Inter-organ handling of Fibroblast Growth Factor-23 in humans

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Abstract. Fibroblast Growth Factor-23 (FGF-23) accumulates in blood of patients with chronic kidney disease (CKD) and is associated both with cardiovascular complications and disease progression. However, our knowledge of the sites and mechanisms which regulate plasma FGF-23 is still incomplete. We measured plasma intact FGF-23 across the kidney, splanchnic organs and lung in eleven patients (eGFR 60 ± 6 ml/min) during elective diagnostic cardiac catheterizations. In these patients FGF-23 was removed by the kidney, with a fractional extraction (FE) of ~22%. The FE of FGF-23 across the kidney was similar to that of creatinine (~17%, p=NS). In addition, the FGF-23 FE by the kidney was significantly directly related to eGFR (r = 0.709 p = 0.018) and to kidney creatinine FE (r=0.736 p=0.013) but only as a trend, to plasma phosphate levels (r=0.55, p=0.18). There was no difference in FGF-23 levels in blood perfusing splanchnic organs and cardiopulmonary bed. However, the arterial-venous difference of FGF-23 across the lung was directly related to FGF-23 pulmonary artery levels, suggesting that the lung, and possibly the heart, participate in the homeostasis of plasma FGF-23 when its systemic levels are increased.

Our data show that the human kidney is the only site for FGF-23 removal from blood and suggest that FGF-23 is predominantly removed by glomerular filtration. The kidney ability to remove FGF-23 from the circulation likely accounts for the early increase in blood of FGF-23 in patients with CKD.

Introduction

Fibroblast Growth Factor-23 (FGF-23) is a 32-kDa glycoprotein, bone-derived hormone which circulates as an active full-length protein and shorter, inactive fragments (1, 26, 28). Intact FGF-23 activates FGF receptors (FGFR) 1, 3, and 4 in the presence of the co-receptor Klotho. FGF-23 induces phosphaturia and inhibits the conversion of 25 (OH) vitamin D to its active form (21). Circulating FGF-23 rises early, before phosphate, parathyroid hormone, and 1,25 (OH) vitamin D
alterations, and progressively accumulates in blood of patients with chronic kidney disease (CKD) (18). On one hand the increase in circulating FGF-23 is considered to play a major role in the maintenance of the homeostasis of plasma phosphate in CKD (18, 33); on the other, its blood increase appears to be maladaptive, since it is associated with disease progression (2), left ventricular hypertrophy (5, 8), and excess mortality in hemodialysis patients (9,10). In addition, it is increasingly being recognized that the measure FGF-23 adds value to risk assessments for CKD development and cardiovascular complications (11,16, 29).

While the mechanisms by which FGF-23 might accelerate the progression of CKD are still elusive, those underlying its effects on left ventricular hypertrophy include the activation of a FGFR 4-dependent pathway in cardiac myocytes (5). The reason(s) why FGF-23 accumulates in blood of CKD patients are still a matter of discussion (21). Because FGF-23 is constantly produced and exported by the osteocytes, it must also be constantly cleared for plasma levels to remain within 30% of baseline values, as they do in healthy human subjects (4). However, sites and mechanisms which maintain plasma FGF-23 levels are not completely known. Available data support the concept that the control of FGF-23 production in bone and its plasma homeostasis is a multi-level process, with some influences affecting FGF-23 transcription and some post-translational modification of the secreted, bioactive protein (4). Additionally, the actions of FGF-23 on its target tissues via its co-receptor Klotho, are subject to regulatory events (4). The mechanisms which have been suggested to account for the increase of FGF-23 in blood of patients with CKD include hyperphosphatemia and the uremia-associated decrease in FGF-23 receptors (15, 25). These mechanisms imply accelerated production/export from the osteocyte to the plasma compartment and/or a decrease in FGF-23 renal clearance. Otherwise, extra-renal routes of elimination and/or extra-skeletal sites of FGF-23 synthesis may be important determinants in the control of FGF-23 levels in CKD. With this regard, the expression of FGF-23 has also been reported in the liver, thymus, lymph nodes, spleen, small intestine, kidney, and heart, even if at a low level (28).
FGF-23 has most receptors in the kidney, gut, parathyroid and heart (17, 20), suggesting that these organs play a major role in the regulation of its plasma levels. Consistent with the hypothesis that the human kidney is a major site for the regulation of plasma FGF-23, serum FGF-23 levels are inversely correlated with GFR in CKD patients with eGFR<70 ml/min (21). However, the molecular weight of FGF-23 renders unlikely the possibility that glomerular filtration rate plays a major role in its removal. In addition, although FGF-23 can be detected in human urine, urinary excretion appears to play a minor role in its regulation (21). In addition, both residual clearance by the kidney and FGF-23 removal by dialysis do not appear to modify serum FGF-23 to a significant extent in patients with ESRD (21). In spite of these findings, the measure of FGF-23 in renal artery and vein of rodents has recently shown a substantial, unexpected, renal single pass extraction of FGF-23 (~40%) (24), suggesting that the kidney is able to clear large amounts of FGF-23 from blood. In addition, albeit to a lower extent, a removal of FGF-23 by the kidney has been very recently shown to occur also in humans (31).

