

Oral and Intravenous Iron Therapy Differentially Alter The on- and Off-Tumour Microbiota in Anaemic Colorectal Cancer Patients.

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Abstract

Background Iron deficiency anaemia is a common complication of colorectal cancer and may require iron therapy. Oral iron can increase iron available to gut bacteria and may alter the colonic microbiota. We performed an intervention study to compare oral and intravenous iron therapy on the colonic tumour-associated (on-tumour) and paired non-tumour-associated adjacent (off-tumour) microbiota. Anaemic patients with colorectal adenocarcinoma received either oral ferrous sulphate (n=16) or intravenous ferric carboxymaltose (n=24). On- and off-tumour biopsies were obtained post-surgery and microbial profiling was performed using 16S ribosomal RNA analysis.

Results Off-tumour α - and β -diversity were significantly different between iron treatment groups. No differences in on-tumour diversity were observed. Off-tumour microbiota of oral iron-treated patients shows higher abundances of the orders Clostridiales, Cytophagales and Anaeroplasmatales compared to intravenous iron-treated patients. The on-tumour microbiota was enriched with the orders Lactobacillales and Alteromonadales in the oral and intravenous iron groups, respectively. The on- and off-tumour microbiota associated with intravenous iron-treated patients infers increased abundances of enzymes involved in iron sequestration and anti-inflammatory/oncogenic metabolite production, compared to oral iron-treated patients. Paired on- and off-tumour microbiota show large taxonomic differences in intravenous iron-treated patients and limited differences in oral iron-treated patients.

Conclusion Oral iron shows a large shift in the off-tumour microbiota, but a more limited change in on-tumour microbiota. The on- and off-tumour microbiota in intravenous iron-treated patients infers a microbiota associated with anti-inflammatory and tumour protective pathways. Suggesting intravenous iron may be a more appropriate therapy to limit adverse microbial outcomes, compared to oral iron.

Trial registration: NCT01701310. Registered 21 March 2012, <https://clinicaltrials.gov/ct2/show/NCT01701310>

Introduction

Perturbations in gut bacterial populations are characteristic of colorectal cancer, with the presence of pathogenic indigenous bacterial species at the expense of protective probiotic species [1]. Driver bacteria are involved in the initiation of colorectal cancer. Which are gradually outcompeted by opportunistic passenger bacteria, which have a selective advantage within the newly defined tumour-microenvironment. These passenger bacteria can potentially either promote or hinder tumour progression dependent on whether pathogenic or probiotic bacterial populations flourish [2]. Hence, external factors that can influence gut bacterial growth, such as iron supplementation in anaemic colorectal cancer patients, can potentially support colorectal cancer progression dependent on the bacterial populations they promote [2, 3].

Iron is essential for the growth and development of the vast majority of gut bacteria, with pathogenic bacteria tending to have heightened iron acquisition mechanisms [4]. Hence, in anaemic colorectal cancer

patients the use of oral iron supplementation has the potential to alter bacterial populations of the colorectal tumour-associated (on-tumour) microbiota, as well as the non-tumour-associated mucosal (off-tumour) microbiota [5, 6]. This may suggest that a parenteral route of iron administration may be more beneficial to treat anaemia, without increasing iron availability to colonic bacteria. Initial data from the intravenous iron in colorectal cancer associated anaemia (IVICA) trial suggests that intravenous iron may be more beneficial than oral iron in treating pre-operative anaemia [7]. However, results from the preoperative intravenous iron to treat anaemia before major abdominal surgery (PREVENTT) trial has put doubt over the efficacy of intravenous iron pre-operatively [8]. Hence, which is the most beneficial treatment of anaemia pre-operatively, oral or intravenous iron, remains uncertain. The purpose of this study is to determine if patients treated with oral iron have a more procarcinogenic microbiota compared to those treated with intravenous iron.

Conflicting evidence exists regarding the effect of iron supplementation in murine studies of gut bacterial diversity [9, 10]. However, to our knowledge a comparison of the gut microbiota following oral and intravenous iron therapy in human studies of colorectal cancer has yet to be explored. This pilot intervention study aims to compare the outcomes of oral and intravenous iron therapy on the on- and off-tumour microbiota in colorectal cancer patients with iron deficiency anaemia.

Methods

Study population

From the IVICA trial a total of 40 anaemic patients with non-metastatic colorectal adenocarcinoma were randomised to receive either oral ferrous sulphate 200mg twice a day (n=16) or intravenous iron (ferric carboxymaltose - Ferinject™; Vifor Pharma, Glattbrugg, Switzerland) dosed by weight and haemoglobin in accordance with the summary of product characteristics (n=24) [7]. Treatment was administered at least 2 weeks pre-operatively and anaemia was defined as having a haemoglobin level 10 g/l below the sex-specific World Health Organisation definition (women \leq 120 g/l, men \leq 130 g/l). Duration of iron treatment and inclusion haemoglobin for treatment groups are presented in the patient demographics in table 1. To limit the inclusion of non-iron deficient anaemic individuals, those with pre-existing haematological disease, renal failure or undergoing current chemotherapy were not eligible for the trial. Colorectal tumour biopsies and paired tumour-adjacent colonic mucosal tissue biopsies were obtained post-surgery.

