

Applied methodology

## The effect of amino acid deprivation on the transfer of iron through Caco-2 cell monolayers

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### Abstract

Iron (Fe) metabolism is modified by many nutritional factors. Amino acids (AA) play a central role in various biological processes, such as protein synthesis and energy supply. However, the influence of AA status on iron metabolism has not been investigated. Here, we test whether AA alters iron metabolism in an intestinal cell model. Both Fe uptake and transfer across the cell monolayer were significantly increased by non-essential AA deficiency (both  $p < 0.001$ ) while only Fe transfer was increased by essential AA deficiency ( $p < 0.0001$ ). Both essential and non-essential AA deficiency decreased DMT1 ( $\pm$ IRE) exon1A mRNA expression (respectively  $p = 0.0007$  and  $p = 0.006$ ) and increased expression of ferritin heavy chain. DMT1 + IRE (also expressing exon1A or 1B) mRNA levels were decreased by essential AA deficiency ( $p = 0.012$ ). The mRNA levels of total DMT1 were also decreased by essential, but not non-essential, AA deficiency ( $p = 0.006$ ). Hepcidin levels were increased significantly by non-essential amino acid deprivation ( $p = 0.047$ ). Protein levels of ferroportin and/or ferritin heavy chain were not altered by AA deficiency, suggesting that they had no effect on Fe efflux or storage in the cell, though iron content of ferritin could be increased. Our data demonstrate, for the first time, that AA status affects iron transport and the expression of genes related to iron metabolism in Caco-2 cells, although the changes observed are not sufficient to explain the alteration in iron transport. We hypothesise that the effect on Fe transfer is mediated through an increased movement across the cell layer, rather than transfer across the cell membranes.

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**Keywords:** Amino acid deprivation; Nutrient transfer; [Gut](#) transport

## 1 Introduction

Iron is a key trace element, involved in different processes of metabolism such as respiration, energy production, DNA synthesis and cell proliferation. However, given its capacity for changing valency, it also has potential for toxicity at excessive levels. Consequently, a tight regulation of absorption and excretion has evolved.

Many studies have examined the way other nutrients and micronutrients interact with iron metabolism. These interactions can be both direct and indirect. For example, vitamin C or cysteine and histidine can bind iron atoms, increasing bioavailability and increasing iron absorption [1-3]. Alternatively, copper deficiency can negatively impact iron flux from the liver by decreasing the expression of ceruloplasmin, required for iron incorporation into transferrin [4]. Nutrients that alter expression of some of the iron regulatory pathways can also modify iron metabolism [5]. These compounds have proved particularly valuable in elucidating the regulation of iron.

Dietary iron is found in two forms; haem iron and non-haem (inorganic) iron. These two forms of iron have separate absorption mechanisms across the gut [6-8]. Haem iron is transported either by a haem

importer, possibly HCP1 (haem carrier protein 1, SLC46A1), though it may primarily be a folate transporter [9,10], or by a receptor-mediated endocytotic pathway [11]. Once internalized, haem iron is released as ferrous iron by hemoxygenase, mainly hemoxygenase 1 (HOX1) [12], and then enters the same pathways as non-haem iron [13]. Non-haem iron needs to be first reduced from ferric iron (Fe<sup>3+</sup>) to ferrous iron (Fe<sup>2+</sup>) by Dcytb (duodenal cytochrome B) on the gut apical cell membrane. Ferrous iron is then transported into the enterocytes through DMT1 (divalent metal transporter 1, or SLC11A2). In the enterocyte, iron becomes part of the labile iron pool [14], and/or becomes bound to the storage protein ferritin, or is released into the blood stream via ferroportin, a transmembrane transport protein [15]. The iron is exported as Fe<sup>2+</sup>, and is oxidised by hephaestin prior to incorporation into transferrin.

Iron absorption is regulated by a hormone called hepcidin [16-18]. Hepcidin is secreted by the liver and binds to ferroportin in target cells, inducing internalization and degradation of ferroportin [19]. Hepcidin is not only regulated by iron bioavailability and hepatic iron storage but also by other factors including erythropoietic activity, hypoxia and inflammatory or infectious disease.

Four isoforms of DMT1 have been identified [20]. The predominant isoform carries an iron regulator element (IRE) sequence and an exon 1A sequence [21]. This isoform (+IRE/exon1A) has the highest iron uptake rate and is therefore considered as the most active isoform. DMT1(+IRE/exon1A) is present mostly at the apical membrane of enterocytes. An intermediary isoform exhibits the IRE element and an exon1-B sequence. This form (+IRE/exon1B) is mildly active and is found mostly in blood cells [22]. The presence of either exon1A or exon 1-B is thought to have a link with the localization of the DMT1 on different membranes. The two other isoforms have no IRE sequence and can contain either exon1A (-IRE/exon1A) or exon1-B (-IRE/exon1B). They are thought to be localized on the membrane of endosomes [23,24]. The isoforms of DMT1 exhibiting an IRE sequence are regulated in the same way as Tfr1 and their mRNA sequences are stabilized by IRP binding [24]. This means that in presence of high concentration of iron, DMT1 mRNA is degraded, while in iron deficiency, translation of DMT1 mRNA translation is increased.