In this study, to provide a better understanding of sites and mechanisms which regulate FGF-23 metabolism in humans, we measured plasma intact FGF-23 across the kidney and other major organ systems in patients undergoing a cardiac catheterization. Our results show that the human kidney plays a unique role in the removal of FGF-23 from circulation and in the control of plasma FGF-23 levels.

Materials and Methods

Patients and Procedures

Eleven patients (5 males, 6 females, median age 72, range 63-82 years) who were scheduled for elective cardiac catheterization for hemodynamic evaluation or the assessment of coronary heart disease were eligible for enrolment in this protocol at the Department of Internal Medicine, University of Genoa. The study was part of a larger protocol on the study of inter-organ substrate
exchange (7). Most patients were affected by hypertension or valvular disease (Table 1). The patients were enrolled in the study on a consecutive basis if they met the following exclusion criteria: NYHA Class III-IV congestive heart failure, a recent myocardial infarction or pregnancy (7). No subject had evidence of liver disease. Patients were moderately overweight. Their mean eGFR (19) was 60±6 ml/min .1.73 m² (range 31-94). Mean uric acid levels were moderately increased. Blood glucose, albumin, cholesterol, triglycerides, phosphate, calcium, and serum electrolytes were in the normal range. Patients were taking drugs, including antihypertensive drugs, statin, RAS inhibitors, sodium bicarbonate, which were prescribed as appropriate for each individual. The study was approved by the Ethical Committee of the Department of Internal Medicine of the University of Genoa. All subjects were informed about the nature, purposes, procedures, and possible risks of the study, before their informed consent was obtained. The procedures were in accordance with the Helsinki declaration.

The patients were studied in the postabsorptive state. Three sets of blood samples were obtained at ~10-min intervals from the femoral artery as well as from the renal veins, the hepatic veins and the pulmonary artery. The correct position of the catheter in the renal or liver vein was verified by calculating the extraction of creatinine, oxygen, glucose and lactate. FGF-23 was measured in all 10 min interval samples and the results averaged.

Blood samples were collected in cooled ethylenediaminetetraacetate tubes added with aprotinin, which were immediately centrifuged at 6000 rpm for 10 min at +4 °C. Plasma was quickly separated from blood cells and stored at −80° C until assayed. Plasma intact FGF-23 levels were determined using an ELISA assay (Endo Millipore, Darmstadt, Germany) according to the manufacturer's protocol. This assay measures intact FGF-23 by utilising a two-step format, since it employs a polyclonal goat anti-FGF23 capture antibody and biotinylated polyclonal goat anti-FGF23 antibody to which streptavidin-horseradish peroxidase (HRP) conjugate is added. Antibody epitope mapping data are not currently available. For this assay the reported limit of sensitivity is 3.5 pg/ml, and the reported appropriate range from 9.9 pg/ml to 2400 pg/ml. In our laboratory, the lowest limit of detection was 4
pg/ml with an intra-assay coefficient of variation of 5.1 and 7.6 % at 10 and 40 pg/ml, respectively. To minimize variability, samples arterial and venous blood were all run in triplicate. All samples from one individual were always run in the same batch. Blood pH, pO2, pCO2, glucose, lactate and creatinine were measured with an ABL800 Flex apparatus (Radiometer, Copenhagen, Denmark).

