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Journal Title: Journal of Clinical and Translational Endocrinology
Volume: Volume 1, Number 1
Publisher: Elsevier | 2014-01-01, Pages 19-25
Type of Work: Article | Final Publisher PDF
Publisher DOI: 10.1016/j.jcte.2014.01.003
Permanent URL: https://pid.emory.edu/ark:/25593/s95dk

Final published version: http://dx.doi.org/10.1016/j.jcte.2014.01.003

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Accessed September 11, 2019 6:53 AM EDT
Research Paper

The role of vitamin D in regulating the iron-hepcidin-ferroportin axis in monocytes

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A R T I C L E   I N F O

Article history:
Received 13 December 2013
Received in revised form 21 January 2014
Accepted 23 January 2014

Keywords:
Vitamin D
Hepcidin
Ferroportin
NRAMP1
IL-6
IL-1β
Macrophage
Chronic kidney disease
Anemia of inflammation

A B S T R A C T

Chronic kidney disease affects 40% of adults aged 65 and older. Anemia of CKD is present in 30% of patients with CKD and is associated with increased cardiovascular risk, decreased quality of life, and increased mortality. Hepcidin-25 (hepcidin), the key iron regulating hormone, prevents iron egress from macrophages and thus prevents normal recycling of the iron needed to support erythropoiesis. Hepcidin levels are increased in adults and children with CKD. Vitamin D insufficiency is highly prevalent in CKD and is associated with erythropoietin hypersensitivity. Recently, hepcidin levels were found to be inversely correlated with vitamin D status in CKD. The aim of this study was to investigate the role of vitamin D in the regulation of hepcidin expression in vitro and in vivo. This study reports that 1,25-dihydroxyvitamin D3 (1,25(OH)2D3), the hormonally active form of vitamin D, is associated with decreased hepcidin and increased ferroportin expression in lipopolysaccharide (LPS) stimulated THP-1 cells. 1,25(OH)2D3 also resulted in a dose-dependent decrease in pro-hepcidin cytokines, IL-6 and IL-1β, release in vitro. Further, we show that high-dose vitamin D therapy impacts systemic hepcidin levels in subjects with early stage CKD. These data suggest that improvement in vitamin D status is associated with lower systemic concentrations of hepcidin in subjects with CKD. In conclusion, vitamin D regulates the hepcidin-ferroportin axis in macrophages which may facilitate iron egress. Improvement in vitamin D status in patients with CKD may reduce systemic hepcidin levels and may ameliorate anemia of CKD.

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Introduction

Vitamin D insufficiency is common in patients with chronic kidney disease (CKD) [1], with a prevalence rate of up to 80% of all patients with CKD stage 3 or worse [2]. Optimal vitamin D status is important in patients with CKD to regulate parathyroid hormone (PTH) concentrations [3–5] for optimal bone health and prevention of osteomalacia and for potential cardioprotective effects [3,6,7]. Recent reports have established an association between vitamin D insufficiency and anemia in patients with CKD [8–10]; however, the role for vitamin D in the regulation of anemia has not been fully explained.

Iron is one of the key nutrients involved in the pathophysiology of anemia of CKD. The absorption and recycling of iron is under control of the hepcidin-ferroportin axis in humans [11,12]. Elevated hepcidin level inhibits iron uptake from the gut and sequesters iron in the reticuloendothelial system [13]. Macrophages engulf senescent red blood cells and, therefore, play a central role in iron recycling. Hepcidin retains iron in macrophages by binding to its receptor ferroportin, the only iron exporter, causing its internalization and degradation, consequently preventing iron egress from macrophages to circulation [14]. In inflammatory states such as CKD, hepcidin antimicrobial peptide (referred to as hepcidin) is elevated [15,16]. Two cytokines (IL-1β and IL-6) are commonly elevated in CKD and stimulate hepcidin production from the liver and macrophages [17–20]. Hepcidin prevents iron egress from macrophages and thus prevents normal recycling of the iron needed to support erythropoiesis [21–23]. Additionally, reduced kidney function likely prevents efficient hepcidin clearance from the plasma [8,24]. Recent investigations show that vitamin D concentrations [assessed by serum 25-hydroxyvitamin D (25(OH)D)] are inversely associated with hepcidin concentrations and positively associated with hemoglobin and iron concentrations [8–10,24,25].

