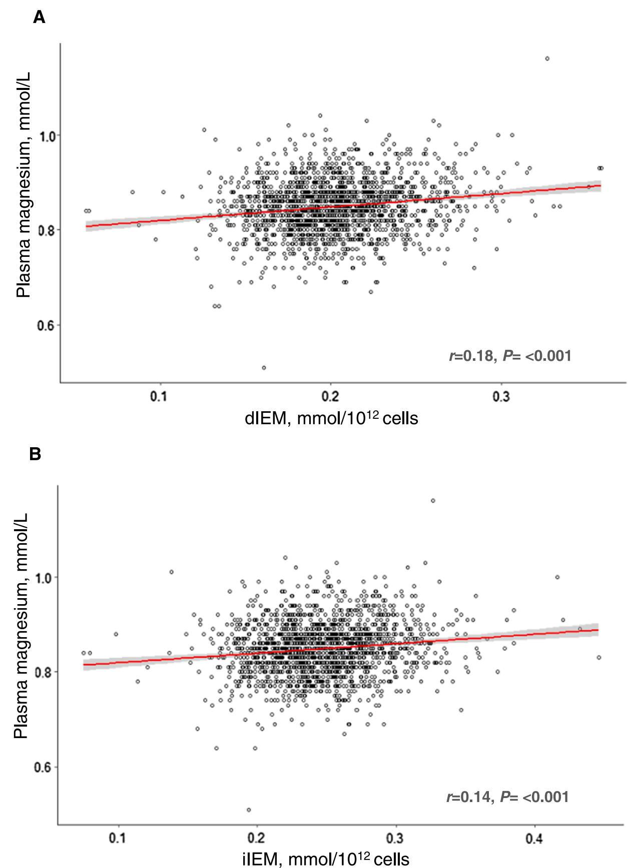

Fig. 2. Scatterplots for the associations between plasma magnesium and dIEM (A), plasma magnesium and iIEM (B). dIEM; direct intra-erythrocyte magnesium, iIEM; indirect intra-erythrocyte magnesium.

Table 2
Univariable and multivariable linear regression analyses with dIEM, iEM and plasma magnesium as dependent variable.