Calculations and statistical methods

The A-V difference of FGF-23 across the splanchnic organs, kidney and heart/lung was calculated as: [A] – [V], where [A] and [V] are the concentrations of metabolites in arterial and venous plasma. As for the cardio-pulmonary circulation, the A-V differences were calculated as Pulmonary Artery-Arterial systemic differences. Fractional extraction (FE) across the kidney were calculated as 100 x ([A] – [V])/[A]. Statistical analysis was performed using Wilcoxon matched-pairs signed-ranks test to compare arterial data with venous data (Statview Statistical Package, Abacus, Berkeley, CA, USA). The reported p values are based on a two-tailed calculation. Spearman r correlation was employed to evaluate the relation between two variables. A p value of <0.05 was considered statistically significant. All data are expressed as means ± SEM.

Results

Handling of FGF-23 by the kidney, splanchnic organs and cardiopulmonary bed

Individual arterial and venous levels of FGF-23, as well as its arterial-venous differences across the kidney, splanchnic organs and cardiopulmonary bed are reported in Table 1 2. In accordance with the moderate impairment of GFR shown by patients studied here, their arterial FGF-23 levels were still in the normal range (10, 12, 29). Renal vein FGF-23 concentrations were remarkably lower (by ~22 %) than the corresponding arterial values, indicating that plasma FGF-23 decreases substantially after a single pass across the kidney. The FE of FGF-23 across the kidney
was similar (p=NS) to that of creatinine. FGF-23 level in the liver veins was quite similar to that of arterial FGF-23.

The FGF-23 FE by the kidney was significantly directly related to eGFR (r = 0.709 p = 0.018) (Figure 1A) and to kidney creatinine fractional extraction (FE) (r = 0.736, p = 0.013) but only as a trend, to plasma phosphate levels (r = 0.55, p = 0.18). The ratio of FGF-23 to creatinine FE across the kidney was also directly related to eGFR (r = 0.691, p = 0.023) (Figure 1B).

Although 10% lower, arterial FGF-23 levels were not statistically different from systemic venous (pulmonary artery) whole body levels. However, a direct association was observed between systemic venous FGF-23 levels and the arterial-venous difference of FGF-23 across the cardiopulmonary circulation (Figure 2).

**DISCUSSION**

The aim of this study was to learn more about the sites and mechanisms which regulate plasma levels of FGF-23 in humans. To this end, we measured FGF-23 concentrations in venous effluents from different organs, including the kidney, the splanchnic organs and the cardiopulmonary bed, as well as in arterial blood, in a cohort of patients undergoing a right-sided cardiac catheterization. These specific measures were chosen in light of the evidence derived from studies in animals showing a role of the kidney and also extra-renal sites, such as the gut and the heart, in the action and metabolism of FGF-23.

Three major observations are made from this study. First, somewhat unexpectedly, but in accordance with a previous study (31), the single-pass extraction of FGF-23 across the human kidney is high. Second, as a new finding, the FE of FGF-23 is related to eGFR and to the FE of creatinine, suggesting that FGF-23 is predominantly removed by glomerular filtration. Third, our
study also shows that the kidney is the only site for the disposal of FGF-23 in humans, a finding which accounts for the early increase in blood of FGF-23 in patients with CKD.

In the cohort of patients studied here, who were mainly made of elderly subjects with a moderate impairment of kidney function, the extraction of FGF-23 by the kidney represented ~22% of its arterial plasma concentration, a figure which is lower than that recently reported in the rodent kidney (24), and close to the value of 17% recently reported by van Ballegooijen et al. (31). As a new finding, we observed that renal FGF-23 FE is directly related to eGFR. In addition, when the relationship between renal FGF-23 FE and eGFR is examined (Figure 1A), it appears that the renal FGF-23 FE drops from ~45% to values close to zero when eGFR declines from ∼90 to ∼30 ml/min. As a matter of fact the FGF-23 FE is even greater (ratio ~2) than creatinine FE when eGFR is ∼90 ml/min, but declines steeply along with the decrease in eGFR (Figure 1B). Therefore, our data are in accordance with the hypothesis that in patients with CKD the decrease in renal FGF-23 removal is the major mechanism causing the early increase in FGF-23 levels (18).