Amino acid absorption across the gut is also very tightly regulated by a variety of different signaling pathways, the most important one being mTOR. Depletion of amino acids alters significantly mTOR and GCN2/eIF2 $\alpha$  pathway, both responsible for major protein synthesis changes and shut-down [25,26]. The inhibition of mTOR decreases phosphorylation of different actors involved in ribosome recruitment (4E-BPS and downstream targets) and elongation (S6Ks and downstream targets). The activation of GCN2 leads to the inhibition of an elongation factor required for protein synthesis (eIF2B) while up-regulation of ATF4 and ATF2 by eIF2 $\alpha$  increase the transcription of targeted genes via binding to an Amino Acid Regulatory Element (AARE). We have shown that iron is intimately involved in this process, and deficiency results in changing of activity of phosphorylation pathways [27]. Despite the fact that they share regulatory signaling pathways, such as mTOR, the possibility of an inverse relationship between amino acid metabolism and iron metabolism – that changing status of amino acids alters iron metabolism – has never been studied. Given the central role they both play in metabolism, this is perhaps surprising. Amino acids are categorized as essential or non-essential according to their ability to be synthesized by human cells. We tested the hypothesis that altering their levels will, in turn, affect iron metabolism in gut cells.

## 2 Material and methods

### 2.1 Caco-2 cell line

The Caco-2 line TC-7 strain was kindly provided by the National Institute of Food and Nutrition (INRAN), Rome, Italy (RRID:CVCL\_0233). The protocols used for maintenance and experiments are derived from the European project EU FP6 Project “LIINTOP”, which has been developed as a gold standard approach and to give the most reliable results [28]. The cells were maintained in 56.7 cm<sup>2</sup> plates (Nunclon, Nunc, cat n°172958) in DMEM (Dulbecco’s Modified Eagle Medium, 4.5 g/L Glucose, 11 g/L sodium pyruvate, no L-Glutamine, Gibco Life Technologies, Ref 21969-035) supplemented with 10% FCS (Fetal Calf Serum, heat inactivated, Hyclone Laboratories, Inc.), penicillin (100kU/L), streptomycin (100 g/L) (Pen Step, Gibco Life Technologies, cat n°10378-06), glutamine (4 mM) (100x, Gibco, Life Technologies, cat n°25030-024) and 1% non-essential amino acid solution (Sigma Aldrich, M7145). This DMEM preparation is referred as ‘complete DMEM’ below.

### 2.2 Passaging and viability

Cells were passaged routinely at  $4.5 \times 10^3$  cells/cm<sup>2</sup> for normal line maintenance, allowing them to grow to approximately 50% confluence between passages in a humidified incubator at 37 °C under a 10% CO<sub>2</sub> atmosphere [28,29]. Cells were used for experiments between passage 10 and passage 33 as recommended [30,31].

For each transport experiment, cells were seeded at  $3 \times 10^5$  cells/cm<sup>2</sup> on 1.12 cm<sup>2</sup> inserts (Snapwell™ permeable support, 0.4  $\mu$ m polycarbonate membrane, Corning Star, ref 3407). After one week, the apical medium routinely used for maintenance was DMEM without FCS, while complete DMEM was still used as basolateral medium [28,32]. The cells were considered as differentiated and usable for experiments when the transepithelial electric resistance (TEER,  $\Omega$ -cm<sup>2</sup>) reached 240  $\Omega$ -cm<sup>2</sup> reached 240  $\Omega$ -cm<sup>2</sup>, which takes about 21 days [33].

### 2.3 Amino acid depleted media

The AA deprived media were prepared using HBSS (Hank's balanced Salt Solution), 1 mM sodium pyruvate, 1 x vitamin solution (Sigma Aldrich x100, M6895), 270 USP units/L insulin (Human recombinant, zinc solution, Gibco Life technologies, cat n°12585-014), 5.5 mg/L transferrin (Human, T8158, Sigma Aldrich), 0.029 µM sodium selenite, 100 µg/mL streptomycin, 100 kU/L of penicillin (Pen Strep, see above), 4 mM L-Glutamine. Non Essential Amino Acids Solution and Amino Acids Solution (M5550) were used at different concentrations for amino acid deprivation of the cells (Table 1). When the concentrations of essential amino acids were studied, the concentration of non-essential amino acids was kept constant at 100% and vice versa. All media were adjusted to a pH of 7.4.

**Table 1** Final concentrations (µmol/L) of amino acids in the control medium (100% AA), essential amino acid depleted (0% EAA) and non-essential amino acid depleted (0% NEAA) media. Glutamine is added separately to control and essential amino acid depleted media, and is considered as a non-essential amino acid.

alt-text: Table 1

	100% AA (µM)	0% EAA (µM)	0% NEAA (µM)
ESS and COND ESS (M5550)			
L-Arginine	126.4	0.0	126.4
L-CYSTEINE•L-cysteine•2HCl	31.3	0.0	31.3
L-HISTIDINE•HCl•L-Histidine•HCl•H2O	42.0	0.0	42.0
L-Isoleucine	52.5	0.0	52.5
L-Leucine	52.4	0.0	52.4
L-LYSINE•L-Lysine•HCl	72.5	0.0	72.5
L-Methionine	15.1	0.0	15.1
L-Phenylalanine	33.0	0.0	33.0
L-Threonine	47.6	0.0	47.6
L-TRYPTOPHAN•L-Tryptophan	10.2	0.0	10.2
L-Tyrosine	36.0	0.0	36.0
L-Valine	46.8	0.0	46.8
NON ESS (M7145)			
L-Alanine	8.9	8.9	0.0
L-ASPARAGINE•L-Asparagine•H2O	15.0	15.0	0.0
L-Aspartic Acid	13.3	13.3	0.0
L-Glutamic Acid	14.7	14.7	0.0
Glycine	7.5	7.5	0.0
L-Proline	11.5	11.5	0.0
L-Serine	10.5	10.5	0.0
L-Glutamine	584.6	584.6	0.0

## 2.4 <sup>59</sup>Fe transepithelial transport

After differentiation, cells were incubated overnight (17 h) in media containing different concentrations (0–100% control) of either essential or non-essential amino acids. The following morning, the cells were washed three times in PBS (Phosphate Buffered Saline) prior to addition of the experimental media.

