

# Novel Iron-Whey Protein Microspheres Protect Gut Epithelial Cells from Iron-Related Oxidative Stress and Damage and Improve Iron Absorption in Fasting Adults

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## Keywords

Absorption · Amorphous formulation · Clinical study · Iron · Modified release · Whey protein

## Abstract

**Background:** Iron food fortification and oral iron formulations are frequently limited by poor absorption, resulting in the widespread use of high-dose oral iron, which is poorly tolerated. **Methods:** We evaluated novel iron-denatured whey protein (Iron-WP) microspheres on reactive oxygen species (ROS) and viability in gut epithelial (HT29) cells. We compared iron absorption from Iron-WP versus equimolar-dose (25 mg elemental iron) ferrous sulphate (FeSO<sub>4</sub>) in a prospective, randomised, cross-over study in fasting volunteers ( $n = 21$  per group) dependent on relative iron depletion (a ferritin level  $\leq / > 30$  ng/mL). **Results:** Iron-WP caused less ROS generation and better HT29 cell viability than equimolar FeSO<sub>4</sub>. Iron-WP also showed better absorption with a maximal  $149 \pm 39\%$  increase in serum iron compared to  $65 \pm 14\%$  for FeSO<sub>4</sub> ( $p = 0.01$ ). The response to both treatments was dependent on relative iron depletion, and multi-variable analysis showed that better absorption with Iron-WP was independent of baseline serum iron, ferritin, transferrin sat-

uration, and haemoglobin in the overall group and in the sub-cohort with relative iron depletion at baseline ( $p < 0.01$ ).

**Conclusions:** Novel Iron-WP microspheres may protect gut epithelial cells and improve the absorption of iron versus FeSO<sub>4</sub>. Further evaluation of this approach to food fortification and supplementation with iron is warranted.

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## Introduction

More than 1.6 billion people worldwide have anaemia, and half of this burden is caused by iron deficiency [1, 2]. The commonest causes of iron deficiency in the general population are inadequate intake, poor absorption of iron and, in particular, menstruation [1–5]. This helps explain the increased daily iron requirement and higher deficiency rates in women versus men [5–8]. Supplementation with oral iron is well established to prevent and treat iron deficiency anaemia and symptomatic fatigue [5]. How-

Trial registration: EUDRACT 2016-003303-57, retrospectively registered.

ever, iron food fortification and oral iron supplements are frequently limited by poor absorption and poor tolerability [3, 4, 7, 8].

Poor absorption of inorganic iron is explained by a complex interplay of pH effects on iron oxidation state and solubility, limiting the availability of divalent (ferrous) iron at the divalent metal transporter (DMT-1) found on intestinal enterocytes [9, 10]. The pharmaceutical approach to this problem has been to increase the dose, which results in dose-dependent adverse effects with little difference in clinical practice between available salt forms [11, 12]. Ferrous sulphate ( $\text{FeSO}_4$ ) continues to be the only oral iron recommended by the World Health Organisation (WHO) Essential Medicines List [13]. It is inexpensive and has better absorption relative to other inorganic ferrous and ferric salts, but is also poorly tolerated [11, 12, 14, 15]. Modified-release formulations as well as novel iron salts have been developed to overcome iron intolerance [16–18]. However, to date, there are no better-absorbed, well-tolerated formulations of inorganic iron relative to the gold standard,  $\text{FeSO}_4$ .

Several groups have evaluated whey protein (WP) hydrogels as carriers for iron [19, 20], although none has been tested clinically. The purpose of this work was to characterise and clinically evaluate a novel, amorphous formulation of microspheres of iron in denatured WP, which has shown features predictive of high pharmacokinetic oral bioavailability. The formulation releases iron under simulated intestinal conditions and protects gut epithelial cells from iron-related oxidative stress and damage. In preliminary clinical testing, it produced unexpectedly high pharmacokinetic serum iron bioavailability compared to  $\text{FeSO}_4$ .

## Material and Methods

### *Materials, Manufacture, and Characterisation of Iron-Denatured WP Microspheres*

Details of the materials, manufacture, and characterisation of Iron-WP microspheres are provided in the online supplementary file sections 1 and 2 (for all online suppl. material, see [www.karger.com/doi/10.1159/000480632](http://www.karger.com/doi/10.1159/000480632)). The manufacturing process for Iron-WP microspheres in this study was adapted to a scalable spray-drying or drip-casting technique and produced in accordance with ISO9002 quality standards and HACCP.

### *Impact of Iron-WP Microspheres on Gut Epithelial Cells*

We examined the impact of Iron-WP microspheres versus  $\text{FeSO}_4$  alone and also versus an admixture of  $\text{FeSO}_4$ , vitamin C, and denatured WP on gut epithelial cell production of ROS and cell viability (by MTT assay).

