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## Proposing a Caco-2/HepG2 cell model for *in vitro* iron absorption studies<sup>☆</sup>

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### Abstract

The Caco-2 cell line is well established as an *in vitro* model for iron absorption. However, the model does not reflect the regulation of iron absorption by hepcidin produced in the liver. We aimed to develop the Caco-2 model by introducing human liver cells (HepG2) to Caco-2 cells. The Caco-2 and HepG2 epithelia were separated by a liquid compartment, which allowed for epithelial interaction. Ferritin levels in cocultured Caco-2 controls were  $21.7 \pm 10.3$  ng/mg protein compared to  $7.7 \pm 5.8$  ng/mg protein in monocultured Caco-2 cells. The iron transport across Caco-2 layers was increased when liver cells were present ( $8.1 \pm 1.5\%$  compared to  $3.5 \pm 2.5\%$  at  $120 \mu\text{M}$  Fe). Caco-2 cells were exposed to 0, 80 and  $120 \mu\text{M}$  Fe and responded with increased hepcidin production at  $120 \mu\text{M}$  Fe ( $3.6 \pm 0.3$  ng/ml compared to  $2.7 \pm 0.3$  ng/ml). The expression of iron exporter ferroportin in Caco-2 cells was decreased at the hepcidin concentration of 3.6 ng/ml and undetectable at external addition of hepcidin (10 ng/ml). The apical transporter DMT1 was also undetectable at 10 ng/ml but was unchanged at the lower concentrations. In addition, we observed that sourdough bread, in comparison to heat-treated bread, increased the bioavailability of iron despite similar iron content (53% increase in ferritin formation, 97% increase in hepcidin release). This effect was not observed in monocultured Caco-2 cells. The Caco-2/HepG2 model provides an alternative approach to *in vitro* iron absorption studies in which the hepatic regulation of iron transport must be considered.

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**Keywords:** Caco-2; HepG2; Hepcidin; Coculture; Intestinal; Cell model

### 1. Introduction

Iron deficiency is one of the most prevalent nutritional deficiencies. It is also the major cause of anemia. Several factors can contribute to iron deficiency, but low bioavailability of iron in the diet is one of the most important. Iron bioavailability depends on host factors, such as iron status, and the composition of foods, including the presence of inhibitory or enhancing factors for iron absorption. Studies of approaches to combat iron deficiency need to explore the effects of discrete compounds and interactions of iron with other micronutrients or organic chelators. Performing such studies in humans and animals is expensive, time-consuming and associated with a number of problems such as compliance and ethical issues. An important tool is the use of cell models to determine intracellular effects and regulatory mechanisms involved with iron absorption.

The Caco-2 cell model has commonly been used for iron bioaccessibility studies after it was first proposed as such in 1998 by Glahn *et al.* who postulated a combined *in vitro* digestion/Caco-2 cell model [1]. Subsequently, several research teams have validated Caco-2 cell models and used them for iron uptake studies [2–4] and iron transporter studies [5–7]. Unfortunately, Caco-2 cells have several limitations as a model for the intestinal border including

the absence of mucus-producing goblet cells. There have been attempts to cocultivate Caco-2 cells with goblet cells [8,9]. Another concern raised against Caco-2 cells, as a model for the human intestine, is that the epithelium lacks communication with other organs that are involved in the regulation of nutrient absorption *in vivo*. In particular, the liver plays such a role. Of major importance in the regulation of iron absorption is the peptide hormone hepcidin, which is secreted by the liver into the portal circulation in response to high iron levels [10,11]. Hepcidin interacts with enterocytes and thereby decreases iron absorption by affecting the transport of iron through ferroportin [12]. The iron is then trapped inside the enterocytes until ferroportin levels are restored or iron is released back into the intestinal lumen by means of enterocyte turnover. Haptoglobin is another important iron regulator secreted by the hepatic epithelium, and it also modulates iron absorption via ferroportin [13]. However, the importance of haptoglobin in the regulation of iron transport has not yet been well established.

In the present study, we aimed to develop the Caco-2 cell model by providing the Caco-2 cells with a functional liver epithelium (HepG2) expressing hepcidin. We investigated the responsiveness of the model to iron by measuring the ferritin formation in the Caco-2 cells and iron transport across the basolateral border of the Caco-2 cells. We also investigated how the hepatic epithelium interacts with the Caco-2 cells via hepcidin signaling through the liquid compartment separating the epithelia. In addition, the cytokines interleukin (IL)-1 and IL-6 were used to test their ability to provoke hepcidin production in the model. To demonstrate that the Caco-2/hepG2

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model also responds to a complex food sample, we compared the models' responses toward two *in vitro* digested bread samples with similar iron content.

## 2. Methods and materials

### 2.1. Experimental design

Experiments were run to evaluate the Caco-2/HepG2 assay and to compare its performance against the standard Caco-2 model: (a) Hepcidin levels in the basal medium and ferritin levels in the Caco-2 cells of both assays were tested after exposure to iron at varying concentrations (Fe-free control, Fe 80  $\mu$ M and Fe 120  $\mu$ M). (b) Iron transport across the basolateral border of Caco-2 cells in both models was measured. (c) The Caco-2 levels of the basolateral iron transporter ferroportin and apical iron transporter DMT1 were estimated in response to hepcidin. (d) Cytokines IL-1 and IL-6 were used to stimulate hepcidin release from the HepG2 cells in the Caco-2/HepG2 model. (e) Ferritin and hepcidin levels were assessed in both cell models after exposure to complex digested food samples (sourdough and scalded breads).

