Iron Deficiency and Erythropoietin Excess: Two Sides of the Same Coin?

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DOI:
10.33612/diss.98865528

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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C-Terminal Fibroblast Growth Factor 23, Iron Deficiency, and Mortality in Renal Transplant Recipients

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Iron deficiency (ID) is independently associated with an increased risk of death in renal transplant recipients (RTRs). ID promotes production and cleavage of intact fibroblast growth factor 23 (iFGF23) into C-terminal fibroblast growth factor 23 (cFGF23), elevated levels of which are also prospectively associated with adverse outcomes. We hypothesized that in RTRs, the relationship between ID and mortality is mediated by FGF23. We measured plasma iFGF23 and cFGF23 levels in 700 stable RTRs at a median of 5.4 years following transplantation. Median cFGF23 concentrations were higher in iron deficient compared to non-iron deficient RTRs (223 [131 – 361] vs. 124 [88 – 180] RU/mL; \( P < 0.001 \)), whereas iFGF23 concentrations were similar between groups. In multivariable-adjusted Cox regression analyses, ID was associated with increased mortality (81 events; hazard ratio [HR], 1.95; 95% confidence interval [CI], 1.22-3.10; \( P = 0.005 \)). However, this association lost significance after additional adjustment for cFGF23 levels (HR, 1.45; 95%CI 0.87-2.51; \( P = 0.15 \)). In further mediation analysis, we found that cFGF23 explained 46% of the association between ID and mortality, whereas iFGF23 did not mediate this association. In conclusion, we found that cFGF23 levels are increased in iron-deficient RTRs and that most probably, the underlying biological process driving production and cleavage of iFGF23, or alternatively the increased level of cFGF23 fragments is an important mediator of the association between ID and mortality. Our results underline the strong relationship between iron and FGF23 physiology, and provide a potential mechanism explaining the relationship between ID and adverse outcome in RTRs.
INTRODUCTION

Iron deficiency (ID) is highly prevalent among renal transplant recipients (RTRs) and an important contributor to post-transplant anemia.\cite{1,2} In addition to its role in hemoglobin synthesis, iron also plays a pivotal role in oxygen sensing, synthesis of DNA, electron transport and cellular proliferation and differentiation.\cite{3} Independent of anemia, ID is a known risk factor for mortality in RTRs, though the underlying mechanisms are unclear.\cite{4}

Recent studies suggest that ID is crucially involved in fibroblast growth factor 23 (FGF23) production and metabolism. FGF23 is an osteocyte-derived hormone, and an essential regulator of phosphate metabolism, among others by influencing vitamin D homeostasis. Higher plasma FGF23 levels are associated with an increased risk of mortality in RTRs,\cite{5,6} which may be explained, at least in part, by off-target effects of high FGF23 levels on the heart and other organs.\cite{7,8} Experimental animal models revealed that ID stimulates FGF23 transcription accompanied by increased intracellular cleavage of FGF23, which results in elevated circulating C-terminal FGF23 (cFGF23) concentrations, but relatively normal intact FGF23 (iFGF23) concentrations.\cite{9} Observational studies in humans have also demonstrated an inverse relationship between markers of iron status and cFGF23 levels.\cite{10-13} Moreover, administration of intravenous iron in iron-deficient anemic women resulted in markedly decreased circulating cFGF23 concentrations,\cite{14} consistent with the notion that ID is an important physiologic regulator of increased cFGF23 levels.

To date, it is unknown whether an association exists between FGF23 and ID in RTRs and whether FGF23 modulates the increased mortality risk observed in RTRs with ID. Therefore, the aim of the present study was to elucidate whether ID influences FGF23 levels, and whether the association of ID with all-cause mortality is mediated by FGF23.

RESULTS

Baseline characteristics

We included 700 RTRs at a median of 5.4 (interquartile range [IQR], 1.9-12.0) years after transplantation. Mean age was 53±13 years; 57% of participants were male; mean body mass index (BMI) was 26.7±4.8 kg/m². Additional baseline characteristics are shown in Table 1.

