

Blood Free Carnitine Level is an Independent Determinant of Erythrocyte Membrane Homogeneity and Osmotic Resistance in Maintenance Hemodialysis Patients

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**Blood Free Carnitine Level is an Independent Determinant of
Erythrocyte Membrane Homogeneity and Osmotic Resistance in
Maintenance Hemodialysis Patients**

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Abstract

18 **Background**

19 Carnitine deficiency is highly prevalent in chronic maintenance hemodialysis (HD)
20 patients and is clinically implicated in erythropoietin resistance and impaired cell
21 survival. However, whether carnitine status directly governs red blood cell (RBC)
22 membrane properties independently of major clinical confounders remains
23 unproven. We aimed to evaluate systemic carnitine profiles, RBC osmotic fragility,
24 and physical cell fragmentation to clarify their independent relationships.

25 **Methods**

26 In this prospective observational study of 29 maintenance HD patients, blood
27 carnitine fractions (free carnitine [FC], total carnitine, acylcarnitine) were
28 quantitatively measured. RBC osmotic fragility was evaluated using a 96-well
29 microplate-based Parpart assay, and individual hemolysis curves were fitted to a
30 two-parameter sigmoidal regression model to extract curve slope (α , reflecting
31 membrane homogeneity) and 95% complete hemolysis concentration (x_{comp}).
32 Automated fragmented RBCs (FRC%) were simultaneously quantified.
33 Multivariable linear regression models adjusted for age, sex, HD duration, C-
34 reactive protein (CRP), and type 2 diabetes mellitus (T2DM).

35 **Results**

36 Systemic carnitine depletion was widespread, with 89.6% of patients meeting the
37 criteria for absolute (24.1%, FC < 20.0 $\mu\text{mol/L}$) or probable (65.5%, FC 20.0–36.0
38 $\mu\text{mol/L}$ with acylcarnitine/FC ratio ≥ 0.4) carnitine deficiency. Multivariable linear
39 regression analyses demonstrated that higher FC levels were independently and
40 significantly associated with an increased OFT slope α (Model 1A [adjusted for
41 age, sex, HD duration, CRP]: $\beta = 0.6090$, $P = 0.036$; Model 1B [Model 1A +
42 T2DM]: $\beta = 0.5970$, $P = 0.045$) and a higher x_{comp} (Model 2A: $\beta = 0.0009$, $P =$
43 0.042; Model 2B: $\beta = 0.0009$, $P = 0.041$), irrespective of clinical confounders.
44 FRC% exhibited a significant positive correlation with slope α ($\rho = 0.398$, $P =$
45 0.032), reflecting dynamic intravascular selective destruction of fragile cells. In
46 ESA-treated patients ($n = 18$), x_{comp} correlated with the ESA resistance index (ρ
47 = 0.474, $P = 0.047$) and maintained a consistent positive linear relationship in
48 adjusted models ($\beta = 0.327$ – 0.356).

49 **Conclusions**

50 Blood FC sufficiency is an independent determinant of RBC membrane
51 homogeneity and complete hemolysis resistance in chronic maintenance HD
52 patients. Monitoring and optimizing systemic carnitine status represents a

promising adjunctive therapeutic strategy to preserve erythrocyte quality and enhance renal anemia management.

Keywords: Carnitine deficiency, Erythrocyte osmotic fragility, Hemodialysis, Red blood cell membrane, Fragmented red blood cells, Multivariable linear regression

Background

Systemic carnitine deficiency is highly prevalent in chronic hemodialysis (HD) patients due to reduced endogenous synthesis, restricted dietary intake, and continuous dialytic clearance of free carnitine (FC) through high-flux dialyzers ¹⁻³. In routine clinical nephrology practice, blood carnitine status is not included in monthly laboratory evaluations ⁴⁻⁶, and carnitine supplementation is typically reserved for patients exhibiting overt clinical symptoms, such as intradialytic hypotension, severe muscle cramps, or refractory ESA resistance ^{1-3,5,7,8}. Consequently, a substantial proportion of hemodialysis patients is thought to remain in an undiagnosed and untreated state of severe carnitine deficiency ^{1,2}. Although carnitine plays a pivotal role in acyl-CoA transport and membrane

phospholipid fatty acid turnover, which is essential for maintaining erythrocyte membrane viscoelasticity and structural resilience ^{1,3,9–11}, clinical evidence directly linking this widespread, unappreciated carnitine depletion to erythrocyte fragility in modern hemodialysis patients remains remarkably scarce ^{1,12}.

