# Shortened red blood cell age in patients with end-stage renal disease receiving haemodialysis: a cross-sectional study

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# Abstract

**Background:** Cause of anaemia in end-stage renal disease is not only due to relative deficiency in erythropoietin production, but also to the complex clinical conditions. We sought to investigate the underlying mechanisms of anaemia in patients with end-stage renal disease undergoing maintenance dialysis by measuring erythrocyte creatine.

**Methods:** In a cross-sectional study, we evaluated 69 patients with end-stage renal disease receiving haemodialysis (n = 55) or peritoneal dialysis (n = 14). Erythrocyte creatine, a quantitative marker of mean red blood cell (RBC) age, was measured.

**Results:** Mean RBC age was significantly shorter in haemodialysis patients than in those with peritoneal dialysis (47.7 days vs. 59.8 days, p<0.0001), although haemoglobin levels were comparable between the groups. Spearman correlation coefficient analysis revealed that transferrin saturation (r = 0.54), ferritin (r= 0.47), and haptoglobin (r = 0.39) were positively, whereas reticulocyte (r = -0.36), weekly dose of erythropoiesis-stimulating agent (r = -0.62), erythropoietin resistance index (r = -0.64), and intradialytic ultrafiltration rate (r = -0.32) were inversely related to shortened RBC age.

**Conclusions:** Shortened RBC age was observed in patients receiving maintenance haemodialysis. Shortened RBC age was associated with iron deficiency, greater haptoglobin consumption, higher ESA requirements, and poor erythropoietin responsiveness as well as greater intradialytic fluid extraction.


# Background

Anaemia is a common complication in patients with end-stage renal disease and is associated with poor long-term survival [1]. Cause of anaemia in end-stage renal disease is not only due to a relative deficiency in erythropoietin production, but also to the complex clinical conditions including iron deficiency, inflammation, and haemolysis [2]. However, development of anaemia related to complex clinical conditions in end-stage renal disease especially haemodialysis is undetermined. Labelling erythrocytes with radioactive chromium ($^{51}$Cr) is the standard method to estimate RBC age, which requires exclusive equipment for radioactive materials and a prolonged examination period with serial blood withdrawals from the patients [3]. Compared to the $^{51}$Cr-labelling method, erythrocyte creatine is a simple, rapid, and economically favourable marker that uses a single blood sample examination. Erythrocyte creatine is deemed as a quantitative marker to determine mean RBC age, because young RBCs contain substantially higher creatine levels than older RBCs, and creatine contents in RBCs decrease gradually with advancing cell age; an elevation of erythrocyte creatine reflects shortened RBC age [4-6]. Moreover, in contrast to reticulocyte levels, which reflects present erythropoiesis, erythrocyte creatine levels reflects average or cumulative erythropoiesis up to the present [5,7]. Accordingly, we utilized erythrocyte creatine to elucidate the incidence and underlying mechanisms associated with development of anaemia in patients with end-stage renal disease on maintenance haemodialysis or peritoneal dialysis.

# Methods

## Study design

We assessed haemolysis in out-patients with end-stage renal disease recruited from the dialysis unit at Kansai Medical University Hospital from May to November 2019. Patients aged over 20 years who had been established on maintenance haemodialysis 3 times a week or peritoneal dialysis therapy for at least 6 months were included in this cross-sectional study. The exclusion criteria were as follows: patients undergoing both haemodialysis and peritoneal dialysis, patients with a bleeding event within the last 3 months, blood transfusion within the last 3 months, concurrent malignancy, haemolytic disease, mechanical heart valves. The study protocol was approved by the ethics committee of Kansai Medical University (No.2018233) and was registered in the University Hospital Medical Information Network (UMIN) clinical trial registry (URL: https://www.umin.ac.jp/ctr/, Unique Identifier: UMIN000036418). All patients gave written informed consent and the investigation conforms to the principles outlined in the Declaration of Helsinki.