### 2.2. Materials

Polystyrene disposables for cell cultivation were produced by Corning (Tewksbury, MA, USA). Media, supplements and other reagents for cell culture maintenance were obtained from PAA (Pasching, Austria). Reagents used for the cell experiments were bought from Sigma-Aldrich (Schnelldorf, Germany). Ascorbic acid was used in the form of sodium ascorbate (Sigma-Aldrich). Iron was added as Fe(II)Cl<sub>2</sub>·4H<sub>2</sub>O (Sigma-Aldrich). Hepcidin-25 (1  $\mu$ g/ml) was obtained from Bachem (Torrance, CA, USA).

### 2.3. Cell lines

The established cell lines Caco-2 (HTB-37) and HepG2 were obtained from the American Type Culture Collection (Rockville, MD, USA) at passage 20 and 74, respectively. Both cell lines were verified to be mycoplasma-free at the time of purchase, and no further testing specifically for mycoplasma occurred. Stock cultures of Caco-2 were maintained in Dulbecco's modified Eagle's medium, and HepG2 cells were cultured in modified Eagle's medium (MEM) with Earle's salt. Both media were supplemented with fetal bovine serum (FBS) (16%). The cell cultures were incubated at 37°C in humidified air (95%) and CO<sub>2</sub> (5%). The medium was changed every second or third day, and the cells were passaged at approximately 80% confluence. At passage 29–39, the Caco-2 cells were seeded in 12-well plates on Transwell polycarbonate or polyethylene inserts (0.4  $\mu$ m) at 60,000 cells/insert. Simultaneously, the HepG2 cells were seeded at the bottom of 12-well plates at passage 78–80 at 200,000 cells/well. An antibacterial, antifungal and antimycoplasma agent (Normocin; Invivogen, CA, USA) was added to the medium after plating.

### 2.4. Setup of the cell models

Thirteen days after seeding, the Caco-2 inserts were transferred to the HepG2 wells, and both cell lines were incubated together in fresh MEM supplemented with FBS (1%). The cell system was allowed to equilibrate for 24 h before the experiments, and the trans-epithelial electrical resistance was measured to verify the intactness of the transferred Caco-2 cell layers. The resistance across the layers was typically 450  $\pm$  60  $\Omega$ . In the standard Caco-2 cell model, the Caco-2 inserts were prepared in exactly the same way as the inserts for the Caco-2/HepG2 assay. The only difference of the setups was the absence of HepG2 cells in the basal chamber of the standard Caco-2 cell model.

### 2.5. Preparation of bread samples

Two varieties of breads, one sourdough and one nonsourdough, were used for the experiments. The ingredients and total iron content were similar for the two breads. The sourdough bread was made from a sourdough starter (10%) and fermented for 24 h before baking. Nonsourdough bread was made from rye flour, which was heat-treated (100°C) to eliminate its endogenous phytase (phytic acid degrading enzyme) activity. Freeze-dried samples of breads (1 g) was suspended in double distilled water (10 ml) and digested with pepsin solution (0.3 ml/sample) containing 0.16 mg pepsin/L of HCl (0.1 M) at pH 2 for 1 h at 37°C. The pH was adjusted to 7 by the addition of NaHCO<sub>3</sub> (1 M), and the sample was further digested with pancreatin (1.7 ml/sample of a solution containing 4.0 g pancreatin/L) for 0.5 h. The digested samples were filtered through a membrane with a 10-kD cutoff (Millipore) at 37°C and 2000g for 45 min to imitate the filtering effect of the mucus layer. Filtered and unfiltered samples were analyzed for the soluble iron content by high-performance ion chromatography according to Fredriksson *et al.* [14] before being transferred to the cells.

### 2.6. Cell experiments for ferritin and hepcidin analysis

Caco-2/HepG2 cells in quadruples were treated at the apical surface with iron, sodium ascorbate or bread samples. The iron-free control contained less than 0.9  $\mu$ M Fe. Filtered bread samples (250  $\mu$ l) were added to the apical medium (250  $\mu$ l). After

incubation (3 h), the apical medium (500  $\mu$ l) was aspirated. The cells were washed in PBS and incubated in fresh medium (MEM, 1% FBS) for another 21 h to allow for ferritin formation in the Caco-2 cells and hepcidin production in the HepG2 cells. Basal medium was collected, and cells were lysed in radioimmunoassay buffer (Sigma-Aldrich). Aliquots were frozen at –80°C for later analysis. Aspirated medium samples were streaked on tryptone glucose extract agar plates to verify the absence of bacterial/fungal infections.