Median plasma iFGF23 and cFGF23 concentrations were 62 (IQR 43-99) pg/mL and 140 (95-233) RU/ml, respectively. Mean hemoglobin concentration was 13.2±1.7 g/dL; median ferritin concentrations were 118 (54-222) µg/L; and mean TSAT was 25.4±10.8 %. ID, defined as transferrin saturation (TSAT) <20% and ferritin <300 µg/L, was present in 208 (30%) patients. Significant differences in baseline characteristics between RTRs
with versus without ID were noted with respect to gender, BMI, smoking status, time since transplantation, diabetes mellitus, hemoglobin concentration, mean corpuscular volume (MCV), ferritin, TSAT, proteinuria, high-sensitivity C-reactive protein (hs-CRP), and use of angiotensin converting enzyme (ACE)-inhibitors and diuretics (Table 1). Increased cFGF23 levels were noted in the iron deficient compared to non-iron deficient RTRs (223 [131 – 361] vs. 124 [88 – 180] RU/mL; \( P < 0.001 \)), whereas iFGF23 levels were similar between groups (Table 1).

Table 1. Baseline characteristics of renal transplant recipients according to iron deficiency

<table>
<thead>
<tr>
<th>Variables</th>
<th>Total population (n=700)</th>
<th>No iron deficiency (n=492)</th>
<th>Iron deficiency (n=208)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yr)</td>
<td>53±13</td>
<td>53±13</td>
<td>54±12</td>
<td>0.37</td>
</tr>
<tr>
<td>Male sex (n, %)</td>
<td>398 (57)</td>
<td>299 (61)</td>
<td>99 (48)</td>
<td>0.001</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>26.7 ± 4.8</td>
<td>26.3 ± 4.6</td>
<td>27.5 ± 5.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Body surface area (m²)</td>
<td>1.9 (1.8 - 2.1)</td>
<td>1.9 (1.8-2.1)</td>
<td>2.0 (1.8-2.1)</td>
<td>0.15</td>
</tr>
<tr>
<td>Alcohol use (n, %)</td>
<td>569 (82)</td>
<td>393 (80)</td>
<td>176 (85)</td>
<td>0.16</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
<td></td>
<td></td>
<td>0.02</td>
</tr>
<tr>
<td>Never smoker (n, %)</td>
<td>317 (45)</td>
<td>232 (47)</td>
<td>85 (41)</td>
<td></td>
</tr>
<tr>
<td>Former smoker (n, %)</td>
<td>299 (43)</td>
<td>194 (39)</td>
<td>105 (51)</td>
<td></td>
</tr>
<tr>
<td>Current smoker (n, %)</td>
<td>84 (12)</td>
<td>66 (13)</td>
<td>18 (9)</td>
<td></td>
</tr>
<tr>
<td>Primary glomerular disease (n, %)</td>
<td>197 (28)</td>
<td>132 (27)</td>
<td>65 (31)</td>
<td>0.24</td>
</tr>
<tr>
<td>Glomerulonephritis (n, %)</td>
<td>53 (8)</td>
<td>40 (8)</td>
<td>13 (6)</td>
<td></td>
</tr>
<tr>
<td>Tubulo-interstitial disease (n, %)</td>
<td>83 (12)</td>
<td>63 (13)</td>
<td>20 (10)</td>
<td></td>
</tr>
<tr>
<td>Polycystic renal disease (n, %)</td>
<td>145 (21)</td>
<td>98 (20)</td>
<td>47 (23)</td>
<td></td>
</tr>
<tr>
<td>Dysplasia and hypoplasia (n, %)</td>
<td>29 (4)</td>
<td>23 (5)</td>
<td>6 (3)</td>
<td></td>
</tr>
<tr>
<td>Renovascular disease (n, %)</td>
<td>40 (6)</td>
<td>27 (6)</td>
<td>13 (6)</td>
<td></td>
</tr>
<tr>
<td>Diabetic nephropathy (n, %)</td>
<td>35 (5)</td>
<td>20 (4)</td>
<td>15 (7)</td>
<td></td>
</tr>
<tr>
<td>Other or unknown cause (n, %)</td>
<td>118 (17)</td>
<td>89 (18)</td>
<td>29 (14)</td>
<td></td>
</tr>
<tr>
<td>History of cardiovascular disease (%)</td>
<td>96 (14)</td>
<td>54 (11)</td>
<td>42 (20)</td>
<td>0.007</td>
</tr>
<tr>
<td>Time since transplantation (years)</td>
<td>5.4 (1.9 - 12.0)</td>
<td>6.2 (2.6-13.0)</td>
<td>4.3 (1.1-10.0)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Acute rejection (%)</td>
<td>186 (27)</td>
<td>130 (26)</td>
<td>56 (27)</td>
<td>0.89</td>
</tr>
<tr>
<td>Diabetes mellitus (n, %)</td>
<td>170 (24)</td>
<td>97 (20)</td>
<td>73 (35)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Systolic blood pressure (mmHg)</td>
<td>136 ± 17</td>
<td>135 ± 17</td>
<td>137 ± 17</td>
<td>0.23</td>
</tr>
<tr>
<td>Diastolic blood pressure (mmHg)</td>
<td>83 ± 11</td>
<td>82 ± 11</td>
<td>83 ± 11</td>
<td>0.20</td>
</tr>
</tbody>
</table>
During a median follow-up of 3.1 (2.7–3.9) years, 81 (12%) RTRs died, of whom 38 (47%) died from cardiovascular causes. Other causes of death were infection (24%), malignancy (16%), and miscellaneous causes (14%).