Determinants	dIEM, mmol/10^{12} cells		iIEM, mmol/10^{12} cells		Plasma magnesium, mmol/L	
	Univariable St. β (95% CI)	Multivariable St. β (95% CI)	Univariable St. β (95% CI)	Multivariable St. β (95% CI)	Univariable St. β (95% CI)	Multivariable St. β (95% CI)
Age, years	0.15 (0.10; 0.19)**	0.11 (0.05; 0.17)**	0.13 (0.08; 0.18)**	0.11 (0.05; 0.16)**	0.08 (0.03; 0.13)*	0.03 (−0.03; 0.09)
Sex, female	−0.003 (−0.05; 0.05)	0.05 (−0.004; 0.10)	0.03 (−0.07; 0.13)	0.13 (0.03; 0.23)*	−0.03 (−0.08; 0.02)	−0.02 (−0.07; 0.04)
Anthropometry						
Body mass index, kg/m^2	0.05 (0.01; 0.10)*	0.04 (−0.01; 0.08)	0.09 (0.05; 0.14)**	0.08 (0.03; 0.13)*	−0.05 (−0.10; −0.004)*	−0.07 (−0.12; −0.02)*
Waist circumference, cm	0.06 (0.02; 0.11)*	−0.01 (−0.11; 0.09)	0.11 (0.07; 0.16)**	0.10 (−0.01; 0.20)	−0.002 (−0.05; 0.05)	−0.04 (−0.10; 0.01)
Glucose metabolism						
Log_{10} glucose, mmol/L	0.03 (−0.02; 0.08)	−0.01 (−0.07; 0.05)	0.04 (−0.01; 0.09)	0.003 (−0.05; 0.06)	−0.14 (−0.19; −0.09)**	−0.17 (−0.23; −0.11)**
Log_{10} HbA1c, mmol/mol	−0.001 (−0.05; 0.05)	−0.07 (−0.12; −0.02)*	0.04 (−0.01; 0.08)	−0.02 (−0.07; 0.04)	−0.10 (−0.15; −0.05)**	−0.15 (−0.20; −0.09)**
Lipid metabolism						
Total cholesterol, mmol/L	0.12 (0.07; 0.16)**	0.07 (0.02; 0.12)*	0.12 (0.07; 0.17)**	0.08 (0.03; 0.12)*	0.12 (0.08; 0.17)**	0.11 (0.06; 0.16)**
LDL cholesterol, mmol/L	0.10 (0.06; 0.15)**	0.07 (0.02; 0.12)*	0.09 (0.04; 0.14)**	0.06 (0.01; 0.11)*	0.13 (0.08; 0.18)**	0.12 (0.07; 0.16)**
HDL cholesterol, mmol/L	−0.02 (−0.07; 0.03)	−0.05 (−0.10; 0.01)	−0.03 (−0.08; 0.02)	−0.06 (−0.12; −0.01)*	−0.002 (−0.05; 0.05)	−0.03 (−0.09; 0.03)
Log_{10} triglycerides, mmol/L	0.11 (0.07; 0.16)**	0.10 (0.05; 0.15)**	0.15 (0.10; 0.19)**	0.14 (0.09; 0.19)**	0.05 (0.002; 0.10)*	0.06 (0.01; 0.11)*
Blood pressure						
Systolic, mm Hg	0.04 (−0.004; 0.09)	−0.01 (−0.07; 0.04)	0.07 (0.03; 0.12)*	0.01 (−0.04; 0.07)	0.01 (−0.04; 0.06)	−0.001 (−0.06; 0.06)
Diastolic, mm Hg	0.05 (−0.002; 0.09)	0.02 (−0.03; 0.07)	0.06 (0.01; 0.11)*	0.03 (−0.02; 0.08)	0.06 (0.01; 0.11)*	0.07 (0.01; 0.12)*
Renal function						
eGFR < 90 ml/min/1.73 m^2	0.16 (0.06; 0.26)*	−0.01 (−0.12; 0.11)	0.13 (0.03; 0.23)*	−0.03 (−0.14; 0.09)	0.17 (0.07; 0.28)*	0.13 (0.003; 0.25)*
Creatinine clearance, mL/min	−0.05 (−0.10; 0.002)	−0.003 (−0.06; 0.05)	−0.04 (−0.09; 0.01)	−0.01 (−0.07; 0.05)	−0.08 (−0.13; −0.03)*	−0.05 (−0.11; 0.01)
Plasma levels						
Sodium, mmol/L	0.15 (0.10; 0.20)**	0.13 (0.09; 0.18)**	0.14 (0.10; 0.19)**	0.13 (0.08; 0.18)**	0.14 (0.09; 0.19)**	0.13 (0.08; 0.18)**
Potassium, mmol/L	0.13 (0.08; 0.18)**	0.11 (0.06; 0.16)**	0.07 (0.02; 0.12)*	0.04 (−(−0.01; 0.09)	0.08 (0.03; 0.13)*	0.06 (0.01; 0.11)*
Hematology						
Hemoglobin, mmol/L	0.08 (0.03; 0.13)*	0.11 (0.05; 0.17)*	0.08 (0.03; 0.12)*	0.11 (0.05; 0.18)**	0.11 (0.06; 0.16)**	0.15 (0.09; 0.21)**
Hematocrit, v/v	0.06 (0.01; 0.10)*	0.05 (−0.01; 0.11)	0.04 (−0.01; 0.08)	0.03 (−0.03; 0.09)	0.13 (0.08; 0.18)**	0.17 (0.11; 0.23)**
MCV, fL	0.27 (0.22; 0.32)**	0.24 (0.20; 0.29)**	0.26 (0.22; 0.31)**	0.24 (0.19; 0.28)**	0.01 (−0.04; 0.06)	−0.02 (−0.07; 0.04)

Multivariable models were adjusted for age, sex, BMI, eGFR (< 90 ml/min/1.73 m^2), plasma sodium and plasma potassium, and smoking and alcohol consumption. Abbreviations: dIEM, direct intra-erythrocyte magnesium; iIEM, indirect intra-erythrocyte magnesium; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c, MCV, mean corpuscular volume.

* $p < 0.05$.

** $p < 0.001$

Table 3
Subgroup analyses[1] with dIEM, iEM and plasma magnesium as dependent variable (stratified for age and sex).