Two buffers were used to conduct the transport experiments. The apical buffer consisted of 20 mM HEPES, 5 mM Glucose, 1 mM Phenol Red in 1xBSS (pH7.4). The basolateral buffer was made of 20 mM HEPES, 5 mM Glucose in 1xBSS (pH7.4). For all these experiments, 6  $\mu\text{Ci/mL}$  of  $^{59}\text{Fe}$  was added to the apical medium as Fe-NTA (1 M ratio, 6  $\mu\text{Ci/mL}$ ). Transfer of iron across the cell layer was measured by taking samples from the basolateral side, replacing the sample volume with fresh buffer, every 30 [see](#).

Following incubation, the cells were washed three times in BSS and lysed in 0.05 M NaOH. Cells were lysed for 15 min on ice. The lysate was then heated for 30 min at 90° C to denature the DNA. The radioactivity of each sample (basolateral media and cell lysates) was measured in a gamma counter (Wizard 3" 1480, Automatic gamma counter, Perkin Elmer) calibrated against standards of the incubation medium.

## 2.5 RT-qPCR

After treatment, cells were washed with PBS. Total RNA was isolated from trypsinised cells using an RNeasy kit (Qiagen Cat N°74104) added to an RNase-Free DNase set (Qiagen Cat N°79254). RNA (200 ng) was reverse transcribed with a Taqman reagent kit (Applied Biosystems, cat N°4312704) according to the following cycle: 25 °C for 10 min, 37 °C for 60 min and 95 °C for 5 min. The cDNA was amplified with Power SYBR Green Master mix (Applied Biosystems, Life Technologies, cat N°4309155). Real time qRT-PCR was performed using Applied Biosystem 7500 Fast thermal cycler according to the following cycle: 95 °C for 10 min, 1 repeat; 95 °C for 15 [seconds](#) and 60 °C for 1 min, 40 repeats. Specific primers were designed with the Primer BLAST tool from NCBI and checked for single amplification ([Table 2](#)). DMT1 primers were designed to differentiate isoforms according to the presence of IRE (DMT1 +IRE (exon1A or 1B)), the absence of IRE (DMT1 –IRE (exon 1A or 1B)), the presence of exon 1A (DMT1 ( $\pm$ IRE) exon1A), or the presence of exon 1B (DMT1 ( $\pm$ IRE) exon1B).

**Table 2** Nucleotide sequence of primers used to determine levels of the genes measured in this paper.

alt-text: Table 2

Gene of interest	Type of sequence	Nucleotide sequence
DMT1(+IRE)	Forward	ACC TTA GAA CTG GAT TAG GGT
	Reverse	AAG GAT AAA CTG AGC TCG CCC
DMT1(-IRE)	Forward	AAA TGT AGG TCA GAG ACC CAC
	Reverse	AAT CCC AGA GTC CAA GAC ACA
DMT1(exon1A)	Forward	AGG CAG CTC CAC ACT GTG AAC
	Reverse	ACA TCT TCT GTT CAG GAC CCA
DMT1(exon1b)	Forward	TGG CGC TGG CTC CCG GAA TAT
	Reverse	GAA TAT GAT TCT TAC CAG CTC
18S	Forward	AGA AAC GGC TAC CAC ATC CCA
	Reverse	CAC CAG ACT TGC CCT CCA

The other primers were purchased from Qiagen (Quantitect primers) ([Table 3](#)). Expression data were normalized using Ct values of the internal control 18S. Fold differences were calculated using the  $\Delta\Delta\text{Ct}$  method ([34](#)).

**Table 3** Product number and company of purchase of commercially available primers.

alt-text: Table 3

Gene of interest	Company of purchase	Catalogue number
Ferritin Light (Hs_FTL_1_SG)	Qiagen (Quantitect primers)	QT00055860
Ferritin Heavy (Hs_FTH1_1_SG)	Qiagen (Quantitect primers)	QT00072681
Ferroportin (Hs_SLC40A1_1_SG)	Qiagen (Quantitect primers)	QT00094843
Hepcidin (Hs_HAMP_1_SG)	Qiagen (Quantitect primers)	QT00200522
Hephaestin (Hs_HEPH_1_SG)	Qiagen (Quantitect primers)	QT00008106

## 2.6 Protein expression of ferroportin and intracellular and extracellular ferritin heavy chain by ELISA

After differentiation, cells were treated overnight with various concentrations (0–100%) of essential and non-essential amino acids. The medium was recovered for each sample to measure extracellular levels of ferritin. Cells were washed, scraped into PBS and collected by centrifugation for 3 min at 3000xg. The cell pellets were washed, resuspended in PBS and ultra-sonicated at low amplification 3 times for 5 sec. The suspension was then centrifuged at 1500xg for 10 min at 4 °C to remove cellular debris. The supernatant was then transferred into a fresh tube. The proteins in recovered medium were concentrated using Microcon® Centrifugal Filter Devices (Merck Millipore).

