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Metabolic and hepatic effects of bloodletting in dysmetabolic iron overload syndrome: a randomized controlled study in 274 patients.

Authors

Fabrice Lainé MD [1-3], Marc Ruivard PhD [4], Véronique Loustaud-Ratti PhD [5], Fabrice Bonnet PhD [1,6-7] Paul Calès MD [8], Edouard Bardou-Jacquet PhD [1-3,7] Sylvie Sacher-Huvelin MD [9], Xavier Causse MD [10], Christine Beusnel MD [11], study group*, Alain Renault PhD [1,7], Eric Bellissant PhD [1, 7] and Yves Deugnier MD [1,7].

Institutions

[1] INSERM CIC 1414, Rennes, France.

[2] INSERM U 991, Rennes, France.

[3] CHU Rennes, Liver Unit, Rennes, France.

[4] CHU Estaing, Department of Internal Medicine and Clermont University, Auvergne University, EA 4681, PEPRADE, Clermont-Ferrand, France

[5] CHU Limoges, Liver-Gastroenterology Department, INSERM U850, Limoges, France

[6] CHU Rennes, Department of Endocrinology-Diabetology, Rennes, France

[7] University of Rennes 1, Rennes, France

[8] University Hospital, Liver-Gastroenterology Department and UPRES 3859, LUNAM University, Angers, France

[9] INSERM CIC 1413, Nantes, France; CHU, IMAD, Nantes, France

[10] CHR Orléans, Liver-Gastroenterology and oncology Department, France

[11] CHR Saint-Malo, Saint-Malo, France

*Members of the study group. All authors are members of the study group. Additional members of the group are Dominique Guyader, Romain Moirand, Caroline Jézéquel, Anne Guillygomarc'h and Pierre Brissot (CHU Pontchaillou, Liver Unit, France), Agnès Benses, Nolwenn Boissel, Marion Couegnat, Elise Thébault, Viviane Fortuna, Catherine Massart, Cécile Réminiac and Chloé Rousseau (INSERM CIC 1414, Rennes, France), Armand Abergel (CHU d'Estaing, Clermont Ferrand, France), Gérard Le Dréau (Lorient Hospital, France), Roger Faroux (La Roche sur Yon Hospital, France), Bruno Turlin and Danielle Le Quilleuc (Centre of Biological Ressources, CHU Pontchaillou, Rennes, France).

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Corresponding author:

Fabrice Lainé, INSERM CIC 1414, CHU Pontchaillou, 35000 Rennes, France

E-mail: fabrice.laine@chu-rennes.fr

Phone: (33) 299289199; Fax: (33) 299289541

Abbreviations

DIOS: dysmetabolic iron overload syndrome, NAFLD: non alholic fatty liver disease, LFDA**:** lifestyle and diet advice, AST: aspartate aminotransaminase, ALT: alanine aminotransaminase, GGT: gamma-glutamyl transferase, MRI: magnetic resonance imaging, BMI: body mass index

Declaration of interests

All authors declare no competing interests.

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Summary (275 words)