Unaddressed carnitine deficiency and the resulting erythrocyte membrane fragility pose pathophysiological challenges in end-stage renal disease, contributing to shortened red blood lifespan, blunted erythropoiesis-stimulating agent (ESA) responsiveness, and persistent renal anemia ^{1,3,5}. Although L-carnitine supplementation has been suggested to reduce ESA requirements ^{1,3}, direct clinical evidence linking blood carnitine fractions with quantitative erythrocyte osmotic fragility curve parameters and automated fragmented red blood cells (FRC) in modern maintenance HD cohorts remains scarce ¹².

Therefore, we hypothesized that unappreciated carnitine deficiency directly contributes to increased erythrocyte membrane fragility in chronic maintenance HD patients. To test this, we conducted a prospective observational study of 29 patients, evaluating erythrocyte osmotic fragility via the Parpart assay and automated fragmented red blood cell (FRC) quantification. Our primary objective was to establish the comprehensive relationship between blood carnitine profiles

(FC, total carnitine [TC], acylcarnitine [AC], and the AC/FC ratio) and red cell fragility markers, thereby clarifying the clinical relevance of carnitine status to erythrocyte quality in hemodialysis care. Additionally, the prevalence of carnitine deficiency was classified according to the 2018 Japanese guidelines.

Methods

Study Population and Patient Selection

The study population consisted of end-stage renal disease patients undergoing maintenance HD at Seseragi Hospital (Isesaki, Gunma, Japan). Enrollment was conducted in August 2026. The inclusion criteria were: (1) age between 20 and 80 years at the time of providing written informed consent; (2) receiving stable maintenance HD (three times weekly, 4 hours per session) for at least 6 months at Seseragi Hospital; and (3) voluntary written informed consent. Exclusion criteria were active infection, malignant tumor, severe clinical complications, history of blood transfusion within 3 months, administration of L-carnitine formulations within 3 months, or significant adjustments in ESA (exceeding 10 µg) or iron dosage within 3 months. Based on a previous domestic study evaluating blood carnitine levels and erythrocyte osmotic fragility (n = 26)

105 ¹², the target sample size was set at 30 cases to ensure adequate statistical power.

106 Among 30 initially enrolled hemodialysis patients, one was excluded due to
107 delayed sample transport (>24 hours). The remaining 29 patients (23 males, 6
108 females) completed the protocol and were analyzed.

109 **Ethical Considerations**

110 This study was conducted in strict accordance with the Declaration of
111 Helsinki and the "Ethical Guidelines for Medical and Health Research Involving
112 Human Subjects" in Japan. The study protocol was approved by the Ethics
113 Committee of Juntendo University School of Medicine (Approval No. E26-0103;
114 Date of Approval: June 3, 2026) and the Ethics Committee of Seseragi Hospital
115 (Approval No. RI2026-8; Date of Approval: July 27, 2026). All participating
116 patients, or their legally authorized representatives if the patient had cognitive
117 limitations, provided voluntary written informed consent prior to enrollment.

118 **Blood Sample Collection and Laboratory Assays**

119 Prior to the HD session, 10 mL of blood was collected and aliquoted into
120 heparinized tubes (2 mL for OFT), EDTA-2K tubes (2 mL for complete blood count,
121 FRC, and reticulocytes), and serum separator tubes for carnitine fraction analysis.

122 Blood samples were refrigerated at 4°C and transported to a commercial
123 laboratory (Sanritsu Co., Ltd., Chiba, Japan). Carnitine profiles (FC, TC, and AC)
124 and the AC/FC ratio were determined using an enzymatic cycling method.
125 Complete blood counts, reticulocytes, and fragmented red blood cells (FRC%
126 and FRC#) were measured using an automated XN-9000 analyzer (Sysmex Co.,
127 Kobe, Japan).

128 Heparinized blood samples for OFT were stored at 4°C and hand-delivered
129 under continuous refrigeration to our laboratory at Juntendo University (Chiba,
130 Japan). All OFT measurements were performed within 24 hours of collection to
131 prevent *ex vivo* artifacts, after which the samples were discarded.

132 **Erythrocyte Osmotic Fragility Test and Data Analysis**

133 Erythrocyte osmotic fragility was evaluated using a modified microplate
134 Parpart method ¹³ with the Parpart Test Kit (Muto Pure Chemicals Co., Ltd., Tokyo,
135 Japan). Briefly, 5 µL of room-temperature whole blood was mixed in duplicate
136 with 150 µL of PBS across 12 NaCl concentrations (0.85%, 0.75%, 0.70%, 0.65%,
137 0.60%, 0.55%, 0.50%, 0.45%, 0.40%, 0.35%, 0.30%, and 0.10%). After a 60-min
138 incubation at room temperature and centrifugation (480 × *g*, 8 min), 70 µL of
139 supernatant was transferred to a flat-bottom plate to measure absorbance at 540

140 nm using a microplate reader.

