Sickle cell disease
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Chapter 6

Urinary zinc loss in sickle cell disease primarily due to increased bone degradation

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Zinc deficiency is common in patients with sickle cell disease (SCD). To investigate mechanisms leading to this deficiency, we studied relations between urinary zinc levels, adjusted for urinary creatinine, urinary markers of bone resorption, urinary markers of renal damage and plasma markers of hemolysis in two cohorts of sickle cell patients. In the primary cohort, urinary zinc levels were higher in steady state sickle cell patients (1.80 (IQR 1.49-2.39) µmol/mmol; n=38) as compared to healthy controls (0.84 (IQR 0.72-1.13) µmol/mmol; n=25, *P* <0.001) and showed a further increment during painful crisis (3.15 (IQR 1.74-4.14) µmol/mmol; n=27, *P* =0.004). During painful crisis urinary zinc levels were related to urinary levels of bone degradation marker pyridinoline (PYD), but not deoxypyridinolone (DPD). Urinary zinc levels were not related to serum zinc levels or markers of hemolysis. Increased urinary excretion of zinc in steady state patients with further increments during painful crisis were confirmed in the second cohort of patients. In the second cohort of sickle cell patients, a gradual increment of urinary zinc levels during subsequent days of painful crisis was observed. Urinary zinc levels during painful crisis in this cohort were related to both PYD and DPD. No correlations with tubular damage markers Kidney Injury Molecule-1 or Heart-type Fatty Acid Binding Protein were found. In conclusion, ongoing bone degradation due to (a-)symptomatic vaso-occlusive ischemia leads to increased release of zinc from bones. Together with the reduced concentrating capacity of the kidneys in SCD, this results in zinc loss in sickle cell patients.
INTRODUCTION

Sickle cell disease (SCD) is a common inherited hemoglobinopathy with the highest incidence in people of African descent. SCD is characterized by chronic hemolytic anemia and (recurrent) vaso-occlusive events, resulting in ischemic bone degradation and other organ complications such as nephropathy. SCD is associated with zinc deficiency, which can be found in 15-80% of steady state sickle cell patients. Renal loss of zinc seems to increase further during painful crisis. Zinc is a vital component of many metalloproteinases and is required for cellular structure and metabolism and has been demonstrated to be essential for normal growth, sexual development and function of the immune system. Interestingly, chronic zinc deficiency has been related to an increased inflammatory state. Zinc deficiency in sickle cell patients may result in reduced growth and sexual development and increased susceptibility to infections. Correction of zinc deficiency seems to improve growth in children with SCD and reduces the incidence of infections in these patients. Previous studies indicate that zinc deficiency in patients with SCD is caused by increased urinary zinc loss. The exact pathogenesis of this urinary loss is unclear. Renal loss due to tubular injury as a result of chronic oxidative stress and inflammation, especially during painful crisis with increased ischemia-reperfusion damage, has been suggested to limit tubular zinc resorption, leading to increased renal loss of zinc in SCD. Others have suggested that an altered tubular handling of zinc is the main cause of urinary zinc loss in SCD. Alternatively, increased bone degradation due to recurrent bone ischemia, especially during painful crisis, may also contribute to the increased urinary zinc loss in sickle cell patients since bone is the primary site of zinc storage in the human body. Furthermore, ongoing hemolysis in SCD could also contribute to the increased zinc loss in SCD as 75% of the zinc content in the circulation is found in erythrocytes.

The aim of the current study was to investigate whether zinc deficiency in SCD is related to ischemia-induced increased bone degradation, chronic intravascular hemolysis or disturbed renal handling of zinc. Therefore, we analyzed urinary levels of zinc in relation to markers of bone degradation, hemolysis and renal tubular damage in sickle cell patients in steady state and during painful crisis.
METHODS

The study was designed as a cross-sectional cohort study and executed in 2 cohorts of sickle cell patients and a supplementary cohort of healthy controls. In the primary cohort, urinary zinc levels were determined in patients with SCD in steady state and during painful crisis and compared to levels in healthy controls. Urinary zinc levels in this cohort were related to markers of hemolysis and bone resorption. In the secondary cohort, changes in urinary zinc levels in sickle cell patients during painful crisis were evaluated longitudinally and urinary zinc levels were analyzed in relation to markers of renal tubular damage and bone degradation. In a third cohort with healthy volunteers the effect of sodium-chloride (NaCl) infusion on the fractional excretion of urinary sodium and zinc in healthy volunteers was assessed.