### 2.7. Iron uptake and basal transfer

In these experiments, iron uptake and basal transfer in the Caco-2/HepG2 as well as the Caco-2 model were measured using radiolabeled iron (<sup>55</sup>Fe). Fourteen days after seeding, including 24-h equilibration time for Caco-2/HepG2 cells, iron solutions (5  $\mu$ l, pH 7) were added to the apical medium, giving final concentrations of 80 and 120  $\mu$ M. Iron solutions contained <sup>55</sup>FeCl<sub>3</sub> and FeCl<sub>2</sub>·4H<sub>2</sub>O stabilized in nitrilotriacetate buffer (NaHCO<sub>3</sub>, 2.05 g/L and NTA, 32 g/L) to prevent precipitation of the ferric isotope. The total contribution of <sup>55</sup>Fe to iron solutions was 37 kBq (0.0023  $\mu$ M) at 80  $\mu$ M and 55.5 kBq (0.0035  $\mu$ M) at 120  $\mu$ M. The incubation persisted for 3 h, after which the medium and lysates were collected and diluted in scintillation buffer (Ultima-Flo AP, Perkin Elmer, Boston, MA). Samples were counted in a Tri-Carb 2800TR liquid scintillation analyzer (Perkin Elmer). The uptake was estimated as a percentage of the apical medium containing the iron solutions, and the basal transfer was estimated as a percentage of intracellular iron levels. Finally, the percentage transport was normalized to Caco-2 total protein levels.

### 2.8. Cytokine stimulation of hepcidin release

The effects of the inflammatory cytokines IL-1 and IL-6 were tested in the Caco-2/HepG2 model by incubating the basal medium with IL-1 or IL-6 (10 ng/well) for 6 h. After the incubation, the medium was aspirated, and fresh MEM (1% FBS) was added. The cells were put back into the incubator for another 18 h before lysis and collection of the basal medium for hepcidin analysis.

### 2.9. Analysis of hepcidin levels in the basal medium

Hepcidin levels in the basal medium were measured in duplicates for all wells (except for the ones in the <sup>55</sup>Fe transport studies) using a commercial sandwich enzyme-linked immunosorbent assay (ELISA) (Bachem, Torrance, CA, USA) according to the manufacturer's instructions. An antibody-coated plate was incubated with samples, antiserum and a biotinylated tracer for 2 h at room temperature. After washing, the wells were incubated with streptavidin-conjugated horseradish peroxidase (HRP) for 1 h. Finally, the wells were washed and incubated with 3,3',5,5'-tetramethyl-benzidine (TMB), a commonly used substrate for HRP. The reaction was stopped with HCl, and the signals were detected by absorbance measurements at 450 nm using a microplate reader (Tecan GmbH, Austria).

### 2.10. Analysis of ferritin and total protein in Caco-2 cells

Intracellular ferritin formation in the Caco-2 cells was estimated by sandwich ELISA (DRG, GmbH, Germany). Briefly, an antiferritin immunoglobulin-G-coated plate was incubated for 1 h with samples, standards and controls together with ferritin antibodies conjugated with HRP. After washing, the TMB substrate was added, and the plate was incubated for a further 10 min (in the dark). The reaction was stopped with HCl, and the absorbance was read at 450 nm with a microplate reader (Tecan, GmbH). Total cellular protein content was estimated by the bicinchoninic acid assay (Pierce, Rockford, IL, USA), which is based on the color change associated with the reaction of bicinchoninic acid with Cu<sup>2+</sup> formed by the biuret reaction in the first step. Absorbance was measured at 562 nm.

### 2.11. Western blot analysis of ferroportin and DMT1 expression in Caco-2 cells

Cell lysates to be analyzed for ferroportin levels were diluted in 2 $\times$  Laemmli sample buffer with  $\beta$ -mercaptoethanol and heated at 90°C for 2 min. Cell lysates measured for DMT1 levels were heated at 100°C for 10 min. Samples (20  $\mu$ g protein) were loaded on TGX-gels (any kD; Bio-Rad, Sundbyberg, Sweden) and electrophoresed with Tris/glycine/sodium dodecyl sulfate buffer at 250 V. The molecular weight standard was a Precision Plus Protein Western C standard (Bio-Rad). After electrophoresis, the separated proteins were blotted to a polyvinylidene difluoride membrane using the Trans-Blot Turbo system with prepacked transfer packs with a 4-min protocol at 2.5 A and 25 V (Bio-Rad). The blotted membranes were blocked for unspecific binding in blocking buffer (Sigma-Aldrich) for 60 min at room temperature or overnight at 4°C. Primary antibodies [rabbit anti-human SLC40A1, NBP1-21502; Novus Biologicals, Littleton, CO, USA, (C-terminal) rabbit anti-human DMT1, NRAMP22-A; Alpha diagnostics, San Antonio, TX, USA or (N-terminal) rabbit anti-human DMT1, NBP1-59869; Novus Biologicals, Littleton, CO, USA] were added at 1  $\mu$ g/ml. The primary antibody to ferroportin used in Fig. 4B was ab85370 (Abcam, Cambridge, MA, USA), which yields a band approximately 10 kD heavier than NBP1-21502 used in Fig. 4A.  $\beta$ -Actin at 0.2  $\mu$ g/ml (Abcam, Cambridge, MA, USA) was added as the loading control, and the membranes were rocked for 1 h, followed by vigorous

washing for 20 min in Tris-buffered saline Tween. The secondary antibody (goat anti-rabbit HRP; Bio-Rad) at 0.5  $\mu\text{g/ml}$  was added together with a Strep-Tactin-HRP conjugate (0.1  $\mu\text{g/ml}$ ) to enhance the signal of the molecular weight standard. Following incubation for 1 h, a final washing step was performed after which the blots were soaked in lumol/hydrogen peroxide (1:1) and immediately analyzed with a chemiluminescence detection system (Chemidoc XRS+, Bio-Rad) and ImageLab software (Bio-Rad).