For the ROS production experiment, cells were plated into 96-well plates at a concentration of  $6 \times 10^5$  cells/mL (HT29 gut epithelial cells) in 100- $\mu\text{L}$  volume. After incubation for 24 h, the medium was removed carefully and the cells were incubated with 100  $\mu\text{L}$  25  $\mu\text{M}$  DCF-DA (2',7'-dichlorofluorescein-diacetate) phenol red-free medium for 45 min. The medium was then removed and cells were washed with KRB buffer twice (containing 115 mM NaCl, 5.9 mM KCl, 1.2 mM  $\text{MgCl}_2$ , 1.2 mM  $\text{NaH}_2\text{PO}_4$ , 1.2 mM  $\text{Na}_2\text{SO}_4$ , 2.5 mM  $\text{CaCl}_2$ , 25 mM  $\text{NaHCO}_3$ , 10 mM glucose, adjusted pH 7.4). The cells were incubated with the phenol red-free medium containing 10 mM Iron-WP microspheres,  $\text{FeSO}_4$  and a mixture of whey protein,  $\text{FeSO}_4$  and vitamin C, respectively. The negative controls were incubated with phenol red-free medium only and the positive controls contained 100  $\mu\text{M}$  Luperox. After 4 and 16 h, the plate was read for fluorescence (excitation 485 nm and emission 520 nm) and the results were normalised to the negative control (100%).

Gut epithelial cell damage was assessed using the MTT cell viability assay. This assay assesses NAD(P)H-dependent cellular enzyme reduction of (4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide to insoluble formazan. Again, cells were plated into 96-well plates at a concentration of  $6 \times 10^5$  cells/ml (HT29) in 100- $\mu\text{L}$  volume. After incubation for 24 h, the medium was carefully removed, and the cells were treated with Iron-WP,  $\text{FeSO}_4$ , and the admixture of denatured WP,  $\text{FeSO}_4$ , and vitamin C at 10 mM in supplement-free medium, respectively. The positive controls were the blank wells containing only medium and the negative controls were the empty wells. The plates were incubated at 37°C for 4 and 16 h. After incubation, 20  $\mu\text{L}$  of MTT solution (2.5 mg MTT in 1 mL PBS) were added to each well and incubated for another 3 h at 37°C. The medium was gently aspirated from the wells, and 100  $\mu\text{L}$  of DMSO was added to each well to lyse the cells. The plates were covered with aluminium foil, shaken for 15 min, and then analysed using a plate reader at a wavelength of 590 nm. Cell survival rates were calculated by the following formula: cell viability % =  $(A_{\text{sample}} - A_{\text{nc}})/(A_{\text{pc}} - A_{\text{nc}}) \times 100$ , where  $A_{\text{sample}}$  is the absorbance of the samples,  $A_{\text{nc}}$  is the absorbance of the negative controls, and  $A_{\text{pc}}$  is the absorbance of the positive controls.

### *Clinical Evaluation of Iron-WP Microspheres versus $\text{FeSO}_4$ Study Design*

A randomised, prospective, double-blind, controlled clinical study with at least 28-day cross-over was designed to evaluate the clinical performance of Iron-WP microspheres versus equimolar doses of  $\text{FeSO}_4$  providing an oral dose of 25 mg of elemental iron in fasting subjects. The dose of 25 mg elemental iron was selected based on previous work with an aqueous formulation of  $\text{FeSO}_4$  in healthy volunteers, and also the knowledge that relative absorption of supplemental doses of elemental iron can be assessed when the dose in fasting subjects is >10 mg of elemental iron [21].

### *Population*

Participants were between 18 and 40 years of age, non-pregnant if female, generally in good health, and willing and able to give written informed consent. Women of child-bearing age were included only if the study visits were >7 days before or after the expected first day of menstruation. All women returned to the clinic for subsequent follow-up visits at the same time point in their menstrual cycle. These women were also required to abstain from

sexual intercourse and/or use adequate contraception during the study. Subjects were excluded if they were hypersensitive to any of the components of the test product, suffered from a metabolic disorder, had any evidence of current infection (viral, bacterial, or other) at screening, were taking any iron supplements, had a significant acute or chronic co-existing cardiovascular, gastrointestinal, endocrine, immunological, or metabolic illness, suffered any condition which would exclude them from the investigator's judgement, or had a condition or were taking a medication that the investigator believed would interfere with the objectives of the study, pose a safety risk, or confound the interpretation of the study results. In addition, participants were excluded if they self-reported consumption of >21 alcohol units/week for males and >14 units/week for females.

#### Study Visits

Subjects underwent an initial phone-screening to determine study eligibility. Study participation involved 3 study visits over a period of 6–10 weeks and eligible subjects were scheduled for a screening visit. The participants were free to withdraw from the study at any time. Due to the cross-over study design, only fasting subjects evaluable on both study days (baseline and follow-up) were included in the analysis. Subjects were withdrawn if they had inter-current infection on a study day, which limits iron absorption, or if they had baseline serum iron >50% above the upper limit of normal (i.e., indicating a non-fasting status).

Selected participants attended the hospital clinic for a screening visit, where the details of the study were explained and their informed consent was obtained. This included consent for the publishing of data without participant identifiers. Demographic data, vital signs, body weight, body mass index (BMI), medical history and general health were recorded. A gastrointestinal symptom screening questionnaire was administered to identify subjects with any pre-existing chronic inflammatory bowel conditions. For women of child-bearing age, a urine sample was collected and a pregnancy test performed. A fasting venous blood sample (8 mL) was collected and a full blood count was performed, along with measurements of serum iron, transferrin saturation (TSAT), and ferritin.

