Systemic iron reduction by venesection alters the gut microbiome in Haemochromatosis patients

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Data availability statement: all research data outlined in this paper can be made available to collaborating researchers.

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 improvement in liver function tests, the reversal of liver scarring, and a reduced risk of liver cancer. During iron depletion, iron absorption from the gastro-intestinal (GI) tract increases to compensate for iron lost with treatment. Iron availability is limited in the GI tract and is critical 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 which to date had not been studied in Haemochromatosis patients.

Abstract

Background & Aims: Iron reduction by venesection has been the cornerstone of treatment of Hemochromatosis for decades; its reported health benefits are many. Repeated phlebotomy can lead to a compensatory increase in intestinal iron absorption, reducing intestinal iron availability. As 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 Haemochromatosis patients undergoing iron reduction therapy. Results: Here we show that systemic iron reduction is 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 *F. prausnitzii*, a bacteria associated with improved colonic health, were increased by 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 may 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 commonest iron overload condition. Venesection typically involves the removal of 500mls of blood (equivalent to 250mg of iron) weekly from patients until normal iron levels are achieved. Repeated phlebotomy in HH has been associated with an enhanced quality of life and increased energy levels, an improvement in liver function tests and a reversal of liver fibrosis, as well as a reduction in cancer risk.(1-3) The factors mediating these benefits are largely unknown.

Studies have indicated that patients undergoing venesection experience increased intestinal iron absorption during treatment,(4) leading to reduced faecal iron excretion.(5) As iron is a vital nutrient for the growth of many gut bacteria,(6) changes in intestinal iron availability during phlebotomy could have implications for the 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) while 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 and therefore, reducing colonic iron may confer a therapeutic benefit.(9-11)

In this study, we aimed to determine the relationship between gut bacteria and faecal iron levels before and during phlebotomy. Stool samples were collected from patients initiating treatment (characteristics outlined in supplementary table 1), 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.

Methods

Study Population and Design

20 patients (10 C282Y homozygotes, 4 compound C282Y/H63D heterozygotes, 6 with non-HFE hyperferritinaemia) initiating therapeutic venesection at the John Radcliffe Hospital, Oxford were enrolled alongside standard clinical care. Written informed consent was obtained from all study subjects. The study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, and patients were recruited through the Oxford GI Biobank (Gastrointestinal Illness in Oxford: *prospective cohort for outcomes, treatment, predictors and biobanking* REC Ref: 16/YH/0247). 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 >300µg/L in men and >200µg/L in women in combination with an elevated fasting transferrin saturation (>45%) for men and women. All patients were treated by a nurse-led venesection service, aiming to achieve an initial serum ferritin target of 50-100µg/L through weekly or fortnightly venesection. Each venesection would typically involve in the removal of 500mls blood (250mg iron). Venesection intervals would be increased, or less volume of blood would be removed should the patient not tolerate phlebotomy (due to weakness, hypotension, or the development of anaemia, for instance).

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 approximately 200mg of each, with an additional bead beating step using FastPrep.(12)

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 Illumina Miseq platform. Sequencing produced 980,0284 high-quality reads, with an average of 224,980 ± 50072 reads per sample. Data analysis was performed using the Quantitative Insights into Microbial Ecology (QIIME, V1.9) 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)(13), was employed to identify and characterise the differences in abundance of genera between faecal samples according to response to venesection. Differentially abundant taxa were identified using non-parametric Kruskal-Wallis sun-rank test ($p=0.05$), followed by linear discriminant analysis (LDA) estimating effect size of each differentially abundant genus. Differences in abundance were considered statistically significant if logarithmic LDA score was >2.0.

Short chain fatty acid quantification in cultured microbiomes

Briefly, 0.2g of faecal sample was mixed with 12x volume of NMR buffer, and 1mM 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. The vast majority of iron within the colonic lumen is derived from the diet, with approximately 90% of dietary iron in the non-haem iron form; iron may is bound to non-haem compounds (ferritin) or haem compounds (haemoproteins) or haem enzymes. Total faecal iron includes both bound and unbound iron. (14).

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 re-measured again to calculate water content, transferred into glass crucibles and ashed in a muffle furnace for 48 hrs at 600°C. The ashed sample was resuspended in 20% 16M HNO_3 and crucibles were then placed on a hot plate until 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 248.3nm.