Inclusion criteria were: patients ≥ 18 years with sickle cell anemia (HbSS) or compound heterozygous states HbSβ0-thalassemia (HbSβ0-thal), HbSβ+thalassaemia (HbSβ+-thal) and sickle-hemoglobin C (HbSC) as confirmed by high-performance liquid chromatography (HPLC) and/or electrophoresis in combination with determination of erythrocyte mean corpuscular volume. Exclusion criteria were: inflammatory auto-immune diseases, active infection, pregnancy and blood transfusion within 3 months prior to inclusion.

Patients

Primary cohort

Consecutive adult sickle cell patients in steady state visiting the out-patient clinic or patients admitted with painful crisis at the Academic Medical Center or Slotervaart Hospital (Amsterdam, The Netherlands) were approached for participation. Healthy race and age-matched volunteers with normal HbAA genotype were included as controls. Blood and urinary samples during painful crisis were obtained the morning following hospital admission.

Secondary cohort

In the secondary cohort, patients with SCD admitted for painful crisis were included and urinary samples were obtained in the morning of the following days during admission. On the first morning after admission also 24-hour urine collection was started. Patients were allowed to participate in this study for a maximum of two
times. All admitted patients were seen at the out-patient clinic at least 4 weeks after discharge for collection of control samples in steady state.

**Third cohort with healthy volunteers**

To exclude the possibility that zinc loss during painful crisis is a result of the saline infusion that patients with SCD admitted with painful crisis receive (3 liters 0.9% NaCl/24 hours), we also determined urinary sodium and zinc levels in samples from healthy volunteers that were loaded with intravenously administered hypertonic 2.7% NaCl (5 mmol sodium/liter body water) administered in 30 minutes.

From all patients a written informed consent was required before any study procedure was carried out. All protocols were approved by the Medical Ethical Committees of participating centers and conducted in agreement with the Helsinki declaration.

**Laboratory analysis**

Blood samples were taken by venipuncture of the antecubital vein. Blood vials were centrifuged once or twice (citrated plasma) at 4°C for 15 minutes at 3000 x g and stored in small aliquots at -80°C until further analysis. Hematology parameters and hemolysis parameters were measured in EDTA-anticoagulated and heparinized plasma respectively. Serum free zinc was measured using the colorimetric method 5-Br-PAPs kit (Instruchemie, Delfzijl, The Netherlands). Zinc levels were measured spectrophotometrically at wavelength 560 nm on a Shimadzu UV spectrophotometer.

**Urinary processing**

Urinary samples were collected in the morning between 08.00 and 10.00 a.m. and stored aliquoted unprocessed at -80°C. Urinary levels of phosphate, magnesium and sodium were determined using standardized methods measuring absorbance on the automated ARCHITECT cSystems. Urinary markers of bone degradation pyridinoline (PYD) and deoxypyridinoline (DPD) were determined by HPLC using commercial reagents (ChromSystems, Munchen, Germany). After acid hydrolysis interfering fluorophores were removed by solid-phase extraction. Total PYD and DPD in the eluates was quantified by HPLC with fluorescence detection (excitation 290 nm, emission 395 nm). For zinc measurement, urine was acidified by adding <1% of a 25% hydrochloric acid solution. Subsequently, urine was centrifuged for 5 minutes at 2000 g. Supernatant was deproteinized and further processed using the colorimetric method.
5-Br-PAPs kit (Instruchemie, Delfzijl, The Netherlands). Levels of zinc were measured (wavelength 560 nm) on a Shimadzu UV spectrophotometer.

Urinary levels of Kidney Injury Molecule -1 (KIM-1), a marker for renal proximal tubular damage, were determined using enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, US) according to manufacturer’s instructions. Levels of Heart type fatty acid binding protein (H-FABP), a marker for renal distal tubular injury, were determined using ELISA (Haemoscan, Groningen, The Netherlands) with the use of capture antibody clone 10E1 and a HRP-labeled detection antibody 9F3 (both Hytest, Turku, Finland). To adjust for the degree of urinary dilution, creatinine levels were measured in all samples and all urinary markers are expressed as ratio in relation to creatinine levels.

**Statistical analysis**

For data analysis a commercial statistical package (IBM SPSS Statistics 21.0, US) was used. Descriptive statistics were performed primarily. Non-normally distributed data are expressed as median with interquartile range (IQR). Mann-Whitney rank sum test, or Kruskal-Wallis test when appropriate, was used to assess differences between groups. For repeated sample analysis the related- samples Wilcoxon Signed rank test was used. Correlation analysis was done using Spearman’s rank correlation (Sr). A $P$ value < 0.05 was considered statistically significant. Bonferroni correction for multiple testing was applied when appropriate.