## Haemodialysis and peritoneal dialysis

Haemodialysis was performed via native arterio-venous fistulas utilizing a dual plastic needle with a 16-gauge cannula size. Haemodialysis patients were uniformly administered with a dialysate (D-dry, Nikkiso Co., Ltd, Tokyo, Japan) and an anticoagulant with heparin sodium. Bolus heparin sodium 500 to 1000 units was intravenously administrated at the start of haemodialysis, followed by 500 to 1000 units continuous administration to maintain 1.5 to 2 times upper level of pre-haemodialysis activated partial thromboplastin time. Dialysate temperature of extracorporeal circulation was strictly maintained at 36 to 38 °C. Nocturnal intermittent peritoneal dialysis (Baxter Healthcare, Tokyo, Japan) was performed in all patients with peritoneal dialysis. Evaluation and treatment of anaemia, including erythropoiesis-stimulating agent (ESA) and iron therapy, were prescribed according to the KDIGO Clinical Practice Guideline 2012 [8]. Iron administration therapy was performed using intravascular supplement (40mg of iron/week) in haemodialysis patients and oral supplement (100mg of iron/day) in peritoneal dialysis. Utilized ESA therapy employed darbepoetin alfa in haemodialysis patients and epoetin beta pegol in peritoneal dialysis. Quantity of blood flow (mL/min) and intradialytic ultrafiltration rate (mL/h/kg) were collected to assess haemodialysis conditions, which were calculated by an average of 3 consecutive haemodialysis sessions. One of the following dialysis membrane was utilized in haemodialysis patients by dialysis unit physician: cellulose [FB-Uβ (Nipro Corporation, Osaka, Japan)], polysulfone [ABH-PA (Asahi Kasei Corporation, Tokyo, Japan); APS-EA (Asahi Kasei Corporation, Tokyo, Japan); NV-X (Toray Medical Co.,Ltd., Tokyo, Japan); NVF-H (Toray Medical Co.,Ltd., Tokyo, Japan); VPS-VA (Asahi Kasei Corporation, Tokyo, Japan)], polyethersulfone [MFX-S (Nipro Corporation, Osaka, Japan); PES-D (Nipro Corporation, Osaka, Japan)], polymethylmethacrylate [NF-H (Toray Medical Co.,Ltd., Tokyo, Japan)], or acrylonitrile-co-methallyl sulfonate [H12-4000 (Baxter, Tokyo, Japan)].

## Measurements

Body weight was obtained pre- and post-dialysis in haemodialysis patients. In peritoneal dialysis patients, body weight was measured after discarding dialysate from the peritoneal cavity. After enrolment, blood samples were drawn from all patients to examine erythrocyte creatine, haemolytic markers (reticulocyte count, haptoglobin and lactate dehydrogenase) and other laboratory parameters (haemoglobin, haematocrit, albumin, transferrin saturation and ferritin). Blood sample was obtained immediately before receiving haemodialysis in patients with haemodialysis. A weekly dose of erythropoiesis-stimulating agents (ESA) was administered as a darbepoetin alfa equivalent dose. ESA was converted using the following formula: darbepoetin alfa (μg) = epoetin beta pegol (μg) × 0.8 = epoetin (U) × 200, based on previous reports [9-10]. ESA responsiveness was assessed using an erythropoietin resistance index, which was calculated using the following formula: erythropoietin resistance index (U/kg/week/g/dL) = weekly dose of epoetin (U/week)/(Body weight (kg) × Haemoglobin (g/dL)) [11]. Post-haemodialysis weight was extracted as a body weight in patients receiving haemodialysis.

### Erythrocyte creatine

Creatine in human packed erythrocytes indicate the mean age of an RBC population [4]. Erythrocyte creatine was assayed enzymatically in accordance with previous reports [12]. Briefly, blood was collected in ethylenediamine tetra-acetic acid-containing tubes and centrifuged to remove the plasma and the buffy coat. After lysis and deproteinization of packed erythrocytes, the supernatant was obtained by centrifugation and filtration. Creatine concentration in the supernatant was measured using the enzymatic assay method. Mean RBC age (days) was obtained by $-22.84 \times \log_e(\text{erythrocyte creatine}) + 65.83$ [6]. Erythrocyte creatine levels represent average or cumulative erythropoiesis up to the present. Therefore, erythrocyte creatine levels are indicative of a chronic rather than an acute haemolytic condition. RBC age in 305 normal subjects was extracted as a healthy control from our previous report [5,6].

### Statistical analysis

Continuous variables are presented as medians and interquartile ranges, and categorical variables are presented as numbers and percentages. Differences between the 2 groups were analysed using the Wilcoxon rank-sum tests for continuous variables and the chi-squared tests for categorical variables. The relationship between the clinical covariates and erythrocyte creatine was explored through Spearman correlation analysis. A p-value < 0.05 was considered significant. The JMP 14.2.0 software (SAS Institute Inc., Cary, NC, USA) was used for all statistical analyses.

