JHEP|Reports

Systemic iron reduction by venesection alters the gut microbiome in patients with haemochromatosis

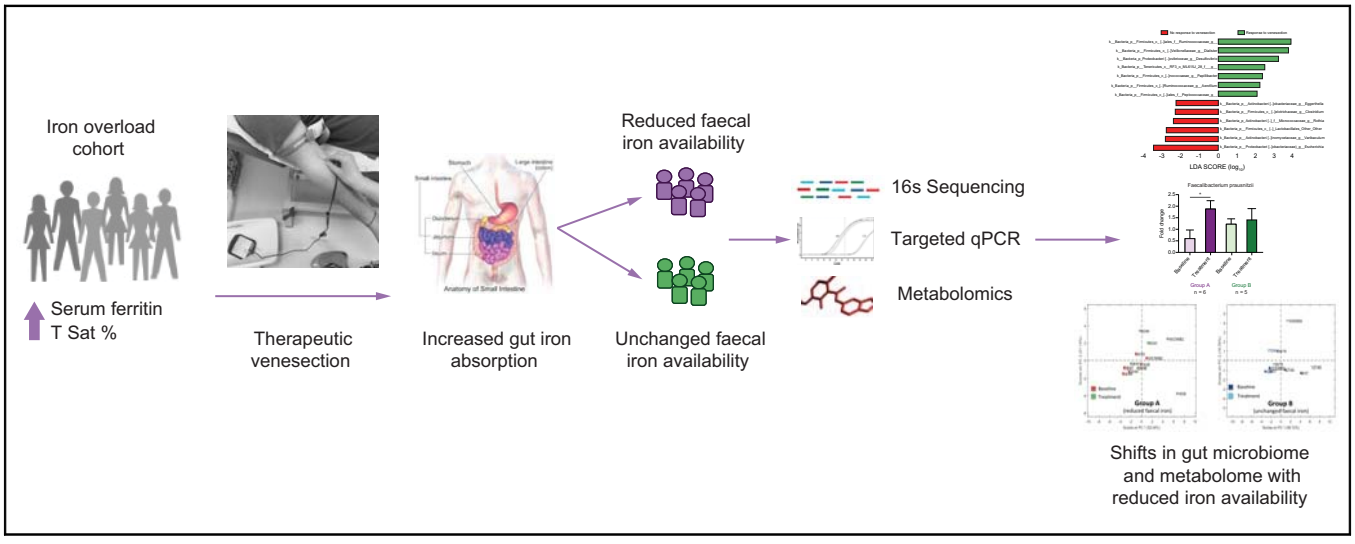
Authors

Bhavika Parmanand, Michael Watson, Karen J. Boland, Narayan Ramamurthy, Victoria Wharton, Alireza Morovat, Elizabeth K. Lund, Jane Collier, Gwenaelle Le Gall, Lee Kellingray, Susan Fairweather-Tait, Jeremy F. Cobbold, Arjan Narbad, John D. Ryan

Correspondence

john.ryan@ndm.ox.ac.uk (J.D. Ryan).

Graphical abstract



Highlights

- Venesection is the cornerstone of haemochromatosis treatment.
- Venesection leads to a compensatory increase in intestinal iron absorption.
- Reduced faecal iron availability leads to shifts in human colonic microbial composition.
- Changes in the human colonic metabolome occur with reduced faecal iron availability.

Lay summary

Iron depletion by repeated venesection is the mainstay of treatment for haemochromatosis, an iron-overload disorder. Venesection has been associated with several health benefits, including improvements in liver function tests, reversal of liver scarring, and reduced risk of liver cancer. During iron depletion, iron absorption from the gastrointestinal (GI) tract increases to compensate for iron lost with treatment. Iron availability is limited in the GI tract and is crucial to the growth and function of many gut bacteria. In this study we show that reduced iron availability in the colon following venesection treatment leads to a change in the composition of the gut bacteria, a finding that, to date, has not been studied in patients with haemochromatosis.

https://doi.org/10.1016/j.jhepr.2020.100154

Short communication

JHEP|Reports

Systemic iron reduction by venesection alters the gut microbiome in patients with haemochromatosis