DNA extraction and 16S rRNA amplicon sequencing

Microbial DNA was extracted from colorectal tumour biopsies and paired tumour-adjacent mucosal colonic tissue biopsies using a modified protocol of Qiagen All Prep DNA/RNA Mini Kit (Qiagen, Hilden, Germany). Biopsies were mechanically lysed using 5mm steel bead (Qiagen) and 0.1mm Zirconia/Silica beads (Strateck, Suffolk, UK) with a TissueLyser (Qiagen), followed by enzymatic and heat lysis. Extracted microbial DNA was used for 16S ribosomal RNA (rRNA) gene amplification and sequencing to determine the mucosal-adherent microbiota according to the Earth Microbiome project protocol [11].

Using primers targeted to the V4 region (515F-806R), the 16S rRNA genes were amplified in technical triplicates. This was performed using a single-step, single-indexed polymerase chain reaction (PCR). DNA extraction and 16S rRNA gene PCR were both performed in batch with appropriate multiple-reagent based negative controls. Paired-end sequencing (2x250 base pairs) was completed in a single batch using the Illumina MiSeq platform (Illumina, San Diego, USA).

Statistical analysis

Microbial analysis was performed using the Quantitative Insight Into Microbial Ecology 2 (QIIME2) pipeline [12]. Bacterial taxonomy was assigned using the Silva-132-99% operational taxonomic units (OTUs) database [13]. Intra-sample comparisons (α -diversity) were assessed using Mann-Whitney U test comparing variation in Abundance-based Coverage Estimator (ACE), Chao1, Shannon diversity index, Faith's phylogenetic diversity and Observed OTUs. Inter-sample comparisons (β -diversity) were assessed using permutational multivariate analysis of variance (PERMANOVA) comparing Bray-Curtis dissimilarity and Jaccard similarity using principal coordinate analysis (PCoA). α -diversity indices and PCoA were plotted using the R package "ggplot" [14]. Comparison of relative abundances of taxa between treatment groups and sample types were performed using a linear discriminant analysis (LDA) effect size (LEfSe), taxa with an LDA score greater than 2 with a p -value ≤ 0.05 were considered statistically significant [15]. Predictive functional profiles from microbial populations were derived using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2), using enzyme classification pathway analysis. Differences in predictive enzyme abundances were assessed using Statistical Analysis of Metagenomic Profiles (STAMP). Corrected q -values were calculated following multiple testing correction using Storey false discovery rate. q -values ≤ 0.05 between treatment groups were considered statistically significant [16-18].

Results

On- and off-tumour bacterial diversity following iron therapy

A total of 7.9 million reads (109,777 reads/sample) and 2,367 features were obtained following quality control, with a sampling depth of 8,000 reads/sample. Comparison of α -diversity metrics shows that patients treated with oral iron have a significantly higher bacterial diversity (Shannon Diversity and Faith's Phylogenetic Diversity), richness (Observed OTUs), and abundance (Chao1 and ACE) within their off-tumour microbiota, relative to those treated with intravenous iron therapy (Figure 1; $p < 0.05$). Consistent with this Jaccard similarity and Bray-Curtis dissimilarity assessed β -diversity between treatment groups and show that within the off-tumour microbiota, oral and intravenous iron form significantly different bacterial community clusters (Figure 2; $p < 0.05$).

The on-tumour microbiota of these patients shows no differences in α -diversity between iron treatment groups (Figure 1; *ns*). Along with this, no significant differences in β -diversity were observed (Figure 2: *ns*). Taken together this suggests that oral iron and intravenous iron-treated patients show significantly

different bacterial diversity in their tumour-adjacent microbiota. However, the tumour-associated microbiota shows a consistent bacterial diversity between iron treatments.

Oral and intravenous iron-treated patients show differing bacterial communities

Differences in microbial populations between oral and intravenous iron-treated patients were assessed using LEfSe to determine bacterial taxa that are significantly enriched between treatments (Figure 3). The off-tumour microbiota of oral iron-treated patients shows a greater abundance of the Clostridia class, Clostridiales, Anaeroplasmatales and Cytophagales orders, *Lactobacillaceae* and *Anaeroplasmataceae* families and *Lactobacillus*, *Agathobacter*, *Coproccoccus 3*, *Eubacterium eligens* groups, *Eubacterium xylanophilum* group, *Lachnospiraceae* ND3007 group, *Ruminococcaceae* NK4A214 group, *Ruminococcus 1*, *Ruminococcus 2* and *Anaeroplasma* genera. Whereas in the intravenous iron group there was higher abundance of *Lachnospiraceae* NK4A136 group genus (Figure 3a, b).