Before using the concentrators, bovine serum albumin (Sigma Aldrich, BSA, P5369) (final concentration 0.1 mg/mL) was added to the samples to reduce non-specific binding. Samples were concentrated using either 30 K or 10 K Microcon® Centrifugal Filter Devices (Merck Millipore) which were used according to manufacturer's instructions. The volume of concentrated medium obtained for each sample was recorded in order to normalise the results of the ELISA.

Ferroportin expression levels were measured using a sandwich enzyme immunoassay kit (SEC498Hu 96 tests, Enzyme-linked Immunosorbent Assay Kit for Ferroportin (FPN), Homo sapiens (human) from Cloud-Clone Corp). Intracellular and extracellular ferritin expression levels were measured using a sandwich enzyme immunoassay kit (SED021Hu 96 tests, Enzyme-linked Immunosorbent Assay Kit for Ferritin, Heavy Polypeptide (FTH), Homo sapiens (human) from Cloud-Clone Corp).

After bringing samples to room temperature, ferritin heavy chain extracellular and intracellular levels were measured using manufacturer's instructions. DNA assays were performed to normalise the protein levels, and data were then expressed as percentage of the control (100% amino acids).

## 2.7 Statistical analysis

All experiments were repeated three times and values are expressed as means ± S.E.M. Values were normalized by the DNA content of each insert monolayer and expressed as % of control (100% amino acids at 30 min for transfer experiments, 100% amino acids for RT-qPCR and protein expression).

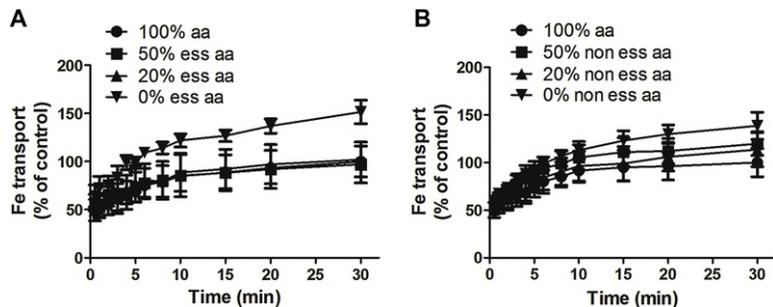
When two parameters were tested (time and concentrations) data were analysed using a two-way ANOVA or a corresponding non-parametric test when equality of variances was not verified. When only one parameter varied, statistical significance was determined by one-way ANOVA or corresponding non-parametric test when equality of variance was not verified.

All tests were performed using Graph-Pad Prism 5 statistical software (<http://www.graphpad.com/>, RRID:SCR\_002798) and  $p \leq 0.05$  was considered as significant.

## 3 Results

### 3.1 <sup>59</sup>Fe transepithelial transport is increased by both essential and non-essential amino acid deprivation

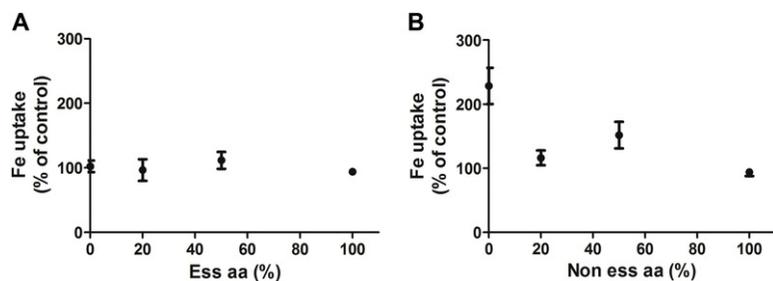
<sup>59</sup>Fe across the cells monolayer was linear across 30 min incubation. The transfer was temperature dependent, as would be expected of an active transport process. Reducing the concentration of either essential or non-essential amino acids increased transfer across the cells ( $p < 0.0001$  in both cases) (Fig. 1A and B), without a change in the TEER or an increase in Fe levels early in the incubation period, as might be expected if the reduction in amino acid supply were increasing passive permeability of the cell layer. Further, phenol red levels in the basolateral medium remained undetectable under all experimental conditions and DNA content on the filters was not altered by the treatments (Appendix A, Fig. A1).



**Fig. 1** Effect of changing amino acid concentrations on iron transport through a Caco-2 cell monolayer. Caco-2 cells were incubated overnight with differing concentrations of (A) essential amino acids or (B) non-essential amino acids. After washing,  $^{59}\text{Fe}$ -NTA was introduced in the apical side and  $^{59}\text{Fe}$  transported through the monolayer was measured from the basolateral side 30 min after addition. Values were normalized to the DNA content of each insert monolayer and expressed as % of control (100% AA at 30 min). Values are mean  $\pm$  S.E.M.,  $n = 6$  per treatment/time point. Statistical significance was determined by a two-way ANOVA followed by a Bonferroni post-hoc,  $p \leq 0.0001$  for both essential and non-essential amino acid treatments. ess aa: essential amino acids, non ess aa: non-essential amino acids, aa: amino acids.

alt-text: Fig. 1

Reduction of essential amino acid concentrations did not affect iron content of the cells (one-way ANOVA  $p = 0.72$ ) (Fig. 2A). In contrast, depletion of non-essential amino acids resulted in a significant increase in iron content ( $p < 0.0001$ ) (Fig. 2B).