### 2.12. Statistics

Four sets of cells were analyzed for each cell experiment, and every experiment was repeated on three occasions ( $n=12$ ) if not otherwise stated. Calculated protein concentrations (including ferritin and hepcidin) are thus the mean of 12 individual samples  $\pm$  standard deviation (S.D.). The significance of the difference between treatment and control was analyzed by an unpaired, two-tailed, Student's *t* test using Microsoft Office Excel 2011. Differences were considered significant at  $P<.05$ .

## 3. Results

### 3.1. The effect of iron on hepcidin release

In the first experiments, the aim was to evaluate if iron, given at the apical surface of Caco-2 cells as  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ , would be transported across the basal border at a sufficient rate to be able to affect hepcidin release from HepG2 cells in the basal chamber. We observed that, at 120  $\mu\text{M}$  Fe, there was a significant increase (36%,  $P=.02$ ) in hepcidin levels in the basal medium compared with the iron-free control ( $3.6 \pm 0.3$  ng/ml compared to  $2.7 \pm 0.3$  ng/ml, Fig. 1). There was no significant change in hepcidin levels versus iron-free control at 80  $\mu\text{M}$  (as  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ ). Medium from Caco-2 cells alone and also the contribution of hepcidin from pure medium and FBS 1% were evaluated as controls. There was a small contribution from all controls ( $0.68$ – $1.0$  ng/ml). The difference between controls was insignificant ( $P=.09$ ).

### 3.2. Ferritin formation in Caco-2 cells in the presence and absence of HepG2 cells

Intracellular ferritin formation is a measure of the iron status of the cell and is often used as a surrogate measure of iron uptake [1]. In both the Caco-2 and the Caco-2/HepG2 models, ferritin formation was proportional to the concentration of iron in the system ( $P<.05$ , Fig 2). However, in the Caco-2/HepG2 model, ferritin formation in the Caco-2 cells was significantly higher than in the absence of HepG2 cells. The levels of iron-free Caco-2 controls were  $7.7 \pm 5.8$  ng/mg and  $21.7 \pm 10.3$  ng/mg for the Caco-2 controls of the Caco-2/HepG2 cell coculture (significant difference between controls;  $P=.02$ ).

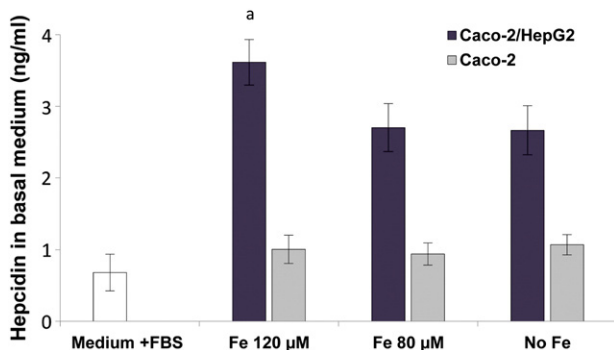


Fig. 1. Hepcidin levels in the basal medium of cocultured and monocultured Caco-2 cells. The Caco-2 cells were treated with iron as  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ . Hepcidin in FBS-supplemented medium was included as control. Data are means of 12 samples  $\pm$  S.D. ( $n=12$ ). The letter a indicates a significant difference ( $P<.05$ ) compared to control (no Fe).

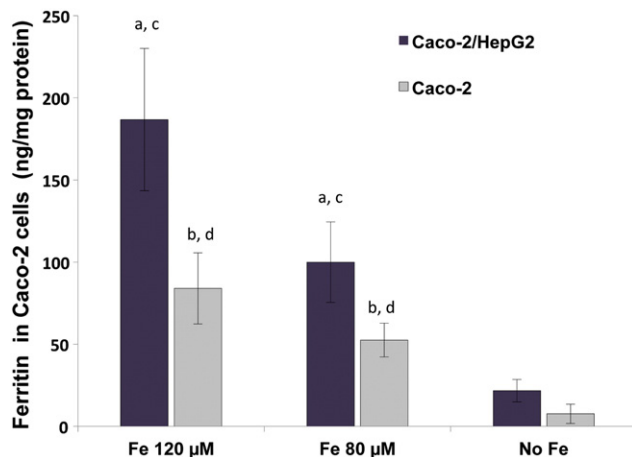


Fig. 2. Ferritin levels in cocultured and monocultured Caco-2 cells. The Caco-2 cells were treated with iron as  $\text{FeCl}_2\cdot 4\text{H}_2\text{O}$ . Ferritin levels are means of 12 samples  $\pm$  S.D. ( $n=12$ ). Letters a and b indicate a significant difference ( $P<.05$ ) compared to control (no Fe). Letters c and d indicate that  $P<.05$  between treatments.