Bhavika Parmanand,[1,2] Michael Watson,[3,4] Karen J. Boland,[5] Narayan Ramamurthy,[3] Victoria Wharton,[3,4] Alireza Morovat,[6] Elizabeth K. Lund,[2] Jane Collier,[3,4] Gwenaelle Le Gall,[2] Lee Kellingray,[1] Susan Fairweather-Tait,[2] Jeremy F. Cobbold,[3,4] Arjan Narbad,[1] John D. Ryan[3,7,*]

[1]*Quadram Institute, Norwich, UK;* [2]*University of East Anglia, Norwich, UK;* [3]*Translational Gastroenterology Unit, University of Oxford, Oxford, UK;* [4]*NIHR Oxford Biomedical Research Centre, Oxford, UK;* [5]*Department of Gastroenterology, Beaumont Hospital/Royal College of Surgeons in Ireland, Dublin, Ireland;* [6]*Department of Clinical Biochemistry, Oxford University Hospitals Foundation Trust, Oxford, UK;* [7]*Hepatology Unit, Beaumont Hospital/Royal College of Surgeons in Ireland, Dublin, Ireland*

JHEP Reports **2020.** https://doi.org/10.1016/j.jhepr.2020.100154

Background & Aims: Iron reduction by venesection has been the cornerstone of treatment for haemochromatosis for decades, and its reported health benefits are many. Repeated phlebotomy can lead to a compensatory increase in intestinal iron absorption, reducing intestinal iron availability. Given that most gut bacteria are highly dependent on iron for survival, we postulated that, by reducing gut iron levels, venesection could alter the gut microbiota.
Methods: Clinical parameters, faecal bacterial composition and metabolomes were assessed before and during treatment in a group of patients with haemochromatosis undergoing iron reduction therapy.
Results: Systemic iron reduction was associated with an alteration of the gut microbiome, with changes evident in those who experienced reduced faecal iron availability with venesection. For example, levels of *Faecalibacterium prausnitzii*, a bacterium associated with improved colonic health, were increased in response to faecal iron reduction. Similarly, metabolomic changes were seen in association with reduced faecal iron levels.
Conclusion: These findings highlight a significant shift in the gut microbiome of patients who experience reduced colonic iron during venesection. Targeted depletion of faecal iron could represent a novel therapy for metabolic and inflammatory diseases, meriting further investigation.

Introduction

Venesection is the standard therapy for patients with hereditary haemochromatosis (HH), the most common iron-overload condition. Venesection typically involves the removal of 500 ml of blood (equivalent to 250 mg of iron) weekly from patients until normal iron levels are achieved. Repeated phlebotomy in HH has been associated with enhanced quality of life, increased energy levels, improvement in liver function tests and a reversal of liver fibrosis, as well as a reduction in cancer risk.[1–3] However, the factors mediating these benefits are largely unknown.

Research indicates that patients undergoing venesection experience increased intestinal iron absorption during treatment,[4] leading to reduced faecal iron excretion.[5] Given that iron is a vital for the growth of many gut bacteria.[6] changes in intestinal iron availability during phlebotomy could have implications for microbes residing in the colon. Conversely, oral iron supplementation has been shown to adversely affect the composition and function of the human gut microbiome,[7] whereas differences in gut bacteria have been demonstrated comparing iron-deficient with iron-replete individuals.[8] Moreover, increased colonic iron has been associated with colonic inflammation and oxidative stress; therefore, reducing colonic iron could confer a therapeutic benefit.[9–11]

In this study, we determined the relationship between gut bacteria and faecal iron levels before and during phlebotomy. Stool samples were collected from patients initiating treatment (characteristics outlined in Table S1 in the supplementary information online), with paired samples obtained from 11 of these patients during follow-up. Faecal iron levels were measured, and their relationship with the gut microbiome was assessed by 16S metagenomic sequencing and metabolomic analysis of faecal water.

Patients and methods

Study population and design

In total, 20 patients [10 C282Y homozygotes, 4 compound C282Y/H63D heterozygotes and 6 with non-hyperferritinaemia (HFE)] initiating therapeutic venesection at the John Radcliffe Hospital, Oxford, UK were enrolled alongside standard clinical care. Written informed consent was obtained from all study subjects. The study conformed to the ethical guidelines of the

Keywords: Microbiome; Iron; Venesection; Haemochromatosis.
Received 24 October 2019; received in revised form 29 June 2020; accepted 15 July 2020; available online 28 July 2020
* Corresponding author. Address: Hepatology Unit, Beaumont Hospital, Royal College of Surgeons in Ireland, Dublin 9, Ireland.
E-mail address: john.ryan@ndm.ox.ac.uk (J.D. Ryan).