In unadjusted Cox regression analysis, ID (hazard ratio [HR], 2.04 for present vs. absent; 95% confidence interval [CI], 1.31-3.16; p<0.001), cFGF23 (HR, 1.61 per SD; 95%CI 1.35-1.91; P<0.001), and iFGF23 (HR, 1.33 per SD; 95%CI 1.11-1.60; P=0.002) were associated with all-cause mortality. The simulation study showed that this difference in HRs could not be explained by the differences in intra-assay coefficients of variation (CVs) (HR cFGF23, 1.60 per SD; 95% CI 1.35-1.90). We observed no effect modification of the
association between ID and mortality by age, sex, eGFR, proteinuria, history of cardiovascular disease, time since transplantation, smoking status, BMI, presence of diabetes, hs-CRP, serum calcium, phosphate, PTH, use of ACE-inhibitors, use of diuretics, cFGF23, and iFGF23 concentrations \( (P_{\text{interaction}} > 0.10 \text{ for all}) \). In multivariable Cox regression analysis, the association between ID and mortality remained significant after adjustment for age, sex, eGFR, proteinuria, time since transplantation, primary renal disease, history of cardiovascular disease, and smoking status (HR 1.95; 95% CI, 1.22-3.10; \( P < 0.001 \)). Further adjustment for iFGF23 did not affect the association between ID and mortality (HR 1.94; 95% CI, 1.22-3.10; \( P = 0.005 \)). In contrast, adjustment for cFGF23 abolished the association between ID and mortality such that it was no longer significant (HR 1.45; 95% CI, 0.87-2.51; \( P = 0.15 \)) (Table 2). The correlation coefficient of ID with cFGF23 levels was \( r = 0.35 \), \( P < 0.001 \) and the variance inflation factor (VIF) was 1.1, indicating absence of relevant co-linearity.

**Table 2.** Univariate and multivariate-adjusted associations between iron deficiency and all-cause mortality

<table>
<thead>
<tr>
<th>Model</th>
<th>HR (95% CI)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Univariate</td>
<td>2.04 (1.31-3.16)</td>
<td>0.001</td>
</tr>
<tr>
<td>Model 1</td>
<td>1.94 (1.25-3.01)</td>
<td>0.003</td>
</tr>
<tr>
<td>Model 2</td>
<td>1.95 (1.22-3.10)</td>
<td>0.005</td>
</tr>
<tr>
<td>Model 3</td>
<td>1.94 (1.22-3.10)</td>
<td>0.005</td>
</tr>
<tr>
<td>Model 4</td>
<td>1.45 (0.87-2.51)</td>
<td>0.15</td>
</tr>
</tbody>
</table>

Model 1: Adjustment for age and sex; Model 2: Model 1 + adjustment for eGFR, proteinuria, time since transplantation, primary renal disease, history of cardiovascular disease, and smoking status; Model 3: Model 2 + adjustment for iFGF23; Model 4: Model 2 + adjustment for cFGF23; cFGF23, iFGF23, and time since transplantation were ln-transformed before adding to the Cox regression analysis due to skewed distribution; HR, hazard ratio; ID, iron deficiency.

**Mediation analysis**

In mediation analyses, iFGF23 was not found to be a significant mediator (\( P \) value for indirect effect >0.05) of the effect between ID and mortality. In contrast, cFGF23 was a significant mediator (\( P \) value for indirect effect <0.05; 46% of the association between ID and mortality was explained by cFGF23) (Table 3). Differences in intra-assay CVs could not explain this difference in mediation effects either (proportion of mediation from simulated data, 45.7%).