### 3.3. Iron uptake and basolateral transport

Iron uptake at the apical surface of the Caco-2 cells was higher in the Caco-2/HepG2 cell model at 80  $\mu\text{M}$  Fe compared to monocultured Caco-2 cells ( $8.3\% \pm 1.4\%$  compared to  $4.9\% \pm 1.3\%$   $^{55}\text{Fe}$  uptake/mg protein, Fig. 3A). In the Caco-2 model, the percentage iron uptake at 120  $\mu\text{M}$  was slightly, but insignificantly, higher compared to iron at 80  $\mu\text{M}$ , while the uptake at 120  $\mu\text{M}$  in the Caco-2/HepG2 model decreased compared to 80  $\mu\text{M}$ . This suggests that iron uptake reached the plateau level in which further uptake was inhibited. To find a possible reason for this observation, the levels of the apical iron transporter DMT1 were estimated. We also measured basolateral iron transport in the two cell models, which was found to follow the expected trends indicated by the ferritin levels. Iron transport was significantly increased at incubation with 120  $\mu\text{M}$  compared to 80  $\mu\text{M}$  in the Caco-2/HepG2 system ( $8.1\% \pm 1.5\%$  and  $4.4\% \pm 1.5\%$  per mg protein, respectively; Fig. 3B). In the Caco-2 model, the higher iron concentration affected iron transport, but not significantly ( $3.5\% \pm 2.5\%$  compared to  $2.9\% \pm 0.2\%$  per mg protein).

### 3.4. Hepcidin down-regulates both DMT1 and ferroportin in the Caco-2/HepG2 model

The levels of the Caco-2 apical iron transporter DMT1 were completely undetectable when the cells were incubated with externally added hepcidin at 10 ng/ml (Fig. 4A). Interestingly, a small fragment of DMT1 at 20 kD was observed. There was no significant difference between the iron-treated cells and iron-free controls (hepcidin at 3.6–2.7 ng/ml). The ferroportin levels of the Caco-2 cells in the Caco-2/HepG2 system followed the same pattern as the hepcidin levels in the medium with a change occurring at 120  $\mu\text{M}$  Fe and 3.6 ng/ml hepcidin, which was associated with a decrease ( $20\% \pm 10\%$ ,  $n=9$ ) in ferroportin levels normalized to the cellular expression of  $\beta$ -actin (Fig. 4A). External hepcidin addition to the basal medium at 10 ng/ml (4 nM) rendered ferroportin levels undetectable. A dose-response experiment with external addition of hepcidin to mono-cultured Caco-2 cells was made for comparison (Fig. 4B). In monocultured Caco-2 cells, ferroportin levels were not significantly altered by hepcidin at 10–100 ng/ml (4–40 nM,  $n=6$ ) when the cells were incubated in serum-free medium for 24 h before the experiment. We did not observe sufficient levels of the DMT1 protein during the present iron load. We have previously observed the DMT1 protein at measurable levels in monocultured Caco-2 cells after 48–72

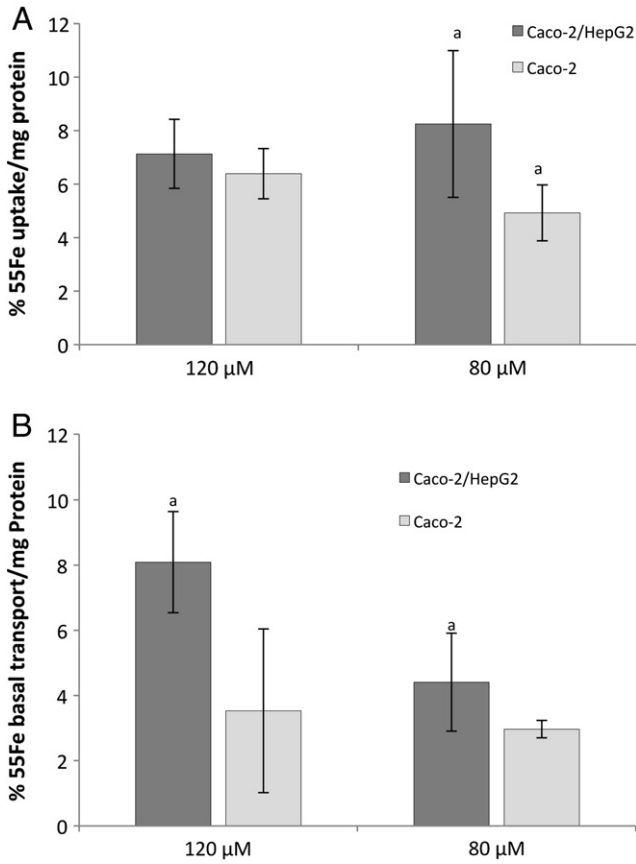


Fig. 3. Iron uptake and basal transfer in the Caco-2 vs. Caco-2/HepG2 model. (A) Uptake at the apical surface of the Caco-2 cells was estimated using the radioisotope <sup>55</sup>Fe. The cells were incubated with iron (80 or 120 μM) for 3 h. The hepcidin level in the basal medium of the cocultured cells was insignificantly affected 3 h after the iron addition. Iron transport is expressed as the percentage of the added iron-containing medium. Levels are means±S.D. (n=6). (B) Iron (<sup>55</sup>Fe) transport across the basolateral border of Caco-2 cells in the presence and absence of HepG2 cells. Levels are means±S.D. (n=6).