1975 Declaration of Helsinki, and patients were recruited through the Oxford GI Biobank.[12] Patients were requested not to modify their diet during the study period. No patients reported antibiotic use within 6 weeks before, or during, the study period. Stool samples from 5 healthy controls were obtained for comparison of baseline faecal iron levels.

Treatment protocol

Venesection was initiated according to unit protocol; individuals were deemed to have iron overload by their treating hepatologist, with a serum ferritin level >300 μg/L in men and >200 μg/L in women in combination with an elevated fasting transferrin saturation (>45%) for both men and women. All patients were treated by a nurse-led venesection service, aiming to achieve an initial serum ferritin target of 50–100 μg/L through weekly or fortnightly venesection. Each venesection would typically involve the removal of 500 mL blood (250 mg iron). Either venesection intervals were increased or a lower volume of blood was removed if the patient did not tolerate phlebotomy (e.g., because of weakness, hypotension or development of anaemia).

Metagenomic DNA extraction

DNA was extracted from all samples using a commercially available kit (FastDNA spin kit for soil; MP Biomedicals, USA, Cat No. 6560200). Samples were thawed on ice, homogenized, and DNA was extracted from ∼200 mg of each sample with an additional bead-beating step using FastPrep.[13]

16S rRNA gene amplification and sequencing

The impact of iron on the composition of the human gut microbiome was investigated using high-throughput 16S rRNA gene (V4 region) sequencing using the MiSeq platform (Illumina, San Diego, USA). Sequencing produced 9,800,284 high-quality reads, with an average of 224,980 ± 50,072 reads per sample. Data analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME, version 1.9) pipeline. ChimeraSlayer was used to filter trimmed reads for chimeric sequences.

Comparison of taxa composition according to response to venesection by LEfSe

Linear discriminant analysis effect size (LEfSe)[14] was used to identify and characterise the differences in abundance of genera between faecal samples according to response to venesection. Differentially abundant taxa were identified using nonparametric Kruskal–Wallis sun-rank test ($p = 0.05$), followed by linear discriminant analysis (LDA) estimating the effect size of each differentially abundant genus. Differences in abundance were considered statistically significant if the logarithmic LDA score was >2.0.

Short-chain fatty acid quantification in cultured microbiomes

Briefly, 0.2 g of faecal sample was mixed with 12× volume of NMR buffer, and 1 mM sodium 3-(Trimethylsilyl)-propionate-*d*4 (TSP) as a chemical shift reference. The ^{1}H NMR spectra were recorded at 600 MHz on a Bruker AVANCE spectrometer (Bruker BioSpin GmbH, Germany) running Topspin 2.0 software. The metabolites were quantified using the software Chenomx® NMR Suite 7.0™.

Measuring total iron concentrations in stool samples

Free faecal iron refers to unbound iron, which is typically kept at very low levels to prevent the production of toxic reactive oxygen species, or its use by pathogens for growth. Most iron within the colonic lumen is derived from the diet, with ∼90% of dietary iron in the non-haem iron form; iron can be bound to non-haem compounds (ferritin), haem compounds (haemoproteins) or haem enzymes. Total faecal iron includes both bound and unbound iron.[15]

Flame atomic absorption spectrophotometry (FAAS) was used to determine the concentration of iron in faecal samples. Faecal samples were thawed, weighed and then dried at 110°C in an oven. The sample was remeasured to calculate water content, transferred into glass crucibles and ashed in a muffle furnace for 48 h at 600°C. The ashed sample was resuspended in 20% 16M HNO_3 and crucibles were then placed on a hot plate until the sample had almost evaporated. This was then diluted to a final volume of 25 ml of 1M HCL. The spectrophotometer (Perkin Elmer Atomic Absorption Spectrophotometer Model 3300) was calibrated against a range of iron standards and samples were measured at an absorption wavelength of 248.3 nm.