## Results

A total of 80 outpatients aged over 20 years were considered for this study. Of these, patients undergoing both haemodialysis and peritoneal dialysis (n = 6) and patients with a bleeding event within the last 3 months (n = 1), mechanical heart valves (n = 2) and no written informed consent (n = 2) were excluded.

Finally, 55 patients receiving haemodialysis and 14 patients receiving peritoneal dialysis were included for the final analysis.

There were no significant differences in patient characteristics between the groups (Table 1). Although there was no significant difference in haemoglobin level between the groups, patients receiving haemodialysis had significantly lower transferrin saturation and ferritin than those receiving peritoneal dialysis. The weekly dose of ESA and the erythropoietin resistance index were significantly higher in patients receiving haemodialysis compared to those receiving peritoneal dialysis.

RBC age is shown in Figure 1. Patients receiving haemodialysis had significantly shorter RBC age than in patients with peritoneal dialysis [47.7 (43.2–52.8) days vs. 59.8 (56.0–66.2) days, $p<0.0001$]. RBC age in healthy control was 60.5 days which was comparable in patients with peritoneal dialysis.

When haemodialysis patients were stratified by median RBC age (47.7 days), haemodialysis patients with shortened RBC age had lower transferrin saturation, ferritin, and haptoglobin compared to those with preserved RBC age, despite higher administration rate of iron (Table 2). The weekly dose of ESA, the ESA resistance index, and the intradialytic ultrafiltration rate were all significantly higher in haemodialysis patients with shortened RBC age than those with preserved RBC age. On the other hand, there was no significant difference in type of dialysis membranes between the groups.

To investigate clinical covariates to correlate mean RBC age, Spearman correlation coefficient analysis was conducted (Table 3). Transferrin saturation, ferritin, and haptoglobin had positive correlations to RBC age, whereas reticulocyte, weekly dose of ESA, erythropoietin resistance index, and intradialytic ultrafiltration rate had negative correlations to RBC age.

# Discussion

In the present study, RBC age was measured in patients with end-stage renal disease receiving dialysis therapy, and we found significant shortening of RBC age in patients receiving haemodialysis compared to those receiving peritoneal dialysis, despite no significant differences in haemoglobin levels between the 2 groups. Moreover, spearman correlation coefficient revealed that shortened RBC age was associated with iron deficiency, greater haptoglobin consumption, higher ESA requirements, and poor ESA responsiveness as well as greater intradialytic ultrafiltration rate.

A prospective small study using radioactive chromium ($^{51}Cr$), investigated shortening of RBC age in end-stage renal disease patients receiving haemodialysis and peritoneal dialysis, and in healthy volunteers with preserved glomerular filtration rate (> 60 mL/min/1.73m$^2$) [13]. RBC age was shortened by 20% in end-stage renal disease compared with healthy volunteers, but there was no significant difference in RBC age between haemodialysis and peritoneal dialysis. Due to a small number of patients in each group and a lack of haemolytic markers, they did not analyse the mechanisms of RBC age shortening in patients receiving dialysis. In our study, shortening of RBC age was observed in patients receiving haemodialysis compared to those receiving peritoneal dialysis, whereas RBC age was comparable between patients

receiving peritoneal dialysis and healthy control. Although this discrepancy is not fully elucidated, our study showed that patients receiving haemodialysis had greater iron consumption and required higher ESA dosage compared to those receiving peritoneal dialysis, indicating that patients receiving haemodialysis were accompanied by absolute or functional iron deficiency. RBC age shortening leads to systemic tissue hypoxia which stimulates endogenous erythropoietin production and enhances iron availability [1]. Persistent RBC age shortening is attributable to absolute or functional iron deficiency and relative ESA hyporesponsiveness. Therefore, iron deficiency and requiring higher ESA dosage in patients receiving haemodialysis indicate existence of persistent RBC age shortening.