Participants returned to the hospital clinic for the baseline study visit within 2 weeks of the screening visit. They attended the clinic having fasted from 22:00 the previous night, and were randomised into 1 of the 2 cross-over treatment groups (Iron-WP microspheres to FeSO<sub>4</sub> or FeSO<sub>4</sub> to Iron-WP microspheres). A baseline blood sample was taken and participants were given a single dose of the supplement (Iron-WP or FeSO<sub>4</sub> equivalent to 25 mg elemental iron), mixed with either 100 mL of water or apple juice. They were required to remain in the hospital clinic for the duration of the 6-h, post-dose study period. Additional blood samples were collected at 2, 4, and 6 h after dosing. Participants were required to continue fasting for 4 h post-dose and at 4–6 h, they were offered a light snack with a low iron content (<2 mg elemental iron). Water was consumed ad libitum. Subjects were questioned about any adverse events at the end of the 6-h study period.

Participants returned to the hospital clinic for the follow-up study visit following at least a 4-week washout for the cross-over follow-up day. Subjects were given a single dose of the alternative randomly allocated supplement (25 mg elemental iron), and the study procedures of the baseline visit were repeated.

#### Biochemical Analyses

Biochemical analyses were conducted by the ISO 15189-accredited laboratory, Biomnis, in Dublin, Ireland. Serum iron and unbound iron binding capacity (UIBC) were measured using a MULTIGENT iron kit (Abbott Diagnostics, Abbott Park, IL, USA) for the direct colorimetric determination of iron without deproteination in human serum on the Abbott ARCHITECT cSystems. Further details are provided in the online supplementary file section 3.

#### End Points

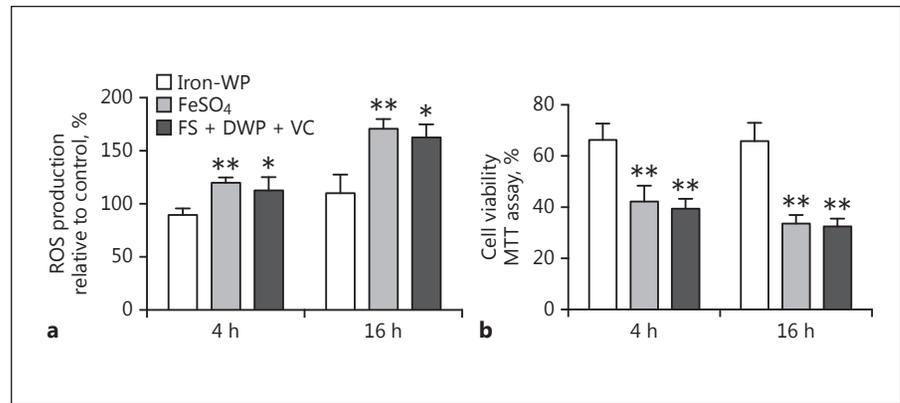
The primary endpoint was the assessment of relative absorption, using the serum iron trough-to-peak ratio (TPR) over 0–6 h post-dosing, expressed as a percentage. This measure represents the maximum increase in serum iron over the specified period, and was chosen because it helps account for the high (65%) intra-individual and between-day variability in fasting morning serum iron [20]. Secondary end points included the comparison of test articles with the following measures: change in serum iron at 2, 4, and 6 h; TPR difference (TPD) or peak serum iron increase; serum iron bioavailability (measured as area under the curve using the trapezoidal rule); change in UIBC; and TSAT level (TSAT %). In addition, we compared end points in subjects taking the Iron-WP microspheres with baseline ferritin levels > and < the median, including linear regression on peak serum iron increase and both baseline ferritin and baseline haemoglobin (Hb). We carried out a multivariable analysis to control for the impact of baseline serum iron, TSAT %, and Hb on end points. This study was not designed to formally evaluate safety criteria, but, as an exploratory objective, any adverse events during the study period (defined as any untoward medical occurrence in a clinical investigation subject following the administration of an investigational product and which does not necessarily have to have a causal relationship with this treatment) were documented and evaluated.

#### Sample Size and Statistical Analysis

From a screening cohort of 40 people, it was estimated that up to 24 healthy subjects would be eligible for randomisation and that this would result in 20 evaluable subjects. Based on previous clinical data using serum iron measurements in fasting subjects with normal Hb and ferritin levels <100 ng/mL over 6 h, it was expected that the TPR % of the Iron-WP microspheres would be >50% greater than FeSO<sub>4</sub> in the paired analyses. The mean  $\pm$  standard deviation (SD) TPR % of FeSO<sub>4</sub> was assumed to be 60  $\pm$  50%, and with 85% power and a 2-sided  $\alpha$  = 0.05, 6 paired analyses of participants in either group were required. In addition, the study was powered on the secondary end point of TPD. In this case, it was also assumed that the TPD of the Iron-WP microspheres would be >50% greater than FeSO<sub>4</sub> in the paired analyses. The expected mean value of serum iron measurements for Iron-WP microspheres would be 20.0  $\pm$  3.0  $\mu$ mol/L. With 85% power and a 2-sided  $\alpha$  = 0.05, we required 6 subjects per group. With an expected dropout rate of 10%, we wanted sufficient power to evaluate subjects according to ferritin status at baseline, so we screened 40 subjects and aimed to include 24 subjects per group in the cross-over study.