Measuring Available Iron in Stool Samples

0.2g faecal sample was homogenised with a known volume of Milli-Q water, mixed on a rotator stirrer for 30 min at the room temperature and centrifuged at 3,000g for 15 min at 4^oC. Supernatants were analysed using the Ferrozine assay, where iron in the sample is reduced using an Fe reducer provided by the kit 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 dysfunction such as obesity and type 2 diabetes mellitus (Qiagen) and Microbial DNA qPCR Assay for Hs.GAPDH. Data was analysed using the 2-ΔΔCt method with GAPDH as internal control gene and using the mean of the control duplicates as control.

Statistical Analyses

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

Results and Discussion

Free iron levels in stool samples were significantly higher in venesection patients at baseline when compared with that of healthy controls (figure 1). As expected, treatment with venesection was associated with a significant reduction in serum ferritin levels, and an improvement in liver enzymes (figure 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), while no change was observed in samples from 5 patients (group B, unchanged faecal iron; characteristics outlined in supplementary table 2). No significant differences in age or gender, baseline or treatment serum ferritin, or baseline faecal free iron levels were noted between groups, although comparisons were limited by small sample size. However, individuals in the group A had significantly more iron removed by phlebotomy [2.7g (+/-0.8) vs. 1.1g (+/-0.7)] and were all homozygous for C282Y mutation in the *HFE* gene; only these patients experienced significant biochemical improvements when compared with group B, including reductions in ALT [43 (+/-11) IU/ml to 28 (+/-4) IU/ml, vs. 39 (+/-28) IU/ml to 22 (+/-9) IU/ml; supplementary figure 1a] 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; supplementary figure 1b), while no significant relationship was evident in group B. No significant relationship between markers of colonic inflammation such as CRP or

WCC was evident (these parameters were largely normal in the study cohort; suppl. table 1).

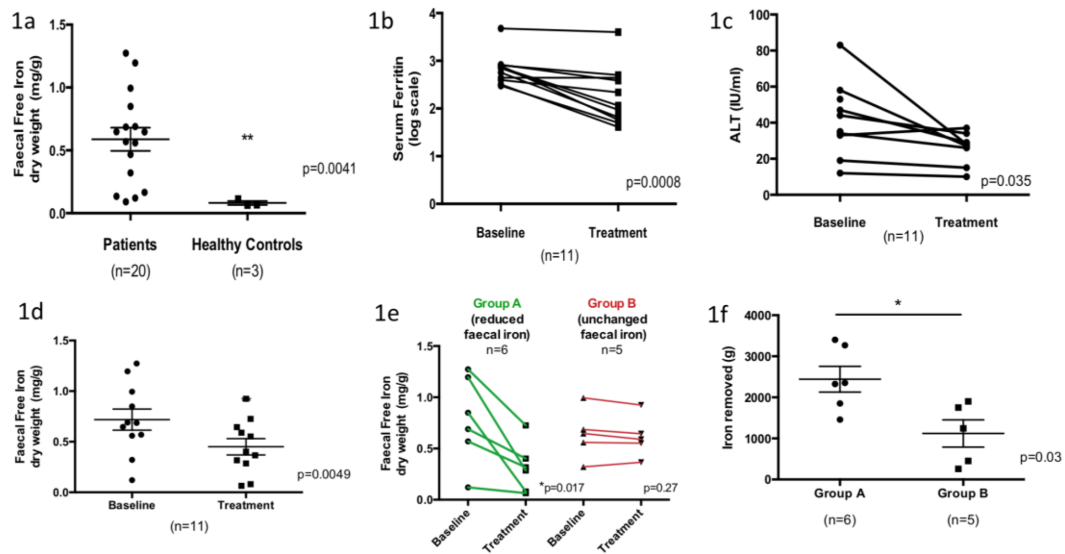

Fig. 1. Baseline and on treatment iron and biochemical changes. 1a Patients with iron overload had significantly higher faecal free iron levels than healthy controls. Significant reductions in serum ferritin and ALT were seen in paired samples on treatment (1b and 1c; note: one ALT datapoint missing in treatment group). Free faecal iron fell significantly with treatment (1d). 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, 1e). Group A had significantly more iron removed by venesection than group B (1f). Graphs are presented as mean +/-SEM. *$p<0.05$ **$p<0.005$.