**RESULTS**

**Primary cohort**

In the primary cohort 38 sickle cell patients (27 HbSS/HbSβ⁰-thalassemia and 11 HbSC/HbSβ⁺-thalassemia) during steady state, 27 patients (21 HbSS/HbSβ⁰-thalassemia and 6 HbSC/HbSβ⁺-thalassemia) during painful crisis and 25 healthy race-matched controls were included. The baseline characteristics are presented in Table 1. Compared to healthy volunteers (0.84 (IQR 0.72-1.13) μmol/mmol creatinine), urinary zinc levels were higher in sickle cell patients in steady state (1.80 (IQR 1.49-2.39) μmol/mmol creatinine, $P < 0.001$) with further increments during painful crisis (3.15 (IQR 1.74-4.14) μmol/mmol creatinine, $P = 0.004$). This increment during painful crisis was seen only in patients with the HbSS/HbSβ⁰-thalassemia genotype (Figure 1).
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| Table 1. Baseline characteristics. Results are shown as median with interquartile range. |
|---------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
| | Healthy controls | Sickle cell disease Asymptomatic | Sickle cell disease Painful crisis | Sickle cell disease Asymptomatic | Sickle cell disease Painful crisis |
| Demographics | | | | | |
| HbSS-HbSβ0 // HbSC-HbSβ+ | N/A | 27/11 | 21/6 | 13/8 | 22/10 |
| Age (y) | 33 (23-37) | 28 (21-35) | 24 (20-35) | 26 (22-30) | 26 (21-30) |
| Female/male ratio | 18/7 | 26/12 | 27/10 | 10/11 | 16/16 |
| On hydroxycarbamide (%) | N/A | 25% | 30% | 29% | 25% |
| Laboratory measurements | | | | | |
| White blood cells (10e9/l) | 5.1 (4.3-7.1) | 8.1 (6.4-10.1)* | 11.8 (9.6-14) † | 8.6 (6.1-9.6) | 9.9 (7.2-12.7) |
| Hemoglobin (mmol/l) | 7.8 (7.4-8.5) | 5.9 (5.2-6.5)** | 5.5 (4.7-6.1) | 6.0 (5.2-7.0) | 6.1 (4.7-6.9) |
| Bilirubin Total (µmol/l) | 9.0 (6.0-16.0) | 39.0 (27.5-63.0)** | 53.5 (31.5-94.8) | 26 (19-40) | 30 (23-55) |
| LDH (U/l) | 177 (145-197) | 367 (244-475)** | 442 (319-641) | 362 (218-435) | 405 (260-492) |
| Creatinine (µmol/l) | 68 (61-83) | 52 (42-62)** | 52 (40-61) | 61.0 (51.0-73.5) | 63 (53-72) |
| CRP (mg/l) | ND | 3.7 (2.2-9.0) | 4.8 (1.9-15.4) | 2.1 (0.8-6.2) | 3.7 (1.5-10.6) |

*Significant difference between healthy controls and asymptomatic patients with sickle cell disease, P < 0.05. ** P < 0.001
† Significant difference between asymptomatic patients with sickle cell disease and patients with sickle cell disease in painful crisis P < 0.05. †† P < 0.001
Zinc levels in serum were comparable between healthy controls and steady state sickle cell patients (17.9 (IQR 17.3-18.6) µmol/l vs. 18.9 µmol/l (IQR 16.6-19.1), *P* = 0.436). During painful crisis, serum levels of zinc in the HbSS/HbSβ^0^-thalassemia patients showed a significant drop compared to steady state levels (17.1 µmol/l (IQR 15.6-18.1) vs 18.9 (17.5-19.5), *P* = 0.032). No significant change in zinc levels during painful crisis was observed in the HbSC/HbSβ^+-thalassemia group (17.4 µmol/l (15.9-17.9) versus 18.9 (15.5-19.5), *P* = 0.039). Both fractional excretion of zinc and fractional excretion of sodium were higher in patients during painful crisis when compared to steady state values. (Figure 2A)

Urinary levels of zinc were not related to serum levels of zinc in patients both in steady state and during painful crisis. Also urinary levels of zinc did not correlate with established plasma markers of hemolysis such as reticulocyte counts, LDH and bilirubin. In patients during painful crisis a significant relation was observed between urinary zinc and urinary PYD levels (Sr 0.417, *P* = 0.030) but not with DPD levels.