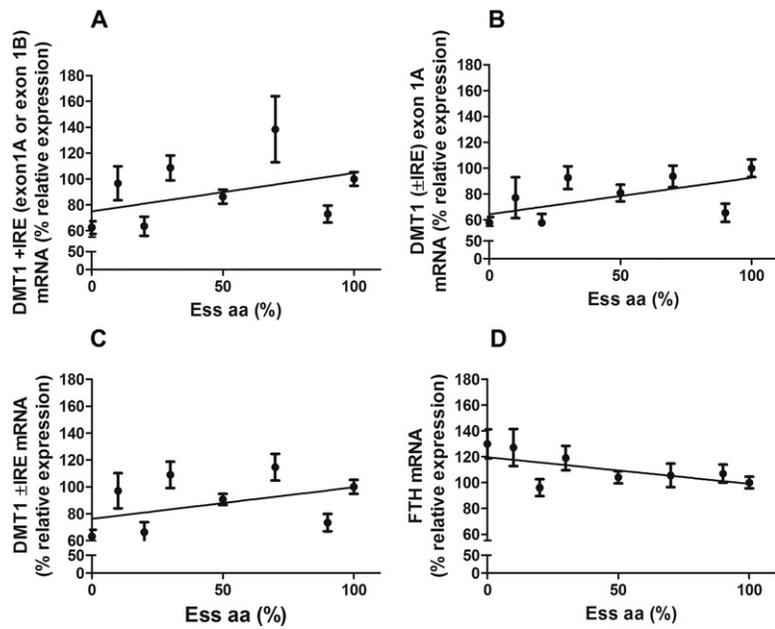
**Fig. 2** Effect of changing amino acid concentrations on iron levels within the Caco-2 cell monolayer. Caco-2 cells were incubated overnight with differing concentrations of (A) essential amino acids or (B) non-essential amino acids. After washing,  $^{59}\text{Fe}$ -NTA was introduced to the apical side. After 30 min, cells were treated as described in Materials and Methods and  $^{59}\text{Fe}$  levels within the cell monolayer were determined. Values were normalized to the DNA content of each insert and expressed as % control (100% AA). Values are mean  $\pm$  S.E.M.,  $n = 6$ . Statistical significance was determined by either a one way ANOVA ( $p = 0.0720$ ) (A) or a non-parametric Kruskal-Wallis test ( $p \leq 0.0001$ ). NTA: nitrotriacetate, ess aa: essential amino acids, non ess aa: non-essential amino acids.

alt-text: Fig. 2

### 3.2 Gene expression

The expression of different genes involved in iron metabolism was measured following essential or non-essential amino acid depletion.

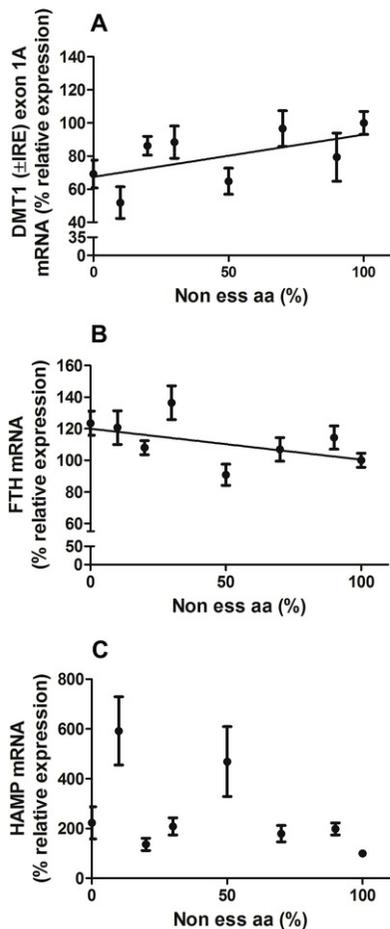
There are four isoforms of DMT1, with exons 1A or 1B, both either with or without an iron regulatory element (+IRE or -IRE respectively). As explained in material and methods, we measured the presence or absence of one feature at a time. When measuring the presence of IRE, the isoform expresses exon1A and 1B, and is referred as +IRE (exon1A and 1B). When measuring the presence of exon1A, the isoform expresses either IRE or not and is referred as ( $\pm$ IRE) exon1A. Essential amino acid depletion resulted in a decrease in expression of both +IRE (exon 1A or 1B) and ( $\pm$ IRE) exon1A (respectively,  $p = 0.012$  and  $p = 0.0007$ ) (Fig. 3A and B). The mRNA levels of total DMT1 showed an overall significant decrease in expression ( $p = 0.006$ ) (Fig. 3C). In contrast to DMT1, ferritin heavy chain mRNA levels significantly increased with essential amino acid depletion ( $p = 0.01$ ) (Fig. 3D).



**Fig. 3** Effects of various concentrations of essential amino acids treatment on the mRNA levels of various iron metabolism genes in differentiated Caco-2 cells: (A) DMT1 + IRE, (B) DMT1-exon1A, (C) Total DMT1 (addition of relative expression of DMT1 +IRE and -IRE) and (D) Ferritin Heavy Chain. Cells were grown on inserts and left to differentiate until the TEER reaches  $240 \Omega \cdot \text{cm}^2$ . Cells were then treated for 17 h with either 0, 10, 20, 30, 50, 70, 90 and 100% essential amino acids solutions. Levels of mRNA expression were measured by RT-qPCR and normalized against 18S as housekeeper and expressed as percentage of control (100% amino acids solution). Values are mean  $\pm$  S.E.M.,  $n = 12$  or 18, statistical significance was determined by linear regression, (A)  $p = 0.012$ , (B)  $p = 0.0007$ , (C)  $p = 0.0058$  and (D)  $p = 0.01$ . Linear regression was used to explore the relationship between essential amino acid deficiency and gene expression. The true relationship between these variables can be expected to be more complex but given the high noise in the measurements and the small sample size linear regression can be regarded as a first order approximation of the unknown true model, that allows us to decide whether there is a general trend of protein expression increasing or decreasing depending on amino acid deficiency. TEER: ~~Trans~~trans-epithelial electric resistance, DMT1: divalent metal transporter 1, IRE: iron responsive element, FTH: ferritin heavy chain, 18S: ribosomal RNA 18S. RT-qPCR: quantitative real time polymerase chain reaction, ess aa: essential amino acids.