h of serum-free conditions [5]. If monocultured Caco-2 cells were cultured in medium supplemented with FBS 1%, both ferroportin and DMT1 levels were undetectable (data not shown). Comparing data presented in Fig. 4A and B, it is evident that ferroportin and DMT1 proteins are more abundant in Caco-2 cells of the coculture system, which is also supported by the data in Fig. 3 showing that iron uptake and transport are higher in the cocultured cells compared to monocultured cells. A possible explanation for the higher ferroportin and DMT1 levels in the coculture system, despite higher levels of baseline hepcidin, is that the Caco-2 cells may experience a lower iron load since the HepG2 cells absorb iron from the medium and are covering a three-times greater surface area than the Caco-2 cells, and thus, the HepG2 iron usage is relatively high.

### 3.5. IL-6, but not IL-1, induced hepcidin release into the medium

The cytokine IL-6 is a well-known hepcidin inducer in plasma, which causes the associated anemia of inflammatory conditions. The basal medium of the Caco-2/HepG2 system was treated with IL-6 (10 ng/well), which induced a 2.4-fold increase (P=.04) in hepcidin secretion (from 0.36±0.06 to 0.86±0.2 ng/ml; Fig. 5). Another cytokine, IL-1, which is less associated with hepcidin levels *in vivo*, was added to the basal medium at 10 ng/ml. Hepcidin levels were 0.26±0.04 ng/ml in IL-1-treated medium, which is not significantly different compared to untreated cells (P=.11).

### 3.6. Hepcidin and ferritin levels increase in response to sourdough bread

We also compared samples of two types of bread (sourdough and nonsourdough) containing the same ingredients in the two cell models. Both types of bread contained similar amounts of soluble iron after *in vitro* digestion and filtration (0.193 and 0.190 mg Fe/L, respectively). Baking with sourdough has been shown to increase iron bioavailability [15]. The formation of ferritin was higher in Caco-2 cells of the Caco-2/HepG2 system in response to the sourdough bread (29.3±12.4 ng/mg protein) than with the nonsourdough bread

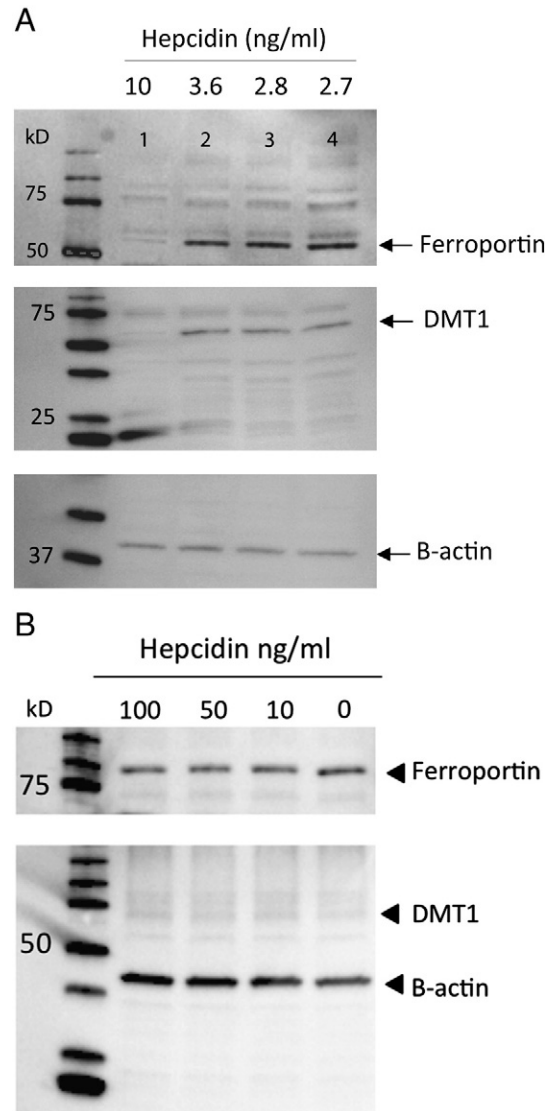


Fig. 4. Hepcidin regulation of DMT1 and ferroportin protein levels in HepG2 cocultured Caco-2 cells. (A) Western blot of ferroportin and DMT1 levels in cocultured Caco-2 cells. Lane 1: The basal medium was exogenously added with hepcidin-25 at 10 ng/ml as a positive control, lane 2: 120 μM Fe in the apical medium, lane 3: 80 μM Fe in the apical medium, lane 4: no Fe. DMT1 and ferroportin transporters were absent in the presence of exogenously added hepcidin (10 ng/ml). Ferroportin levels decreased at apical addition of 120 μM Fe (20%±10%, n=9), which generated hepcidin levels of 3.6 ng/ml in average. (B) Western blot of ferroportin and DMT1 levels in monocultured Caco-2 cells in response to external addition of hepcidin-25 to the basal medium. The cells were cultured in serum-free medium 24 h before the experiments. Lane 1: 100 ng/ml, lane 2: 50 ng/ml, lane 3: 10 ng/ml, lane 4: no treatment (control). Ferroportin levels were not significantly changed in response to hepcidin addition (n=6) in monocultured Caco-2 cells. DMT1 was undetectable at the present iron load and experimental conditions. All blots were made with 20 μg of total protein and detected using chemiluminescence.