The Shapiro-Wilks test of normality was applied to all of the data before making comparisons between groups. Between-group comparisons were carried out using the paired-sample Student *t* test for normally distributed continuous variables and the Wil-

**Fig. 1. a** Gut epithelial cell (HT29) production of reactive oxygen species (ROS) relative to the negative control from iron-denatured whey protein (Iron-WP) microspheres, ferrous sulphate ( $\text{FeSO}_4$ ), and an admixture of  $\text{FeSO}_4$ , denatured WP, and vitamin C (FS + DWP + VC). All conditions were set at an iron concentration of 10 mM ( $n = 3$  per group) and experiments were carried out over 4 h. **b** Gut epithelial cell viability using the MTT assay from the same iron-containing conditions. \*  $p < 0.05$ , \*\*  $p < 0.01$ , for Iron-WP microspheres vs. other conditions.



coxon rank-order test for non-normal distributions;  $\chi^2$  analyses were used to compare categorical variables. Multi-variable linear regression was used to investigate the relationship between dependent variables (TPR %, TPD, and AUC) and independent variables (baseline serum iron, TSAT, ferritin, and Hb). In order to see if there was an independent treatment group difference, the model was adjusted by a group categorical variable. This model was also applied to the 2 sub-cohorts, in which the ferritin level was  $<$  or  $>$  the baseline median. All analyses were 2-sided, and significance was set at  $\alpha = 0.05$ . Analyses were carried out using R statistical software and GraphPad Prism v6.0e. Data is presented as the mean value  $\pm$  standard error of the mean (SE) for normally distributed continuous variables, and median (interquartile range, IQR) for non-normally distributed continuous variables, unless otherwise stated; frequencies and percentages are used for categorical variables.

## Results

### Impact on Gut Epithelial Cells

Experiments with gut epithelial cells in culture indicated lower toxicity when iron is applied in Iron-WP microspheres compared to  $\text{FeSO}_4$  solution or the admixture of  $\text{FeSO}_4$ , WP, and vitamin C. The results show significantly lower ROS production in HT29 gut epithelial cells after 4 h of incubation with Iron-WP microspheres (10 mM iron) versus  $\text{FeSO}_4$  (10 mM) or the admixture of  $\text{FeSO}_4$  (10 mM), WP, and vitamin C (Fig. 1). These differences persisted for up to 16 h after treatment. Furthermore, the MTT assay results indicated that cells treated with Iron-WP microspheres have significantly better viability at 4 and 16 h than cells treated with  $\text{FeSO}_4$  or the admixture of  $\text{FeSO}_4$ , WP, and vitamin C (Fig. 1). The model was developed to show iron-related cellular damage, and the concentration of iron used was calibrated to achieve this. This pro-apoptotic effect was attenuated in

the presence of components of the Iron-WP matrix used but was not abolished. There was, however, significantly more cellular death in the cells treated with iron alone or with the mixture of  $\text{FeSO}_4$ , WP, and vitamin C (all  $p < 0.01$  Iron-WP microspheres vs. the other conditions).

### Clinical Study

#### Study Population

The average age of the study population was  $32.9 \pm 6.3$  years and the participants were mostly female. Apart from 1 withdrawal by a participant for personal reasons following the administration of  $\text{FeSO}_4$  (who was then excluded from the analysis), there were no adverse events reported with either test article.

The baseline demographic and haematinic characteristics of the total population are presented in Table 1. We pre-specified end-point evaluation dependent on baseline ferritin. We carried out analyses according to whether participants had a ferritin level  $>$  or  $<$  the median at baseline (34 ng/mL), resulting in 2 sub-cohorts, which are described and compared in Table 1. The first had relative iron depletion and ferritin  $\leq 30$  ng/mL ( $n = 10$ ), and the second was relatively iron-replete with ferritin  $> 30$  ng/mL ( $n = 11$ ). As expected, the participants with relative iron depletion had lower ferritin, TSAT %, UIBC, and Hb. Of a total of 24 participants screened, 21 were included in the analysis. Three subjects were excluded due to major deviations from protocol: 1 withdrew from the study following the treatment with  $\text{FeSO}_4$  and did not attend the follow-up visit; 1 had baseline serum iron  $> 50\%$  of the upper limit of normal on day 28; and 1 had persistent thrombocytosis at the baseline and follow-up clinic visits and an elevated white blood cell count at follow-up. The average BMI was  $27.2 \pm 3.8$ , no participants were current smokers, none had a medical history of note, and 2 were anaemic.