Haptoglobin level was significantly lower in patients receiving haemodialysis compared to those receiving peritoneal dialysis. Meyer C et al. investigated haemodialysis-induced haemolysis using free haemoglobin [14]. They calculated free haemoglobin pre- and post-haemodialysis and found that free haemoglobin level was significantly increased in post-haemodialysis. These data suggest that haemodialysis-induced haemolysis is one of the underlying mechanisms of shortened RBC age because mechanical stress caused by flow resistance and turbulence during extracorporeal circuit often contribute to haemodialysis-induced haemolysis. Toshner et al. investigated alteration of haptoglobin and lactate dehydrogenase levels between pre- and 8-hour post-haemodialysis in 12 patients, and found that both parameters did not change during this period [15]. They concluded that RBC damage due to mechanical stress of extracorporeal circuit was a negligible factor contributing to persistent anaemia. However, baseline haptoglobin level was quite uneven (9 to 210 mg/dL for baseline haptoglobin level) among the study population. This difference in haptoglobin level was also observed in our study. Moreover, haptoglobin level was significantly lower in haemodialysis patients with shortened RBC age compare to those with preserved RBC age, indicating potential relation between haptoglobin and shortening RBC age.

Sixteen-gauge plastic needle was utilized in this study and median blood flow ranged from 200 to 220 ml/min. A previous study reported that there was no difference in haemolysis markers when 15-gauge needle with blood flow of 400 ml/min were compared with 16-gauge needle with blood flow of 300ml/min [16]. Likewise, no significant differences of haemolytic markers were observed between 14-gauge with 500ml/min blood flow and 17-gauge needle with 250ml/min blood flow, suggesting that the size of puncture needle does not affect haemolysis [17]. Interestingly, a greater intradialytic ultrafiltration rate, not an increased quantity of blood flow, was observed in haemodialysis patients with shortened RBC age, suggesting that greater ultrafiltration volume through the dialysis membrane rather than intraluminal blood velocity was one of the underlying mechanism associated with increased shear stress of the circulating erythrocyte causing haemolysis.

Haemodialysis patients with shortened RBC age had greater iron deficiency despite higher rate of iron supplementation compared to those with preserved RBC age. Despite adequate intravenous iron therapy has been widely accepted to optimize ESA responsibility, recent European national study demonstrated that intravenous or oral iron supplementation was used only in 19% of end-stage renal disease [18]. Adequate iron therapy may improve ESA responsiveness especially haemodialysis patients with shortened RBC age.

In addition to high prevalence of known risk factors for cardiovascular disease, haemolysis-associated endothelial dysfunction has been reported [14,19,20]. Release of free haemoglobin induced by haemolysis scavenges and reduces the bioavailability of nitric oxide, which leads to impaired vascular endothelial function. Impaired endothelial function due to haemodialysis-induced haemolysis could lead to an increased risk of cardiovascular complications. Several complex clinical conditions including iron deficiency, ESA responsiveness, and haemolysis involve in the development of anaemia in end-stage renal disease. Therefore, correct recognition of shortened RBC age is important to reduce the risk of future unfavourable cardiovascular events in patients receiving maintenance haemodialysis.

## Limitation

Three limitations of the present study should be noted. First, this study includes relatively small sample size, and there is a discrepancy in the number of patients in the haemodialysis and peritoneal dialysis group. Therefore, investigations using a larger sample size are needed to verify this result. Second, tissue hypoxia due to low cardiac output, hypotension, or anaemia leads to increase in endogenous erythropoietin production and enhance iron availability. Increase in endogenous erythropoietin production contributes to accelerate erythropoiesis, which result in higher erythrocyte creatine values. Third, we excluded patients with unstable systemic circulation such as bleeding event, blood transfusion, malignant disease, or initiated dialysis within 6 months, but erythrocyte creatine has potential limitation to define RBC age in patients with end-stage renal disease.

## Conclusions

Shortened RBC age was observed in patients receiving maintenance haemodialysis. Iron deficiency, erythropoietin hyporesponsiveness, haemolysis as well as greater intradialytic fluid extraction were related to shortened RBC age.

## Abbreviations

RBC: red blood cell.

## Declarations

# Ethics approval and consent to participate:

The study protocol was approved by the ethics committee of Kansai Medical University (No.2018233).

# Consent for publication:

Patients signed informed consent regarding publishing their data.

# Availability of data and material:

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

# Competing interests:

The authors declare that they have no competing interests.

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This study was funded by the Nakatani foundation. The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report.