Dysmetabolic iron overload syndrome (DIOS) is a common cause of hyperferritinaemia accounting for mild increase of iron stores in insulin-resistant subjects. Iron removal could improve insulin sensitivity. We performed a prospective, randomized, controlled trial (NCT01015525) in nondiabetic DIOS patients with hepatic iron >50µmol/g at MRI to compare the metabolic and hepatic outcomes of one-year maintenance of serum ferritin levels <50µg/l by bloodletting associated with lifestyle and diet advice (LFDA) to those of LFDA only. Patients were randomly assigned (1:1) with stratification by centre (n=8) and hyperglycaemia (>5.6mmol/L). Sample size was calculated to provide 90% power and a difference in fasting glycaemia of 0.25mmol/l. Analysis was done in intention-to-treat. In 2010-2014, 146 patients were randomly assigned to receive venesections with LFDA and 128 to LFDA only. At end of study, comparison of iron-depleted patients and controls showed: ferritin levels 71±48µg/l after removal of 4.9±1.6L of blood versus 733±277µg/l (p<0.0001), glycaemia 5.44±0.7 versus 5.49±0.7mmol/l (p=0.57), body weight +0.5±4.3% versus -0.6±3.3% (p=0.03), HOMA 3.39 versus 2.40 (p=0.002), ALT 33±22 versus 37±21IU/L (p=0.10), AST 27±13 versus 27±10IU/L (p=0.81), GGT 54±138 versus 49±35IU/L (p=0.72), Fatty Liver Index 58.9±24.6 versus 61.2±22.9 (p=0.37), and FIB-4 score 1.5±0.6 versus 1.30±0.6 (p=0.51). Fatigue occurred in 25.3% of venesected patients versus 2.3 % of controls (p<0.0001). In the subgroup of patients who lost weight, glycaemia, HOMA, serum ferritin, lipid profile and liver function tests improved irrespective of bloodletting. **Conclusion.** In DIOS patients, iron depletion by bloodletting does not improve metabolic and hepatic features, is associated with weight gain, and is not as well tolerated as expected. Sustained modification of diet and life-style habits remains the first therapeutic intervention in DIOS.

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Introduction

Insulin resistance-associated hepatic iron overload also coined as the dysmetabolic iron overload syndrome (DIOS) was first described in the late 90's [1,2]. It corresponds to mild increase of both liver and body iron stores [3] associated with various components of the metabolic syndrome in the absence of any identifiable cause of iron excess. It accounts for most cases of hyperferritinaemia referred to outpatient clinics and is much more frequent than genetic hemochromatosis [4]. The liver may be either normal or steatotic with non-alcoholic fatty liver injury (NAFLD) [5]. DIOS shares the same cause as NAFLD, i.e. insulin-resistance, and about 10% of DIOS patients have type 2 diabetes [6]. The role of iron excess in the development or the worsening of insulin resistance and, then, in the occurrence of diabetes and of NAFLD remains debated. Epidemiological studies have shown that elevated serum ferritin values were associated with a higher risk of developing type 2 diabetes [7] and were correlated with serum insulin and serum glucose levels [8], the best biological predictive factor of incident diabetes [9,10]. In early and small studies, achievement of low body iron stores by bloodletting [11-13] or low iron diet [14] was associated with decreased insulin resistance. This was not confirmed by a recent Australian study from Adams et al [15] who failed to find any improvement in both insulin-resistance and steatosis in phlebotomized NAFLD patients. However, as stressed by Ryan et al [16], only a few patients had increased hepatic iron in this study.

The aim of the present study was to assess, in a large population of DIOS patients with well-defined hepatic iron excess, whether iron depletion by sustained phlebotomies may improve insulin resistance and liver tests.

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Patients and methods

Study design and patients

The SAIGNEES study was a multicentre, open-labelled, randomized, controlled phase 3 trial of one-year maintenance of serum ferritin levels lower than 50µg/l by bloodletting associated with lifestyle and diet advices (LFDA) versus LFDA only in nondiabetic DIOS patients. Adult subjects with DIOS were recruited from January 2010 to May 2014 in 8 French hospitals (Angers, Clermont-Ferrand, La Roche Sur Yon, Limoges, Nantes, Orleans, Rennes and Saint-Malo). DIOS was diagnosed as follows: i) liver iron concentration, measured by magnetic resonance imaging [17], higher than 50 µmol/g dry weight, ii) presence of one of the following metabolic features at least: body mass index (BMI) >25 kg/m², waist size >80 cm in females and >93 cm in males, blood pressure higher than or equal to 130/85 mmHg or antihypertensive drug therapy, fasting blood glucose higher than 5.6 mmol/l but lower than 7 mmol/l, serum triglycerides levels higher than 1.7 mmol/l, serum high-density lipoprotein cholesterol levels lower than 1.29 mmol/l for females and lower than 1.03 mmol/l for males or cholesterol-lowering therapy and (iii) absence of the following conditions: diabetes mellitus, alcohol consumption >20g/day in females and >30g/day in males, homozygosity for the C282Y mutation of the *HFE* gene, aceruloplasminaemia, haematological disorder, acute or chronic inflammatory disease, viral hepatitis, auto-immune or cholestatic liver disease. The study (Clinical Trials.gov NCT01045525) was approved by the review board of the ethics committee of Rennes. All patients provided a written informed consent.