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http://dx.doi.org/10.1016/j.jcte.2014.01.003
Given the high prevalence of vitamin D insufficiency in patients with CKD and the potential link between vitamin D and anemia, iron and hepcidin concentrations, we hypothesized that vitamin D therapy could improve expression of iron regulating proteins in macrophages in vitro which would translate into improved circulating hepcidin concentrations in humans. We examined the three key iron regulating proteins, hepcidin, NRAMP1 (the endosomal iron transporter that transfers recycled iron from the late endosome to the cytosol) [26,27], and ferroportin, the only known cellular iron exporter [28,29], in addition to other pro-hepcidin cytokines in monocytic cell cultures in vitro. In order to translate these findings to humans, we conducted a pilot study to examine the impact of high-dose vitamin D on circulating hepcidin concentrations.

Materials and methods

Macrophage cell culture and stimulation

THP-1 macrophage-like monocytic cells obtained from ATCC (Manassas, VA) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 50 μg/ml penicillin and 50 IU/ml of streptomycin. Freshly grown cells were harvested and adjusted to 1 million cells/ml and transferred into 12-well tissue culture plates of streptomycin. Freshly grown cells were harvested and adjusted to 2 ml/well. THP-1 cells were cultured with 1,25(OH)2D3 (Sigma, St. Louis, MO, USA) in TBS for 1 h at 37°C. LPS from Neisseria meningitidis serogroup B was purified and quantified as previously described [30]. Cell suspensions were centrifuged and supernatants were removed and saved at −20°C for cytokine measurements. Harvested THP-1 cells were washed with phosphate buffered saline (PBS) then placed in RLT buffer (Qiagen; Hilden, Germany) containing 1% β-mercaptoethanol, passed over QiaShredder columns, and the resulting lysates were saved at −80°C for mRNA extraction.

RNA isolation, quantitative real-time PCR and gene expression analysis

RNA was isolated using RNasy Mini kits (Qiagen) following the manufacturer’s instructions, as previously described [32]. Briefly, cell lysates saved in RLT buffer were mixed in 70% ethanol then passed over RNasy columns. Columns were washed and treated with 10 μl of RNase-free DNase (Qiagen) for 15 min at room temperature prior to RNA extraction, followed by additional washing and centrifugation. RNA was eluted in 35 μl of RNase-free water, then was reverse transcribed to cDNA using QuantiTect® Reverse Transcription kit (Qiagen) following the manufacturer’s instructions. Relative gene expression was determined by quantitative RT-PCR performed on resulting cDNA using SYBR Green (Promega; Madison, WI) following the manufacturer’s instructions. The mRNA level was calculated in reference to β-actin, and fold change gene expression was calculated in reference to vehicle treated controls using the ΔΔCT method. Results were normalized to vehicle-treated controls which were used as controls for basal gene expression level. The following primers were used for qRT-PCR reactions: human hepcidin 5′-GACCAGTGCCCT GTGTTTTCC-3′ and 5′-CACATCCCAACTTGTGATC-3′; human NRAMP1 5′-GGAGGCTGTCCCATCTCCTAC-3′ and 5′-GTGTCACC AGTGGTGGAG-3′; human IL-37 5′-CAACAGACTCCAAGGG ATGG-3′ and 5′-GCCTGGTGGAGGTGCT-3′; human β-actin 5′-TCTCTAGCTTCCTTCCT-3′ and 5′-AGCAGGCTTGGCGTACAG-3′. Ferroportin Quant iTect primers (HS_SLC40A1_1_SG) were purchased from Qiagen.

Cytokine release quantification

Cytokines IL-6 and IL-1β released from THP-1 cells were quantified by DuoSet ELISA (R&D Systems, Minneapolis, MN) as previously described [31,33].

Hepcidin-25 measurements

Antibody labeling: Anti-hepcidin monoclonal antibodies were adjusted to an approximate concentration of 2 mg/ml and were Biotin- and MSD-SulfoTag (Mesoscale Discovery (MSD), Gaithersburg, MD, USA) labeled according to manufacturer’s protocols. Capture antibody was biotin-labeled with Thermo no-weigh EZ Link Sulfo-NHS-LC Biotin with a 20-fold molar excess of biotin. Conjugate antibody was labeled with MSD SulfoTag NHS Ester with a 12-fold molar excess of ruthenium. Following the labeling reactions, antibodies were extensively dialyzed to remove unbound label.