The on-tumour microbiota in oral iron-treated patients shows a greater abundance of the Bacilli class, Lactobacillales order and *Prevotella 7*, *Butyrivibrio*, *Coproccoccus 1*, *Hungatella*, *Lachnospiraceae* ND3007 group, *Eubacterium ventriosum* group, *Ruminococcaceae* NK4A214 group and *Pleomorphomonas* genera. Whereas the on-tumour microbiota of intravenous iron-treated patients show higher abundance of the Alteromonadales order and *Alloprevotella* and *Enhydrobacter* genera (Figure 3c, d). These results suggest that the on- and off-tumour microbiota show differential responses to iron therapy. With oral iron leading to a more prominent change in bacterial taxa in the tumour-adjacent compared to the tumour-associated microbiota.

Differential predictive enzymatic pathways between iron treatment groups

In order to infer the metagenomic pathways associated with microbial profiles, we performed a PICRUSt2 metagenomic analysis using predicted enzyme classification abundances based on 16S rRNA bacterial populations. These show a large difference in predicted microbial enzymes between iron treatments in the off-tumour microbiota (Figure 4a), while fewer differences in the on-tumour microbiota were observed (Figure 4b). The off-tumour microbiota is associated with increased abundance of iron-related enzymes, Bacterial non-haem ferritin and Ferric-chelate reductase, in intravenous iron-treated patients compared to oral iron. Along with this, the off-tumour microbiota of intravenous iron-treated patients shows increased abundance of enzymes involved in the production of anti-inflammatory metabolites, including Lactaldehyde dehydrogenase and 2-iminobutanoate/2-iminopropanoate deaminase (Figure 4a).

The on-tumour microbiota is associated with increased abundance of enzymes involved in anti-inflammatory and colorectal cancer protective metabolite production, including Cellulose synthase and N-sulfoglucosamine sulfohydrolase, in intravenous iron-treated patients (Figure 4b). Overall, the predictive metagenomic results suggest that the on- and off-tumour microbiota profiles associated with intravenous iron-treated patients are involved in regulating iron metabolism and production of metabolites involved in inhibiting intestinal inflammatory and colorectal cancer, compared to oral iron-treated patients.

Paired on- and off-tumour microbiota show varying microbial communities following oral and intravenous iron therapy

As the tumour-associated and tumour-adjacent microbiota show differing outcomes following iron therapy in respect to bacterial taxa, we aimed to assess if the method of iron administration led to changes between paired on- and off-tumour microbiota (Figure 5a). Patients treated with oral iron show their off-tumour microbiota being enriched with the *Bacteroidaceae* family and *Bacteroides* genus, while their on-tumour microbiota shows a higher abundance of *Nocardiodaceae*, *Intrasporangiaceae* and *Brevibacteriaceae* families and *Prevotella*, *Nocardioides*, *Kocuria*, *Brevibacterium*, *Veillonella* and *Catenibacterium* genera (Figure 5c, d).

In contrast, patients treated with intravenous iron show their off-tumour microbiota being enriched with the Firmicutes phylum and Clostridia class, along with a greater abundance of the Clostridiales and Sphingomonadales orders, *Sphingomonadaceae* family and *Paraprevotella* genus. Whereas the on-tumour microbiota of patients treated with intravenous iron shows a higher abundance of the Epsilonbacteraeota phylum, Campylobacteria class, Campylobacterales order, *Campylobacteraceae*, *Propionibacteriaceae* and *Porphyromonadaceae* families and *Campylobacter*, *Porphyromonas* and *Cutibacterium* genera (Figure 5a, b). These results suggest that patients treated with oral iron have a more consistent tumour-associated and tumour-adjacent microbiota, showing only small changes at lower taxonomic levels. Whereas patients treated with intravenous iron show a much greater difference between their tumour-associated and tumour-adjacent microbiota, with major differences at the phylum, class, and order levels.

Discussion

A high incidence of colorectal cancer-associated iron deficiency leads to the requirement of therapeutic iron to correct anaemia, with oral iron being the standard treatment due to low cost and convenience [19, 20]. However, oral iron is associated with gastrointestinal side-effects and can increase colonic iron concentration, which can contribute to oncogenic signalling and colitis [21-23]. However, to our knowledge this is the first study to investigate microbial outcomes of oral iron supplementation in anaemic colorectal cancer patients.

This study shows that the on- and off-tumour microbiota shows differential outcomes following oral and intravenous iron supplementation, demonstrating differences in α - and β -diversity, bacterial taxa, and predictive metagenomics. Off-tumour bacterial diversity is significantly different between oral and intravenous iron treatments, supporting a defined off-tumour microbial profile following each therapy. This is consistent with large differences in bacterial taxa, showing greater abundance of Clostridia class, Anaeroplasmatales, Clostridiales and Cytophagales orders and *Lactobacillaceae* and *Anaeroplasmataceae* families in oral iron-treated patients. In contrast, the on-tumour microbiota is more consistent between iron treatments, showing no difference in diversity metrics. Potentially pre-existing tumour microbial alterations may prevent oral iron from leading to major shifts in bacterial populations.

However, differences in bacterial taxa are present between treatment groups, including a higher abundance of Bacilli class and Lactobacillales order in the oral iron group and higher abundance of Alteromonadales order in the intravenous iron group.