Measuring available iron in stool samples

To measure available iron in each sample, a 0.2 g faecal sample was homogenised with a known volume of Milli-Q water, mixed on a rotator stirrer for 30 min at room temperature and centrifuged at 3,000 g for 15 min at 4°C. Supernatants were analysed using the ferrozine assay, where iron in the sample is reduced using an iron reducer provided by the Iron Assay Kit (Abcam, Cambridge, UK), after which iron reacts with Ferene S (an iron chromogen) to produce a stable coloured complex and give absorbance at 593 nm.

Gene expression analysis

Profiling of gut bacterial species was performed using the Metabolic Disorders qPCR array for microbial DNA testing of 45 specific bacterial species implicated in metabolic dysfunctions, such as obesity and type 2 diabetes mellitus (Qiagen) and the microbial DNA qPCR assay for high-sensitivity GAPDH. Data were analysed using the 2-ΔΔCt method, using GAPDH as the internal control gene and the mean of the control duplicates as a control.

Statistical analyses

Continuous, normally distributed variables are reported as mean ± standard deviation (SD), or median (range) for non-Gaussian distributions. Categorical variables are presented as *n* (%). Comparison between groups was performed using Student's *t* test or Mann-Whitney *U* test, and analysis of variance (ANOVA) or Kruskal-Wallis test as appropriate, for continuous variables. Correlations were performed by Pearson's correlation or Spearman Rank methods. Data were analysed using Graphpad Prism 6.0. A *p* value <0.05 was considered statistically significant.

Results

Free iron levels in stool samples were significantly higher in patients undergoing venesection at baseline compared with those of healthy controls (Fig. 1). As expected, treatment with venesection was associated with a significant reduction in serum ferritin levels, and an improvement in liver enzymes (Fig. 1). Of the 11 patients with paired samples from baseline and during treatment, no significant change in total faecal iron levels was noted with treatment (data not shown). However, free faecal iron levels decreased significantly on follow-up in 6 patients (Group A, reduced faecal iron), whereas no change was observed in samples from 5 patients