Determinants	Subgroup	dIEM, mmol/10^{12} cells		iIEM, mmol/10^{12} cells		Plasma magnesium, mmol/L	
		Univariable St. β (95% CI)	Multivariable St. β (95% CI)	Univariable St. β (95% CI)	Multivariable St. β (95% CI)	Univariable St. β (95% CI)	Multivariable St. β (95% CI)
Age, years	Males	0.06 (−0.02; 0.13)	0.05 (−0.04; 0.14)	0.06 (−0.01; 0.14)	0.07 (−0.02; 0.16)	−0.05 (−0.12; 0.03)	−0.05 (−0.14; −0.05)*
	Females	0.22 (0.16; 0.29)**	0.16 (0.08; 0.24)**	0.19 (0.13; 0.26)**	0.14 (0.07; 0.22)*	0.18 (0.12; 0.25)**	0.11 (0.03; 0.19)*
Sex, female	Age < 55	−0.05 (−0.11; 0.01)	0.01 (−0.05; 0.08)				
	Age ≥ 55	0.08 (0.002; 0.16)*	0.10 (0.01; 0.18)*				
Body mass index, kg/m^2	Males	−0.01 (−0.09; 0.08)	−0.01 (−0.10, 0.07)				
	Females	0.08 (0.02; 0.14)*	0.06 (−0.003; 0.11)				
	Age < 55					−0.03 (−0.08; 0.03)	−0.05 (−0.11; 0.01)
	Age ≥ 55					−0.11 (−0.20; −0.02)*	−0.11 (−0.20; −0.02)*
Waist circumference, cm	Males	0.01 (−0.07; 0.10)	0.02 (−0.15; 0.18)				
	Females	0.11 (0.04; 0.17)*	−0.004 (−0.12; 0.12)				
	Age < 55					0.04 (−0.02; 0.10)	−0.02 (−0.09; 0.04)
	Age ≥ 55					−0.11 (−0.20; −0.02)*	−0.08 (−0.18; 0.01)
Log_{10} glucose, mmol/L	Age < 55					−0.14 (−0.20; −0.07)**	−0.17 (−0.24; −0.10)**
	Age ≥ 55					−0.23 (−0.31; −0.15)**	−0.17 (−0.26; −0.07)*
Log_{10} HbA1c, mmol/mol	Age < 55					−0.06 (−0.12; −0.001)*	−0.09 (−0.15; −0.03)**
	Age ≥ 55					−0.25 (−0.34; −0.17)**	−0.20 (−0.30; −0.11)**
	Males					−0.21 (−0.29; −0.14)**	−0.22 (−0.31; −0.14)**
	Females					−0.003 (−0.07; 0.06)	−0.09 (−0.16; −0.01)*
Log_{10} triglyerides, mmol/L	Age < 55	0.10 (0.04; 0.15)*	0.08 (0.02; 0.14)*			0.08 (0.02; 0.13)*	0.07 (0.01; 0.13)*
	Age ≥ 55	0.10 (0.004; 0.19)*	0.11 (0.02; 0.21)*			−0.04 (−0.14; 0.07)	0.01 (−0.09; 0.12)
Renal function							
eGFR < 90 ml/min/1.73 m^2	Age < 55					0.21 (0.06; 0.37)*	0.18 (0.02; 0.34)*
	Age ≥ 55					0.08 (−0.11; 0.26)	0.17 (−0.03; 0.36)
	Males					−0.05 (−0.21; 0.12)	0.01 (−0.19; 0.21)
	Females					0.33 (0.20; 0.47)**	0.20 (0.05; 0.35)*
Creatinine clearance, mL/min	Males					−0.03 (−0.10; 0.05)	−0.05 (−0.13; 0.03)
	Females					−0.17 (−0.25; −0.09)**	−0.08 (−0.16; 0.04)
Plasma sodium, mmol/L	Age < 55	0.15 (0.09; 0.22)**	0.13 (0.06; 0.19)**				
	Age ≥ 55	0.12 (0.05; 0.20)*	0.12 (0.04; 0.19)*				
Hemoglobin, mmol/L	Age < 55	0.10 (0.05; 0.16)**	0.12 (0.04; 0.20)*	0.11 (0.05; 0.16)**	0.14 (0.07; 0.22)**		
	Age ≥ 55	0.01 (−0.08; 0.10)	0.09 (−0.01; 0.19)	0.00 (−0.09; 0.09)	0.05 (−0.05; 0.16)		
	Males			0.04 (−0.06; 0.13)	0.04 (−0.06; 0.13)		
	Females			0.23 (0.15; 0.31)**	0.17 (0.09; 0.25)**		
Hematocrit, v/v	Age < 55	0.08 (0.02; 0.13)*	0.05 (−0.03; 0.13)				
	Age ≥ 55	−0.01 (−0.09; 0.08)	0.02 (−0.08; 0.12)				
	Males			−0.04 (−0.13; 0.05)	−0.05 (−0.14; 0.04)		
	Females			0.16 (0.08; 0.24)**	0.09 (0.004; 0.17)*		