alt-text: Fig. 3

Non-essential amino acid depletion had the same effect as essential amino acid depletion on expression on DMT1 ( $\pm$ IRE) exon1A ( $p = 0.006$ ) (Fig. 4A). However, it did not affect any other isoform. In contrast to the effect of essential amino acid depletion, the expression levels of total DMT1 were not changed (data not shown). Ferritin heavy chain expression and hepcidin also increased with non-essential amino acid depletion (respectively  $p = 0.01$  and  $p = 0.047$ ) (Fig. 4B and C).



**Fig. 4** Effects of various concentrations of non-essential amino acids treatment on the mRNA levels of various iron metabolism genes in differentiated Caco-2 cells: (A) DMT1  $\pm$ IRE, (B) Ferritin Heavy Chain and (C) Hepcidin. Cells were grown on inserts and left to differentiate until the TEER reaches 240  $\Omega$  cm<sup>2</sup>. Cells were then treated for 17 h with either 0, 10, 20, 30, 50, 70, 90 and 100% non-essential amino acids solutions. Levels of mRNA expression were measured by RT-qPCR and normalized against 18S as housekeeper and expressed as percentage of control (100% amino acids solution). Values are mean  $\pm$  S.E.M., n = 12 or 18, statistical significance was determined by linear regression, (A) p = 0.006, (B) p = 0.01 and (C) p = 0.0475. Linear regression was used to explore the relationship between non-essential amino acid deficiency and gene expression. The true relationship between these variables can be expected to be more complex but given the high noise in the measurements and the small sample size linear regression can be regarded as a first order approximation of the unknown true model, that allows us to decide whether there is a general trend of protein expression increasing or decreasing depending on amino acid deficiency. TEER: ~~Trans~~trans-epithelial electric resistance, DMT1: divalent metal transporter 1, FTH: ferritin heavy chain, FTL: ferritin light chain, 18S: ribosomal RNA 18S, RT-qPCR: quantitative real time polymerase chain reaction, non ess aa: non-essential amino acids.

alt-text: Fig. 4

We hypothesized that the/increase in iron transport and/or cell content were due to an increase in ferritin excretion from the cells and/or changed expression of ferroportin, which is regulated by ubiquitinylation rather than at mRNA level [12]. However, protein levels of ferritin and of ferroportin were not significantly altered by any of the treatments (Appendix A Fig. A2 and Fig. A3).

## 4 Discussion

In this paper, we have shown that depletion of either essential or non-essential amino acids induces an increase in iron transfer from the apical to the basolateral side of a Caco-2 cell layer. The changes cannot be accounted for by alterations in the cell layer permeability, since they are both temperature and time dependent, there is no alteration in the transepithelial electrical resistance, no change in DNA content on the

filters and no significant increase in marker dye concentration in the basolateral medium. Consequently, we hypothesized that some stage in the iron transfer mechanism had to be modified by amino acid depletion. We measured mRNA levels of the genes of iron metabolism known to be regulated at this level, either by transcriptional or post-transcriptional mechanisms. We also measured protein levels of ferroportin, since, as it is regulated by ubiquitinylation, mRNA is not necessarily an accurate reflection of activity

The first stage in iron transfer is the uptake into the cell through DMT1. mRNA levels of total DMT1 decreased as a consequence of depletion of essential, but not non-essential, amino acids. Both treatments reduced the expression of both DMT1 ( $\pm$ IRE) exons 1A isoforms (Figs. 3B and 4A), while essential amino acid deprivation also reduced +IRE (exon 1A or 1B) expression (Fig. 3A). Given that DMT1 (+IRE/exon1A) is the major isoform present in the small intestine, it seems logical that alterations in exon1A and +IRE isoforms would induce changes in the levels of total DMT1 (Fig. 3C) and hence DMT1 protein levels, since the two correlate well in gut cells [35,36]. If DMT1 were the rate limiting step for transport, then a decrease, rather than an increase in iron transfer would be predicted. However, results from different studies suggest that DMT1 is not the limiting step in iron transport in the brain [37-40] and our data support this contention in the gut. Ferroportin is considered to be the limiting step for iron export from the cell. However, the increased transport of iron in both types of AA depletion cannot be explained by changes in ferroportin expression, which remained constant in our study.