**Table 1.** Baseline demographic and haematinic characteristics of the study population and sub-populations with above and below median ferritin levels at baseline

	Total cohort <i>n</i> = 21	Ferritin ≤30 ng/mL <i>n</i> = 10	Ferritin >30 ng/mL <i>n</i> = 11	<i>p</i> value
Age, years	32.9±6.3	31.4±7.5	34.2±4.8	ns
M/F gender, <i>n</i>	4/17	1/9	3/8	ns
Serum iron, μM	16.0±7.1	11.7±6.0	19.9±5.8	0.005
UIBC, μM	42.0±10.9	48.9±10.6	35.6±6.5	0.002
TIBC, μM	58.0±8.1	60.6±8.0	55.5±7.8	ns
TSAT, %	28.1±12.2	19.8±10.1	35.7±8.5	0.001
Ferritin, ng/mL (IQR)	34 (22–60)	19.5 (11.8–25.5)	60 (46–103.5)	<0.0001
Hb, g/dL	13.7±1.1	13.2±0.9	14.2±1.2	0.04
WCC, 10 <sup>9</sup> /L	5.86±1.4	6.01±1.15	5.71±1.63	ns
RCC, 10 <sup>12</sup> /L	4.51±0.43	4.40±0.21	4.61±0.56	ns
HCT, L/L	0.42±0.03	0.40±0.02	0.43±0.03	ns
MCV, fL	92.5±4.9	91.4±3.7	93.4±5.8	0.045
MCH, pg	30.5±1.8	30.0±1.9	30.9±1.7	ns
MCHC, g/dL	32.9±0.8	32.8±1.0	33.1±0.5	ns
RDW, %	13.2±1.0	13.4±1.3	13.0±0.7	ns
Platelets, 10 <sup>9</sup> /L	240±49	252±60	230±36	ns

Values are expressed as mean ± SD, unless otherwise indicated. UIBC, unbound iron binding capacity (latent capacity); TIBC, total iron binding capacity; TSAT, transferrin saturation; Hb, haemoglobin; WCC, white blood cell count; RCC, red blood cell count; HCT, haematocrit; MCV, mean corpuscular volume; MCH, mean corpuscular haemoglobin; MCHC, mean corpuscular haemoglobin content; RDW, red blood cell distribution width; M, male; F, female; ns, not significant.

**Table 2.** End points for study population and sub-populations above and below the median ferritin level at baseline over 0–6 h

	Total cohort		Ferritin ≤30 ng/mL		Ferritin >30 ng/mL	
	Iron-WP <i>n</i> = 21	FeSO <sub>4</sub> <i>n</i> = 21	Iron-WP <i>n</i> = 10	FeSO <sub>4</sub> <i>n</i> = 10	Iron-WP <i>n</i> = 11	FeSO <sub>4</sub> <i>n</i> = 11
TPR, %	149±39*	65±14	239±50* <sup>§</sup>	105±17 <sup>§</sup>	68±9*	29±5
TPD, μM	13.4 (7.3–20.5)*	7.2 (3.4–9.8)	22.8 (12.4–38.6)* <sup>§</sup>	8.6 (7.3–17.1) <sup>§</sup>	10.5 (6.2–16.1)*	4.2 (2.2–8.1)
UIBC peak change, μM	12.0 (7.0–17.0)*	7.0 (5.0–9.0)	19 (13.5–37.5)* <sup>§</sup>	7.0 (6.0–14.8) <sup>§</sup>	8.0 (6.5–13.5)*	6.0 (4.5–9.0)
Peak TSAT increase, %	27.4±4.5*	14.6±2.0	38.7±5.4* <sup>§</sup>	17.8±2.1 <sup>§</sup>	19.3±2.6*	12.4±1.9
AUC, μM/h	49.5 (25.4–89.1)*	29.2 (13.0–42.7)	102.7 (49.2–178.2)* <sup>§</sup>	34.5 (29.4–31.0) <sup>§</sup>	25.6 (24.5–64.9)*	17.2 (7.8–33.9)

AUC, area under the curve; TPR, trough-to-peak ratio; TPD, TPR difference (peak serum iron increase); UIBC, unbound iron binding capacity (latent capacity); TSAT, transferrin saturation. \* *p* < 0.05 for Iron-WP vs. FeSO<sub>4</sub>; <sup>§</sup> *p* < 0.05 for ferritin ≤30 ng/mL vs. ferritin >30 ng/mL.

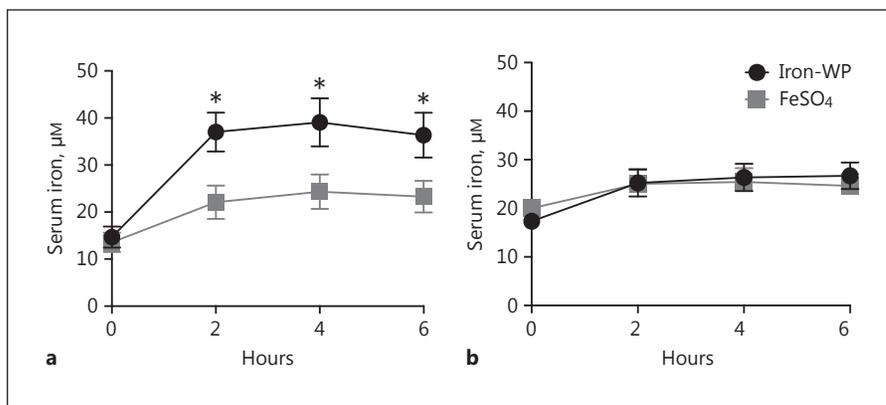
The full baseline and serum iron data over follow-up per participant is presented in online supplementary Table S1.