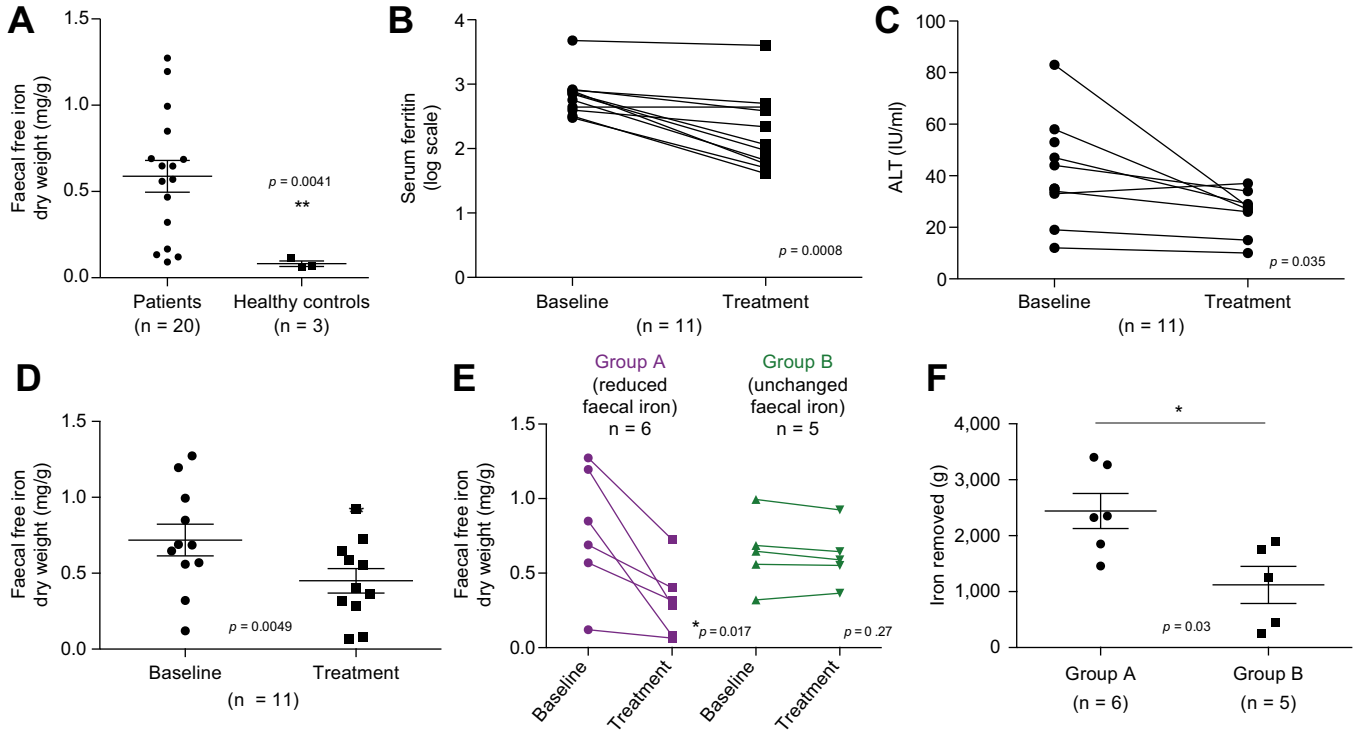


Fig. 1. Iron and biochemical changes at baseline and after treatment. (A) Patients with iron overload had significantly higher faecal free iron levels compared with healthy controls. Significant reductions in serum (B) ferritin and (C) ALT were seen in paired samples on treatment (note that one ALT data point is missing in the treatment group). Free faecal iron fell significantly with treatment (D). Of these, 6 individuals had a significant reduction in faecal free iron (group A; reduced faecal iron), while 5 did not (group B; unchanged faecal iron) (E). Group A had significantly more iron removed by venesection compared with group B (F). Graphs are presented as mean ± SEM. $^{*}p$ <0.05 $^{**}p$ <0.005. Differences between groups were assessed using Mann-Whitney *U* test or Wilcoxon tests as appropriate. ALT, alanine transferase.

(Group B, unchanged faecal iron; characteristics outlined in Table S2 in the supplementary information online). No significant differences in age, gender, baseline or treatment serum ferritin, or baseline faecal free iron levels were noted between groups, although comparisons were limited by the small sample size. However, individuals in Group A had significantly more iron removed by phlebotomy (2.7g [± 0.8] *vs.* 1.1 g [± 0.7]) and were all homozygous for C282Y mutation in the *HFE* gene; only these patients experienced significant biochemical improvements compared with Group B, including reductions in alanine aminotransferase (ALT) (43 [± 11] IU/ml to 28 [± 4] IU/ml, *vs.* 39 [± 28] IU/ml to 22 [± 9] IU/ml; Figure S1a in the supplementary information online) and HbA1c levels (33 [± 4] mmol/mol to 27 [± 4] mmol/mol *vs.* 33 [± 4] mmol/mol to 34 [± 1] mmol/mol). Free faecal iron correlated with ALT levels in Group A (rho = 0.76, p = 0.0052; Figure S1b in the supplementary information online), whereas no significant relationship was evident in Group B. No significant relationship between markers of colonic inflammation, such as C-reactive protein (CRP) or white cell count (WCC) was evident (these parameters were largely normal in the study cohort; Table S1 in the supplementary information online).

The effect of treatment on the gut microbiota was compared between groups to assess the impact of changes in faecal free iron. Although no difference in phylogenetic beta diversity was evident between baseline and treatment in both groups, significant changes in microbiome community composition were found on 16S sequencing using LEfSe analysis (Fig. 2). Furthermore, levels of 3 bacterial species (of 45 assayed by qPCR), namely *Faecalibacterium prausnitzii*, *Dorea formicigenerans* and *Collinsella aerofaciens*, were increased in those patients with reduced faecal iron during venesection (Fig. 2).

Similarly, significant changes in microbial metabolites after treatment were only evident in Group A, where increases in pyruvate, tyrosine, methionine, glycine and aspartate were observed (Fig. S1 in the supplementary information online). In these patients, there was a greater separation in the metabolome, where a shift was observed towards a more positive metabolomic profile with treatment compared with baseline. By contrast, a less distinct shift in the metabolomic profile was evident in Group B (Fig. S1 in the supplementary information online).