Hepcidin electrochemiluminescence [34] immunoassay: The hepcidin sandwich assay [29] was performed on MSD Streptavidin 96-well plates that were washed three times with TBST (Tris buffered saline containing 10 mmol/l Tris pH 7.40, 150 mmol/l NaCl with 1 ml Tween 20/l) and blocked with 1% Bovine serum albumin (Sigma, St. Louis, MO, USA) in TBS for 1 h at room temperature. Following washing of the plate, 25 μl of biotin-labeled capture antibody (4 μg/ml) was added and allowed to bind to the plate for one hour with gentle shaking. Afterward, the wells were washed three times with TBST, and 100 μl of hepcidin standards consisting of varying concentrations of hepcidin protein in assay buffer consisting of 50 mmol/l HEPES, pH 7.40, 150 mmol/l NaCl, 1 ml/l Triton X-100, 5 mmol/l EDTA, and 5 mmol/l EGTA and 0.1% BSA, which was supplemented with 100 μg/ml Heterophilic Blocking Reagent (Scantibodies, Santee, CA, USA) were added to the wells to generate a calibration curve. Plasma samples were diluted 1:50 in the same assay buffer, added to their respective wells, and incubated for 1 h at room temperature with gentle rocking. Following aspiration, wells were washed 3 times with TBST, and 25 μl of 0.1 μg/ml ruthenium-labeled conjugate hepcidin-specific detection antibody were added to the wells, which were incubated for 1 h at room temperature. The plate was again washed three times with TBST, and 150 μl of 2X-MSD Read Buffer T was added to the wells. The plate was then read on an MSD SECTOR Imager 6000 reader, which recorded ruthenium electrochemiluminescence. Concentrations of hepcidin in samples were interpolated against a standard curve made up of reference standard hepcidin (Eli Lilly and Company, Indianapolis, IN, USA) using a 4 PL fit (Mesoscale Discovery Workbench).

Pilot clinical study design

We obtained serum from subjects with early stage CKD (stages 2/3) who completed an IRB-approved, double-blind, randomized, placebo-controlled trial of oral vitamin D3 (cholecalciferol, 50,000 IU weekly for 12 weeks, followed by 50,000 IU every other week for 40 weeks) or matching placebo for one year. CKD staging was defined by an estimated glomerular filtration rate (eGFR) of 60–89 ml/min/1.73 m² and 30–59 ml/min/1.73 m² for stages 2 and 3, respectively, calculated using the Modification of Diet in Renal Disease Study equation [35]. The primary endpoint of this study was serum 25(OH)D and PTH, the results of which have been published [36]. All subjects provided informed consent for evaluation of their blood samples for future sub-studies to explore the impact of high-dose vitamin D on a variety of health outcomes. The clinicaltrials.gov registration number was NCT00781417. This sub-study includes only subjects with available paired serum
specimens for hepcidin measurements at baseline and a three month follow-up visit \((N = 38)\). Serum 25(OH)D was measured with a chemiluminescent assay (Immunodiagnostic Systems iSYS automated machine; Fountain Hills, AZ).

**Statistical analysis**

The mean values ± SD and \(P\) values (Student \(t\) test) of at least three independent determinations were calculated with Microsoft Excel software for the in vitro data. For the clinical data, descriptive statistics were performed. Differences between groups (vitamin D vs placebo) were determined with two-group \(t\)-tests, Mann–Whitney \(U\) tests (for variables that were not normally distributed), or chi-square tests. Spearman’s rank correlation was used to determine the relationship between the percent change in serum 25(OH)D3 and the percent change in serum hepcidin. Clinical data were analyzed with JMP® Pro 10.0.0 (SAS Institute Inc., Cary, NC, USA) using two-sided tests and assuming a 5% significance level.

**Results**

Vitamin D \((1,25(OH)_{2}D_{3})\) is associated with decreased hepcidin and increased ferroportin mRNA expression in THP-1 cells exposed to LPS

Lipopolysaccharide is a known inducer of hepcidin expression in macrophages and hepatocytes [18,19]. The effect of the hormonally active vitamin D \((1,25(OH)_{2}D_{3})\) on regulating hepcidin and ferroportin gene expression in LPS-stimulated THP-1 cells was investigated. We found that 1,25(OH)_{2}D_{3} suppressed hepcidin mRNA expression in LPS-stimulated THP-1 cells in a dose-dependent manner (Fig. 1A). In contrast, 1,25(OH)_{2}D_{3} significantly increased ferroportin mRNA expression at 40 nM dose but not at lower doses (Fig. 1B). These data suggest that vitamin D regulates the hepcidin-ferroportin axis in macrophages exposed to LPS, thereby facilitating iron transport during states of inflammation.
Vitamin D (1,25(OH)2D3) is associated with increased expression of NRAMP1, the endosomal iron transporter.