### End Points of the Study

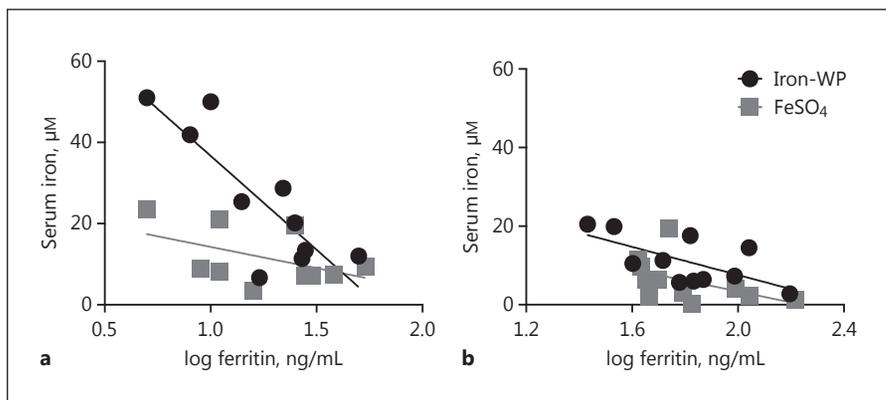
The primary end point in this study was serum iron TPR % over 0–6 h; Iron-WP microspheres showed better absorption with a maximal 149 ± 39% increase compared to 65 ± 14% for FeSO<sub>4</sub> (*p* < 0.001). Other end points are presented in Table 2 for the study population and the sub-groups according to baseline ferritin. These results show a significantly greater maximal UIBC decrease, and an

increase in peak serum iron (TPD) and TSAT % in participants treated with Iron-WP compared to those treated with FeSO<sub>4</sub>. Multi-variable analysis shows that better absorption of Iron-WP was independent of baseline serum iron, Hb, TSAT %, and ferritin for TPR % (*p* = 0.02), peak serum iron increase (TPD, *p* = 0.002), and pharmacokinetic bioavailability over 0–6 h (serum iron AUC, *p* = 0.006). We analysed the relationship between baseline ferritin and Hb versus peak serum iron increase, and found significant differences between the Iron-WP and FeSO<sub>4</sub> treatments overall (online suppl. Fig. S2). There

**Fig. 2.** Serum iron curves over 6 h in fasting subjects following the administration of Iron-WP microspheres containing 25 mg elemental iron and FeSO<sub>4</sub> (25 mg elemental iron) in participants (*n* = 10) with relative iron depletion (**a**, ferritin ≤30 ng/mL, \* *p* < 0.05 for between-group comparisons) and in those (*n* = 11) who were relatively iron-replete (**b**, ferritin >30 ng/mL) at baseline.



**Fig. 3.** Linear regression of peak serum iron increase and ferritin following the administration of Iron-WP microspheres containing 25 mg elemental iron and FeSO<sub>4</sub> (25 mg elemental iron) in participants (*n* = 10) with relative iron depletion (**a**, ferritin ≤30 ng/mL, Iron-WP slope  $-46.2 \pm 10.7$  vs. FeSO<sub>4</sub> slope  $-10.4 \pm 6.7$ , *p* = 0.01) and those (*n* = 11) who were relatively iron-replete (**b**, ferritin >30 ng/mL, Iron-WP slope  $-17.9 \pm 6.9$  vs. FeSO<sub>4</sub> slope  $-14.0 \pm 8.4$ , *p* = ns) at baseline. For overall combined results, see online supplementary Figure S2.



were no within-group differences between the peak serum increase (TPD) and the maximal decrease in UIBC for any treatment overall or in any sub-population (for all: *p* = ns). The relative pharmacokinetic serum iron bioavailability from the Iron-WP microspheres was 240% (IQR 130–346) of FeSO<sub>4</sub> over 0–6 h (*p* = 0.01).

A better response to both treatments was observed in the 10 participants with relative iron depletion (ferritin ≤30 ng/mL) versus the 11 participants higher iron stores (ferritin >30 ng/mL) (Table 2). Serum iron curves over 6 h and the linear regression of peak serum iron increased with log<sub>10</sub> ferritin, respectively, in fasting subjects, following the administration of FeSO<sub>4</sub> (25 mg elemental iron) and Iron-WP microspheres containing 25 mg elemental iron (Fig. 2, 3). The serum iron curves and the linear regression of the ferritin versus peak serum iron increase were different in the sub-cohort with low ferritin at baseline only. Analysis of the relationship between Hb at baseline and peak serum iron increase in these sub-cohorts follows a similar pattern; the slope of the linear regression line for Iron-WP was significantly different from that for FeSO<sub>4</sub> ( $-13.6 \pm 3.4$  vs.  $-1.2 \pm 2.7$  respectively, *p* = 0.01) in