# Authors' contributions:

K.M., T.O. and T.S.: study design and conduction, data collection, analysis and interpretation, and manuscript writing. N.T., Y.Y., S.K., K.F. and M.O.: study conduction, data analysis and interpretation. M.O. and I.S.: study conduction, data analysis and interpretation. All authors read and approved the final manuscript.

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# Tables

**Table 1** Patient profiles

| | Haemodialysis (n = 55) | Peritoneal dialysis (n = 14) | p value |
|---|---|---|---|
| **Age (years)** | 71 (63–79) | 70 (60–79) | 0.83 |
| **Male** | 33 (60) | 7 (50) | 0.45 |
| **Body mass index (kg/m$^2$)** | 21.3 (19.8–24.3) | 23.4 (21.3–24.5) | 0.12 |
| **Hypertension** | 47 (85) | 13 (93) | 0.46 |
| **Diabetes mellitus** | 32 (58) | 5 (36) | 0.13 |
| **Prior cardiovascular disease** | 14 (25) | 3 (21) | 0.76 |
| **Laboratory data** | | | |
| **Haemoglobin (g/dL)** | 11.0 (10.5–11.7) | 11.0 (10.5–11.8) | 0.78 |
| **Haematocrit (%)** | 33.9 (32.1–36.5) | 33.8 (33.1–35.9) | 0.70 |
| **Albumin (g/dL)** | 3.5 (3.3–3.8) | 3.5 (3.1–3.7) | 0.35 |
| **High-sensitive CRP (mg/dL)** | 0.06 (0.04–0.29) | 0.09 (0.02–0.43) | 0.82 |
| **iPTH (pg/mL)** | 139 (103–190) | 203 (150–240) | 0.04 |
| **Transferrin saturation (%)** | 22 (16–28) | 37 (24–43) | 0.008 |
| **Ferritin (ng/mL)** | 50 (29–71) | 119 (105–161) | < 0.0001 |
| **Reticulocyte (%)** | 1.7 (1.4–2.1) | 1.3 (1.0–1.7) | 0.06 |
| **Haptoglobin (mg/dL)** | 78 (38–121) | 96 (76–184) | 0.04 |
| **Lactate dehydrogenase (U/L)** | 194 (176–212) | 184 (156–225) | 0.50 |
| **Iron supplement** | 18 (33) | 3 (21) | 0.40 |
| **Erythropoiesis-stimulating agent (μg/week)** | 30 (10–45) | 12 (10–20) | 0.02 |
| **Erythropoietin resistance index** | 9.4 (3.4–14.1) | 3.9 (2.7–7.5) | 0.02 |

Data presented as median (25th to 75th percentiles), or number (%).

CRP; C-reactive protein, iPTH; intact parathyroid hormone

**Table 2** Comparison of clinical characteristics in patients with haemodialysis according to median RBC age

| | Preserved RBC age (n = 27) | Shortened RBC age (n = 28) | p value |
|---|---|---|---|
| **Age (years)** | 72 (67–79) | 71 (61–78) | 0.32 |
| **Male** | 16 (59) | 17 (61) | 0.91 |
| **Body mass index (kg/m$^2$)** | 21.3 (19.8–23.6) | 21.3 (19.5–25.2) | 0.89 |
| **Laboratory data** | | | |
| **Haemoglobin (g/dL)** | 10.9 (10.6–11.4) | 11.1 (10.3–11.9) | 0.91 |
| **Hematocrit (%)** | 33 (31–35) | 35 (33–37) | 0.09 |
| **Albumin (g/dL)** | 3.6 (3.3–3.8) | 3.5 (3.3–3.8) | 0.97 |
| **High-sensitive CRP (mg/dL)** | 0.06 (0.04–0.43) | 0.06 (0.04–0.26) | 0.67 |
| **iPTH (pg/mL)** | 135 (98–182) | 143 (104–197) | 0.55 |
| **Transferrin saturation (%)** | 26 (19–39) | 18 (14–26) | < 0.01 |
| **Ferritin (ng/mL)** | 57 (36–122) | 43 (20–65) | 0.04 |
| **Reticulocyte (%)** | 1.7 (1.2–2.1) | 1.7 (1.4–2.1) | 0.72 |
| **Haptoglobin (mg/dL)** | 91 (61–139) | 49 (25–114) | < 0.01 |
| **Lactate dehydrogenase (U/L)** | 194 (176–207) | 198 (177–214) | 0.72 |
| **Iron supplement** | 5 (19) | 13 (46) | 0.03 |
| **Erythropoiesis-stimulating agent (μg/week)** | 20 (10–30) | 33 (20–60) | 0.004 |
| **Erythropoietin resistance index** | 5.0 (2.8–11.8) | 11.4 (6.7–20.8) | 0.01 |
| **Quantity of blood flow (mL/min)** | 220 (200–250) | 200 (200–245) | 0.53 |
| **Intradialytic ultrafiltration rate (mL/h/kg)** | 7.3 (6.0–9.3) | 9.3 (7.0–12.7) | 0.02 |
| **Dialysis membranes** | | | |
| **Cellulose** | 11 (41) | 9 (32) | 0.51 |
| **Polysulfone** | 6 (22) | 10 (36) | 0.27 |
| **Polyethersulfone** | 8 (30) | 7 (25) | 0.70 |
| **Polymethylmethacrylate** | 1 (4) | 0 (0) | 0.23 |
| **Acrylonitrile-co-methallyl sulfonate** | 1 (4) | 2 (7) | 0.57 |
| **Heparin dosage (units/session)** | 2850 (2750–3825) | 3205 (2800–3825) | 0.52 |