NRAMP1, the endosomal iron transporter that plays a significant role in cellular iron homeostasis, is a direct target gene for vitamin D. As a positive control to our results that vitamin D regulates the hepcidin-ferroportin axis in THP-1 cells exposed to LPS, we examined the expression of NRAMP1 and IL-37, genes known to be induced by vitamin D [26,30]. As expected, our data confirm that 1,25(OH)2D3 significantly induced NRAMP1 mRNA expression (Fig. 2A) and potently induced IL-37 mRNA expression in LPS-stimulated THP-1 cells (Fig. 2B).

Vitamin D lowers inflammatory cytokines release from LPS-stimulated macrophages in vitro

LPS is a major inducer of inflammatory cytokine release from macrophages including pro-hepcidin cytokines, IL-6 and IL-1β [33].

We examined whether vitamin D may also decrease the release of these inflammatory cytokines from macrophages. We measured cytokine concentrations in the cultured media from THP-1 cells in the presence of increasing concentrations of 1,25(OH)2D3 prior to LPS exposure and found a dose-dependent decrease in IL-6 (Fig. 3A) and IL-1β release in vitro (Fig. 3B). Therefore, vitamin D may be associated with suppression of hepcidin expression by directly reducing pro-hepcidin cytokines release.

Vitamin D effects on hepcidin concentrations in patients with stages 2 and 3 CKD

Baseline demographic and biochemical characteristics for the subjects evaluated in the pilot study are listed in Table 1. Coincidently, baseline serum 25(OH)D concentrations were lower in the vitamin D supplemented group. Baseline systemic hepcidin levels were similar for the vitamin D supplemented and placebo groups. The percent change from baseline to 3 months in serum 25(OH)D concentrations was inversely associated with the percent change in serum hepcidin concentrations (Spearman ρ = −0.38, P = 0.02) (Fig. 4, open circles represent patients receiving placebo. Filled circles represent patients receiving vitamin D).

Figure 3. Vitamin D reduces pro-hepcidin cytokine release from LPS-stimulated macrophages. THP-1 cells were treated with increasing doses of 1,25(OH)2D3 overnight prior to LPS (20 ng/ml) exposure for 6 h. In vitro 1,25(OH)2D3 (1,25D) reduces IL-6 (A) and IL-1β (B) release from THP-1 cells exposed to 20 ng/ml of LPS. Cytokines release was measured by ELISA. *P < 0.05. 1,25D: 1,25(OH)2D3.

Table 1
Baseline demographic characteristics of subjects with early stage CKD

<table>
<thead>
<tr>
<th>Variable</th>
<th>Vitamin D (n = 19)</th>
<th>Placebo (n = 19)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>62.5 ± 11.0</td>
<td>62.0 ± 8.7</td>
<td>0.89</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>33.2 ± 5.4</td>
<td>32.1 ± 8.0</td>
<td>0.63</td>
</tr>
<tr>
<td>Male [n (%)]</td>
<td>19 (100.0)</td>
<td>17 (89.5)</td>
<td>0.15</td>
</tr>
<tr>
<td>African American (%)</td>
<td>9 (47.4)</td>
<td>9 (47.4)</td>
<td>1.00</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>95.4 ± 14.4</td>
<td>92.3 ± 16.0</td>
<td>0.57</td>
</tr>
<tr>
<td>CKD Stage 2/3 (N)</td>
<td>9/10</td>
<td>8/11</td>
<td>0.74</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>17 (89.5)</td>
<td>17 (89.5)</td>
<td>1.00</td>
</tr>
<tr>
<td>Diabetes (%)</td>
<td>17 (89.5)</td>
<td>13 (68.4)</td>
<td>0.11</td>
</tr>
<tr>
<td>25(OH)D (ng/ml)</td>
<td>27.5 ± 6.3</td>
<td>32.9 ± 8.5</td>
<td>0.03</td>
</tr>
<tr>
<td>Hepcidin (ng/ml)a</td>
<td>8.5 (5.5, 17.7)</td>
<td>11.9 (8.8, 13.8)</td>
<td>0.90</td>
</tr>
</tbody>
</table>

Values are reported as mean ± SD or n (%). P for difference calculated with two-group t-test or chi-square test.

a Reported as median (IQR) and P for difference calculated with Mann–Whitney U test.