On- and off-tumour bacterial genera that are more abundant in oral iron-treated patients include *Coprococcus*, *Prevotella* and *Ruminococcus* (Figure 3a, b). A previous study by Flemer *et al* [24] identified colorectal cancer enriched microbiota, showing that *Coprococcus*, *Prevotella* and *Ruminococcus* were all colorectal cancer enriched genera [24]. Furthermore, a study by Kim *et al* [25] found that the genera *Ruminococcus 2* and *Ruminococcaceae* NK4A214 group, which were more abundant in oral iron-treated patients (Figure 3a, b), were enriched in colorectal cancer patient. Whereas the genus *Lachnospiraceae* NK4A136 group, which was more abundant in intravenous iron-treated patients (Figure 3a), was depleted in colorectal cancer patients [25]. Comparing our results to these studies suggests that oral iron may increase the abundance of colorectal cancer enriched genera, whereas intravenous iron is associated with colorectal cancer depleted genera. However, what is not stated in these studies is whether these are driver or passenger bacteria in colorectal cancer. Potentially beneficial bacterial genera were differentially enriched between iron treatments. Potentially probiotic *Lactobacillus* were more abundant in oral iron (Figure 3a), whereas anti-inflammatory acetic and butyric acid-producing *Alloprevotella* and *Lachnospiraceae* NK4A136 group were more abundant in intravenous iron-treated patients (Figure 3a, b) [26-28]. Collectively the results of our study are consistent with previous findings by Lee *et al* [29], showing the faecal microbiota of oral and intravenous iron-treated inflammatory bowel disease patients had differential bacterial communities. With the authors suggesting intravenous iron may be more beneficial for treating anaemic Crohn's disease patients, in order to limit microbial perturbations [29].

Predictive metagenomics shows the off-tumour microbiota of intravenous iron-treated patients has greater abundance of bacterial enzymes involved in iron metabolism, compared to oral iron (Figure 4). Ferric-chelate reductase is a bacterial enzyme that catalyses the reduction of siderophore-bound ferric-iron to release ferrous-iron [30]. Bacterial non-haem ferritin is a storage protein that binds ferrous-iron keeping it inert intracellularly [31]. Collectively these can contribute to bacterial sequestration of colonic luminal iron, hence not biologically available to contribute to colonic inflammation and tumour initiation [32]. The off-tumour microbiota of intravenous iron-treated patients also shows an increased abundance of Lactaldehyde dehydrogenase, which is involved in the production of anti-inflammatory lactate and pyruvate [33-35]. Likewise, 2-iminobutanoate/2-iminopropanoate deaminase is involved in isoleucine biosynthesis, which can limit intestinal inflammation and inhibit colorectal cancer metastasis [36, 37]. The on-tumour microbiota of intravenous iron-treated patients shows increased abundance of Cellulose synthase. This bacterial enzyme produces cellulose, which can inhibit colonic inflammation through modulating lipid metabolism and inactivating secondary bile acids [38, 39]. N-sulfoglucosamine sulfohydrolase produces glucosamine, which is anti-inflammatory and colorectal cancer protective [40, 41]. Collectively this infers that the on- and off-tumour microbiota in intravenous iron-treated patients is associated with iron sequestration, along with anti-inflammatory and tumour protective metabolite production, compared to oral iron-treated patients. It remains to be investigated whether the PICRUST2 results are functional to further confirm the outcome of this study.

Paired on- and off-tumour microbiota show differentially enriched bacterial taxa in the oral and intravenous iron cohorts. The intravenous iron group shows substantial differences between the on- and off-tumour microbiota at the phylum level. With the off-tumour microbiota being enriched with Firmicutes and the on-tumour microbiota with Epsilonbacteraeota. In contrast, patients treated with oral iron show few differences, primarily at the family and genus level. This suggests that oral iron-treated patients have a more consistent on- and off-tumour microbiota, compared to more diverse in intravenous iron-treated patients.

This pilot study provides novel insights into microbial outcomes of iron therapy in colorectal cancer, suggesting oral iron may be a more deleterious microbial-altering iron therapy. Whereas intravenous iron therapy may be more appropriate in this cohort of anaemic patients. Despite the relatively small sample size of this study, significant differences were observed between iron treatment groups, which can form the foundation for further large-scale explorative studies. These studies could account for confounding variables, such as diet, that have the potential to lead to differential microbial populations, as well as including comparator colonic mucosal microbiota from healthy controls or pre-iron treatment. The relatively small window of therapeutic intervention in this study may show differential microbial outcomes compared to longer-term iron therapy. Nevertheless, this period relevantly reflects the common clinical intervention in anaemic colorectal cancer patients prior to surgery. Furthermore, this study could be further validated through a more comprehensive study of bacterial metabolomics following iron therapy, as well as addressing the clinical relevance of differential microbial communities on long term outcomes following surgery in these patients.

Declarations

Ethics approval and consent to participate Ethical approval for the IVICA trial was granted by the National Research and Ethics Service (East Midlands - Nottingham 2 Research Ethics Committee, 11/EM/0237). The study was registered with Clinical Trials.Gov (NCT01701310) and the Medicines and Healthcare Products Regulatory Agency (2011-002185-21). The study was undertaken in line with the Declaration of Helsinki.

