Levels of the erythropoietin-responsive hormone erythroferrone in mice and humans with chronic kidney disease

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SUPPLEMENT:

Supplemental Table 1: Characteristics of the non-dialysis and dialysis-dependent CKD cohorts. Data are presented as percentages, mean ± standard deviation, or median (interquartile range). eGFR: estimated glomerular filtration rate, rhEPO: recombinant human erythropoietin.

Supplemental Figure 1: Serum erythroferrone concentrations in humans. Random serum erythroferrone concentrations were measured in adult and pediatric subjects with normal kidney function (non-CKD), non-dialysis CKD (non-end stage kidney disease), and end stage kidney disease (on dialysis). Figure 1A shows adult and pediatric subjects combined, and Figure 1B shows adult and pediatric subjects separated. * denotes a statistically significant ($p<0.05$) pairwise comparison versus the non-CKD group. # denotes a statistically significant ($p<0.05$) pairwise comparison versus the non-dialysis CKD group. Data are presented as medians and interquartile ranges, with box plot whiskers representing the 10th and 90th percentiles.

Complete Methods:

Mouse studies

Experiments were conducted in accordance with UCLA Division of Laboratory Animal Medicine guidelines, and the study protocol was approved by the UCLA Office of Animal Research Oversight. Mice were housed at UCLA, in standard cages with wood chip bedding that was changed twice weekly. Animal housing rooms were temperature and humidity controlled, with a 12-hour light cycle.

Mouse models and experiments

Wild type C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were used for experiments assessing the effects of a single rhEPO dose on ERFE. Mouse diets were from Harlan Teklad (Indianapolis, IN), and contained 50 ppm iron. For groups of mice in which CKD was induced, the diets also contained 0.2% w/w adenine [1-3]. Diets were started at 4-6 weeks of age, and provided ad libitum. Mice remained on the diets for ~5 weeks, then the experiments were conducted. Groups of mice, with and without CKD, received a single intraperitoneal dose of 40 µg/kg (~70 units/gram) rhEPO (BioLegend, San Diego, CA) or saline and were euthanized 6, 24, or 48 hours post-injection. Separate groups that received no injections provided baseline data. At the time of euthanasia, we collected serum for urea nitrogen, ERFE, hepcidin, and iron measurements.

Mouse biochemical parameters

Colorimetric methods were used to assay serum urea nitrogen (BioAssay Systems, Hayward, CA) and iron (Genzyme, Cambridge, MA). Serum erythroferrone and hepcidin levels were measured by ELISA, as previously described [4,5]. Liver *Saa1* mRNA expression, normalized to hypoxanthine-guanine phosphoribosyltransferase (*Hprt*), was assessed as a measure of inflammation.

Human studies

To assess ERFE levels across the spectrum of chronic kidney disease (CKD), we assembled the following three cohorts: healthy patients without CKD, non-dialysis CKD patients (estimated glomerular filtration rate (eGFR) <90 ml/min/1.73m^2), and dialysis-dependent CKD patients. Each cohort included both adult and pediatric patients. We recruited healthy adult and pediatric patients from UCLA-affiliated medical practices, adult and pediatric non-dialysis CKD patients from UCLA general nephrology clinics, and adult and pediatric dialysis patients from UCLA-affiliated Davita dialysis centers. The study was approved by the UCLA Institutional Review

Board, and was performed in accordance with the Declaration of Helsinki. Informed consent was obtained from adult subjects, and informed consent and assent were obtained from parents and pediatric subjects, respectively.

Exclusion criteria were: (1) previously diagnosed non-renal cause of anemia; (2) evidence of active bleeding; (3) blood transfusion within four months of enrollment; (4) history of malignancy, end-stage liver disease, or chronic hypoxia; and (5) hospitalization or infection requiring antibiotics within four weeks of enrollment.

Following inclusion, we obtained demographic data and medication history from the medical records. Dialysis patients receiving rhEPO were on stable doses for at least four weeks prior to study enrollment, and all rhEPO administered was epoetin alfa (Epogen®, Amgen). We collected whole blood and serum from all enrolled subjects. Blood was drawn from dialysis patients at the beginning of their hemodialysis treatments, prior to rhEPO administration. This cohort was first assembled and characterized as part of a cross-sectional assessment of circulating hepcidin levels across the CKD spectrum [6].

Human biochemical parameters

Complete blood counts, including hemoglobin measurements (Sysmex automated hematology analyzer), were performed. In the serum samples, we measured the following parameters: EPO (ELISA, R&D Systems, Minneapolis, MN), ERFE (ELISA, used as previously described [7]), hepcidin (ELISA, used as previously described [8]), iron and total iron binding capacity (Roche cobas® 8000 analyzer Ferrozine colorimetric assay), ferritin (Roche cobas® 8000 analyzer electrochemiluminescence assay), and high sensitivity C-reactive protein (CRP; CardioPhase® hsCRP assay, Dade Behring, Deerfield, IL). Transferrin saturation (TSAT) was calculated by iron divided by total iron binding capacity. In adult patients, eGFR was calculated with the

Modification of Diet in Renal Disease (MDRD) Study equation [9]. In pediatric patients, eGFR was calculated with the revised Schwartz equation [10].

Statistical analysis

Statistical analysis was performed using SigmaPlot 12.5 (San Jose, CA). For the mouse studies, we used the Kruskal-Wallis ANOVA on ranks, with Dunn post-hoc multiple comparison pairwise testing versus the baseline group. At each time point, rank sum tests were used to compare EPO-treated groups to saline-treated groups. To compare ERFE levels among the non-CKD, non-dialysis CKD, and dialysis human cohorts, we used the Kruskal-Wallis ANOVA on ranks, with Dunn post-hoc multiple comparison pairwise testing. Lastly, to assess associations among erythropoietin, ERFE, and hepcidin in the CKD subjects, we used Pearson correlation coefficients.

Supplement References:

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Supplemental Table 1

Parameter	n	Non-dialysis CKD Cohort	n	Dialysis-dependent CKD Cohort
Sex	51	53% male, 47% female	97	67% male, 33% female
Age Group	51	47% pediatric, 53% adult	97	35% pediatric, 65% adult
Age (years)	51	41 ± 28	97	43 ± 26
eGFR (ml/min/1.73m^2)	46	37 ± 18	n/a	n/a
Erythropoietin (mIU/ml)	51	10.7 (7.2, 16.5)	n/a	n/a
Weekly rhEPO dose/kg	n/a	n/a	85	228 (122, 408)
Erythroferrone (ng/ml)	51	6.8 (2.3, 17.1)	97	15.7 (7.9, 32.5)
Hepcidin (ng/ml)	47	183 (80, 290)	94	546 (220, 775)
Iron (mcg/dl)	51	73 ± 40	93	75 ± 39
Transferrin Saturation (%)	50	22 ± 9	92	35 ± 16
Ferritin (ng/ml)	50	72 (34, 155)	94	635 (294, 959)
Hemoglobin (g/dl)	49	12.1 ± 1.7	97	11.9 ± 1.5
Mean Corpuscular Volume (fl)	49	87 ± 5	97	100 ± 73
C-reactive Protein (mg/l)	50	1.9 (0.8, 7.5)	81	3.2 (1.0, 7.2)

Supplemental Figure 1