141 The hemolysis percentage (Hemolysis %) at each NaCl concentration was
142 calculated using:

143
$$\text{Hemolysis (\%)} = \frac{A_{\text{sample}} - A_{0.85\%}}{A_{0.10\%} - A_{0.85\%}} \times 100$$

144 where A is the absorbance at 540 nm (0.85% NaCl: 0% hemolysis; 0.10% NaCl:
145 100% hemolysis).

146 To assess osmotic fragility, hemolysis curves were fitted to a four-parameter
147 sigmoid model using non-linear least squares in R:

148
$$\text{Hemolysis (\%)} = \frac{100}{1 + \exp(a \times (\text{NaCl} - b))}$$

149 Two primary parameters were derived: the curve slope (a), indicating
150 erythrocyte population homogeneity, and the mean corpuscular fragility (MCF, b),
151 defined as the NaCl concentration causing 50% hemolysis. Only fits with standard
152 convergence and $\text{pseudo-}R^2 \geq 0.95$ were included. Threshold concentrations for
153 lysis initiation (5% hemolysis; x_{start}) and completion (95% hemolysis; x_{comp}) were
154 calculated as $x_{\text{start}} = b + \frac{\ln(19)}{a}$ and $x_{\text{comp}} = b - \frac{\ln(19)}{a}$, respectively.

155 Non-linear regression was performed using EZR (version 1.70; Saitama
156 Medical Center, Jichi Medical University) ¹⁴.

Statistical Analysis

Continuous variables are presented as mean $\pm$ standard deviation (SD) or median with interquartile range (IQR), depending on the normality verified by the Shapiro-Wilk test. Group comparisons between sexes were evaluated using Student's t-test, Welch's t-test, or Mann-Whitney U-test for continuous variables, and Fisher's exact test for categorical variables. Correlations were analyzed using Spearman's rank correlation coefficient (ρ). To test the independent contribution of FC to erythrocyte membrane parameters (OFT slope α and complete hemolysis concentration x_{comp}), multivariable linear regression models were performed adjusting for age, sex, HD duration, C-reactive protein (CRP), and type 2 diabetes mellitus (T2DM). Statistical significance (two-tailed) was set at $P < 0.05$. All statistical analyses were conducted using SPSS version 29.0 (Japan IBM, Tokyo, Japan).

Results

Baseline Demographic and Clinical Characteristics

The baseline demographic and clinical characteristics of the 29 analyzed patients are summarized in **Table 1**. The study cohort comprised 23 males and 6

females, with a mean age of 61.3 ± 13.2 years and a median HD duration of 5.3 years. Major comorbidities included hypertension (93.1%), T2DM (72.4%), secondary hyperparathyroidism (69.0%), and dyslipidemia (41.4%). All patients were clinically stable with no active infections (mean CRP: 0.268 ± 0.352 mg/dL). Anemia therapies included ESA (62.1%; mean darbepoetin dose: 16.3 ± 11.3 µg/week) and hypoxia-inducible factor prolyl-hydroxylase inhibitor (HIF-PHI) (20.7%).

Table 1. Baseline Demographic and Clinical Characteristics of Hemodialysis Patients by Sex

Parameter	Unit	All (n = 29)	Male (n = 23)	Female (n = 6)	P-value
Demographics & Clinical Background					
Age	years	61.3 ± 13.2	62.2 ± 12.7	57.8 ± 15.7	0.482 ^a
HD vintage	years	6.80 ± 5.66	7.12 ± 5.98	5.58 ± 4.50	0.609 ^b
Height	cm	167.2 ± 9.1	169.9 ± 8.1	157.0 ± 3.3	< 0.001 ^{a **}
Dry Weight (DW)	kg	68.0 ± 15.3	70.2 ± 15.9	59.5 ± 9.3	0.128 ^a
Body Mass Index (BMI)	kg/m ²	24.2 ± 4.1	24.2 ± 4.1	24.3 ± 4.4	0.962 ^a
Systolic Blood Pressure	mmHg	161.4 ± 22.6	161.4 ± 20.8	161.3 ± 31.0	0.996 ^a
Diastolic Blood Pressure	mmHg	81.3 ± 12.6	80.8 ± 11.7	83.2 ± 16.7	0.688 ^a
C-Reactive Protein (CRP)	mg/dL	0.268 ± 0.352	0.280 ± 0.383	0.226 ± 0.214	0.854 ^b
Comorbidities (Under Treatment)					
Hypertension (HT)	n (%)	27 (93.1%)	22 (95.7%)	5 (83.3%)	0.377 ^c
Type 2 Diabetes Mellitus (T2DM)	n (%)	21 (72.4%)	20 (87.0%)	1 (16.7%)	0.003 ^{c **}
Secondary Hyperparathyroidism	n (%)	20 (69.0%)	16 (69.6%)	4 (66.7%)	1.000 ^c
Dyslipidemia (DL)	n (%)	12 (41.4%)	10 (43.5%)	2 (33.3%)	1.000 ^c
Anemia Therapies					
ESA Therapy	n (%)	18 (62.1%)	13 (56.5%)	5 (83.3%)	0.377 ^c
HIF-PHI Therapy	n (%)	6 (20.7%)	6 (26.1%)	0 (0.0%)	0.301 ^c
Free (No Erythropoietic Agent)	n (%)	5 (17.2%)	4 (17.4%)	1 (16.7%)	1.000 ^c
Darbepoetin alfa dose	µg/week	16.3 ± 11.3	17.3 ± 13.1	13.8 ± 2.5	0.613 ^b