Consent for publication Not applicable.

Availability of data and material The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests MJB's research department has received grant support from Tillots Pharma and Vifor Pharma (Switzerland). MJB has received honoraria and travel support for consulting or lecturing from Vifor Pharma, Tillots Pharma, and Abbvie. The funders had no role in the writing of the manuscript.

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Authors' contributions Study design: HOA, HS, AGA, ADB and MJB. Data acquisition: OP, HOA and MNQ. Statistical analysis: OP and MNQ. Data interpretation and manuscript writing: OP, HOA, MNQ, EAD, JS, HS, AK, AGA, ADB, MJB.

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Tables

Table 1: Patient cohort demographics. Categorical variables are presented with percentages. Continuous variables are presented as mean value [standard deviation] or *median value [interquartile range]. Hb, haemoglobin. CR-POSSUM, ColoRectal Physiological and Operative Severity Score for the enumeration of Mortality and morbidity. ASA, American Society of Anaesthesiologist.

	Oral Iron (n = 16)	Intravenous Iron (n = 24)
Age	74.9 [7.4]	74.9 [9.5]
Male	8 (50%)	16 (67%)
Female	8 (50%)	8 (33%)
Height, m	1.66 [0.1]	1.70 [0.9]
Weight, kg	72.7 [17.4]	79.3 [17.3]
Inclusion Hb, g/l	100.3 [10.6]	98.8 [13.1]
Recruitment ferritin, µg/l *	24.5 [11.1-37.3]	23 [10-48.3]
Recruitment transferrin saturation, % *	2.9 [2.5-3.3]	2.8 [2.4-3.3]
Duration of iron treatment, days *	26.5 [15-43]	23.5 [15-40.5]
Tumour Features		
Tumour size, mm	48.5 [25.3]	42.4 [23.2]
<i>Tumour Stage</i>		
T _{≤2}	1 (6.25%)	3 (12.5%)
T3	10 (62.5%)	18 (75%)
T4	5 (31.25%)	3 (12.5%)
<i>Tumour Location</i>		
Right colon	14 (87.5%)	17 (71%)
Left colon	2 (12.5%)	7 (29%)
Preoperative Risk Assessment		
<i>ASA fitness status classification</i>		
I-II	12 (75%)	10 (42%)
III-IV	4 (25%)	14 (58%)
CR-POSSUM mortality score, % *	3.6 [2.8-9.3]	3.5 [2.6-8.6]