**Sensitivity analyses**

In sensitivity analyses, we first assessed the prospective association between ID and mortality using an alternative definition of ID: TSAT<20% and ferritin <200µg/L. The association between ID and mortality remained significant independent of adjustment for age, sex, eGFR, proteinuria, time since transplantation, primary renal disease, history of
cardiovascular disease and smoking status (HR, 1.87; 95%CI, 1.16-3.01, P=0.01). Similar to the primarily used definition of ID, the association persisted after adjustment for iFGF23 (HR, 1.87; 95% CI, 1.16-3.02; P=0.01), whereas adjustment for cFGF23 abolished the association (HR, 1.40; 95%CI, 0.81-2.40, P=0.23).

Second, we assessed whether the association between ID and mortality remained independent of adjustment for markers related to mineral metabolism other than FGF23, i.e. serum calcium and serum PTH. In these analyses, the association between ID and mortality remained materially unchanged independent of further adjustment for serum calcium in addition to adjustment for age, sex, eGFR, proteinuria, time since transplantation, primary renal disease and smoking status (HR, 1.90; 95%CI 1.23-3.13; P=0.005 (see table 2, model 2 for comparison)).
Furthermore, we assessed whether the association between ID and mortality remained independent of adjustment for angiotensin converting enzyme (ACE) inhibitors and angiotensin II (All)-antagonists, hemoglobin levels, acute rejection, presence of diabetes, use of iron supplements, or hs-CRP. In these analyses, the association between ID and mortality remained materially unchanged independent of further adjustment for use of ACE-inhibitors and All-antagonists (HR, 1.97; 95%CI 1.23-3.16; $P=0.005$ (see table 2, model 2 for comparison)) in addition to adjustment for age, sex, eGFR, proteinuria, time since transplantation, primary renal disease and smoking status. The same was true with further adjustment for hemoglobin (HR, 1.76; 95%CI 1.09-2.84; $P=0.02$), acute rejection (HR, 1.93; 95%CI 1.21-3.09; $P=0.006$), presence of diabetes (HR, 1.83; 95%CI 1.13-2.94; $P=0.01$), use of iron supplements (HR, 2.06; 95%CI 1.29-3.29; $P=0.003$), and hs-CRP (HR, 1.86; 95%CI 1.15-2.99; $P=0.01$).

**Figure 1.** Associations of low serum ferritin and low transferrin saturation with all-cause mortality are abrogated by adjustment for C-terminal fibroblast growth factor 23. Data were fit by a Cox proportional hazard regression model based on restricted cubic splines. For both variables the median was utilized as reference (4.8 µg/L for naturally logarithmic ferritin, 24% for transferrin saturation). The black line represents the hazard ratio. The grey area the 95% confidence interval. Panels A and C show univariate analyses between serum ferritin and TSAT and all-cause mortality. Models B and D show adjustment for cFGF23. Abbreviations: cFGF23, C-terminal fibroblast growth factor 23; TSAT, transferrin saturation.
Finally, we assessed the associations between the individual iron status components and mortality. The relationship between serum ferritin and mortality demonstrated a non-linear relationship, as shown by cubic restricted splines (Figure 1A). When divided in quintiles and adjusted according to model 2, the lowest and the highest quintiles of serum ferritin were associated with an increased risk of mortality (HR, 2.82; 95%CI, 1.20-6.63; P=0.02, and HR, 2.49; 95%CI 1.10-5.65; P=0.03, respectively), compared with the fourth quintile. Adjustment for iFGF23 did not materially alter the association of the lowest quintile (HR, 2.79; 95%CI 1.19-6.59; P=0.02) and the highest quintile (HR, 2.54; 95%CI 1.12-5.77; P=0.03) of ferritin with mortality. In contrast, after adjustment for cFGF23, the association of the lowest quintile with mortality was markedly weakened and no longer significant (HR, 1.67; 95%CI 0.66-4.22; P=0.27), but the highest quintile of ferritin was still significantly associated with mortality (HR, 2.78; 95%CI 1.22-6.37; P=0.02; Figure 1B).