the group with low ferritin at baseline. In the participants that were relatively iron-replete, the regression slopes for Iron-WP were similar to those for FeSO<sub>4</sub> ( $0.38 \pm 1.87$  vs.  $0.09 \pm 1.74$  respectively, *p* = NS). Multi-variable analysis in these sub-groups also demonstrated that better absorption of Iron-WP over FeSO<sub>4</sub> was independent of baseline serum iron, Hb, TSAT %, and ferritin for TPR % (*p* = 0.046), peak serum increase (TPD, *p* = 0.007), and AUC over 0–6 h (*p* = 0.009) in the group with relative iron depletion (ferritin ≤30 ng/mL). However, in the relatively iron-replete group (ferritin >30 ng/mL), better absorption of Iron-WP over FeSO<sub>4</sub> was independent of baseline serum iron, Hb, TSAT %, and ferritin for TPR % (*p* = 0.02) but not peak serum increase (TPD) or pharmacokinetic bioavailability (AUC) over 0–6 h (for both: *p* = ns).

## Discussion

The prevention and treatment of iron deficiency is a major public health goal, particularly for women and young children [1–5]. However, oral iron remains poorly

absorbed despite decades of food fortification and supplementation, and has resulted in the widespread use of poorly tolerated, high-dose formulations [3, 4, 11, 12, 14]. This is the first clinical report of iron absorption from Iron-WP microspheres. Using either a drip-casting or spray-drying manufacturing method, we report on stable, free-flowing, amorphous Iron-WP microspheres containing approximately 1–7% w/w elemental iron. Clinical evaluation of this formulation shows a significantly greater absorption of iron than of the gold standard, FeSO<sub>4</sub>, when compared across a range of outcome measures, including peak changes in serum iron, UIBC, TSAT, and pharmacokinetic bioavailability measured by serum iron AUC over 0–6 h. In vitro studies showed less ROS generation and better HT29 cell viability when iron-WP was administered than with equimolar FeSO<sub>4</sub>.

As only 2 subjects in the study were anaemic and many subjects were relatively iron-replete, we carried out sub-analyses in the participants with > and < the median level of ferritin at baseline (34 ng/mL). As expected, we demonstrated a better response to both treatments in the 10 participants with relative iron depletion (ferritin ≤30 ng/mL) than in the 11 participants with higher iron stores (ferritin >30 ng/mL). Furthermore, the multi-variable analysis showed that Iron-WP treatment was associated with a greater peak serum iron increase independent of baseline serum iron, ferritin, TSAT, and Hb in the overall group and in the sub-cohort with relative iron depletion at baseline ( $p < 0.01$ ). However, we observed differences in the linear regression between the peak serum iron increase and both ferritin and Hb only in the sub-cohort with relative iron depletion. This is important for 2 reasons. First, the measurement of post-dose serum iron increase in iron-replete individuals may not be a good marker for bioavailability (defined as red blood cell incorporation of iron), as the validated algorithms that correlate serum iron maximal increase with erythrocyte incorporation have only been done for FeSO<sub>4</sub> in iron-depleted individuals. Second, the similar linear regression slopes of the 2 treatments in participants that were iron-replete do not suggest a potential for non-transferrin-bound iron formation. In this regard, it is noteworthy that there were no within-group differences between peak serum iron increase and maximal drop in UIBC.

Iron deficiency is usually diagnosed in general practice in response to symptoms reflecting iron deficiency anaemia (extreme fatigue, pallor, weakness, shortness of breath, dizziness, and palpitations or chest pain) [3, 11]. It is also associated with poor immunity, frequent infections, headache, hair loss, brittle nails, and cognition

problems [3, 6, 23]. The median baseline ferritin level in our fasting subjects was 34 ng/mL. While some biochemical diagnostic thresholds usually involve a serum ferritin level of <15 ng/mL, other guidelines have suggested ferritin thresholds of 30 ng/mL and/or fasting TSAT levels of <20% to confirm an iron deficiency [23, 24]. Accordingly, we described our sub-group with a ferritin level < the median at baseline as having relative iron depletion. In selected populations with high underlying inflammation, such as heart failure patients, a ferritin level <100 ng/mL, and/or a fasting TSAT level of <20% are consistent with iron deficiency and may warrant intervention [25]. Prevalence rates are dependent on the diagnostic criteria and the population studied, but the reported US prevalence of iron deficiency ranges from 4.5 to 18.0% [2].

The widespread use of high-dose iron supplements in primary care partly reflects a poor absorption of iron from fortified foods [3, 4]. In addition, insufficient dietary intake is a factor, not only in low- and middle-income countries. For example, in a study in France [26], >90% of women were found to have insufficient dietary iron intake. However, high oral doses cause additional gastrointestinal problems. High levels of intestinal iron can promote ROS-mediated mucosal damage and modify the gut microbiome [4, 9]. Adverse effects include nausea, vomiting, eructation, constipation, and diarrhoea, which impact on quality of life and therapy adherence [11, 27].