Figures

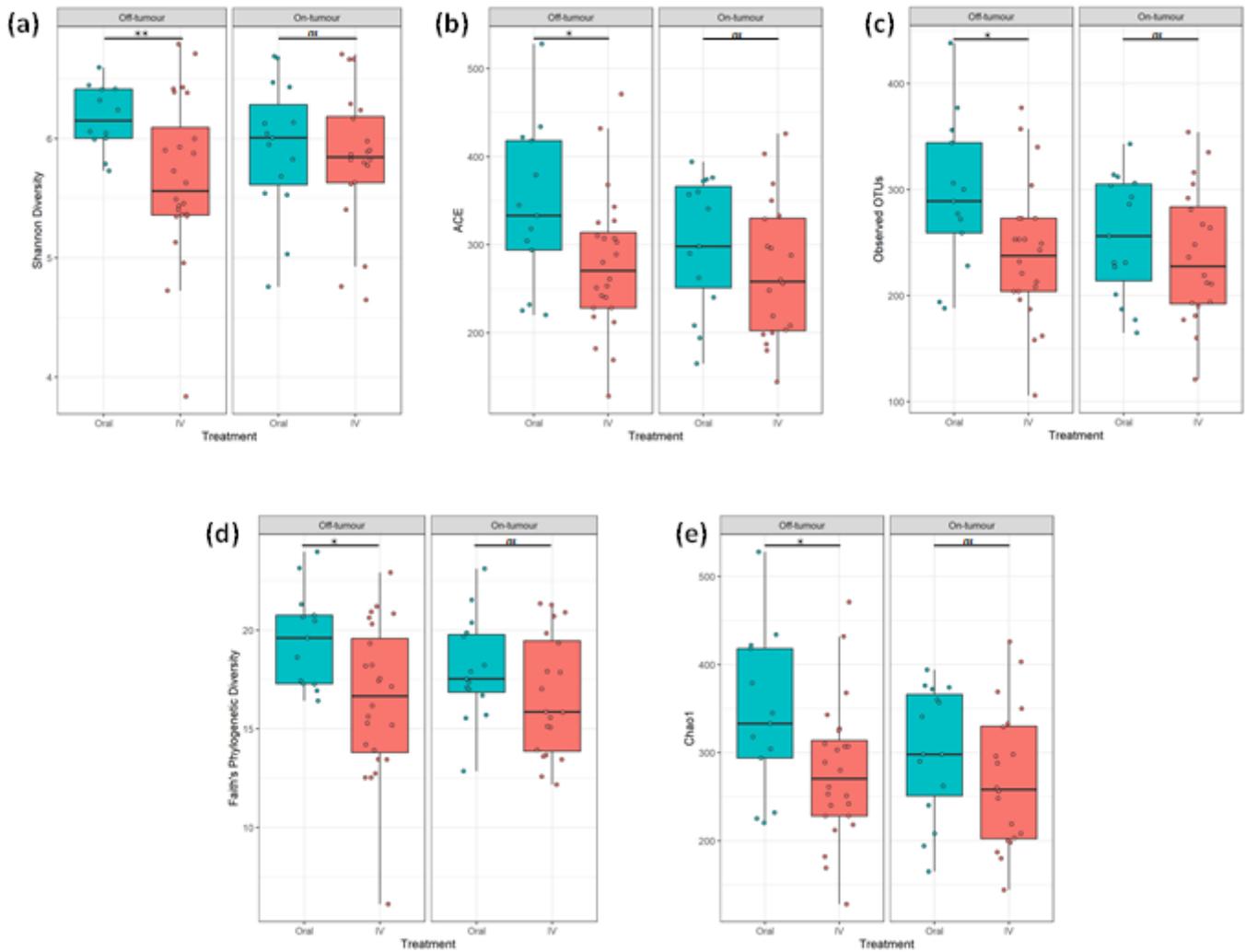


Figure 1

On- and off-tumour α -diversity is differentially altered following iron therapy. α -diversity metrics (a) Shannon Diversity, (b) Abundance-based coverage estimate (ACE), (c) Observed operational taxonomic units (OTUs), (d) Faith's Phylogenetic Diversity and (e) Chao1. Off-tumour diversity metrics were significantly greater in oral iron compared to intravenous (IV) treated patients. On-tumour diversity showed no significant differences between oral and IV iron-treated patients (* $p \leq 0.05$, ** $p \leq 0.01$, ns $p > 0.05$).