[1] Associations presented in this table were significantly modified by age and/or sex (all associations: $P_{interaction} < 0.1$). Multivariable models were adjusted for age, sex, BMI, eGFR (< 90 ml/min/1.73 m^2), plasma sodium and plasma potassium, and smoking and alcohol consumption. Abbreviations: dIEM, direct intra-erythrocyte magnesium; iIEM, indirect intra-erythrocyte magnesium; eGFR, estimated glomerular filtration rate; HbA1c, hemoglobin A1c, MCV, mean corpuscular volume.

* $p < 0.05$.

** $p < 0.001$.

3.4. Determinants of dIEM and iIEM

Determinants of dIEM and iIEM are presented in Table 2. HbA1c was inversely associated with dIEM after adjustment for potential confounders. Among lipids, we found that higher total- and LDL cholesterol and triglyceride concentrations were associated with higher dIEM, even after adjustment for potential confounders. Also, higher plasma concentrations of sodium and potassium were associated with higher dIEM. iIEM was positively associated with BMI, waist circumference, total- and LDL cholesterol, triglycerides, plasma sodium and potassium. Subgroup analyses are presented in Table 3. Higher age was associated with higher iIEM concentrations in females. Similar associations were found between the determinants and iIEM and dIEM expressed as mmol per liter packed blood cells (Supplemental Table 3).

3.5. Determinants of plasma magnesium

Determinants of plasma magnesium are shown in Table 2. BMI, as well as glucose and HbA1c were inversely associated with plasma magnesium. Higher total- and LDL- cholesterol and triglyceride concentrations, as well as higher diastolic blood pressure were associated with higher plasma magnesium concentrations. Finally, eGFR < 90 ml/min/1.73 m^2 was associated with higher plasma magnesium concentrations. Subgroup analyses are presented in Table 3. Higher age was associated with higher plasma magnesium concentrations in females. In males, however, this association was found to be negative. The association of BMI with plasma magnesium was only present in older subjects (age ≥ 55 years). Furthermore, the association of HbA1c and plasma magnesium was stronger in males and older subjects (age ≥ 55 years). Subgroup analysis revealed that the positive association of triglycerides with plasma magnesium was only present in younger subjects (age < 55 years).

3.6. Determinants of 24-h urinary magnesium excretion

Determinants of 24-h urinary magnesium excretion are presented in Supplemental Table 1. Females showed lower 24-h urinary magnesium excretion compared to males. HDL cholesterol, and plasma potassium were positively associated with 24-h urinary magnesium excretion. eGFR of < 90 ml/min/1.73 m^2 was negatively associated with 24-h urinary magnesium excretion. Subgroup analyses are shown in Supplemental Table 2. BMI and waist circumference were inversely associated with 24-h urinary magnesium excretion only in older subjects (age ≥ 55 years). In males, higher LDL cholesterol was associated with 24-h urinary magnesium excretion. The positive association of plasma potassium with 24-h urinary magnesium excretion was only present in younger subjects (age < 55 years).