Randomisation and masking

Subjects were prospectively randomized in a 1:1 fashion to iron depletion by phlebotomy with lifestyle advice versus lifestyle advice only. Randomization was performed using computer software. Stratification was made according to baseline values of fasting serum glucose (lower than 5.6 mmol/l or not). The study was open-labelled.

Procedures

Iron depletion by bloodletting

Subjects randomized to iron depletion underwent 2 phases of treatment. The initial phase consisted of bi-monthly phlebotomies (ranging from 350 ml to 450 ml according to body weight and sex) with the objective to achieve serum ferritin levels <50 µg/l with haemoglobin levels >11g/dl. Then, the maintenance phase consisted of bi-monthly 350 to 450 ml phlebotomy on the basis of serum ferritin

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measurement every two months in order to maintain serum ferritin lower than 50 µg/l and haemoglobin >11 g/dl.

Lifestyle advice

All subjects received written dietary recommendations promoting an healthy and varied diet, in the form of two books edited by the French National Program of Nutrition and Health (http://www.inpes.sante.fr) and were advised to have physical activity during at least 30 minutes per day. They were left free to consult a dietician.

Clinical assessment

It was performed at baseline and 12 months after randomisation and included medical history, medications, questionnaires about alcohol consumption, eating habits and physical activity, BMI, waist circumference, and systolic and diastolic blood pressure. Waist circumference was measured according to the recommendations of the NHANES III (http://www.cdc.gov). Blood pressure was measured on a subject lying down, at rest for at least 5 minutes. Three successive measurements spaced of 2 minutes were performed using automatic test equipment. The value used was the average of the last two measures.

Biochemical assessment

A fasting venipuncture and a 75 g OGTT were performed at baseline and at 12 months. Biochemical testing included blood cell count, aspartate aminotransaminase (AST), alanine aminotransaminase (ALT), gamma-glutamyl transferase (GGT), HDL- and LDL-cholesterol, triglycerides, glucose, insulin, HbA1c, iron parameters (iron, transferrin, ferritin, and transferrin saturation), C Reactive protein and adiponectin. HOMA was calculated as (insulin T0 x glucose T0)/22.5, modified Matsuda index as $1000/\sqrt{[(\text{glucose T0 x insulin T0}) \text{ x } (\text{glucose T30 x insulin T30})]}$, and insulinogenic index as the ratio of the increment of the insulin concentration over the first 30 minutes of the OGTT to the increment in blood glucose over the same period (insulin T0-T30/glucose T0-T30). Hepatic fibrosis was assessed using the Fib-4 score [18] and steatosis using the Fatty Liver Index [19].

Outcomes

The primary outcome was the mean value of fasting blood glucose one year after randomisation.

Secondary outcomes were 1) variations of metabolic features from baseline to end of treatment, 2) insulin resistance measured by homeostasis model of assessment of insulin resistance (HOMA),

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modified Matsuda index and insulinogenic index based upon a 75g oral glucose tolerance test (OGTT) with measurement of glycaemia and insulinemia at baseline and 30 and 120 minutes after glucose ingestion, one year after randomisation 3) serum adiponectin levels one year after randomisation, 4) serum C reactive protein levels one year after randomisation, 5) liver function tests and 6) FLI and Fib-4 scores.

Statistical analysis

An *a priori* power calculation indicated that a sample size of 270 subjects would provide 90% power to determine a difference in fasting blood glucose of 0.25 mmol/l, assuming a standard deviation of 0.61 mmol/l, a two-sided p value of 0.05 and a drop-out rate of 10%. Finally, standard deviation of fasting glycaemia was 0.71 at the end of the study; *a posteriori* power calculation indicated 80 % power, with a two-sided p value of 0.05 to determine a difference in fasting glycaemia of 0.25mmol/l.