LEfSe analysis of faecal samples before and after venesection demonstrated changes in the faecal microbiome in patients in response to altered iron availability. There was a significant (p <0.05) increased abundance of the pathobiont *Escherichia* in faecal samples of patients who did not response to iron reduction through venesection (Fig. 2b). Interestingly, this mirrored findings from a study reporting a differential reduction in the relative abundance of *Escherichia* in response to iron chelation.[9] In addition, LEfSe analysis identified increased log LDA abundance of *Desulfovibrio* (Fig. 2b), a sulfate-reducing member of the Proteobacteria phylum associated with iron metabolism through reduction of iron oxides.[16] These data, and the changes seen in participant faecal microbiome analysis, support previously published data that propose that haem iron, through its impact on mucosal homeostasis, can alter the colonic luminal environment and, hence, the associated microbiome.[17]

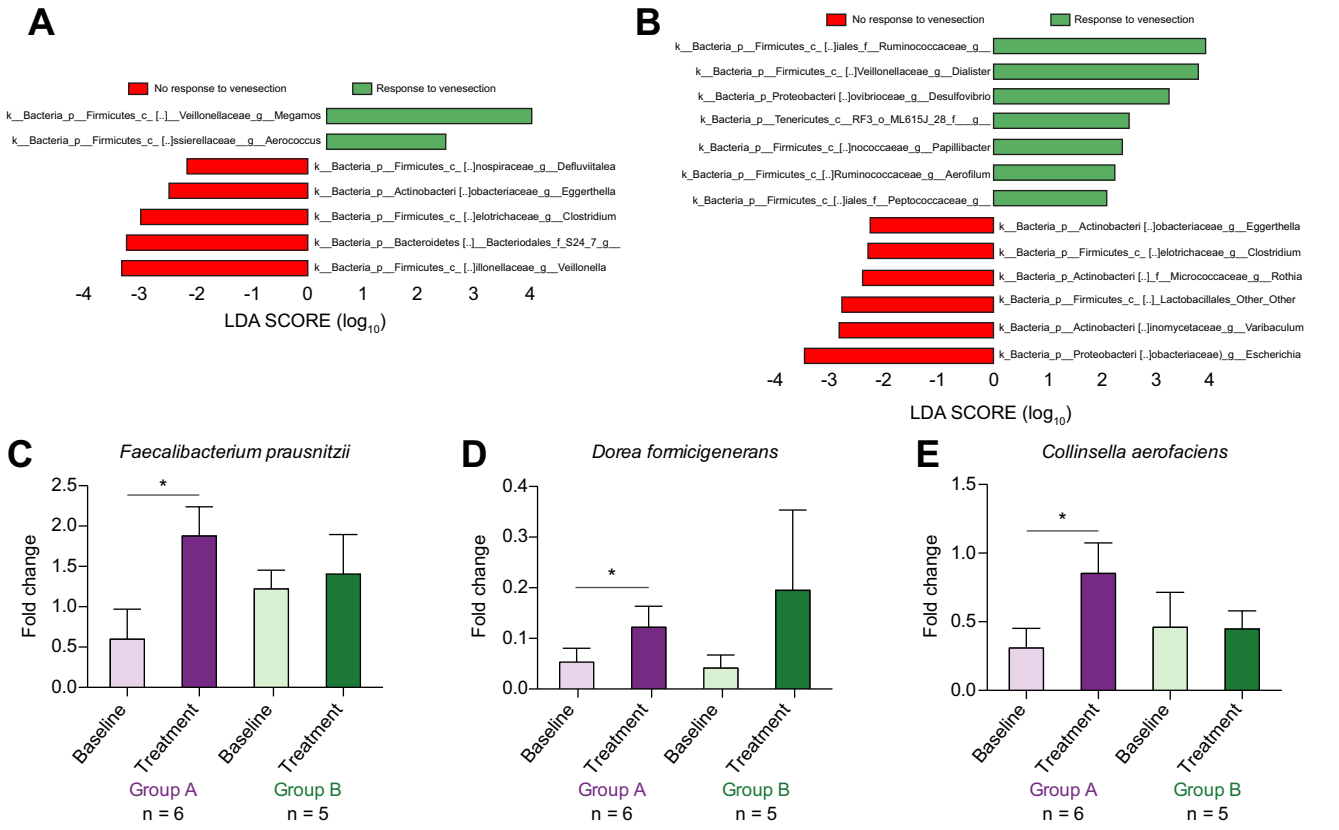


Fig. 2. Changes in bacterial composition with iron reduction. LEfSe analysis of faecal samples stratified by response to venesection. LEfSe identified taxa with differential relative abundance between categories (p <0.05). Data indicate LDA showing an effect size greater than log LDA = 2, which were deemed statistically significant. Baseline faecal samples of patients stratified according to eventual response to venesection (A) and differentially abundant taxa in faecal samples after venesection (B). Significant changes in 3 bacterial species were evident in those with reduced faecal free iron following venesection [Group A, *p <0.05 (C–E)]. Graphs are presented as mean ± SEM. Differences between groups were assessed using Wilcoxon test. LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size.

Strikingly, in a study by Lee *et al.* examining the effect of oral and intravenous iron supplementation on patients with inflammatory bowel disease,[8] oral iron supplementation was associated with decreased abundances of the bacterial species *F. prausnitzii*, *D. formicigenerans* and *C. aerofaciens*; in this study, the reduction in colonic iron was associated with an increase in these exact species, aligning findings with 2 independent approaches. In particular, a depletion of *F. prausnitzii* has been implicated in several diseases, including fatty liver disease and inflammatory bowel disease, and therapies to augment its abundance would be of potential clinical benefit. An increase in the relative abundance of *Collinsella* was also found, and both these bacterial genera are associated with the production of potentially beneficial short-chain fatty acids, such as acetate and butyrate, although no change in these metabolites was noted in the current study.[18,19]