Investigating relations between urinary zinc levels and levels of other urinary electrolytes, magnesium, phosphate and sodium, only high and significant correlations were observed between urinary zinc and sodium levels in healthy controls (Sr 0.726, *P* < 0.001), sickle cell patients in steady state (HbSS/HbSβ^0^-thalassemia Sr 0.520, *P* = 0.004 and HbSC/HbSβ^+-thalassemia Sr 0.755, *P* = 0.007) and HbSS/HbSβ^0^-thalassemia patients during painful crisis (Sr 0.679, *P* = 0.001).
Secondary cohort

In the secondary cohort, the course of urinary zinc excretion in sickle cell patients admitted for painful crisis was investigated. Thirty-two painful crises (22 HbSS/HbSβ0-thalassemia and 10 HbSC/HbSβ+-thalassemia) in 24 patients were included. From 28 of these 32 admissions also steady state samples were obtained. Levels of zinc in the 24-hour urine collections and the urinary samples taken on the second morning after admission correlated strongly ($r = 0.9$, $P = 0.001$). An increase in urinary zinc
excretion was observed on subsequent days of painful crisis (Figure 3A). Urinary zinc levels on the first morning after admission correlated significantly with the duration of admission \( (S_r = 0.437, P = 0.02) \).

While urinary PYD and DPD levels between steady state and the first morning sample after admission were comparable, increasing trends were observed on the subsequent days of painful crisis (Figure 3B and C). Urinary levels of KIM1 were significantly lower in samples taken on the first day of painful crisis (0.0246 (0.001-0.067) µg/mmol) compared to steady state levels (0.124 (0.030-0.170) µg/mmol, \( P = 0.044 \)) and showed a trend to drop further during subsequent days of admission (data not shown). Urinary levels of HFABP were significantly higher in samples taken on the first day of painful crisis (0.074 (0.023-0.158) µg/mmol creatinine) compared to levels in

Figure 3. Levels of zinc (A), pyridinoline, PYD (B) and deoxypiridinoline, DPD (C) during subsequent days of admission. Samples were taken on the first, second, third and fifth morning of painful crisis. Means with SEM, Kruskal Wallis statistics.

A. Levels of zinc show a significant increment during painful crisis. B. Levels of PYD show a trend to increase during painful crisis. After the third morning of admission, levels of PYD diverge in both genotype groups, possibly due to low number of patients still admitted on day 5. C. Levels of DPD show a trend to increase during painful crisis, levels in the different genotype groups show a different pattern.
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steady state (0.033 (0.014-0.048) µg/mmol creatinine, \( P = 0.002 \)), and showed a trend to decline back to baseline levels on subsequent days of admission (data not shown).

In the secondary cohort we investigated relations between urinary zinc levels and markers of renal tubular damage. When applying Bonferroni correction for multiple comparisons no significant correlations between urinary levels of zinc and urinary levels of KIM-1 and HFABP were observed in patients in steady state or painful crisis. When investigating relations between urinary zinc levels and urinary markers of bone resorption PYD and DPD, a strong correlation was found between urinary levels of zinc and PYD in steady state (\( S_r 0.701, \ P = 0.008 \)). During painful crisis, in the first sample taken after admission, urinary levels of zinc were significantly related to urinary levels of DPD (\( S_r 0.439, \ P = 0.012 \)) and PYD (\( S_r 0.388, \ P = 0.028 \)), although the latter just failed to reach statistical significance after applying Bonferroni correction. For relations between steady state and admission days 2, 3 and 5 see supplementary table 1.

Third cohort
In this third cohort, 12 healthy male volunteers (age 21 (IQR 19.5-25.5) years) were intravenously loaded with a mean volume of 542 (SD 6) mL, 2.7% (SD 0.2) NaCl solution. Urinary samples were obtained 120 and 240 minutes after infusion. Baseline urinary zinc levels were 0.911 (IQR 0.80-1.15) µmol/mmol creatinine and showed a decrease over time to 0.66 (IQR 0.54-0.79) µmol/mmol creatinine, \( P = 0.002 \) at 240 minutes after infusion. The fractional excretion of sodium in these healthy controls increased in time after NaCl infusion, while the fractional excretion of zinc decreased. (Figure 2B)

DISCUSSION

Levels of zinc, an essential trace element for various metabolic functions, are decreased in 15-80% of sickle cell patients. [3-6] While previous studies have shown a relation between zinc deficiency and increased urinary zinc excretion in SCD, we demonstrate that this increased urinary excretion may be mediated by elevated zinc loss due to ischemia-induced bone degradation.