Data are presented as Mean $\pm$ SD or n (%). **P < 0.01.

Abbreviations: DW, dry weight; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CRP, C-reactive protein; HT, hypertension; T2DM, type 2 diabetes

mellitus; ESA, erythropoiesis-stimulating agent; HIF-PHI, hypoxia-inducible factor–prolyl hydroxylase inhibitor. Darbepoetin alfa dose was evaluated in patients receiving darbepoetin alfa (n = 15; male n = 11, female n = 4).

^a Analyzed by Student's t-test. ^b Analyzed by Mann-Whitney U-test. ^c Analyzed by Fisher's exact test. ** P < 0.01.

Patient Characteristics and Prevalence of Carnitine Deficiency

Laboratory characteristics, carnitine profiles, and erythrocyte properties are presented in **Table 2**. Mean hemoglobin was 10.8 ± 0.9 g/dL. OFT parameters demonstrated a mean curve slope *a* of 52.1 ± 17.9 and MCF (*b*) of $0.380 \pm 0.018\%$. Male patients exhibited significantly higher fragmented red cells (FRC%: $0.48 \pm 0.57\%$ vs $0.03 \pm 0.04\%$, P = 0.005) than female patients.

Table 2. Clinical and Laboratory Characteristics of Hemodialysis Patients by Sex

Parameter	unit	All (n = 29)	Male (n = 23)	Female (n = 6)	P-value		
Carnitine Profile							
Free carnitine	μmol/L	25.5 ± 12.1	24.0 ± 8.2	31.4 ± 21.6	0.694 ^c		
Total carnitine	μmol/L	40.2 ± 20.9	37.6 ± 14.1	50.4 ± 37.7	0.694 ^c		
Acylcarnitine	μmol/L	14.7 ± 9.1	13.6 ± 6.3	19.1 ± 16.3	0.581 ^c		
AC/FC ratio		0.561 ± 0.131	0.556 ± 0.130	0.582 ± 0.142	0.677 ^a		
Erythrocyte Destruction							
Fragmented red blood cells (FRC)	%	0.39 ± 0.54	0.48 ± 0.57	0.03 ± 0.04	0.005 ^c	**	
FRC count (FRC#)	10 ⁴ /μL	1.24 ± 1.68	1.54 ± 1.78	0.09 ± 0.15	0.005 ^c	**	
Hematological Parameters							
Hemoglobin	g/dL	10.8 ± 0.9	10.8 ± 1.0	10.7 ± 0.6	0.679 ^a		
Hematocrit	%	32.7 ± 2.7	32.8 ± 3.0	32.4 ± 1.2	0.733 ^c		
Reticulocyte	‰	17.5 ± 5.1	17.9 ± 5.3	16.2 ± 4.5	0.896 ^c		
Red blood cell (RBC) count	10 ⁴ /μL	340.7 ± 36.3	342.1 ± 39.7	335.3 ± 20.1	0.691 ^a		
MCV	fL	96.3 ± 5.3	96.3 ± 5.7	96.5 ± 3.6	0.655 ^c		
MCH	pg	31.8 ± 1.8	31.8 ± 1.9	31.8 ± 0.8	0.445 ^c		
MCHC	%	33.0 ± 0.9	33.0 ± 0.8	32.9 ± 1.4	0.806 ^b		
Osmotic Fragility Test (OFT)							
Curve slope (a)		52.1 ± 17.9	53.4 ± 18.8	47.5 ± 14.5	0.484 ^a		
Mean corpuscular fragility (MCF)	%	0.380 ± 0.018	0.383 ± 0.013	0.372 ± 0.030	0.179 ^a		
5% Hemolysis NaCl concentration	%	0.444 ± 0.029	0.445 ± 0.029	0.438 ± 0.030	0.597 ^a		
95% Hemolysis NaCl concentration	%	0.317 ± 0.028	0.320 ± 0.024	0.305 ± 0.039	0.242 ^a		

Data are presented as Mean ± SD. AC/FC, acylcarnitine-to-free carnitine ratio; MCV, mean corpuscular volume; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration. ^a Analyzed by Student's t-test. ^b Analyzed by Welch's t-test. ^c Analyzed by Mann-Whitney U-test. ** P < 0.01.