TSAT was inversely associated with mortality (HR, per 5% increase 0.86, 95%CI 0.77-0.96; P=0.007; Figure 1C). In multivariable analysis, TSAT remained significantly associated with mortality (HR, 0.87, 95% CI 0.78-0.98; P=0.02). Adjustment for iFGF23 did not alter the association of TSAT with mortality (HR, 0.88, 95% CI 0.78-0.98; P=0.02). In contrast, further adjustment for cFGF23 abolished the association (HR, 0.96, 95%CI 0.84-1.08; P=0.45; Figure 1D).

**DISCUSSION**

In this study, we show that in RTRs, ID is accompanied by markedly higher cFGF23 levels than in RTRs without ID, whereas iFGF23 levels were similar in both groups. Importantly, variation in cFGF23 explained a considerable part of the association between ID and mortality. Similar findings were obtained in sensitivity analyses using an alternative definition of ID, and using TSAT and ferritin as individual components of ID. Thus, this study confirms earlier findings that iron plays an essential role in FGF23 production and metabolism, extends these findings to a novel patient setting (i.e. RTRs) and supports the notion that the biological process by which ID simultaneously upregulates FGF23 production and its cleavage or alternatively the increased level of cFGF23 fragments, is an important mediator of the association between ID and mortality.

Post-transplantation anemia is highly prevalent in RTRs, affecting approximately one third of the population. Furthermore, it has been widely documented that post-transplant anemia is associated with poor outcomes. Recently, we showed that ID, independent of anemia, is associated with an increased risk of mortality, shifting the focus from anemia to ID. The etiology of ID-related risk of mortality is unknown. In the present study, we have shown that the association of ID with mortality is largely explained by cFGF23. The extent of this effect is illustrated by the restricted cubic
splines depicting the individual components of iron deficiency (Figure 1). The association between low serum ferritin and low TSAT and mortality is abrogated by adjustment for cFGF23, whereas the association between high serum ferritin levels and mortality was not altered. In mediation analyses, cFGF23 was a prominent statistical mediator of the association between ID and mortality. Therefore, it seems that an ID-induced rise in cFGF23 levels plays an important role in the outcome of RTRs. Indeed, cFGF23 has been shown to be an independent risk factor for death in various patient groups, including post-operative acute kidney injury, non-dialysis CKD, end-stage renal disease, and RTRs.5, 6, 17-20 It seems likely that a, so far unknown, underlying process driving FGF23 production and cleavage plays a role in adverse outcomes of ID, but not iron overload or inflammation. The fact that adjustment for cFGF23 did not materially alter the upper part of the non-linear association that was present in sensitivity analyses on the association of serum ferritin with mortality is supportive of our hypothesis that inflammation is not the driving force behind the association of ID and mortality. In line with this observation, the association of ID with mortality persisted upon adjustment for hs-CRP. Similarly, renal function in itself also does not seem to be the sole underlying factor, since adjustment for eGFR did not materially change the results neither in the current study nor in previous studies addressing the association between cFGF23 and mortality.5, 6 As an alternative to an underlying factor or process mediating both FGF23 cleavage and adverse outcome, it may be that C-terminal fragments in themselves drive adverse outcome. Previously, C-terminal fragments have been found to compete with intact FGF23 for binding to its receptor complex and function as a competitive inhibitor, which may impair phosphaturia and aggravate soft tissue calcification.21

The present data confirm the concept that iron plays an important role in regulating FGF23 production and metabolism. FGF23 is regulated by a complex, partly unrevealed, interplay between local bone factors that modulate bone turnover and mineralization, and systemic factors that regulate mineral metabolism.22 Parathyroid hormone, 1,25-dihydroxyvitamin D, klotho, glucocorticoids, calcium, and phosphate are all known to regulate FGF23 production.23, 24 It has been established that systemic ID stabilizes hypoxia-inducible factor 1-α (HIF-1α) which increases FGF23 transcription9, and simultaneously upregulates furin, which cleaves FGF23.25, 26 Normal osteocytes couple increased FGF23 production with commensurately increased FGF23 cleavage, which ensures normal phosphate homeostasis in the event of ID26 because the intact, biologically active iFGF23 levels remain relatively unchanged. In keeping with this hypothesis, in our iron deficient RTRs, cFGF23 levels were upregulated whereas the levels of iFGF23 were similar in ID and non-ID patients.