Non-essential amino acid depletion induced an increase in hepcidin mRNA levels. However, mRNA levels in Caco-2 cells are very low and it is not clear whether protein produced by these cells will have an effect on iron uptake. Our results would suggest not, since an increased hepcidin production by the liver is associated to a decrease, rather than an increase in iron transfer. Increased levels of hepcidin are associated with inflammation [41]. The mRNA up-regulation of hepcidin, even if only at very low levels, could be reflective of inflammation. This contrasts with previous studies reporting that amino acid starvation is associated with oxidative stress and apoptosis, which in turn decreases levels of hepcidin [5,42]. It should be noted, however, that this effect occurs in liver rather than gut cells. Interestingly, glutamine, a non-essential AA, has been shown to decrease inflammation in the human gut and the lack of glutamine increases interleukin-8 production in Caco-2 cells [43]. It is therefore possible that the lack of glutamine in our model had a pro-inflammatory effect on Caco-2, which in turn led to increased hepcidin production [44]. However the changes in hepcidin production by the enterocyte were not associated with changes in ferroportin expression and its biological role remains to be established.

Expression of ferritin heavy chain mRNA was increased by both essential and non-essential amino acid deprivation (Figs. 2D and 3B). Ferritin is an iron storage protein, so a possible mechanism explaining the increased iron transport with amino acid depletion could be the excretion of ferritin into the basolateral side of the cells. Higher expression of the ferritin heavy chain could also be responsible for higher iron cell content and would explain the result observed with non-essential amino acid depletion. Naturally, the increased levels of iron observed inside the cells could also increase the expression of ferritin heavy chain. In both types of amino acid depletion, ferritin excretion could explain the increased radioactive levels of iron found in the basolateral medium without observing any changes in the mRNA levels of ferroportin. However, the protein levels of ferritin heavy chain (inside the cells, and total) did not change significantly with either essential or non-essential amino acid depletion. The regulation of ferritin by iron is complex. Each molecule of ferritin can hold a variable amount of iron, so iron content could change markedly without changing ferritin protein levels. Similarly, ferritin turnover is altered by many more factors than just iron status. At this stage, the lack of correlation makes it difficult to draw clear conclusions on the relationship between ferritin levels and iron transfer. However, the simplest conclusion is that ferritin is not involved directly in the increase in transfer.

At this stage, therefore, it is not clear what underlies the increased transfer of iron. A link to inflammation, even if hepcidin levels are increased with non-essential amino acid depletion seems unlikely. Rather, inflammation is associated with increased iron storage (which in this case occurs only in non-essential AA depletion) and entrapment of iron inside the cells, together with a decrease in the expression of transferrin and the internalization and degradation of ferroportin, caused by hepcidin up-regulation.

Very little is known about the mechanism underpinning the movement of iron from the apical to the basolateral side of the gut cell. The iron inside the cell is thought to be ferrous iron ( $\text{Fe}^{2+}$ ) and it seems unlikely that this highly reactive and toxic form of iron is left free inside the cell. Different studies have suggested the importance of an apotransferrin cycle in the transport of iron. Moniya, et al., and Nuñez, et al. showed that adding apotransferrin to the basolateral side of Caco-2 cells significantly increased iron transport from the apical to the basolateral side of the cells [45]. They also showed that apotransferrin was stored in vesicles above the nucleus before returning to the basolateral side to be used again [46]. Another study revealed that iron transfer across the cell layer is energy-dependent, while DMT1 is only a proton-dependent transporter. However, DMT1 also depends on the activity of the  $\text{Na}^+/\text{K}^+$  pump which is an ATPase, and therefore energy-dependent [47]. Interestingly, transferrin and DMT1 have been detected in the same endosomes as well [48]. Another group confirmed the involvement of apotransferrin in iron transport in Caco-2 cells but also suggested that up to 50% of iron transport involved vesicles. They used inhibitors of various aspects of vesicular trafficking such as nocodazole and 5'-(4-fluorosulfonylbenzoyl)-adenosine, and showed that the uptake and basolateral transport was reduced up to 50% depending on the inhibitor used independently of apotransferrin [49]. At this stage, we are uncertain whether this mechanism is correct, though it does seem very plausible, and would also help to explain why no "chaperones" of iron have been identified, equivalent to those clearly identified for copper [50,51].

In summary, therefore, we have shown for the first time, that it is possible for iron transfer across the gut layer to increase without concomitant changes in the genes involved in the transfer process. This is an intriguing outcome, and one that deserves further investigation.

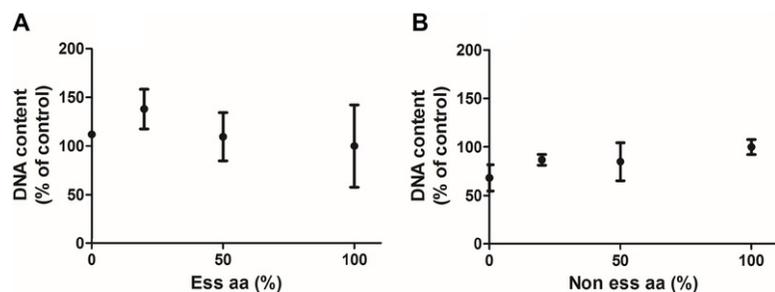
## Funding Source

Rural and Environmental Scientific and Analytical Services, the Scottish Government

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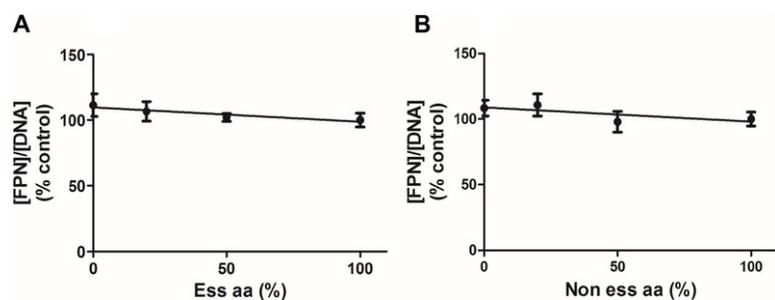
We thank Dr Helen Hayes for her technical support during this project. We also thank Dr Christine Kennedy for her involvement at the beginning of this project. This study was funded by [Rural and Environmental Scientific and Advisory Service of Scottish Government](#).