Based on the 2018 Japanese guidelines ⁸, patients were stratified by carnitine status (**Table 3**): 7 (24.1%) had absolute deficiency (FC < 20.0 μmol/L) and 19 (65.5%) had highly probable deficiency (FC 20.0–36.0 μmol/L with AC/FC ≥ 0.4). Only one patient (3.4%) fell within the normal range, while 2 (6.9%) exhibited sufficient FC (≥ 36.0 μmol/L) with an elevated AC/FC ratio.

215 **Table 3. Classification of Carnitine Status in Hemodialysis Patients Based**
 216 **on the 2018 Guidelines (N = 29)**

Carnitine Status Category	Diagnostic Criteria	Patients, n (%)
Absolute carnitine deficiency	FC < 20.0 µmol/L	7 (24.1%)
Highly probable carnitine deficiency (Relative/Metabolic deficiency)	20.0 ≤ FC < 36.0 µmol/L and AC/FC ratio ≥ 0.4	19 (65.5%)
Normal carnitine range (Non-deficient state)	20.0 ≤ FC < 36.0 µmol/L and AC/FC ratio < 0.4	1 (3.4%)
Sufficient free carnitine with abnormal AC/FC	FC ≥ 36.0 µmol/L and AC/FC ratio ≥ 0.4	2 (6.9%)

217 Note: Classification was performed according to the Guidelines for Diagnosis and Treatment of
 218 Carnitine Deficiency 2018. FC, free carnitine; AC/FC, acylcarnitine-to-free carnitine ratio.

219

220 In exploratory analyses (**Supplementary Table S1**), diastolic blood
 221 pressure negatively correlated with FC ($\rho = -0.446$, $P = 0.015$) and TC ($\rho = -0.410$,
 222 $P = 0.027$), whereas HD duration positively correlated with FC ($\rho = 0.372$, $P =$
 223 0.047). CRP positively correlated with ESA dose ($\rho = 0.438$, $P = 0.017$) and ERI
 224 ($\rho = 0.446$, $P = 0.015$). Higher ESA dose and ERI were significantly associated
 225 with reduced osmotic resistance, specifically 95% complete hemolysis
 226 concentration (ESA dose: $\rho = 0.540$, $P = 0.021$; ERI: $\rho = 0.474$, $P = 0.047$) and
 227 MCF (ESA dose: $\rho = 0.547$, $P = 0.019$). Patient age negatively correlated with dry
 228 weight, BMI, MCF, and 95% complete hemolysis concentration ($P < 0.05$ for all).

Association Between Carnitine Status and Erythrocyte Membrane Properties

Spearman's rank correlation analyses (N = 29) are summarized in **Table 4**, **Figure 1**, and **Supplementary Table S2**. Blood FC level positively correlated with OFT curve slope α ($\rho = 0.387$, $P = 0.038$; Fig. 1A) and 95% complete hemolysis concentration x_{comp} ($\rho = 0.485$, $P = 0.008$; Fig. 1B). Additionally, TC and AC positively correlated with MCHC ($\rho = 0.391$, $P = 0.036$ and $\rho = 0.430$, $P = 0.020$, respectively), with no other significant carnitine correlations (all $P > 0.05$). FRC% and absolute FRC count positively correlated with OFT slope α (FRC%: $\rho = 0.398$, $P = 0.032$; FRC#: $\rho = 0.379$, $P = 0.043$; Fig. 1C). Standard hematological correlations, such as RBC count versus MCV ($\rho = -0.628$, $P < 0.001$) and MCV versus MCH ($\rho = 0.791$, $P < 0.001$), were also confirmed.