How can circulating FGF-23 reach its target sites, the proximal and distal tubules? The most obvious explanation is that FGF-23 is filtered across the glomerular membrane. On the average, in our study the FGF-23 FE across the kidney was similar to the FE of creatinine, suggesting that a similar mechanism accounts for the removal of these molecules. Data presented here are also in accordance with the major role that the human kidney plays in plasma turnover of small proteins, including enzymes, immunoproteins, and peptide-hormones (6, 23). However, FGF-23 has a molecular mass of 32 kDa, which is in the upper limits of the filtration range of normal glomeruli. In the rat glomeruli, the sieving coefficient of molecules such as myoglobin (m.w. 19.6 kDa) and κ-dimer (m.w. 40 kDa) is 0.72 and 0.15, respectively (22). So one would expect very low sieving coefficient for FGF-23. Alternatively, some researchers have proposed that the glomerular filtration barrier is more permeable to plasma proteins than previously thought (14).
Besides glomerular filtration and apical tubule cell uptake, an alternative mechanism of cellular uptake is via the basolateral surface in distal tubule cells, as an escape mechanisms known to provide metabolic substrates to cells when luminal substrate delivery is decreased (27). As a matter of fact, the precise segments of the kidney and receptors that mediate the renal response to FGF-23 are not entirely clear (21). The highest expression of Klotho/FGFR complexes is in the distal tubule, whereas the major biologic effects of FGF-23 are in the proximal tubule (14, 21). In addition, in the distal tubule Klotho is mainly expressed in the cytoplasm, with scarce apical distribution (15, 20).

In humans FGF-23 has a short half life (58 min for the intact assay) (14), which implicates a fast removal from blood. The exact contribution of the human kidney to the FGF-23 removal could not be exactly quantitated in the present study, since the measure of blood flow was not feasible. One could estimate, considering an average kidney plasma flow of 400 to 500 ml/min in patients studied here, that the removal of FGF-23 by the kidney corresponds to ~2000 and ~2500 µg/day, respectively. According to these findings, the extracellular FGF-23 pools (~ 220 µg, considering a plasma FGF-23 concentration of 16 pg/ml and extracellular fluid 14 liters in a 70 kg man), are renewed by the kidney removal 10-12 times a day.

In this study, we were not able to demonstrate a net removal of FGF-23 by splanchnic organs and the cardiopulmonary bed. On one hand, this is in accordance with the low expression of Klotho in lung, liver, spleen, small intestines (17). On the other hand, these findings may represent some inaccuracies in the methods currently available to detect small arterial-venous differences of FGF-23, which can be typical of conditions of high blood flow. However, we observed a positive arterial-venous difference of FGF-23 across the lung circulation in eight out of the eleven subjects studied. In addition, the FGF-23 lung arterial-venous difference was directly related to the pulmonary artery FGF-23 levels (Figure 2), which might suggest that lung/heart play a role in the maintenance of FGF-23 levels when its systemic levels are raised. If so, this would be a more
accommodative than fully compensatory response that is not able to prevent FGF-23 pools from increase largely in CKD, and is also associated with cardiac complications. FGF-23 directly induces left ventricular hypertrophy in normal mice and stimulates hypertrophy of isolated cardiac myocytes, suggesting that the heart is a target tissue for FGF-23 action (5). However, less is known on the FGF-23/Klotho system in the lung. The lung has been recently recognized as an extra-renal target site for FGF-23 mediated regulation of vitamin D (3); in addition Klotho gene expression is essential to maintain pulmonary integrity during postnatal life (30).

Current ELISA kits for plasma intact FGF23 measurement show poor analytical agreement (29). A possible limitation of our study is that we used for the measure of intact FGF-23 a recently marketed assay, for which few clinical studies are available. Despite the patients studied here had reduced eGFR, their concentrations of arterial intact FGF-23 were in the low range of reported normal values for other assays. There are large variations in the FGF-23 results according to the assay used (29). Formally defined reference intervals for plasma FGF-23 concentrations have been described in adult populations using Immutopics and Kainos (12, 29) kits, but not for the Millipore assay.

Another potential limitation of this work is that the results obtained in this cohort of elderly patients could express the combined events occurring in aging, atherosclerosis and cardiopulmonary comorbidities, conditions which per se are potentially associated with altered FGF-23 metabolism. Therefore, the results obtained here are not necessarily representing physiology. In addition, the net balance data obtained from a simple arterial-venous measurement can result from a variety of different rates of uptake and release. Therefore, zero net balance does not imply that uptake and release are also zero, but rather that their difference is such. Although unlikely, since FGF-23 is mainly produced by osteocyte, this means that a given organ still produces and utilizes FGF-23, but at comparable rates.
In conclusion, the present study provides the first report of FGF-23 handling measured simultaneously across the human kidney and major organ systems in humans. Our data demonstrate that the human kidney is the only site for FGF-23 removal. Besides providing a better understanding of pathophysiology of FGF-23 metabolism, the data reported in this study could be useful to understand the alterations in FGF-23 that are observed in CKD and many systemic and organ diseases.