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

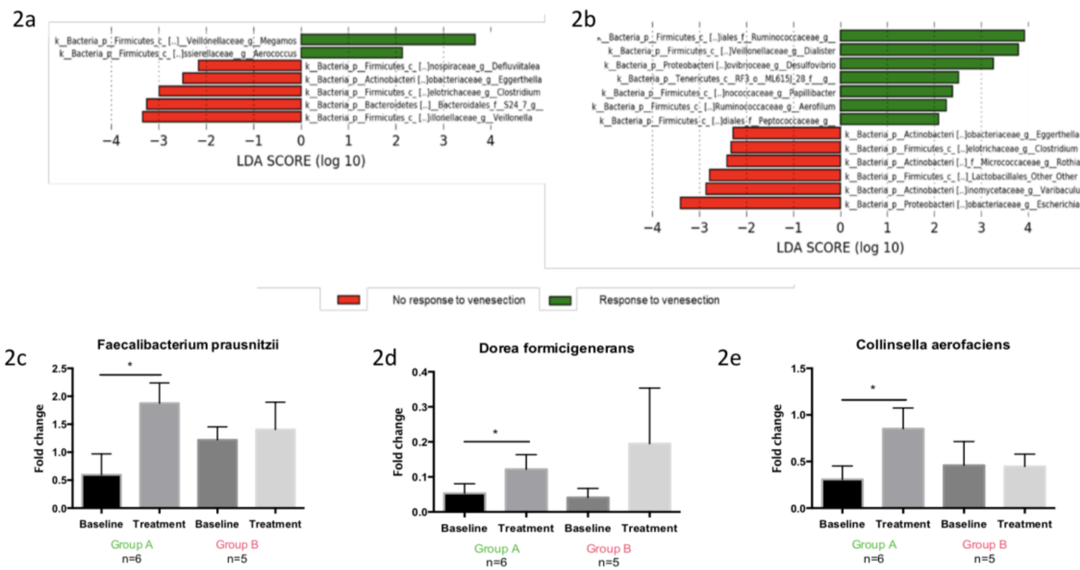

Fig. 2. Changes in bacterial composition with iron reduction. Linear discriminant analysis effect size (LEfSe) analysis of faecal samples stratified by response to venesection. Linear discriminant analysis effect size (LEfSe) identified taxa with differential relative abundance between categories ($p<0.05$). Data indicate linear discriminant analysis (LDA) showing effect size greater than log LDA=2 which were deemed statistically significant. Baseline faecal samples of patients stratified according to eventual response to venesection (2a); Differentially abundant taxa in faecal samples after venesection (2b). Significant changes in 3 bacterial *Species* were evident in those with reduced faecal free iron following venesection (group A, *$p<0.05$; Fig 2c-e). Graphs are presented as mean +/-SEM.

Similarly, significant changes in microbial metabolites after treatment were only evident in group A, where an increase in pyruvate, tyrosine, methionine, glycine, and aspartate was observed (supplementary figure 1). In these patients, a greater separation in the metabolome was illustrated, where a shift was observed towards a more positive metabolomic profile with treatment compared to baseline. In contrast, a less distinct shift in the metabolomic profile was evident in group B (supplementary figure 1).

LEfSe analysis of faecal samples before and after venesection demonstrated changes in the faecal microbiome in patients in response to altered iron availability. A significant increased abundance of the pathobiont Escherichia in faecal samples of patients who did not response to iron reduction through venesection was noted (Figure 2b). Interestingly, this mirrors findings from a study reporting differential reduction in Escherichia relative abundance in response to iron chelation(9). In addition, LEfSe analysis identified increased log LDA abundance of Desulfovibrio, (Figure 2b) a sulphate-reducing member of Proteobacteria phylum associated with iron metabolism through reduction of iron oxides(15). These data, and the changes seen in participant faecal microbiome analysis support previously published data which propose that haem iron, through its impact on mucosal homeostasis, can alter the colonic luminal environment and hence the associated microbiome(16).

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 *Faecalibacterium prausnitzii*, *Dorea formicigenerans* and *Collinsella 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 *Faecalibacterium 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 of these bacterial genera are associated with production of potentially beneficial short chain fatty acids such as acetate and butyrate, although no change in these metabolites was noted in this study.(17, 18)

This is the first description of the gut microbiome in patients with Haemochromatosis, a condition in which excess systemic iron can lead to multi-organ 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. This pilot study does not, however, include mechanistic data to determine whether or not the changes noted in microbial composition could account for improvements in disease.

Conclusion

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

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