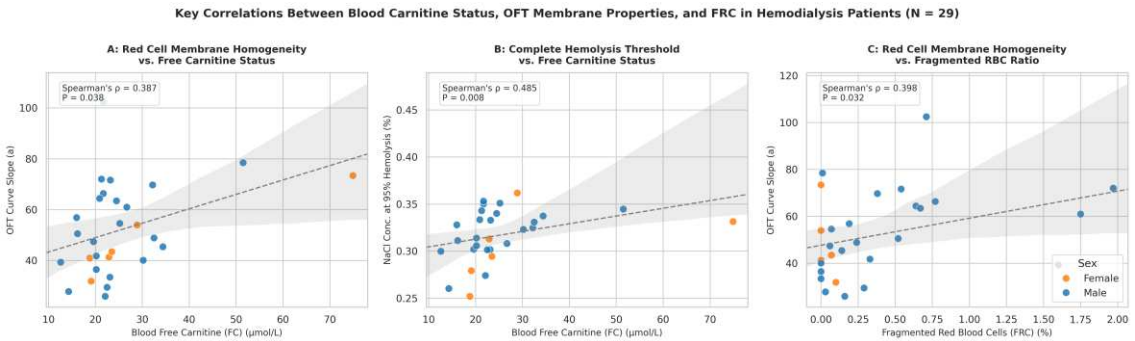


Figure 1. Key Correlations Between Blood Carnitine Status, OFT Membrane Properties, and FRC in Hemodialysis Patients (N = 29). (A) OFT curve slope α versus blood free carnitine (FC) levels ($\rho = 0.387$, $P = 0.038$). (B) NaCl concentration at 95% complete hemolysis versus blood FC levels ($\rho = 0.485$, $P =$

0.008). (C) OFT curve slope α versus fragmented red blood cell percentage (FRC%) ($\rho = 0.398$, $P = 0.032$). The dashed lines indicate the regression lines calculated for the entire cohort ($n = 29$) with 95% confidence intervals (shaded areas). Blue circles indicate male patients ($n = 23$), and orange circles indicate female patients ($n = 6$).

Table 4. Spearman's Correlation Coefficients (ρ) between Carnitine Fractions and Erythrocyte Properties (N = 29)

Carnitine Parameter	Curve slope (α)	MCF (b)	5% Hemolysis	95% Hemolysis	FRC (%)	FRC count	MCHC
Free carnitine	0.387*	0.351	-0.074	0.485**	-0.192	-0.191	0.315
Total carnitine	0.298	0.360	-0.031	0.401*	-0.274	-0.268	0.391*
Acylcarnitine	0.158	0.258	-0.031	0.210	-0.340	-0.337	0.430*
AC/FC ratio	-0.146	-0.052	-0.004	-0.233	-0.283	-0.277	0.342

* $P < 0.05$, ** $P < 0.01$ (based on Spearman's rank correlation analysis). Values represent correlation coefficients (ρ). Significant correlations are highlighted in bold. MCF, mean corpuscular fragility; FRC, fragmented red blood cell.

Multivariable Linear Regression Analysis of Blood Free Carnitine and Erythrocyte Membrane Integrity

Multivariable linear regression analyses were performed to adjust for clinical confounders (**Table 5**). Blood FC level remained an independent positive predictor of OFT curve slope α in Model 1A (adjusted for age, sex, HD duration, and CRP: $P = 0.036$) and Model 1B (+ T2DM: $P = 0.045$). Similarly, FC independently predicted 95% complete hemolysis concentration (x_{comp}) in both

Model 2A ($P = 0.042$) and Model 2B ($P = 0.041$). None of the adjusted confounders showed significant independent effects ($P > 0.20$ for all). These findings confirm that higher FC levels directly contribute to erythrocyte membrane structural homogeneity and osmotic stability, independent of background clinical factors.

270

Table 5. Multivariable Linear Regression Models Evaluating the Independent Association of Blood Free Carnitine with Erythrocyte Membrane Properties

274

Model	Independent Predictor	Unstandardized Coef. <i>B</i>	SE	95% Confidence Interval (CI)	<i>P</i> -value
OFT Curve Slope (<i>a</i>)					
Unadjusted Model	Free carnitine (FC)	0.5670	0.2640	0.026 to 1.108	0.041 *
Model 1A	Free carnitine (FC)	0.6090	0.2740	0.042 to 1.176	0.036 *
	Age	0.2070	0.2510	-0.313 to 0.727	0.419
	Sex (Male vs. Female)	-7.0130	8.0420	-23.650 to 9.624	0.392
	HD duration	-0.7100	0.5860	-1.922 to 0.501	0.238
	CRP	1.4370	9.4410	-18.093 to 20.966	0.880
Model 1B (Model 1A + Type 2 Diabetes Mellitus)					
	Free carnitine (FC)	0.5970	0.2810	0.014 to 1.180	0.045 *
	T2DM (Yes vs. No)	4.2970	11.5640	-19.684 to 28.278	0.714

95% Complete Hemolysis Concentration (x_{comp})

Model 2A	Free carnitine (FC)	0.0009	0.0004	0.0000 to 0.0018	0.042 *
	Age	0.0001	0.0004	-0.0007 to 0.0009	0.773
	Sex (Male vs. Female)	-0.0207	0.0126	-0.0467 to 0.0052	0.112
	HD duration	-0.0001	0.0009	-0.0020 to 0.0018	0.923
	CRP	0.0096	0.0147	-0.0208 to 0.0401	0.519
Model 2B (Model 2A + Type 2 Diabetes Mellitus)					
	Free carnitine (FC)	0.0009	0.0004	0.0000 to 0.0019	0.041 *
	T2DM (Yes vs. No)	-0.0108	0.0180	-0.0481 to 0.0264	0.553

* $P < 0.05$.