The statistical analysis, prospectively defined, was done by intention to treat (i.e., in all analyses, patients were grouped according to their original randomised treatment) with SAS statistical software (version 9.4; Cary, NC, USA). Continuous variables were expressed by mean and standard deviation and categorical variables were expressed by the number of patients and percentages. Baseline characteristics were compared between groups using Student's t-test for continuous variables and chi-squared test or Fisher's exact test for categorical variables. Student's t-tests were also performed between groups on the variation of continuous variables (between the baseline and the end of study).

The primary and secondary outcomes were evaluated first with Student's t-test and chi-square test, followed by analyses of variance with adjustment on the variation of weight. Regarding the insulin-related parameters, analyses of variance with adjustment on the variation of weight and the insulin at baseline, were used. The same analysis was performed in subgroups of patients who lost weight. All statistical tests had a significance level of 0.05.

Results

Patients groups and characteristics at baseline

Between Jan 8, 2010 and May 30, 2014, 274 patients were randomly assigned to receive venesections with LFDA (iron depletion group) and 128 to LFDA only (control group). Seven patients withdrew prior to the end of the study, one in the iron depletion group because he left abroad and 6 in the control arm (one suddenly died and 5 withdrew for personal decision). Finally, 267 patients

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were analysed, 145 in the iron depletion arm and 122 in the control arm (Figure 1). Baseline characteristics are summarized in Table 1. All patients were Caucasians and most were middle-aged males. Mean BMI was 28.5±3.6 kg/m^2. Obesity was present in 31.1% of patients, increased waist circumference in 88%, hypertension in 54 %, elevated serum triglycerides in 55.1%, and hypercholesterolemia in 42.3%. Mean glycaemia was 5.61±0.61 mmol/l. Hyperglycaemia (fasting blood glucose higher than 5.6 mmol/l) was observed in 36.9% of patients. Metabolic syndrome, defined according to the International Diabetes Federation, was present in 59.5 %. Mean alcohol consumption was 90±80 g per week. Increase in serum ALT transaminase levels was present in 29.3 % of patients, but ALT levels were greater than 2 times the upper limit of normal in 7% only. According to Fib-4 score, 3 patients had bridging hepatic fibrosis or cirrhosis. According to Fatty Liver Index, steatosis was present in 59.1% of patients, uncertain in 29.3% and absent in 11.6%. Liver biopsy was performed in 7 patients who all presented with steatosis. Fibrosis (METAVIR) was stage 0 in 1, stage 1 in 3 and stage 2 in 3. Allelic frequency of C282Y mutation of the HFE gene was 27.5% in the venesected group vs 27% in controls (p=0.93).

Iron removal

During the initial phase of treatment, a mean of 12±4 bi-monthly phlebotomies was performed in the iron depletion group. This corresponded to a mean of 4.9±1.6 l of blood removed, i.e. 2.45±0.8 g of iron. At the end of the initial phase, the mean value of serum ferritin levels was 62±37 µg/l. The threshold of 50 µg/l was achieved in 72 (50%) patients, whilst serum ferritin levels were comprised between 50 and 100 µg/l in 55 (38%) and greater than 100 µg/l in 17 patients (12%). During the maintenance phase, 91 (62%) of the iron-depleted patients had a mean of 2±1 phlebotomies. At the end of the study, the threshold of 50 µg/l was achieved in 51 (35%) patients, whilst serum ferritin levels were comprised between 50 and 100µg/L in 73 (50%) and greater than 100µg/L in 21 patients (15%). The main causes of non achievement of the goal of serum ferritin < 50 at the end of treatment were adverse events (43%) and poor adherence to phlebotomies (67%). There was no serious adverse event related to treatment. Dizziness, hypotension and vagal malaise related to iron-depletion therapy were reported in 3, 2 and 5 patients, respectively. Anaemia, defined as haemoglobin level lower than 12 g/dl in females and lower than 13 g/dl in males, was observed in 6 patients. Fatigue was reported in 37 patients (25.3 %) of the iron-depleted group versus 3 patients (2.3 %) in the control group (p<0.0001).