4. Discussion

Circulating magnesium (either plasma or serum) is still the most commonly used laboratory test for the assessment of magnesium status, despite only a small fraction of total body magnesium can be found in the circulation. Previous studies have shown that plasma magnesium concentrations correlate poorly with other magnesium tissue pools [2,8]. In fact, the body can suffer from severe magnesium deficiency while plasma magnesium concentrations are within the normal range [25]. Therefore, we believe that there is a need for a simple and rapid technique to measure intracellular magnesium concentrations. To the best of our knowledge, this is the first study to perform a method comparison between dIEM and iIEM in a large group of healthy participants. The cardinal finding of this study is a strong correlation between dIEM and iIEM, with only a small degree of systematic and proportional bias. These findings indicate that iIEM can be used as a cheaper, less labor intensive alternative for dIEM. In addition, we found that dIEM and iIEM correlated with plasma magnesium, but not with 24-h urinary magnesium excretion. Higher cholesterol and triglyceride concentrations were associated with higher dIEM, iIEM and plasma magnesium, while higher glucose and HbA1c were associated with lower plasma magnesium concentrations.

dIEM requires extensive sample preparation, making it labor-intensive which is accompanied by additional costs, and is therefore not suitable for routine laboratory measurements. Issues regarding labor-intensity and high costs of the dIEM were also reported by Deuster et al. [14]. Particularly, obtaining erythrocytes by means of a washing procedure is time consuming and involves extra costs. In our case, it required two technicians for three hours to prepare fifty samples for dIEM, whereas for iIEM, the same amount of samples could be prepared by one technician in approximately twenty minutes, which makes the sample preparation of dIEM eighteen times more labor-intensive. Overall, personnel costs were four times higher for dIEM, whereas costs for material and additional measurements were comparable between the methods. Altogether, we estimate the total costs of dIEM to be almost twice as high as the total costs of iIEM (US$6.95 per sample vs. US $3.54 per sample, respectively). Thus, iIEM is a simple, rapid, and less expensive technique, which only requires measurement of hematocrit and magnesium in plasma and whole blood, and can therefore be embedded in current routine of clinical measurements. So far, only one study compared dIEM and iIEM [14]. However, they included only a small group of men and women ($n = 10$). The systematic bias that we observed can be attributable to the washing process. Removing all the supernatant PBS from the samples after centrifugation is difficult and requires precision. However, despite the accuracy of the personnel, some PBS could have remained in the samples, which explains the systematic bias and subsequently, the lower concentrations measured by the direct method. The fact that the systematic bias is much lower after adjusting for erythrocyte count supports this hypothesis. The advantages of the indirect method, including lower costs and lower labor intensity, outweigh the relative small bias that was observed.

In a randomized controlled trial, the effect of magnesium supplementation on IEM concentrations was studied. They showed that after a magnesium supplementation period of 3 weeks in women with low IEM concentrations (< 1.97 mmol/L), plasma magnesium and 24-h urinary magnesium excretion were significantly increased, but no significant increase was found in IEM concentrations [26]. The authors concluded that low IEM concentrations may not reflect systemic magnesium deficiency, but rather the activity of metabolic determinants, such as the Na^{+}/Mg^{2+} exchanger [27]. However, the supplementation period of 3 weeks might have been too short to detect differences in erythrocytes concentrations of magnesium. In the present study, dIEM, iIEM and plasma magnesium were not correlated with 24-h urinary magnesium excretion, as estimate of dietary absorption, suggesting that dietary magnesium intake is not directly reflected in the circulation and in cells, at least not in erythrocytes.

Evidence regarding involvement of magnesium in glucose and insulin metabolism is accumulating [7]. Previous studies showed that lower circulating magnesium concentrations were associated with increased risk of T2DM in the general population [4,28]. Furthermore, long-term magnesium supplementation improved insulin sensitivity in T2DM patients [29], suggesting that the previously found associations are causal in nature. Magnesium concentrations in plasma, as well as concentrations in erythrocytes and platelets, were lower in diabetic patients and were even more reduced in diabetic patients with hypertension [30,31]. We therefore hypothesized that glucose and HbA1c are inversely associated with IEM and plasma magnesium concentrations because of its potential to stimulate cellular glucose utilization. In the present study, higher glucose and HbA1c were independently associated with lower plasma magnesium concentrations. This is in line with previous studies in diabetic patients [32,33] and also with our recent findings in the general population [4,34]. Although glucose and HbA1c were inversely associated with plasma magnesium, we have not observed such associations with IEM. Our findings may indicate that