Abbreviations: OFT, osmotic fragility test; FC, free carnitine; HD, hemodialysis; CRP, C-reactive protein; T2DM, type 2 diabetes mellitus; CI, confidence interval; SE, standard error.

Association Between Erythrocyte Osmotic Fragility and ESA Resistance

Index

In univariable correlation analysis among patients receiving ESA therapy ($n = 18$), a significant positive correlation was observed between 95% complete hemolysis concentration (x_{comp}) and ERI ($\rho = 0.474$, $P = 0.047$). In multivariable linear regression analysis, a higher x_{comp} consistently exhibited a positive linear relationship with ERI across the unadjusted model ($\beta = 0.287$, $P = 0.248$), covariate-adjusted Model 3A (age, sex, HD duration, and CRP: $\beta = 0.327$, $P = 0.243$), and Model 3B (Model 3A + T2DM: $\beta = 0.356$, $P = 0.213$) (**Supplementary**

Table S3). However, due to the limited sample size of ERI-evaluable patients ($n = 18$), the multivariable association did not reach statistical significance ($P > 0.05$).

Discussion

In the present study, we evaluated the blood carnitine status and its relationship with erythrocyte osmotic fragility and destruction in chronic maintenance HD patients. The principal finding of this study is that blood FC levels are independently and significantly associated with erythrocyte membrane properties, specifically the homogeneity of erythrocyte membrane stability represented by the OFT curve slope α and the threshold for complete hemolysis (x_{comp}). Importantly, even after adjusting for major clinical confounders—including age, sex, HD duration, CRP, and T2DM—multivariable linear regression analyses demonstrated that higher FC levels remained significantly associated with an increased OFT slope α and a higher 95% complete hemolysis concentration x_{comp} . To our knowledge, this is the first study to demonstrate that FC sufficiency directly contributes to maintaining the structural homogeneity and osmotic resistance of circulating red blood cells independently of background clinical factors in HD patients.

The physiological mechanisms by which FC maintains erythrocyte

306 membrane integrity and homogeneity involve both metabolic and biophysical
307 pathways ^{3,9}. Although mature erythrocytes lack mitochondria, they possess a
308 substantial intracellular carnitine pool and active carnitine palmitoyltransferase
309 (CPT) ^{3,9}. Metabolically, carnitine plays a pivotal role in membrane phospholipid
310 fatty acid turnover, a key repair process against oxidative membrane damage ^{3,9}.
311 Circulating erythrocytes in HD patients are continuously exposed to severe
312 uremic toxins and oxidative stress, leading to lipid peroxidation of membrane
313 phospholipids ^{1,3}. Carnitine facilitates the deacylation-reacylation cycle by
314 buffering the acyl-CoA/free CoA ratio through CPT, providing activated acyl
315 groups to reacylate and repair damaged phospholipids without depleting cellular
316 ATP ^{3,9}. Biophysically, carnitine directly interacts with the membrane cytoskeletal
317 network by strengthening spectrin-actin interactions and reducing the segmental
318 motion of spectrin ^{3,9}. By protecting membrane lipids from oxidative degradation
319 and reinforcing cytoskeletal architecture, sufficient intracellular FC prevents focal
320 membrane weakening and premature spherocytosis across the cell population ³.
321 This dual metabolic and biophysical protection preserves uniform viscoelastic
322 properties among circulating erythrocytes. Crucially, our clinical findings directly
323 support this mechanism, demonstrating that higher blood free carnitine (FC)

levels are significantly associated with both a higher OFT curve slope α and increased resistance to complete hemolysis (X_{comp}).

Maintenance HD patients are continuously subjected to mechanical shear stress generated by blood pumps and dialyzer membranes, which preferentially fragments structurally vulnerable erythrocytes into circulating FRCs^{3,15,16}. In our cohort, FRC% demonstrated a significant positive correlation with the OFT curve slope α . This counterintuitive finding reflects an in vivo 'selective elimination' process: the preferential destruction and reticuloendothelial clearance of the most fragile cells cull the vulnerable population, leaving behind a more uniform cohort of resilient erythrocytes in circulation. Therefore, automated FRC quantification not only marks physical cell destruction but directly reflects the dynamic intravascular selective pressure removing fragile erythrocytes in hemodialysis patients.