Life style & diet

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The percentage of patients who consulted a dietician was identical (12%) in both arms. Physical activity scores during sport and leisure did not significantly change during the study in the 2 groups. Variation in alcohol consumption was not different in the 2 groups with a mean of -10 g per week. Mean variation of BMI was +0.13±1.22 kg/m² in the iron depletion group versus -0.17±0.94 kg/m² in the control group; p=0·03. Mean percentage of variation of body weight was +0.5±4.3% in the iron depletion group vs -0.6±3.3% in the control group (p=0.03). Percentages of patients whose weight decreased, remained stable (±1 kg) or increased were 21%, 41% and 38% respectively in the iron depletion group vs 26%, 53% and 21% respectively in the control group (p=0.009).

Fasting blood glucose

There was no difference in end-of-treatment fasting blood glucose between the iron depletion and control groups (5.44±0.71 vs 5.49±0.70 respectively; p=0.57), even after adjustment for variation of weight (p=0.44). A stepwise multivariate analysis of the variation of glycaemia was performed. The following variables were included in the analysis: treatment group, presence of HTA, variations between baseline and end-of-study of weight, waist circumference, haemoglobin, baseline cholesterol, serum ferritin and liver enzymes values. Weight variations and □-GT values were the only two variables independently and positively associated (p=0.01 and p=0.03 respectively) with the variation of glycaemia. As scheduled, analysis was performed in subgroups of patients according to baseline values of fasting glycaemia. One hundred and thirty-seven (51%) patients had glycaemia greater than or equal to 5.6 mmol/l at inclusion, of whom 74 were randomized in the iron depletion group and 63 in the control group. There was no difference in end-of-treatment fasting blood glucose between both groups (5.81±0.74 vs 5.83±0.77 respectively; p=0.91), even after adjustment for variation of weight (p=0.71). One hundred and thirty (49%) patients had glycaemia lower than 5.6 mmol/l at inclusion of whom 71 were randomized in the iron depletion group and 59 in the control group. There was no difference in end-of-treatment fasting blood glucose between the iron depletion and control groups (5.06±0.4 vs 5.13±0.4 respectively; p=0.27), even after adjustment for variation of weight (p=0.24). *Per protocol* analysis comparing the 51 patients with serum ferritin levels <50µg/l one year after randomization with controls was performed. There was no difference in end-of-treatment fasting blood glucose between the iron depletion and control groups (5.45 ± 0.58 vs 5.49 ± 0.7; p=0.72) even after adjustment on weight variation (p=0.65).

Markers of insulin-resistance

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There was no significant effect of iron depletion on mean serum values of triglycerides, HDL and LDL cholesterol, mean values of systolic and diastolic blood pressure and percentage of hypertensive patients (Table 2) even after adjustment on weight variation. Variations of insulin resistance indexes were compared between the two treatment groups without and with adjustment on variations of weight and adjustment on variations of both weight and baseline values of fasting insulin. Fasting insulinemia and HOMA index were significantly increased in the iron depletion group vs control group (Table 3). A stepwise multivariate analysis of the variation of HOMA was performed. The following variables were included in the analysis: treatment group, presence or absence of HTA, variations between baseline and end-of-study of weight, waist circumference, haemoglobin, baseline cholesterol, serum ferritin and liver enzymes values. Weight variations ($p<0.001$), □-GT values ($p<0.01$), treatment group ($p<0.01$) and arterial hypertension ($p=0.02$) were significantly and positively associated with variation of HOMA. There was no difference in end-of-treatment adiponectin serum levels between iron depletion and control groups (6.2±4.3 vs 6.5±3.9 respectively; $p=0.53$) and in end-of-treatment C reactive protein serum levels between both groups (2.1±1.8 vs 2.4±3.1 respectively; $p=0.24$) even after adjustment for variation of weight ($p=0.70$ and $p=0.20$; respectively).