To our knowledge, this is the first study to investigate the interplay between ID and FGF23 in RTRs. In animal models of chronic kidney disease (CKD), it has been shown that ID stimulates FGF23 production, but also upregulates cleavage, which leads to
increased levels of circulating cFGF23, but normal levels of iFGF23.\textsuperscript{9, 27, 28} In humans, Wolf and colleagues showed that iron deficiency anemia is associated with normal iFGF23 but markedly elevated cFGF23 levels to an extent that is only seen in advanced CKD or in hereditary diseases of FGF23 overproduction.\textsuperscript{14} Moreover, rapid correction of ID with different intravenous iron preparations reduced cFGF23 levels by approximately 80% within 24 hours,\textsuperscript{14} consistent with ID as an important stimulus for elevated cFGF23 levels.

Our study has several strengths as well as limitations. The main strength is the large prospectively followed cohort of stable RTRs in which several markers of ID as well as both iFGF23 and cFGF23 levels were measured. Moreover, end-point evaluation was complete in all participants despite a considerable follow-up period. We also acknowledge several limitations of this study. First, due to the observational status of our single center study, we cannot exclude the possibility of residual confounding. Second, a limitation is that no gold standard for the definition of ID exists.\textsuperscript{29} In the current study, the definition of ID was based on a combination of two commonly used and clinically relevant markers, namely ferritin (iron load) and TSAT (iron transport availability). To increase robustness of our findings, we performed sensitivity analyses where we used an alternative definition of ID and also assessed the association of the individual iron status parameters with mortality, and demonstrated similar results.

In conclusion, we identified that iron deficient RTRs have elevated levels of cFGF23, but not iFGF23, compared with non-iron-deficient RTRs. Importantly, ID was independently associated with mortality, and this association was to a large extent explained by variation in cFGF23 levels. Future studies are needed to unravel the complex interplay between ID, FGF23, and adverse outcomes in RTRs and other populations.

**Concise methods**

**Patient population**

We approached all RTRs (aged ≥18 years) who were at least 1 year post transplantation for participation in the current study. RTRs were approached during outpatient clinic visits between 2008 and 2011, as described previously.\textsuperscript{30} All kidney transplantations took place in the University Medical Center Groningen (Groningen, the Netherlands). Among 817 RTRs who were approached, 707 (87%) chose to participate. We excluded patients with missing data on ID (n=7), resulting in 700 RTRs eligible for analyses. All patients provided written informed consent. All study protocols were approved by the institutional review board (METc 2008/186) and adhered to the principles of the Declaration of Helsinki. The primary end point of this study was all-cause mortality. The continuous surveillance system of the outpatient program ensures up-to-date information on patient status. General practitioners or referring nephrologists were contacted in case
the status of a patient was unknown. End points were recorded until the end of May 2013 with no loss to follow-up.

**Data collection**

The measurement of clinical parameters has been described in detail previously.\(^{31}\) In brief, information on medical history and medication use was obtained from patient records. Participants’ height and weight were measured with participants wearing indoor clothing without shoes. Blood pressure was measured according to a strict protocol as previously described.\(^{31}\) Information on alcohol consumption and smoking behavior was gathered using a questionnaire. Smoking behavior was classified as never, former, or current smoker.

**Laboratory procedures**

Blood was drawn in the morning after an 8-12h overnight fast. Plasma cFGF23 (C-terminal) enzyme-linked immunosorbent assay (ELISA; Immutopics, Inc., San Clemente, CA, USA) in stored plasma samples with intra-assay CVs between 2.2 and 4.4%, and inter-assay CVs between 9 and 16%.\(^{32}\) Intact FGF23 levels were measured in stored plasma samples by ELISA (Kainos Laboratories, Inc., Tokyo, Japan) with intra-assay CVs between 5.3 and 9.7%, and inter-assay CVs between 5.7% and 14%.\(^{32}\) The cFGF23 immunometric assay uses two antibodies directed against different epitopes within the C-terminal part of FGF23 which therefore detects both the intact hormone and C-terminal cleavage products. In contrast, the iFGF23 assay detects only the intact molecule.\(^{26}\) Transferrin was measured using an immunoturbidimetric assay (Cobas c analyzer, Modular P system, Roche diagnostics, Mannheim, Germany). Serum ferritin concentrations were determined using the electrochemiluminescence immunoassay (Modular analytics E170, Roche diagnostics, Mannheim, Germany). Serum iron was measured using photometry (Modular P800 system; Roche diagnostics, Mannheim, Germany). Serum creatinine was measured using an enzymatic, isotope dilution mass spectrometry (IDMS) traceable assay on a Roche P-Modular automated analyzer (Roche diagnostics, Mannheim, Germany). TSAT (%) was calculated as 100 x serum iron (µmol/L)/ 25 x transferrin (g/L).\(^{33}\) ID was defined as TSAT <20% and ferritin <300 µg/L.\(^{34}\) Renal function was determined by estimating GFR by applying the Chronic Kidney Disease Epidemiology Collaboration equation.\(^{35}\) Proteinuria was defined as urinary protein excretion ≥0.5 g/24 h.