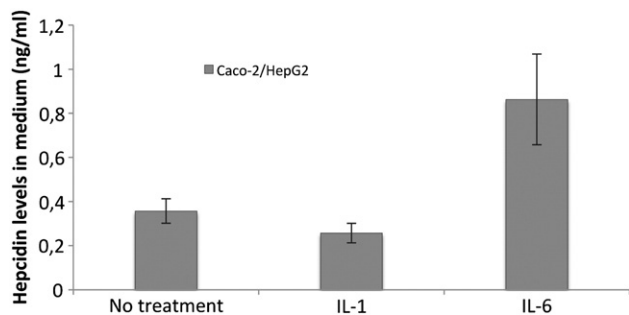


Fig. 5. Induction of hepcidin production with the cytokine IL-6. The basal medium of the Caco-2/HepG2 cells was treated with IL-6 or IL-1 (10 ng/well) to simulate inflammatory effects on the acute phase protein and iron absorption regulator hepcidin. Levels are means  $\pm$  S.D. ( $n=9$ ). The letter a indicates a significant difference ( $P<.05$ ) between the treatments and no treatment).

( $14.2 \pm 0.44$  ng/mg protein, 53%,  $P=.05$ ), despite the soluble iron content being the same (Fig. 6). The increase in Caco-2 cell ferritin was associated with an increase in hepcidin concentration in the basal medium (97%,  $P=.03$ , Fig. 7), indicating an inhibitory effect on ferroportin transport of ferrous iron. These results support that baking with sourdough increases bioavailability of iron [16] since intracellular ferritin levels increased, despite the soluble iron content in the breads being the same. Importantly, this effect is not observed in monocultured Caco-2 cells.

#### 4. Discussion

The Caco-2 cell model has several advantages as an *in vitro* assay of iron uptake through the apical membrane of enterocytes (iron bioaccessibility) [17]. Such advantages include the ability to investigate the effects on iron bioaccessibility of other components in food, such as competing minerals, chelating compounds and intracellular factors of human enterocytes. In general, there is good correlation between iron absorption in *in vitro* Caco-2 cell studies and *in vivo* in humans fed single meals [2,3]. In most cases, the model is able to predict the direction of a response due to iron absorption enhancers or inhibitors, which suggests that the regulation of iron uptake occurs predominantly at the enterocyte

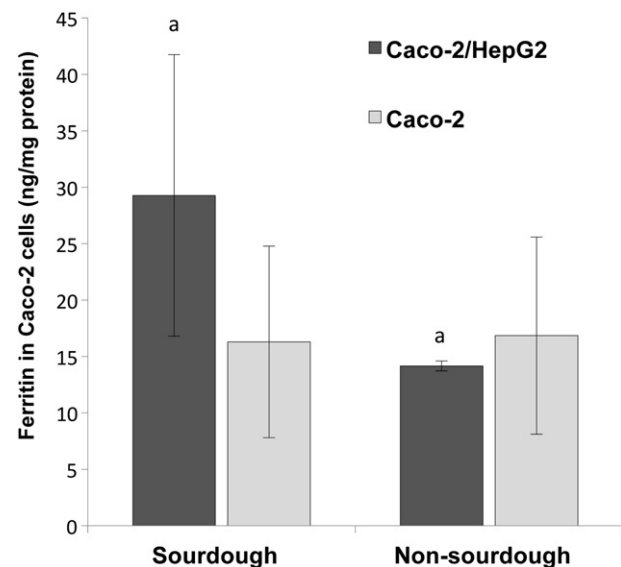


Fig. 6. The ferritin levels in response to *in vitro* digested bread samples were compared in both the Caco-2 and Caco-2/HepG2 cell models. Data are means of 12 samples  $\pm$  S.D. ( $n=12$ ). The letter a indicates a significant difference ( $P<.05$ ) between the breads).

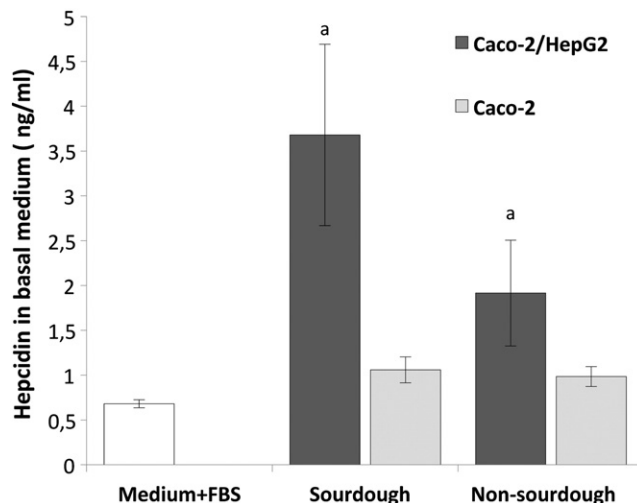


Fig. 7. Hepcidin release into the basal medium after exposure to bread samples. The Caco-2 cells with and without HepG2 cells were exposed to either a sourdough or a scalded bread sample. Data are means of 12 samples  $\pm$  S.D. ( $n=12$ ). The letter a indicates a significant difference ( $P<.05$ ) between the breads).

level in the intestinal wall. However, there are several situations when this is not the case, e.g., when the intestine is exposed to an abnormally high or low iron load and during inflammation or infection [18] in which systemic regulation through hepcidin, secreted by the liver, becomes important.