Figure 4. Relationship between percent change in serum hepcidin and percent change in 25(OH)D from baseline to 3 months. Open circles represent patients receiving placebo. Filled circles represent patients receiving vitamin D. Spearman’s ρ = −0.38, P = 0.02.
Macrophages play a central role in iron metabolism and in host defense [14,37]. Macrophages are also major producers of inflammatory cytokines. Here we report that vitamin D treatment is associated with reduced hepcidin expression in vitro and in vivo. Our in vitro studies show that vitamin D is associated with decreased hepcidin while increasing ferroportin and NRAMP1 mRNA expression in a dose-dependent manner in human monocytic THP-1 cells in the presence of an inflammatory stimulus (i.e. exposure to LPS). This study also shows that vitamin D is associated with reduced concentration of hepcidin stimulatory cytokines, IL-6 and IL-1β, from cultured THP-1 cells exposed to LPS. Taken together, these data suggest that vitamin D may have an important role in regulating cellular iron homeostasis via the hepcidin-ferroportin-NRAMP1 axis in macrophages to facilitate iron egress during inflammation. Our in vivo pilot study shows that an increase in serum 25(OH)D concentration is associated with a decrease in serum hepcidin in subjects with early stage CKD. Our data suggest that vitamin D therapy may improve altered iron homeostasis associated with anemia of CKD in vitamin D deficient patients.

Our study is in agreement with recent studies showing that vitamin D suppresses hepcidin expression in macrophages [38,39]. Bacchetta and co-workers show that vitamin D significantly suppressed hepcidin expression in monocytes by 0.5-fold and provides evidence that vitamin D directly downregulates hepcidin expression as they identified a VDRE binding site on human hepcidin promoter [39]. Adding to the findings of this previous study, we examined the effect of vitamin D on hepcidin expression in monocytes during inflammation (i.e. in LPS-stimulated THP-1 cells). LPS is a well-known inducer of hepcidin and can mimic inflammation in monocytes where microbial translocation is commonly associated with chronic diseases including CKD [40–43]. Our data clearly demonstrate that vitamin D significantly reduced IL-6 and IL-1β release (major inflammatory cytokines that are elevated in CKD) [44–46] from LPS-induced THP-1 cells. Elevated IL-6 levels are associated with poor clinical outcomes and with anemia of chronic disease since IL-6 is a direct inducer of hepcidin [47]. Therefore, we postulate that reducing circulating IL-6 levels would lead to a reduction in hepcidin expression in liver hepatocytes, the major source of hepcidin, as well as in macrophages [48]. Elevation in circulating IL-6 levels have been reported in patients with late stage CKD where anemia of CKD is highly prevalent [47]. Further, LPS is a major component of microbial translocation seen during chronic inflammation [40–43]. LPS induces both hepcidin and IL-6 expression whereas LL-37 binds and neutralizes LPS activity [49]. Therefore, we postulate that high-dose vitamin D therapy suppresses hepcidin expression directly, as shown by Bacchetta et al. [39], and indirectly by reducing pro-hepcidin inflammatory cytokines IL-6 and IL-1β.

Further, we recently reported that vitamin D status assessed as serum 25(OH)D is inversely correlated with the inflammatory chemokine, MCP-1, in vivo and in vitro [3,36]. MCP-1 is found to be associated with serum hepcidin and macrophage iron in patients with metabolic syndrome alterations [50]. Other studies reported that vitamin D suppressed the release of inflammatory cytokines from monocytes and macrophages [51]. The mechanism by which vitamin D exerts anti-inflammatory effects is recently proposed to be mediated by microRNA called miR-155 [52]. MicroRNAs are noncoding RNAs that control gene expression by repressing mRNA translation. Specifically, miR-155 encoded by bic gene is a critical regulator of TLR signaling in macrophages. miR-155 targets SOCS1 (cytokine signaling protein 1), the negative feedback regulator of cytokine release, therefore high miR-155 expression is associated with a heightened cytokine release from macrophages. Increased expression of miR-155 is reported in primary monocytes and macrophages from SLE and in atherosclerotic plaque [53–57]. Vitamin D is reported to promote the negative feedback regulation of LPS-mediated signaling by targeting miR-155-suppressor of cytokine signaling protein 1 (SOCS1) in macrophages since miRNA-155 is highly upregulated by toll-like receptors (TLR) ligands, i.e. LPS and microbial translocation and downregulated by vitamin D [52,58].