**Statistical analyses**

Data were analyzed using IBM SPSS software, version 22.0 (SPSS Inc., Chicago, IL), R version 3.2.3 (Vienna, Austria) and STATA 14.1 (STATA Corp., College Station, TX). Data are expressed as mean ± SD for normally distributed variables and as median (25\(^{\text{th}}\)-75\(^{\text{th}}\)
interquartile range [IQR]) for variables with a skewed distribution. Categorical data are expressed as number (percentage). We evaluated between-group differences comparing RTRs with versus without ID using Student t-test, Mann-Whitney U test, or Chi square test, as appropriate. To study the association between ID and all-cause mortality, we used Cox proportional hazards regression analysis. We performed analyses in which we first adjusted for age and sex (model 1); and additionally for eGFR, proteinuria, time since transplantation, primary renal disease, history of cardiovascular disease, and smoking status (model 2). In further models, we adjusted for iFGF23 (model 3) and cFGF23 (model 4). Due to skewed distribution, iFGF23, cFGF23, and time since transplantation were natural log-transformed. We tested for co-linearity between ID and cFGF23 by calculating a correlation coefficient and a VIF score. A correlation coefficient of $<0.7$ and a VIF$<5$ indicates no evidence for co-linearity. Potential effect modification by age, sex, eGFR, proteinuria, history of cardiovascular disease, time since transplantation, smoking status, BMI, presence of diabetes, hs-CRP, serum calcium, phosphate, PTH, use of ACE-inhibitors, use of diuretics, cFGF23, and iFGF23 concentrations were tested by fitting models containing both main effects and their cross-product terms. In sensitivity analyses, we repeated the Cox regression analysis by using an alternative, frequently used definition of ID: TSAT$<20\%$ and ferritin $<200$ µg/L. We also evaluated ID using the iron status components (i.e., TSAT and ferritin) individually. Splines of individual iron status components with all-cause mortality were fit using a Cox proportional hazards regression model based on restricted cubic splines in univariate analyses and after adjustment for cFGF23. As sensitivity analyses, we performed adjustments of the association of ID with mortality as in table 2, model 2, for serum calcium, serum PTH, use of ACE-inhibitors and AII-antagonists, hemoglobin levels, acute rejection events, presence of diabetes, use of iron supplements, and hs-CRP levels, each time in addition to existing adjustment for age, sex, eGFR, proteinuria, time since transplantation, primary renal disease and smoking status. Finally, we performed mediation analyses with the methods described by Preacher and Hayes, which is based on logistic regression. These analyses allow for testing significance and magnitude of mediation. In all analyses, a two-sided p-value $<0.05$ was considered significant.

**Simulation study**

In order to investigate the effect of differences in intra-assay CVs between cFGF23 and iFGF23 we simulated values of cFGF23 100 times. In each simulation normally distributed noise with a mean of 0 and an SD equal to 4.3% of the cFGF23 value was added to the original cFGF23 value in order to simulate an intra-assay CV between 9.5 and 10%, which is similar to that of iFGF23. The Cox regression and the mediation analyses were repeated for each of these simulated cFGF23 variables and the means of the HRs, their 95%CI, and of the proportion of mediation were calculated.
Acknowledgement
We thank Wendy Dam for measuring cFGF23 levels in the plasma samples of the cohort. Generation of the TransplantLines Food and Nutrition Biobank and Cohort Study (TxL-FN) was funded by the Top Institute Food and Nutrition. M.H.d.B. is supported by a Veni grant (Netherlands Organization for Scientific Research, grant no 016.146.014).

Statement of competing financial interests
C.A.J.M.G. received speaking fees and research funding from Vifor Pharma. The other authors have declared that no conflict of interest exists.
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