plasma magnesium, rather than IEM, is involved in glucose metabolism. However, future studies, preferable in patients with T2DM who are at risk of hypomagnesaemia, should further elucidate whether intracellular concentrations of magnesium are associated with glucose and insulin metabolism. The observed positive associations of cholesterol and triglycerides with IEM and plasma magnesium remain difficult to explain. Recently, Waanders et al. studied determinants of hypomagnesemia in diabetic patients and found that higher LDL cholesterol concentrations, but not triglycerides, were associated with higher plasma magnesium concentrations [33]. Furthermore, serum magnesium was also positively associated with lipoproteins in a large cohort study including healthy individuals [35]. The authors speculated that the positive association might be explained by a binding interaction between serum magnesium and lipoprotein particles. An eGFR below 90 ml/min/1.73 m^2 was associated with increased plasma magnesium concentrations as well as with decreased 24-h urinary magnesium excretion. Indeed, it is known that in patients with end stage renal disease, plasma magnesium concentration may slightly increase as a consequence of a reduced glomerular filtration rate [36]. In the present study, eGFR was not associated with IEM. Finally, IEM was positively associated with age in current study and this association remained significant after adjustment for potential confounders. Interestingly, a previous study found the opposite; intra-erythrocyte magnesium was inversely associated with age [37]. We found no association between age and plasma magnesium.

It should be noted that this study found several differential associations of dIEM and iIEM with their determinants. Most notably, the association with female sex was stronger with iIEM than with dIEM. Similarly, iIEM also associated more strongly with BMI. These findings implicate that, compared to dIEM, iIEM may lead to higher values in females and in obese participants. On the other hand, the association with plasma potassium was stronger with dIEM. Thus, dIEM may yield higher values in participants with higher plasma potassium concentrations. A few limitations should be addressed. The main limitation is that we were unable to demonstrate cause-effect relationships between magnesium concentrations and clinical features. However, low circulating magnesium concentrations and low urinary magnesium excretions at baseline have been previously associated with increased risk of hypertension [38], and ischemic heart disease [39]. Second, previous studies suggested that magnesium is involved in both glucose and insulin metabolism. Unfortunately, insulin was not measured in our cohort and therefore, we are unable to draw conclusions regarding associations between insulin and magnesium. In addition, no data on dietary magnesium intake was available. Instead, however, we used 24-h urinary magnesium excretion as a measure of intestinal absorption [40]. Finally, our cohort consists predominantly of Caucasians (98.7%), which limits external validity of our results.

This study has several strengths. This is the first study that compared two methods for the assessment of IEM in a large population-based cohort study. The measurement of intracellular magnesium has become more popular nowadays and therefore, there is a need for a simple and rapid technique to measure intracellular concentrations without labor-intensive sample preparations. In addition, we used a large cohort study including extensive clinical and biochemical characterization of our subjects to explore associations of clinical parameters with IEM, plasma magnesium and 24-h urinary magnesium excretion.

5. Conclusions

In conclusion, we found a strong correlation between a direct and an indirect method for the assessment of IEM. Our study indicates that iIEM can be used in practice, eliminating labor-intensive washing procedures and high costs required for direct methods. Furthermore, the advantages of iIEM, including lower costs and lower labor intensity, outweigh the relative small bias that was found. Associations between clinical parameters and IEM need to be further explored in well-designed randomized clinical trials and large cohort studies, preferable in study populations that are prone to hypomagnesemia and in which IEM concentrations might be depleted, such as T2DM patients or kidney transplant recipients.

CRediT authorship contribution statement

Joëlle C. Schutten: Investigation, Writing - original draft, Formal analysis. **Adrian Post:** Methodology, Investigation, Writing - review & editing. **Margriet van der Meer:** Investigation. **Jan IJmker:** Investigation. **Frans Goorman:** Writing - review & editing. **Richard M. Danel:** Writing - review & editing. **Marc G. Vervloet:** Writing - review & editing. **Martin H. de Borst:** Conceptualization, Writing - review & editing. **Daan J. Touw:** Resources, Writing - review & editing. **Stephan J.L. Bakker:** Conceptualization, Writing - review & editing, Supervision.

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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Appendix A. Supplementary material

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