We identified critical success factors for a combined intestinal–liver model as: (a) The model should be easy to prepare and minimize additional labor due to the cultivation of the liver cells. (b) The chosen hepatic cell line should be easily accessible for all researchers and possible to purchase. (c) The hepatic cells should have similar nutritional requirements as the Caco-2 cells. (d) The hepatocytes must express hepcidin and be responsive to transferrin-bound iron, which is the prerequisite for the regulation using the SMAD/BMP pathway as reviewed in Ref. [19]. (e) The secretion from the liver cells must be reproducible; the cells should not lose their phenotypes in culture, as is the case with primary hepatic cell lines [20].

In the Caco-2/HepG2 model, we used MEM supplemented with 1% FBS as basal medium since we aimed to simulate the vascular compartment. However, depending on the study, it may be suitable to use a serum-free medium as the apical medium for the last 24 h before the experiments are run to, e.g., decrease the iron contribution. Since Caco-2 cells produce and release transferrin, serum-free conditions may not affect the hepcidin-dependent regulation. If not using serum as medium supplement, the presence of transferrin should be considered when designing the coculture especially if another intestinal cell line than Caco-2 is chosen. Several different serum-free formulations have been reported in the literature, as reviewed by Barnes [21], often containing epidermal growth factor in combination with triiodothyronine and insulin. Addition of insulin has been shown to have no effect on cell growth or differentiation of Caco-2 cells [22]. We used serum-free conditions (for 6 h only) when adding the inflammatory cytokine IL-6 to stimulate hepcidin production in the HepG2 cells. Hepcidin induction by IL-6 is transferrin-independent and activated by the JAK/STAT pathway [23]. After the 6-h incubation, the cells were cultured in serum-containing medium until cell lysis and basal media collection.

The mechanism of hepcidin inhibition of iron absorption has been debated for several years. One of the accepted theories is that hepcidin inhibits iron release from intestinal cells via down-regulation of ferroportin [12]. In a relatively recent study of ferroportin expression

in response to hepcidin in Caco-2 cells, the authors did not observe a down-regulation of the transporter plasma membrane levels, but they did measure a decrease in iron efflux [24]. Ferroportin is considered as the one and only transporter of iron across the basal membrane of intestinal cells.

In addition, the effect of hepcidin in intestinal Caco-2 cells has been ascribed to inhibition of apical uptake of iron by DMT1 [25]. A more recent study reported that an acute increase in hepcidin concentration (200 nM) decreased DMT1 protein levels [26]. These former studies are supported by the present study, in which we observed an almost complete down-regulation of DMT1 at hepcidin levels of 10 ng/ml. We also found a light fragment of DMT1 at 20 kD, which may be a cleavage product and possibly suggests that hepcidin regulates iron uptake by inducing cleavage of the extracellular (N-terminal) domain of DMT1. In addition, there seem to be traces of such a fragment at hepcidin levels of 3.6 ng/ml. Normal serum levels of hepcidin in humans range from 6 to 100 nM [27]. However, DMT1 protein abundance, not necessarily expression, is more sensitive to iron load than ferroportin, which suggests that regulatory events may be first visible in changes in DMT1 levels even if the effect of hepcidin would be mainly at the site of ferroportin.

Ferroportin levels were almost undetectable at 10 ng/ml as observed 24 h after the cells were first incubated. Ferroportin levels were unchanged at 80  $\mu$ M Fe compared to iron-free control, but decreased at 120  $\mu$ M. This change correlated with the increase in hepcidin levels, which increased at 120  $\mu$ M. Similarly, the sourdough bread samples induced higher hepcidin levels in the medium, which correlated with the increased ferritin formation in the Caco-2 cells indicating that iron was confined inside the cells most probably due to a decreased level of ferroportin transporters in the Caco-2 cells. The sourdough bread experiments also show that low concentrations of iron (3.4  $\mu$ M) in a favorable matrix (sourdough) may be more bioavailable than high iron (120  $\mu$ M) distributed as a salt.

With the present work, we propose the Caco2/HepG2 model as an alternative and more physiological approach for *in vitro* studies of mechanisms related to iron absorption, which is dependent on hepcidin production and secretion. The present study serves as an attempt to make the Caco-2 *in vitro* intestinal model more physiological, with a simplified simulation of a systemic response to Fe loading. We also demonstrate that, using the coculture instead of monocultured Caco-2 cells, we could predict the increased iron bioavailability of sourdough breads compared to nonsourdough bread.

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