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Disclosures

There is no information to disclose.

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disease, but does not change in response to variation in phosphate intake in healthy


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*Captions to Figures*
Figure 1. Relationships between (A) the kidney FGF-23 fractional extraction (FE) and (B) the kidney FGF-23/creatinine FE ratio and estimated GFR (eGFR) (n=11; ○ = males, ● = females).

Figure 2. Relationship between the FGF-23 Arterial-Venous difference across the lung and systemic venous (pulmonary artery) FGF-23 levels (n=11; ○ = males, ● = females).
Table 1. Clinical characteristics of patients.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
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<tr>
<td>Number of subjects</td>
<td>11</td>
</tr>
<tr>
<td>Age (years)</td>
<td>72 ± 2</td>
</tr>
<tr>
<td>Gender (M/F)</td>
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</tr>
<tr>
<td>BMI (Kg/m²)</td>
<td>27 ± 2</td>
</tr>
<tr>
<td>Estimated GFR (ml/min 1.73 m²)</td>
<td>60 ± 6</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Haemoglobin (g/dl)</td>
<td>13 ± 0.4</td>
</tr>
<tr>
<td>Phosphate (mg/dl)</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>Calcium (mg/dl)</td>
<td>9.4 ± 0.1</td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>103 ± 9</td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>3.9 ± 0.1</td>
</tr>
<tr>
<td>Cholesterol (mg/dl)</td>
<td>168 ± 12</td>
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<tr>
<td>Triglycerides (mg/dl)</td>
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<tr>
<td>Uric acid (mg/dl)</td>
<td>7.6 ± 0.6</td>
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<tr>
<td>Na⁺ (mEq/L)</td>
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<tr>
<td>K⁺ (mEq/L)</td>
<td>4.0 ± 0.1</td>
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<tr>
<td>Valvular disease</td>
<td>6/11</td>
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<td>Type II diabetes</td>
<td>2/11</td>
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<tr>
<td>RAS blockers</td>
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Data are mean ± SEM. Abbreviations: BMI= Body Mass Index, GFR= Glomerular Filtration Rate.
Table 2. Arterial and Venous FGF-23 across the kidney, splanchnic organs and lung and FGF-23 fractional extraction across the kidney

<table>
<thead>
<tr>
<th>Subject</th>
<th>Gender (M/F)</th>
<th>eGFR (ml/min. 1.73m²)</th>
<th>Arterial FGF-23 (pg/ml)</th>
<th>Renal vein FGF-23 (pg/ml)</th>
<th>FGF-23 Kidney A-V (pg/ml)</th>
<th>FGF-23 kidney FE (%)</th>
<th>Creatinine kidney FE (%)</th>
<th>Liver vein FGF-23 (pg/ml)</th>
<th>FGF-23 Splanchnic A-V (pg/ml)</th>
<th>FGF-23 Lung artery (pg/ml)</th>
<th>FGF-23 Lung V-A (pg/ml)</th>
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<td>1</td>
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<td>+38.1</td>
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<td>19.8</td>
<td>-5.4</td>
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<td>2</td>
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<td>24.2</td>
<td>+8.5</td>
<td>34.7</td>
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</table>

Mean± s.e.m 60 ± 6 16.6 ± 2.1 13 ± 1.9* +3.5 ± 0.8 +21.9 ± 5.2 +17.4 ± 2.0 16.1 ± 1.5 +0.5 ± 1.6 19.0 ± 2.5 +2.5 ± 1.6

The Arterio-Venous (A-V) difference of FGF-23 across the kidney and splanchnic organs was calculated as: [A] – [V], where [A] and [V] are the concentrations of metabolites in arterial and venous plasma. As for the cardio-pulmonary circulation, the differences were calculated as (V-A) Pulmonary Artery-Arterial systemic differences. A positive sign means uptake, while a negative sign means release. FE= fractional extraction; FGF-23= fibroblast growth factor 23; *p<0.01 Artery vs. Vein.
\[ y = 4,6224 \ln(x) - 23,969 \]

\[ R^2 = 0.817 \]

FGF-23 Arterio-venous difference across the lung (pg/ml)

Pulmonary artery FGF-23 (pg/ml)

\[ r = 0.67 \quad p = 0.03 \]