In addition to miR-155, vitamin D may regulate the immune system by inducing autophagy and regulating endoplasmic stress. Autophagy is a conserved process by which cells recycle macromolecules, thus playing an essential role in cellular homeostasis and in host defense [59–62]. Campbell et al. demonstrated that vitamin D enhanced autophagy, which inhibited HIV and Mycobacterium tuberculosis infection in macrophages [61]. Autophagy induction reduces cytokines and other inflammatory mediators release from LPS-stimulated macrophages [63]. Further, endoplasmic reticulum (ER) stress is shown to dysregulate cellular iron homeostasis by inducing hepcidin expression [64]. Recent report shows that vitamin D relieves ER stress, which would be another possible mechanism by which vitamin D reduces hepcidin expression [65,66]. Elucidation of these mechanisms is worthy of further investigation.

In this pilot study we show that an increase in serum 25(OH)D concentrations is associated with a decrease in hepcidin levels in subjects with early stage CKD enrolled in a high-dose vitamin D trial. Our clinical finding supports our in vitro data that suggest vitamin D lowers hepcidin expression directly and/or indirectly by decreasing pro-hepcidin cytokines. Bacchetta and co-workers also demonstrated that high-dose vitamin D therapy reduced blood hepcidin-25 levels in healthy donors [39]. The relationship between vitamin D and hepcidin may explain the link between low vitamin D status and anemia in children [67]. These findings are clinically significant as a large majority of patients with CKD have vitamin D insufficiency [2], and approximately 40% of patients with CKD stage 4 have anemia [68]. Correction of vitamin D as a potential adjunctive therapy in treatment of anemia of CKD is attractive given the relatively inexpensive cost, favorable safety profile, and the potential to reduce the dependence on other more expensive therapies such as erythropoietin stimulating agents. Very few studies have been conducted to evaluate vitamin D as a therapy for anemia of CKD. Goicoechea et al. demonstrated in 28 patients on hemodialysis and severe hyperparathyroidism that intravenous calcitriol, the hormonally active form of vitamin D, reduced the need for erythropoietin therapy [69]. Similarly, Albitar et al. demonstrated in a prospective study of 12 patients, treatment with alfacalcidol, an analog compound of 1,25(OH)2D, improved anemia in patients on hemodialysis [70]. The strength and novelty of this study was the evaluation of the effect of vitamin D on suppressing hepcidin expression in LPS-stimulated monocytes indicating the efficacy of vitamin D on regulating hepcidin during inflammation, a common hallmark of CKD. In addition to hepcidin and ferroportin, this study investigated the impact of vitamin D on NRAMP1, the endosomal iron transporter. A limitation was the use of immortalized human monocytic THP-1 cells rather than primary peripheral monocytes from subjects with CKD. A limitation of our clinical pilot study was that this was a secondary analysis in CKD patients with available serum for measurement of hepcidin; therefore, we may have been limited in statistical power. Regardless, our clinical data support our in vitro findings of a hepcidin-lowering effect of vitamin D.

In summary, this study reports that high-dose vitamin D impacts hepcidin expression in vitro and in vivo. Vitamin D is associated with alterations of the hepcidin–ferroportin axis in monocytes exposed to LPS and leads to a reduction of pro-hepcidin cytokine, IL-6 and IL-1β, therefore facilitating iron egress during inflammation. This in vitro
observation appears to be supported by our early translational pilot study in subjects with early CKD where changes in vitamin D status induced by high-dose oral vitamin D therapy impacted changes in systemic hepcidin levels.

Acknowledgment

This work is supported in part by grants from Emory-Engleston Children’s Research Center and Center for Pediatric Nanomedicine of Emory + Children’s Pediatrics Research Center to S.M.Z. and by T32 DK007298-3251 to J.A.A. S.M.Z. especially acknowledges the support of N. McCarty (Center for Cystic Fibrosis Research, Emory University School of Medicine) for providing the research facility and laboratory space where part of this work was conducted. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

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