Attempts to limit these adverse effects by delaying the release of iron in the stomach have generally resulted in better gastric tolerability but also the persistence of problems in the lower intestine [15–17]. The DMT-1 requires a proton co-factor and is most active early in the duodenum, where it links with the mammalian iron export protein, ferroportin, under regulatory control by ferritin and Hb via hepcidin [28]. Iron release from currently marketed products is generally delayed in the intestine to regions of higher pH, with less DMT-1 activity [10]. Precipitation of insoluble iron salts occurs, resulting in even greater unabsorbed iron in the lower intestinal tract. The Iron-WP formulation studied here provides gut cell protection and increases absorption. This allows lower doses to be administered and, coupled with the established antioxidant properties of whey [29], it can improve the gut tolerability of oral iron.

The need for more effective, better-tolerated oral iron formulations is particularly important in pregnancy, as up to 40% of pregnant women become anaemic [1, 2]. Iron deficiency anaemia, at any time during pregnancy, is associated with premature birth and low birth weight

[30]. Iron needs are increased significantly in pregnancy because of the progressive expansion of maternal blood volume from trimester 1 to trimester 3, and also by the iron requirements of the growing embryo [1, 2]. Moreover, many women enter pregnancy without sufficient stores of iron, aggravated by prior menorrhagia, which has a reported prevalence of 29% in general practice and is highly correlated with iron stores [31]. Finally, the adverse effects of high-dose iron supplements are increased in pregnancy due to the increasing pressure of the gravid uterus on the rectum and stomach as well as hormonal changes, which give rise to nausea, heartburn, and constipation [2, 3, 28, 30].

The approach of microencapsulating bioactives to improve the taste, stability, and/or absorption of iron is not new [31]. The denatured WP in our formulation is an attractive matrix for the formulation of iron because it has low immunogenicity compared to other milk protein fractions when denatured, does not contain lactose, is biocompatible, and can form gel particles in the presence of iron, thereby modifying the unpleasant metallic taste that is associated with iron [19, 20, 32]. With regard to their composition and properties, previously reported Iron-WP formulations differ from the formulation that we used, and they have different characteristics of intestinal release. This distinction may be important because the first physiological barrier to the absorption of inorganic oral iron is the availability of unoxidised ferrous iron II in solution at the DMT-1, active in the early part of the small intestine but not in the stomach [10]. The formulation we used was designed to maximise the release of iron (II) in the gut and reduce the collateral damage to the gut epithelial cells. Inorganic iron absorption and tolerability are related in complex ways, dependent on release but also cellular damage and regulatory control through DMT-1 (SLC11A2) import on the apical membrane and ferroportin export (SLC40A1) on the basolateral membrane of the enterocyte, the latter under control of hepcidin. The novel Iron-WP microsphere formulation reported here may increase iron absorption by preserving soluble ferrous iron close to the DMT-1.

In this study, the increased absorption of iron in subjects with lower ferritin was consistent with the greater iron export activity of ferroportin seen in those with lower iron stores [28]. This, along with the differences in Iron-WP serum iron curves and relationships between peak serum iron increase and baseline ferritin and Hb in people with relative iron depletion versus those with nor-

mal iron stores, suggests that inadvertent overabsorption of iron is unlikely with this formulation.

This work is limited by several features. First, it was a small study in selected, predominantly healthy females. However, as the first clinical evaluation of a novel Iron-WP formulation with improved absorption, the study was adequately powered and carefully controlled to evaluate iron absorption from a single dose. Secondly, our findings warrant further studies in other populations and in comparison with other commonly used oral iron formulations. Finally, the study was not powered to evaluate the gastrointestinal tolerability of the formulation, so more adequately powered studies with higher doses and longer follow-up are required to establish the differences from the high-dose iron supplements currently in use.

## Conclusions

Novel, stable, free-flowing, amorphous microspheres comprising a novel bio-compatible Iron-WP matrix for releasing iron II exhibit reduced potential for intestinal mucosal damage. Clinical studies showed an improved absorption of iron over the that of the gold-standard FeSO<sub>4</sub> and an increased relative absorption in subjects with lower versus higher iron stores. Further evaluation of this approach to food fortification and supplementation with iron is warranted.

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## Statement of Ethics

The study was conducted by Atlantia Food Clinical Research Organisation, Western Gateway Building, University College, Cork, in accordance with the applicable Good Clinical Practice 21 Code of Federal Regulations CFR 50, 56, and 312, International Conference of Harmonisation (ICH) Good Clinical Practice published in the Federal register No. 90 (9 May 1997;62:25691-25709) and the declaration of Helsinki. The study was approved on 6 August 2013 by the Cork University Hospital Medical Ethics Committee (Reference AFCRO-044).

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work. Accordingly, Solvotrin had a role in the design, analysis, and interpretation of the data. The clinical study conduct and data collection was carried out by the Atlantia Food Clinical Research Organisation under contract with Solvotrin.

## Author Contributions

J.F.G. and M.L. are responsible for developing the formulation, the study design, data analysis, funding, and drafting the manuscript. A.O. was involved in study design and drafting the manuscript. A.M.H. was responsible for the design of the solid-state characterisation work, data analysis, and drafting the manuscript. J.W. was responsible for developing the formulation, carrying out the in vitro dissolution, in vitro cell work, data analysis, and drafting of the manuscript. G.R. and M.W. were responsible for developing the formulation, overseeing production, solid-state characterisation, data analysis, and drafting the manuscript.

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