Liver tests and scores

As shown in table 2, end of study AST, ALT, and GGT did not differ between the iron-depleted and control groups. After adjustment for weight variation, serum ALT levels were significantly lower ($p=0.03$) in the iron-depleted group. Fib4 and FLI scores were not different according groups.

Subgroup analysis according to weight loss

Sixty-three patients lost more than 1 kg of body weight; 31 (21%) in the iron depletion group and 32 (26%) in the control group. Glycaemia, insulinemia, HOMA index, HDL and LDL-cholesterol, triglycerides and liver enzymes were or tended to be improved in these patients regardless of the treatment group (Table 4).

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Discussion

The present study demonstrates that, in patients with DIOS, removal of iron excess by phlebotomies has no significant metabolic and hepatic effects. Even more, bloodletting may contribute to weight gain and to the worsening of some insulin-resistance-related features.

Blood glucose was chosen as primary end-point for the following reasons.

First, insulin resistance is the main determinant of both DIOS [1] and NAFLD [20]. Blood glucose is one of the best surrogate markers reflecting the degree of insulin resistance and the risk of forthcoming type 2 diabetes [9]. The epidemiological demonstration of an association between blood glucose and body iron stores concentrations was made by Finnish authors [8]. Since then, a few clinical trials [11-13,15,21-23] have reported conflicting results about the effects of phlebotomy therapy on blood glucose and markers of insulin resistance in patients with various metabolic abnormalities. In all but that conducted by Piperno et al [22], DIOS patients were mixed with NAFLD patients with no iron excess. In addition, most studies were not randomised [11,13,22], included a limited number of patients [11,15,21-24], and mixed diabetics with non-diabetics [11,15,21,22,24].

Second, Fernandez-Real et al reported a positive relationship between serum ferritin levels and insulin resistance and proposed serum ferritin as a component of the insulin resistance syndrome [25]. In a controlled randomised trial conducted in 28 type 2 diabetics with elevated serum ferritin, they found that iron removal improved both insulin sensitivity and beta cell function but had no significant effect on blood glucose [12]. Facchini et al, in a randomized study of 42 patients, mainly diabetics, showed that iron depletion was associated to decrease of both fasting and glucose-stimulated plasma insulin concentrations in a subgroup of 17 hyperferritinemic subjects with non-biopsy proven NAFLD [11]. Piperno et al compared the respective effects of venesection therapy and diet restriction in 44 subjects with DIOS [22]. They found that diet restriction only resulted in a significant decrease in blood glucose. Valenti et al, in a case-control study of 128 non-diabetic patients with NAFLD and either elevated serum ALT levels or increased serum ferritin levels or both, showed that serum insulin, serum glucose and HOMA improved in iron-depleted patients [13]. Finally, Adams et al, in a randomised controlled study, showed that iron depletion failed to improve blood glucose, HOMA and ISI in 61 patients with NAFLD and in a limited subgroup of 36 patients with hyperferritinemia [15]. Compared to these previous studies, we included a much larger sample of patients, which allowed for a satisfactory statistical power. Moreover, all of our subjects had hepatic

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iron excess demonstrated using a well-validated method [17]. Finally, our study was controlled and randomised with stratification on baseline serum glucose. As blood glucose value is associated in a continuous manner with the risk of diabetes [9], the present data supports the conclusion that, in non-diabetic DIOS patients, iron depletion has no effect on the risk of developing diabetes, irrespective of the initial level of that risk.

Third, it would have not been realistic to propose liver biopsy as the primary end-point in our study because, in daily practice, it is rarely performed in DIOS patients. Indeed most of them have no increased serum transaminase levels – as illustrated by the present data – and the risk of severe fibrosis, which is very low in the absence of cofactors of liver disease, can be ruled out by non-invasive tests. Requiring histological end-points would have resulted in a selection bias and then in hardly conclusive results.