Data presented as median (25th to 75th percentiles), or number (%).

CRP; C-reactive protein, iPTH; intact parathyroid hormone, RBC: red blood cell.

**Table 3** Spearman correlations of clinical covariates for mean RBC age

| | r | p value |
|---|---|---|
| **Age (years)** | 0.03 | 0.78 |
| **Haemoglobin (g/dL)** | 0.02 | 0.85 |
| **High-sensitive CRP (mg/dL)** | -0.02 | 0.88 |
| **iPTH (pg/mL)** | 0.08 | 0.52 |
| **Transferrin saturation (%)** | 0.54 | < 0.0001 |
| **Ferritin (ng/mL)** | 0.47 | < 0.0001 |
| **Reticulocyte (%)** | -0.36 | 0.002 |
| **Haptoglobin (mg/dL)** | 0.39 | 0.001 |
| **Erythropoiesis-stimulating agent (μg/week)** | -0.62 | < 0.0001 |
| **Erythropoietin resistance index** | -0.64 | < 0.0001 |
| **Quantity of blood flow (mL/min)** | 0.07 | 0.59 |
| **Intradialytic ultrafiltration rate (mL/h/kg)** | -0.32 | 0.02 |

RBC: red blood cell.

# Figures

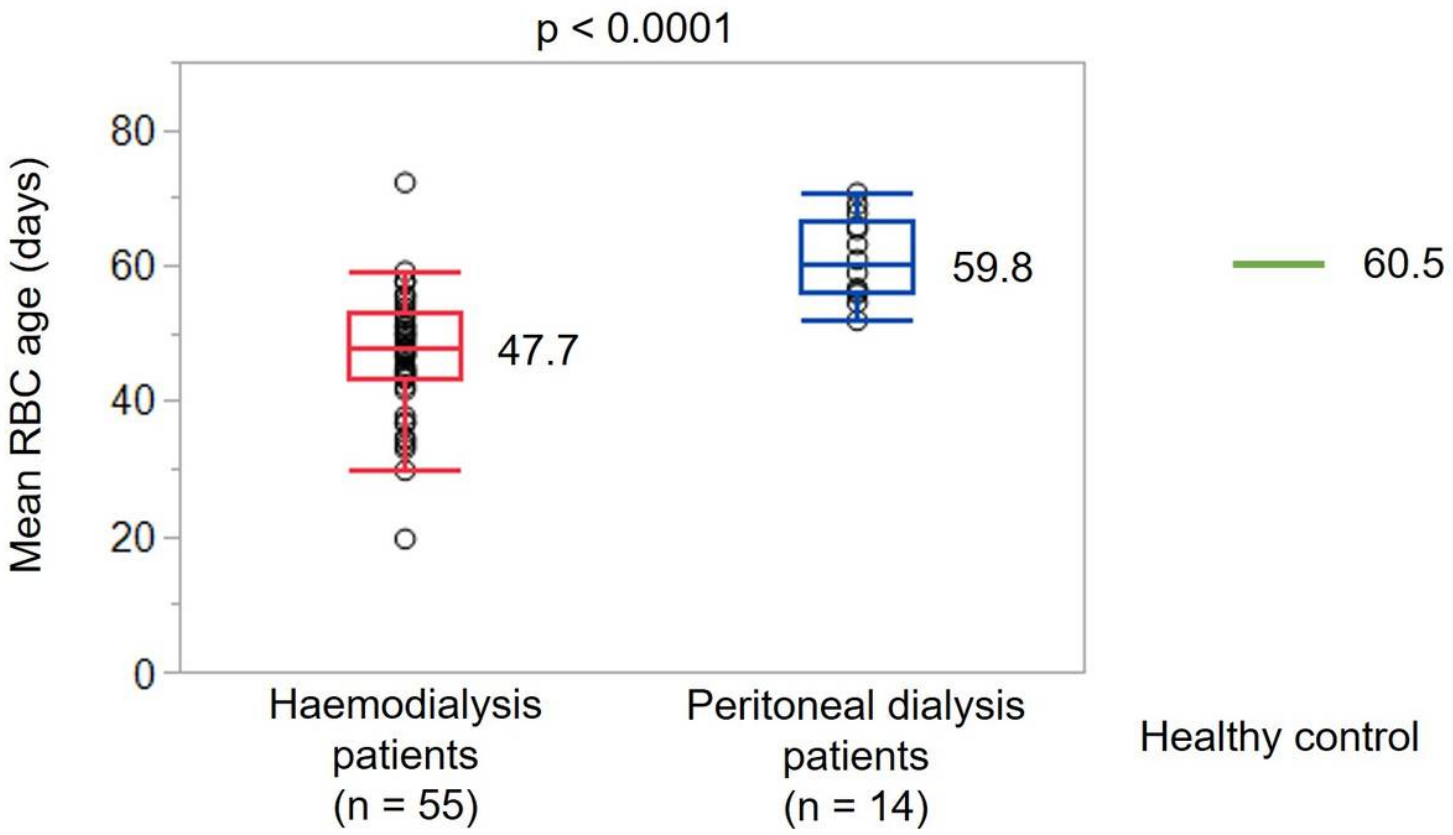


**Figure 1**

Comparison of mean RBC age. Mean RBC age in healthy control is shown as a green line. The box for two groups represents the interquartile range (25th–75th percentile), and the horizontal line in each box represents the median value. RBC: red blood cell.

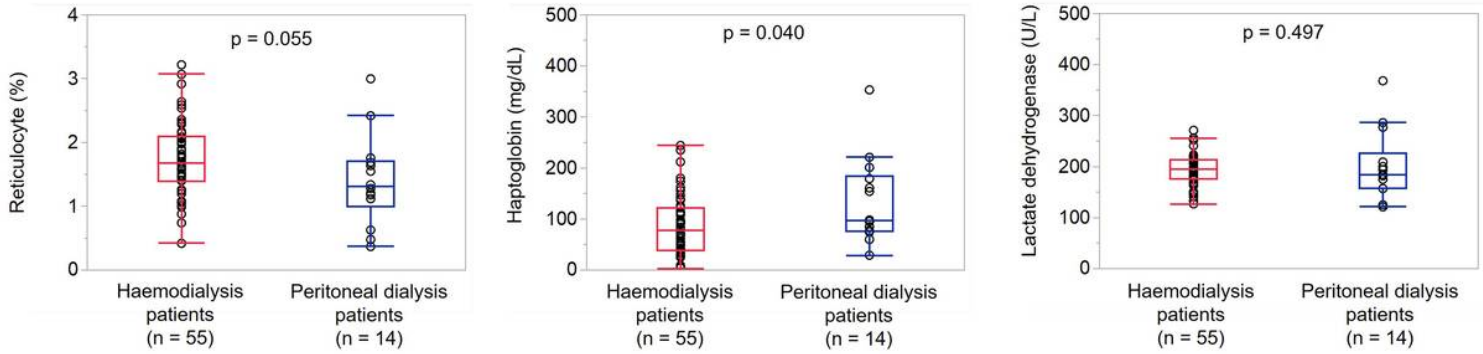


**Figure 2**

Comparison of haemolytic markers in patients receiving haemodialysis and peritoneal dialysis. The box for each group represents the interquartile range (25th–75th percentile), and the horizontal line in each box represents the median value.