We observed a direct relation between urinary levels of zinc and PYD, a specific marker of bone degradation, during painful crisis in the primary cohort. This relation was
confirmed in the secondary cohort, in which not only the excretion of PYD but also DPD correlated with the urinary zinc excretion on subsequent days of painful crises. PYD and DPD, released during bone remodeling, [24] are two of the most extensively characterized bone markers whereby DPD is considered to be more specific for bone resorption as PYD is also a constituent of collagen fibrils in other tissues.[25] Given the fact that this correlation was only found during painful vaso-occlusive crisis, we consider the ongoing ischemia-induced bone degradation to be the main cause of increased zinc excretion in SCD. This may also play a role in the decreased bone mass density observed in the majority of sickle cell patients [26, 27] as urinary zinc levels in postmenopausal women not only correlate with markers of bone resorption but also with the presence of osteoporosis.[28] In fact, urinary zinc levels are considered to be a valuable biomarker in evaluating therapeutic interventions in postmenopausal women with osteoporosis. [29]

Interestingly, urinary zinc excretion increased during painful crisis while serum zinc levels dropped, suggesting active renal involvement in zinc excretion originating from enhanced bone degradation during painful crisis. In general, sickle cell patients develop various changes in renal structure and function due to erythrocyte sickling and local ischemia [30-32] resulting in renal damage[33, 34] ranging from reduced concentrating capacity[33, 35] and micro-albuminuria to renal insufficiency which is observed in up to 20% of homozygous patients. [36] In the current study, we found increased urinary levels of H-FABP during painful crisis, which is a specific marker of distal ischemic tubular injury. In addition, urinary KIM-1 levels, a marker of proximal tubular damage, were twofold higher in steady state sickle cell patients as compared to normal volunteers (< 0.063 µg/mmol creatinine), as described previously.[37, 38] However, in contrast to H-FABP, levels of KIM-1 decreased during painful crisis as compared to steady state levels, which was unexpected, as KIM-1 is normally up-regulated upon acute kidney injury. [39] However, no correlations were observed between urinary zinc levels and the markers of tubular damage, H-FABP or KIM-1 during painful crisis, suggesting that the increased zinc excretion in SCD painful crisis is unrelated to renal tubular injury. Also no relation with markers for hemolysis was found, which is most likely explained by the fact that the amount of zinc in erythrocytes is negligible as compared to the total amount stored in bone tissue.

Because urinary zinc levels showed a strong relation with urinary sodium levels in our primary cohort, we investigated the possibility of urinary zinc loss in response to
intravenous hydration with NaCl that patients with SCD receive during admission for painful crisis. Interestingly, the increase in fractional excretion of sodium between healthy controls treated with NaCl infusion and patients during painful crisis, who were also treated with NaCl infusion, was comparable. However, while the fractional excretion of zinc in healthy controls decreased after NaCl infusion, it increased in patients during painful crisis. Although healthy controls were loaded with a smaller volume of a higher concentration NaCl solution than sickle cell patients, this observation made clear that the increased zinc excretion is specific for SCD and not the result of iatrogenic sodium load.

In conclusion, zinc deficiency in SCD is caused by increased urinary excretion of zinc that is released from bones due to ischemia-induced bone degradation. Whether zinc loss can be reduced by prevention of recurrent ischemia or the use of bisphosphonates should be studied in a prospective clinical trial. Life-long urinary wasting of zinc in SCD may justify supplementation of zinc in sickle cell patients.
REFERENCES


Supplementary table 1. Correlations between urinary levels of zinc and urinary levels of renal tubular markers kidney-injury molecule-1 (KIM-1) and heart-type fatty acid-binding protein (H-FABP) and correlations between urinary levels of zinc and urinary levels of bone degradation markers deoxypyridinoline (DPD) and pyridinoline (PYD).

* Significant correlation $P \leq 0.05$
† Significant correlation when applying Bonferroni correction: $0.05/4 = P < 0.0125$

Significant relations are emphasized using a bold type.

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<th>Sr</th>
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<th>CR day 3</th>
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<td>Zn – HFABP</td>
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<td>-0.306, $p = 0.156$</td>
<td>0.013, $p = 0.956$</td>
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<td><strong>Bone markers:</strong></td>
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<td>Zn – Urinary DPD/creat</td>
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