Our results raised concerns about putative deleterious effects of phlebotomy therapy in DIOS patients. Indeed, despite both groups had comparable baseline BMI and received similar lifestyle advices, BMI decreased by 0.5% in controls whilst it increased by 0.4% in venesected patients (p=0.03). This is in line with the observation of a trend to weight gain in venesected patients in the studies from Valenti et al [13] and from Adams et al [15] that did not reach statistical significance likely due to insufficient sample size. Weight gain could be partly related to looser motivation of venesected patients for lifestyle recommendations, because of the falsely reassuring feeling of being already treated.

Conflicting results about the effects of venesection therapy on HOMA index, a surrogate marker of insulin resistance, have been previously reported. Some authors found that HOMA index decreased in venesected patients [11-13] whilst others did not find any effect of iron removal [15,21,24]. In the present paper, we showed that HOMA index increased in venesected patients, mainly due to increase in serum insulin. This was significant at univariate analysis and confirmed at multivariate analysis which showed that increase in HOMA index was independently explained by both bloodletting and weight gain. Basal hypersecretion of insulin is viewed as an independent abnormality in the pathogenesis of type 2 diabetes and has been shown to predict diabetes development, independently of insulin resistance [26]. This increase in fasting insulin levels besides the improvement of hyperferritinaemia suggests the absence of benefit of iron depletion on the risk of type 2 diabetes. Nevertheless, the deleterious effect of venesection therapy *per se* is likely to remain weak compared to that induced by weight variation.

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Our study demonstrates that weight variation is, in fact, the primary determinant of the evolution of not only HOMA index but also of metabolic features and liver tests in non-diabetic DIOS patients. This was well illustrated by the improvement of blood glucose, LDL and HDL cholesterol, triglycerides, ALAT and GGT in patients who had lost weight and by the absence of clinically significant effect of iron removal on these parameters in these patients (Table 4).

It could be discussed that iron depletion was insufficient to reach significant effects in the present study. However, previous studies had conflicting results independently of bloodletting scheme and endpoints of serum ferritin and serum haemoglobin. Moreover, analysis of the subgroup of our patients having achieved serum ferritin levels < 50 µg/l at one year failed to show any significant effect of venesection therapy on primary and secondary outcomes.

In addition, venesection therapy was not as well tolerated as expected [27]. Indeed, a significant proportion of venesected patients experienced fatigue and mild malaises as previously reported by Piperno et al [22]. This and the poor adherence to phlebotomy we observed render difficult to propose a more intensive scheme of bloodletting in routine practice.

Conclusion

In DIOS patients, iron depletion by phlebotomy has no significant effect on serum glucose level nor on liver biochemical tests and scores, may aggravate insulin-resistance, is associated with weight gain and is poorly tolerated. Weight loss alone is associated with improvement in most metabolic features including glycaemia and insulin resistance, and in serum ferritin and liver tests. Sustained modification of diet and life-style habits remains the first therapeutic intervention in these patients.

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Table 1. Baseline characteristics of trial population.

