Rapid elevation of transferrin saturation and serum hepcidin concentration in hemodialysis patients after intravenous iron infusion

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Supplemental Material and Methods

Patients and study design

The patients included were formally informed and agreed to participate in the study. The protocol was approved by the local hospital committees in agreement with the human rights legislation from the Declaration of Helsinki. Patients underwent dialysis with fiber super high-flux dialyzers (NIKKISO-Medical GmbH, Hamburg, Germany) or capillary low-flux dialyzers (Xenium-LF, Baxter Hellas, Athens, Greece) with γ-sterilized biocompatible polyethersulfone membrane. Dialyzers were heparinized with low molecular-weight heparin as anticoagulant. Patients received an infusion of iron sucrose (Venofer, Vifor France SA, France) during the last 30 minutes of hemodialysis session. Samples were collected 15 min before the initiation and 15 min after the termination of iron administration. In time-course experiments, samples were collected at different time points after iron administration (0, 1, 2, 3, 5, 7, and 9 hours).

Whole blood samples were collected and a part was used for hematological analysis while the rest was used for isolation of serum and standardized routine laboratory analyses. A part of serum was frozen at -20 ^{0}C until further analysis for Tf-Sat by U-PAGE and hepcidin concentration by ELISA.

Estimation of Tf-Sat(%) by U-PAGE

Different transferrin forms were separated and visualized by using a slightly modified methodology, the U-PAGE that was previously described by Makey and Seal.[1] This methodology separates transferrin into its apo-form, the two monoferric C- and N-forms, and the diferric form, according to their electrophoretic mobilities in 6% polyacrylamide gels containing high concentration of urea (6 M). The proteins in the gel were stained with Coomassie brilliant blue R-250 and densitometric image analysis was performed by Quantity One® 1-D analysis software (Bio-Rad Laboratories, CA, US). The relative amount of transferrin saturation was calculated by using the following equation:

$$\text{Tf-Sat}(\%) = [(\text{monoferric} + 2 \times \text{diferric})\text{-band densities}] \times 100 / 2 \times [(\text{apo} + \text{monoferric} + \text{diferric})\text{-band densities}]$$

Serum transferrin forms could be efficiently separated in U-PAGE gels on the condition that the balk of the rest of serum proteins should be precipitated by incubating the samples with Rivanol.

Serum hepcidin measurement

Quantification of secreted hepcidin levels in sera was performed blindly by competitive ELISA, as previously described.[2] Each well was coated with purified recombinant Hepcidin25-His (0.5 μg/ml, at 4ºC for 16 hours). Diluted patient sera (1/8) were incubated with the polyclonal antibody, for 16 h at 4ºC. The complexes formed were added to the coated wells and incubated for 1 h at 37ºC. A goat anti-rabbit, IgG-horseradish peroxidase conjugate was added and the mixture was incubated at 37°C for 1 h before the addition of tetramethylbenzidine (TMB) reagent. Optical densities were measured by a microplate reader at 450 nm.

Statistical analysis

All data are presented as mean ± SD. The paired Mann-Whitney U-Test Student's t-test was used to assess differences between the various parameters before and after dialysis. The association between different parameters was calculated using a nonparametric Spearman rank correlation.

References

1. Makey DG, Seal US. The detection of four molecular forms of human transferrin during the iron binding process. Biochim Biophys Acta. 1976;453(1):250-6.

2. Koliaraki V, Marinou M, Vassilakopoulos TP, Vavourakis E, Tsochatzis E, Pangalis GA, et al. A novel immunological assay for hepcidin quantification in human serum. PLoS One. 2009;4(2):e4581.

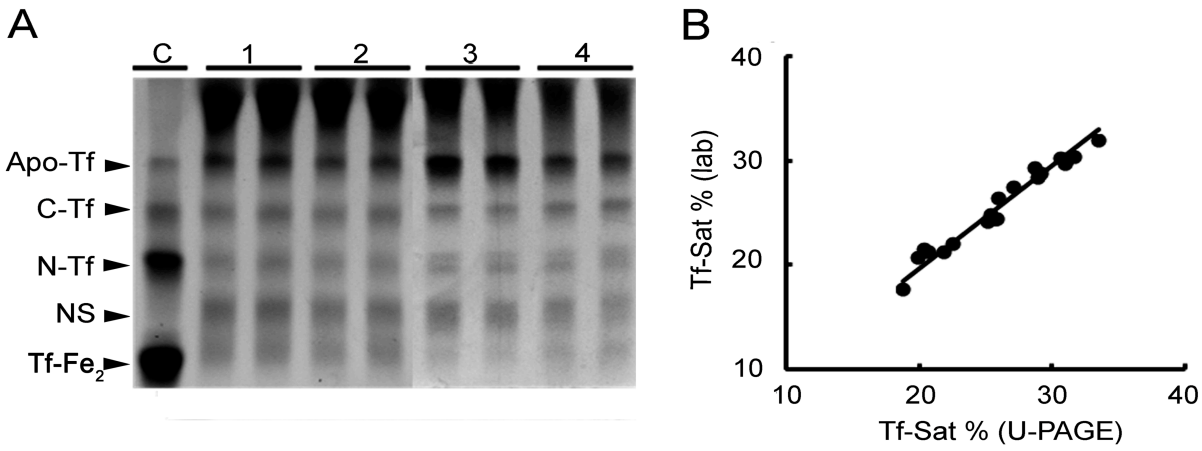
Supplementary Figure legend

Supplementary Figure S1. *Estimation of serum Tf-Sat by urea-PAGE (U-PAGE) and a routine laboratory method.* (A) Representative gel of duplicate serum samples obtained from four healthy individuals, after U-PAGE analysis and Coomassie staining (Double lanes 1, 2, 3 and 4). Purified human Tf incubated with ferric ammonium citrate (30 minutes at 37 ^{0}C) was used as a marker, indicating the different Tf-forms (lane c). (B) Correlation of Tf-Sat determined by a laboratory and U-PAGE assay in the same samples ($r=0.982$; $p < 0.0001$, $n = 20$). Apo-Tf, apotransferrin; C-Tf, C-terminal monoferric-Tf; N-Tf, N-terminal monoferric-Tf; NS, a non-specific protein; Tf-Fe_2, diferric-Tf.

Supplementary Table legend

Supplementary Table S1. *Basal characteristics of the HD patients examined in figure 2.*

Supplementary Figure S1.



Supplementary Table S1.

Patient No	Age (years)	Dialysis (years)	Comorbidity
1	48	1	chronic glomerulonephritis
2	62	17	chronic glomerulonephritis
3	59	7	chronic glomerulonephritis
4	57	6	diabetic nephropathy
5	82	2	hypertensive nephrosclerosis
6	80	5	diabetic nephropathy