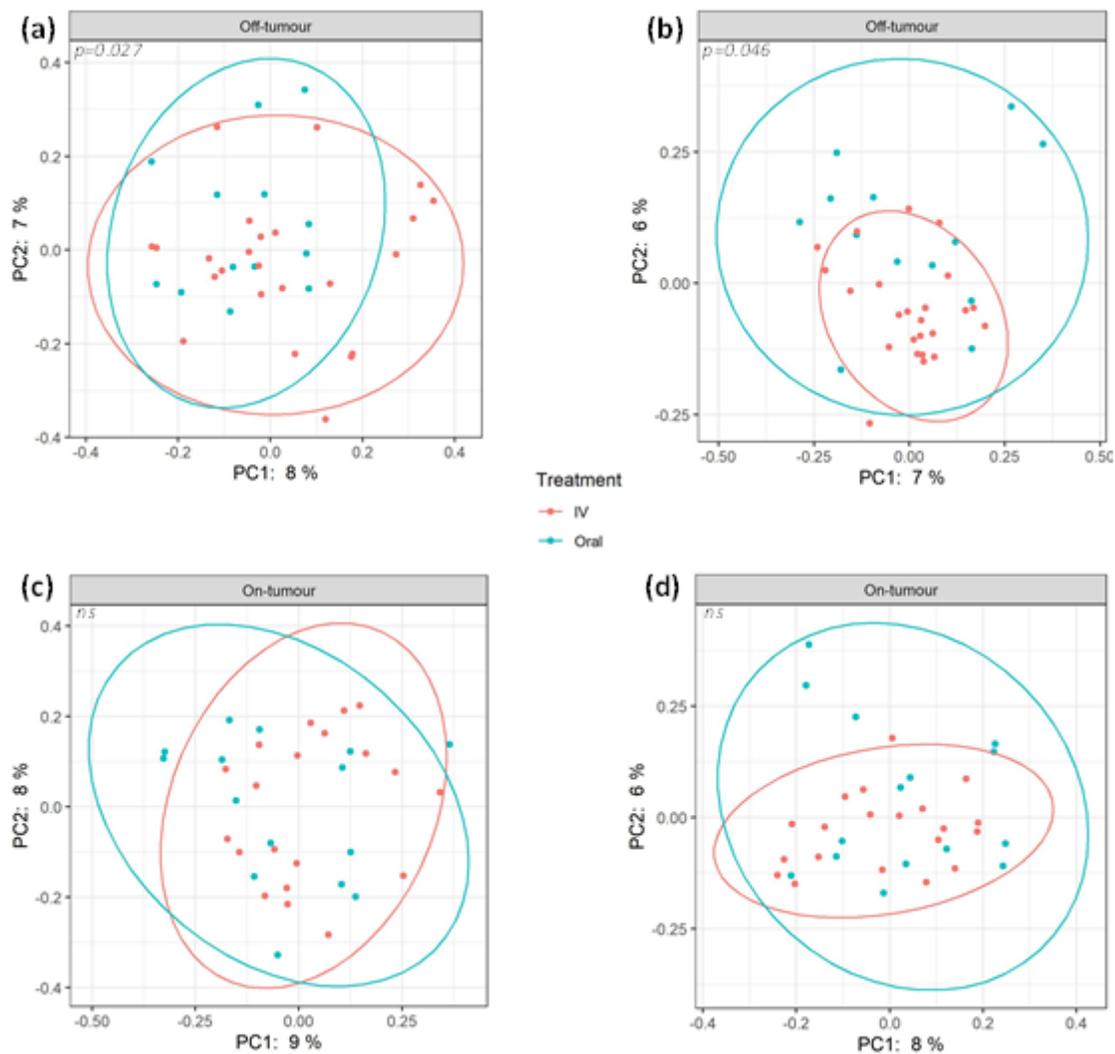


Figure 2

β -diversity of oral and intravenous iron-treated patients differs between on- and off-tumour microbiota. Principle coordinate analysis (PCoA) plots based on Bray-Curtis (a, c) and Jaccard (b, d) distances. Plot ellipses represent 95% confidence incidence for group clusters. The off-tumour β -diversity shows significantly distinct bacterial community clusters between oral and intravenous (IV) iron treatments (a; $p=0.027$, b; $p=0.046$). On-tumour β -diversity shows no significant differences (ns) between iron treatment (c, d).