## Appendix A



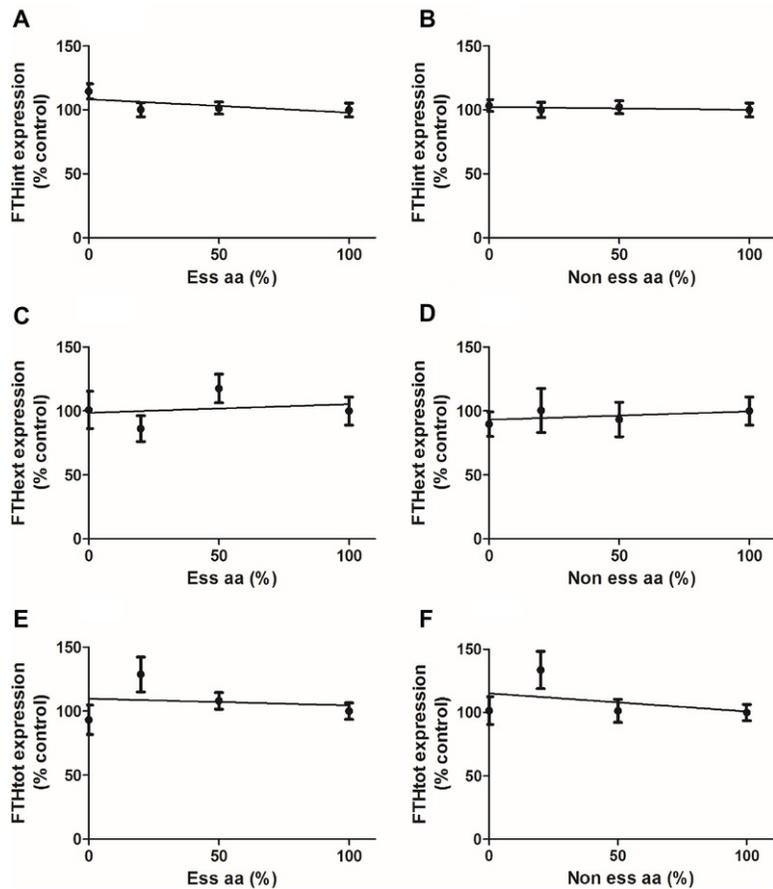
**Fig. A1** Effect of changing amino acid concentration on the DNA content of Caco-2. Caco-2 cells were incubated overnight with differing concentrations of (A) essential amino acids or (B) non-essential amino acids. After washing,  $^{59}\text{Fe}$ -NTA was added on the apical side. After 30 min, cells were treated as described in Materials and Methods and DNA levels within the cell monolayer were measured (in  $\mu\text{gDNA}/\text{mL}$ ). Values were expressed as percentage of the control (100% amino acids). Values are mean  $\pm$  S.E.M.  $n = 3$ , statistical significance was determined by a one-way ANOVA,  $p = 0.43$  (EAA) and  $p = 0.77$  (NEAA). Ess aa: essential amino acids, non ess aa: non-essential amino acids, aa: amino acids, DNA: deoxyribonucleic acid.

alt-text: Fig. A1



**Fig. A2** Effect of changing concentrations of essential (A) and non-essential amino acids (B) on the protein expression of ferroportin in Caco-2 cells. Cells were grown as described in Materials and Methods until the TEER reached  $240 \Omega \text{ cm}^2$ . Cells were incubated overnight with different concentrations of amino acids. After washing, cells were scraped of the plates, centrifuged and ultra-sonicated. Ferroportin protein expression was measured in the cell lysates by ELISA. Values were normalized to DNA content and expressed as % control (100% amino acids). Values are mean  $\pm$  S.E.M.,  $n = 6$ , statistical significance was determined by linear regression,  $p = 0.19$  (EAA) and  $p = 0.24$  (NEAA). TEER: [Trans](#)epithelial electric resistance, FPN: ferroportin, ess aa: essential amino acids, non ess aa: non-essential amino acids.

alt-text: Fig. A2



**Fig. A3** Effects of changing concentrations of essential (1st column) and non-essential amino acids (2nd column) on ferritin heavy intracellular (A, B), extracellular (C, D) and total (E, F) protein levels in Caco-2 cells. Cells were grown as described in Materials and Methods until the TEER reached  $240 \Omega \text{ cm}^2$ . Cells were then incubated overnight with different concentrations of amino acids. Cells were lysed in 50 mM NaOH, centrifuged and ultra-sonicated. Ferritin heavy chain expression was measured in the cell lysates by ELISA. Values were normalized to DNA content and are expressed as percentage of control (100% amino acids). Values are mean  $\pm$  S.E.M., n = 6, statistical significance was determined by linear regression (A) p = 0.16, (B) p = 0.75, (C) p = 0.67, (D) p = 0.71, (E) p = 0.72 and (F) p = 0.36. TEER: *Trans**trans*-epithelial electric resistance, FTHint: intracellular ferritin heavy chain, FTHext: extracellular ferritin heavy chain, FTHtot: total ferritin heavy chain, ess aa: essential amino acids, non ess aa: non-essential amino acids.

alt-text: Fig. A3

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