This is the first description of the gut microbiome in patients with HH, a condition in which excess systemic iron can lead to multiorgan damage, including liver fibrosis and primary liver cancer. Iron reduction by venesection can significantly reduce the risk of these complications. Despite being limited by small numbers, this study reveals a clear effect of colonic iron depletion on the gut microbiome during venesection. Individuals who experienced a beneficial effect of venesection on their microbiome profiles had undergone a greater amount of iron removal than those who did not. This likely reflects a greater initial iron burden, and a better tolerance to venesection, conferred by their HFE genotype. However, this pilot study does not include mechanistic data to determine whether the changes noted in microbial composition could account for improvements in disease.

Discussion

Overall, a general shift towards a healthier systemic and metabolic profile was observed in patients with HH who responded positively to iron reduction via venesection. This was accompanied by an increase in beneficial bacterial species in the large intestine, as well improved metabolomic profiles. Thus, regulating the availability of colonic iron could represent a novel therapy for metabolic and inflammatory disorders through the manipulation of the gut microbiome and, therefore, merits further investigation.

Abbreviations

ALT, alanine aminotransferase; CRP, C-reactive protein; FAAS, flame atomic absorption spectrophotometry; GI, gastrointestinal; HFE, hyperferritinaemia; HH, hereditary haemachromatosis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; TSP, 3-(trimethylsilyl)-propionate-d4; WCC, white cell count.

Financial support

This study was supported by grants from the Oxford Health Service Research Committee, the University of Oxford Medical Research Fund, and the NIHR Oxford Biomedical Research Centre. BP was funded by the UK Biotechnology and Biological Sciences Research Council iCASE studentship (BB/M015122/1).

Conflict of interest

The authors have no conflict of interests to declare in relation to this study.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

BP conducted the research, analysed the data and wrote the manuscript; MW and VW collated the data, co-ordinated the study and reviewed the manuscript; KB analysed the data and reviewed the manuscript; NR, GLG and LK conducted the research and analysed the data; AM, EL, JC, SFT, JFC and AN provided resources and supervision, and reviewed the manuscript; JDR conceptualised the study, acquired funding, analysed the data and wrote the manuscript.

Acknowledgements

The authors wish to thank all the patients who took part in this study. All research data outlined in this paper can be made available to collaborating researchers. Please contact the corresponding author.

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhepr.2020.100154.

References

[1] Powell LW, Dixon JL, Ramm GA, Purdie DM, Lincoln DJ, Anderson GJ, et al. Screening for hemochromatosis in asymptomatic subjects with or without a family history. Arch Intern Med 2006;166:294–301.

[2] Bardou-Jacquet E, Morcet J, Manet G, Laine F, Perrin M, Jouanolle A, et al. Decreased cardiovascular and extrahepatic cancer-related mortality in treated patients with mild HFE hemochromatosis. J Hepatol 2015;62:682–689.