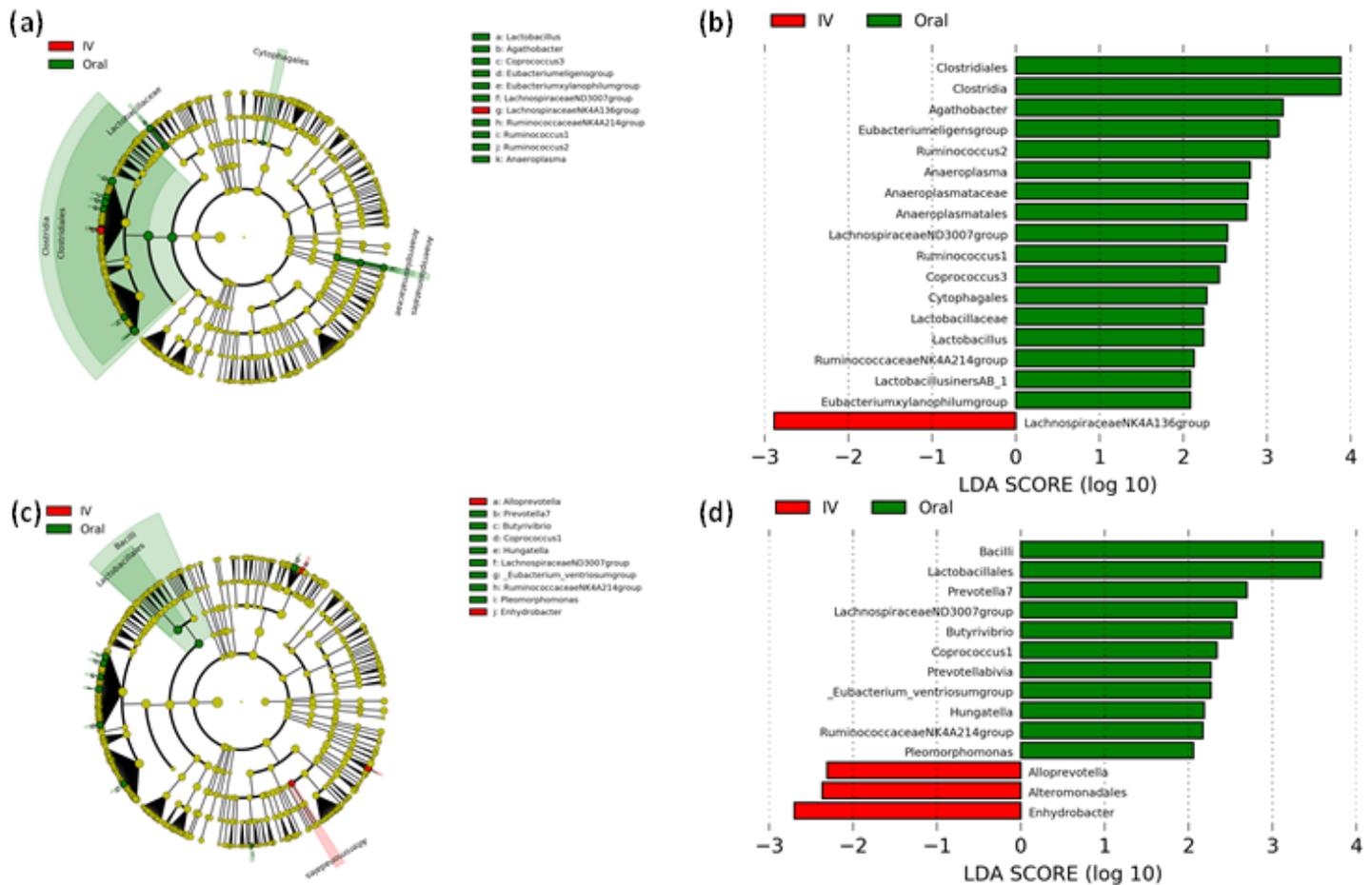


Figure 3

Linear discriminant analysis (LDA) effect size (LEfSe) comparing iron therapy on bacterial taxa in the on- and off-tumour microbiota. Histograms of LDA scores for differentially abundant bacterial taxa between oral and intravenous (IV) iron-treated patients in off-tumour (b) and on-tumour (d) microbiota. LEfSe cladogram representing differentially abundant bacterial taxa in off-tumour (a) and on-tumour (c) microbiota between iron treatments. Differentially abundant taxa from phylum to genus taxonomic levels were included. Taxa and nodes highlighted in green were more abundant in oral and red in IV iron-treated patients. Taxa with an LDA >2 with a p-value ≤ 0.05 were considered statistically significant.

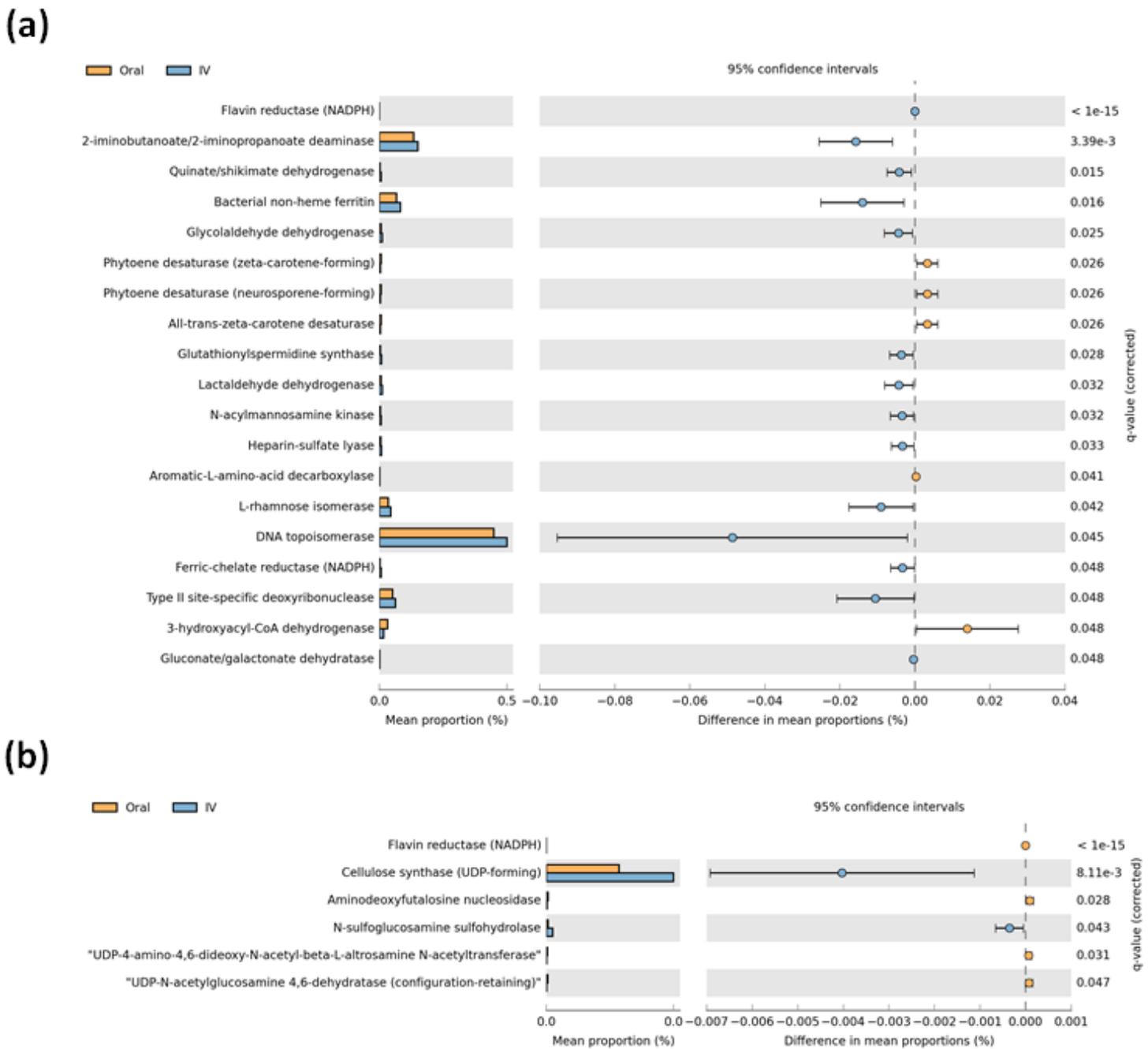


Figure 4

Comparison of predictive metagenomics between iron treatment groups. Predictive metagenomic analysis of enzyme classification abundances between oral and intravenous (IV) iron treatments, based on off-tumour (a) and on-tumour (b) microbial populations. Corrected q-values were calculated following multiple testing correction using Storey false discovery rate. q-values ≤ 0.05 were considered statistically significant.