The relationship between carnitine and erythrocyte osmotic fragility in hemodialysis patients was reported by Matsumura et al. nearly three decades ago, but earlier studies lacked adjustment for modern clinical confounders¹². Overcoming these historical limitations using a modernized microplate Parpart assay with a two-parameter sigmoidal model, our multivariable linear regression

demonstrated that blood FC levels independently predict erythrocyte membrane homogeneity (OFT curve slope α) and complete hemolysis resistance (X_{comp}), regardless of age, sex, HD duration, CRP, or T2DM. Methodologically, this microplate model provides a reproducible quantitative framework for clinical practice. Clinically, these results establish that monitoring and optimizing systemic carnitine status is an independent, effective therapeutic avenue to preserve red blood cell quality and lifespan in hemodialysis patients ¹⁻³.

Several limitations of the present study warrant consideration. First, this was a single-center, cross-sectional observational study with a relatively small sample size ($n = 29$), which inherent to its design, precludes the establishment of direct causal relationships between carnitine deficiency and erythrocyte membrane fragility. Interventional prospective studies, such as randomized controlled trials investigating the effects of L-carnitine supplementation on OFT parameters, are required to confirm causality. Second, we did not directly measure erythrocyte lifespan using radiolabeled methods (e.g., ⁵¹Cr labeling) or evaluate intracellular carnitine content within red blood cells. Third, the multivariable regression analysis for ERI was restricted to a subgroup receiving ESA therapy ($n = 18$), which possessed limited statistical power ($P > 0.05$) despite a consistent positive

linear relationship. Nevertheless, this study provides pivotal clinical and mechanistic insights into modern HD care.

Conclusions

In conclusion, blood FC sufficiency is an independent determinant of erythrocyte membrane homogeneity (OFT curve slope α) and complete hemolysis resistance (X_{comp}) in chronic maintenance HD patients, remaining robust even after controlling for major clinical confounders including microinflammation and diabetes. Therefore, monitoring and optimizing systemic carnitine status may serve as a promising adjunctive therapeutic target to preserve erythrocyte quality and enhance anemia management in maintenance HD patients.

List of abbreviations

AC: Acylcarnitine

AC/FC: Acylcarnitine-to-free carnitine ratio

BMI: Body mass index

CI: Confidence interval

CPC: Coil planet centrifuge

- 377 CPT: Carnitine palmitoyltransferase
- 378 CRP: C-reactive protein
- 379 DW: Dry weight
- 380 ERI: Erythropoiesis-stimulating agent resistance index
- 381 ESA: Erythropoiesis-stimulating agent
- 382 FC: Free carnitine
- 383 FRC: Fragmented red blood cell percentage
- 384 FRC#: Absolute fragmented red blood cell count
- 385 Hb: Hemoglobin
- 386 HD: Hemodialysis
- 387 HIF-PHI: Hypoxia-inducible factor–prolyl hydroxylase inhibitor
- 388 Ht: Hematocrit
- 389 IU: International unit
- 390 MCF: Mean corpuscular fragility
- 391 MCH: Mean corpuscular hemoglobin
- 392 MCHC: Mean corpuscular hemoglobin concentration
- 393 MCV: Mean corpuscular volume
- 394 OFT: Osmotic fragility test

395 RBC: Red blood cell

396 Ret: Reticulocyte

397 SE: Standard error

398 T2DM: Type 2 diabetes mellitus

399 TC: Total carnitine

400

401 **Declarations**

402 **Ethics approval and consent to participate**

403 This study was conducted in strict accordance with the Declaration of
404 Helsinki and the "Ethical Guidelines for Medical and Health Research Involving
405 Human Subjects" in Japan. Under the centralized review system, the study
406 protocol was approved by the Ethics Committee of Juntendo University School of
407 Medicine (Approval No. E26-0103; Date of Approval: June 3, 2026) and the Ethics
408 Committee of Seseragi Hospital (Approval No. RI2026-8; Date of Approval: July
409 27, 2026). Written informed consent was obtained from all individual participants,
410 or their legally authorized representatives if applicable, prior to enrollment in the
411 study.

412 **Consent for publication**

413 Not applicable. This manuscript does not contain any individual person's
414 data in any form.

415 **Availability of data and materials**

416 The datasets used and/or analyzed during the current study are available
417 from the corresponding author on reasonable request.

418 **Competing interests**

419 The authors declare that they have no competing interests.

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425 **Authors' contribution**

426 YI and YS contributed to the conceptualization, data curation, formal
427 analysis, investigation, project administration, resources, validation, visualization,
428 writing – original draft, and writing – review & editing. NM contributed to the

conceptualization, data curation, investigation, project administration, resources, and writing – review & editing. HS contributed to investigation, resources, and writing – review & editing. KF and TK contributed to the conceptualization and writing – review & editing. YS acquired funding and provided overall supervision for the study. All authors read and approved the final manuscript.

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