[3] Ong SY, Gurrin LC, Dolling L, Dixon J, Nicoll AJ, Wolthuizen M, et al. Reduction of body iron in HFE-related haemochromatosis and moderate iron overload (Mi-Iron): a multicentre, participant-blinded, randomised controlled trial. Lancet Haematol 2017;4:e607–e614.

[4] Williams R, Manenti F, Williams HS, Pitcher CS. Iron absorption in idiopathic haemochromatosis before, during, and after venesection therapy. Br Med J 1966;2:78–81.

[5] Pippard MJ, Callender ST, Finch CA. Ferrioxamine excretion in iron-loaded man. Blood 1982;60:288–294.

[6] Andrews SC, Robinson AK, Rodriguez-Quinones F. Bacterial iron homeostasis. FEMS Microbiol Rev 2003;27:215–237.

[7] Jaeggi T, Kortman GA, Moretti D, Chassard C, Holding P, Dostal A, et al. Iron fortification adversely affects the gut microbiome, increases pathogen abundance and induces intestinal inflammation in Kenyan infants. Gut 2015;64:731–742.

[8] Lee T, Clavel T, Smirnov K, Schmidt A, Lagkouvardos I, Walker A, et al. Oral versus intravenous iron replacement therapy distinctly alters the gut microbiota and metabolome in patients with IBD. Gut 2017;66:863–871.

[9] Parmanand BA, Kellingray L, Le Gall G, Basit AW, Fairweather-Tait S, Narbad A. A decrease in iron availability to human gut microbiome reduces the growth of potentially pathogenic gut bacteria; an in vitro colonic fermentation study. J Nutr Biochem 2019;67:20–27.

[10] Lund EK, Wharf SG, Fairweather-Tait SJ, Johnson IT. Oral ferrous sulfate supplements increase the free radical-generating capacity of feces from healthy volunteers. Am J Clin Nutr 1999;69:250–255.

[11] Mahalhal A, Williams JM, Johnson S, Ellaby N, Duckworth CA, Burkitt MD, et al. Oral iron exacerbates colitis and influences the intestinal microbiome. PLoS One 2018;13:e0202460.

[12] Gastrointestinal illness in Oxford: prospective cohort for outcomes, treatment, predictors and biobanking. REC Ref: 16/YH/0247 Available at: https://www.hra.nhs.uk/planning-and-improving-research/application-summaries/research-summaries/gastrointestinal-illness-in-oxford-prospective-cohort-for-biobanking/. [Accessed 21 July 2020].

[13] Kellingray L, Tapp HS, Saha S, Doleman JF, Narbad A, Mithen RF. Consumption of a diet rich in Brassica vegetables is associated with a reduced abundance of sulphate-reducing bacteria: a randomised crossover study. Mol Nutr Food Res 2017;61:1600992.

[14] Segata N, Izard J, Waldron L, Gevers D, Miropolsky L, Garrett WS, et al. Metagenomic biomarker discovery and explanation. Genome Biol 2011;12:R60.

[15] Kortman GA, Raffatellu M, Swinkels DW, Tjalsma H. Nutritional iron turned inside out: intestinal stress from a gut microbial perspective. FEMS Microbiol Rev 2014;38:1202–1234.

[16] Lentini CJ, Wankel SD, Hansel CM. Enriched iron(III)-reducing bacterial communities are shaped by carbon substrate and iron oxide mineralogy. Front Microbiol 2012;3:404.

[17] Martin OCB, Olier M, Ellero-Simatos S, Naud N, Dupuy J, Huc L, et al. Haem iron reshapes colonic luminal environment: impact on mucosal homeostasis and microbiome through aldehyde formation. Microbiome 2019;7:72.

[18] Sokol H, Pigneur B, Watterlot L, Lakhdari O, Bermudez-Humaran LG, Gratadoux JJ, et al. *Faecalibacterium prausnitzii* is an anti-inflammatory commensal bacterium identified by gut microbiota analysis of Crohn disease patients. Proc Natl Acad Sci U S A 2008;105:16731–16736.

[19] Rajilić-Stojanović M, de Vos WM. The first 1000 cultured species of the human gastrointestinal microbiota. FEMS Microbiol Rev 2014;38:996–1047.