	Iron depleted (n=146)	Control (n=128)	*P value*
Demographics			
Age (years)	58 (10)	56 (10)	0.09
Male sex	119 (82%)	114 (89%)	0.08
Iron parameters			
Ferritin (µg/L)	792 $(290)_1$	788 (290)	0.91
Transferrin saturation (%)	37.4 $(10.3)_3$	38.4 (11.8)	0.43
Haemoglobin (g/dL)	15.13 (1.2)	15.26 (1.0)	0.33
MRI estimation of liver iron (µmol/g)	85 $(32)_1$	92 $(39)_2$	0.15
Metabolic factors			
BMI (kg/m²)	28.67 (3.8)	28.41 (3.4)	0.55
Waist circumference (cm)	102 $(10)_2$	101 $(9)_3$	0.56
Hypertension*	105 (72%)	92 $(73\%)_2$	0.84
Triglyceride (mmol/L)	1.47 (0.7)	1.37 (0.6)	0.24
HDL-cholesterol (mmol/L)	1.34 (0.4)	1.38 (0.4)	0.33
Glucose (mmol/L)	5.57 (0.5)	5.65 (0.7)	0.27
HbA1c (%)	5.56 $(0.4)_1$	5.57 (0.4)	0.92
Insulin (µUI/mL)	11.81 (8.3)	10 $(7.2)_1$	0.06
HOMA	2.97 (2.2)	2.58 $(2.0)_1$	0.13
Insulin Sensitivity Index	107 $(271.5)_1$	89.1 $(61.6)_3$	0.47
Liver tests & scores			
ALT (IU/L)	40 $(21)_3$	41 (22)	0.87
AST (IU/L)	28 $(11)_3$	29 (10)	0.73
Gamma GT (IU/L)	49 $(40)_3$	54 (41)	0.34
Prothrombin (%)	101 $(12)_1$	103 $(11)_1$	0.34
Fatty liver index	63.71 $(23.49)_5$	63.89 $(22.97)_3$	0.95
FIB-4 score	1.34 $(0.6)_3$	1.32 (0.6)	0.73

Data are n (%) or mean (SD). HOMA=homeostasis model assessment. *Hypertension was defined as blood pressure > 140/90 mmHg or as receiving an anti-hypertensive drug. Numbers in subscript indicate the number of missing data.

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Table 2. End of study characteristics of trial population.

	Iron depleted (n=145)	Control (n=122)	*P value*	*P value (adjustment on the variation of weight)*
Iron parameters				
Ferritin (µg/L)	71 $(48)_2$	733 (277)	< 0.0001	< 0.0001
Transferrin saturation (%)	26.4 $(11.6)_6$	37.3 $(12.5)_1$	< 0.0001	< 0.0001
Hemoglobin (g/dL)	14.62 (1.2)	15.14 (1.1)	0.0004	0.0002
Metabolic factors				
BMI (kg/m²)	28.76 (3.9)	28.12 $(3.5)_1$	0.17	
BMI change (%)	0.5 (4.3)	-0.6 $(3.3)_1$	0.03	
Waist circumference (cm)	101 $(10)_2$	101 $(10)_1$	0.86	0.72
Hypertension*	103 (72 %)$_3$	93 (76%)	0.49	0.46
Triglyceride (mmol/L)	1.3 $(0.6)_2$	1.37 (0.7)	0.36	0.24
HDL-cholesterol (mmol/L)	1.39 $(0.5)_2$	1.36 (0.4)	0.63	0.56
Glucose (mmol/L)	5.44 (0.7)	5.49 (0.7)	0.57	0.44
HbA1c (%)	5.57 $(0.6)_1$	5.52 $(0.4)_1$	0.50	0.64
Insulin (µUI/mL)	13.57 $(10.5)_3$	9.47 (6.3)	0.0002	0.0006
HOMA	3.39 $(3.0)_3$	2.40 (1.9)	0.002	0.004
Insulin Sensitivity Index	73.76 $(57.3)_4$	86.37 $(52.2)_1$	0.07	0.10
Liver tests & scores				
ALT (IU/L)	33 $(22)_1$	37 (21)	0.10	0.03
AST (IU/L)	27 $(13)_1$	27 (10)	0.81	0.54
Gamma GT (IU/L)	54 $(138)_1$	49 (35)	0.72	0.91
Prothrombin (%)	101 (11)	101(12)	0.75	0.80
Fatty liver index	58.93 $(24.6)_5$	61.58 $(22.9)_2$	0.37	0.12
FIB-4	1.35 $(0.6)_1$	1.30 (0.6)	0.51	0.47

Data are n (%) or mean (SD). HOMA=homeostasis model assessment. *Hypertension was defined as blood pressure > 140/90 mmHg or as receiving an anti-hypertensive drug. Numbers in subscript indicate the number of missing data.

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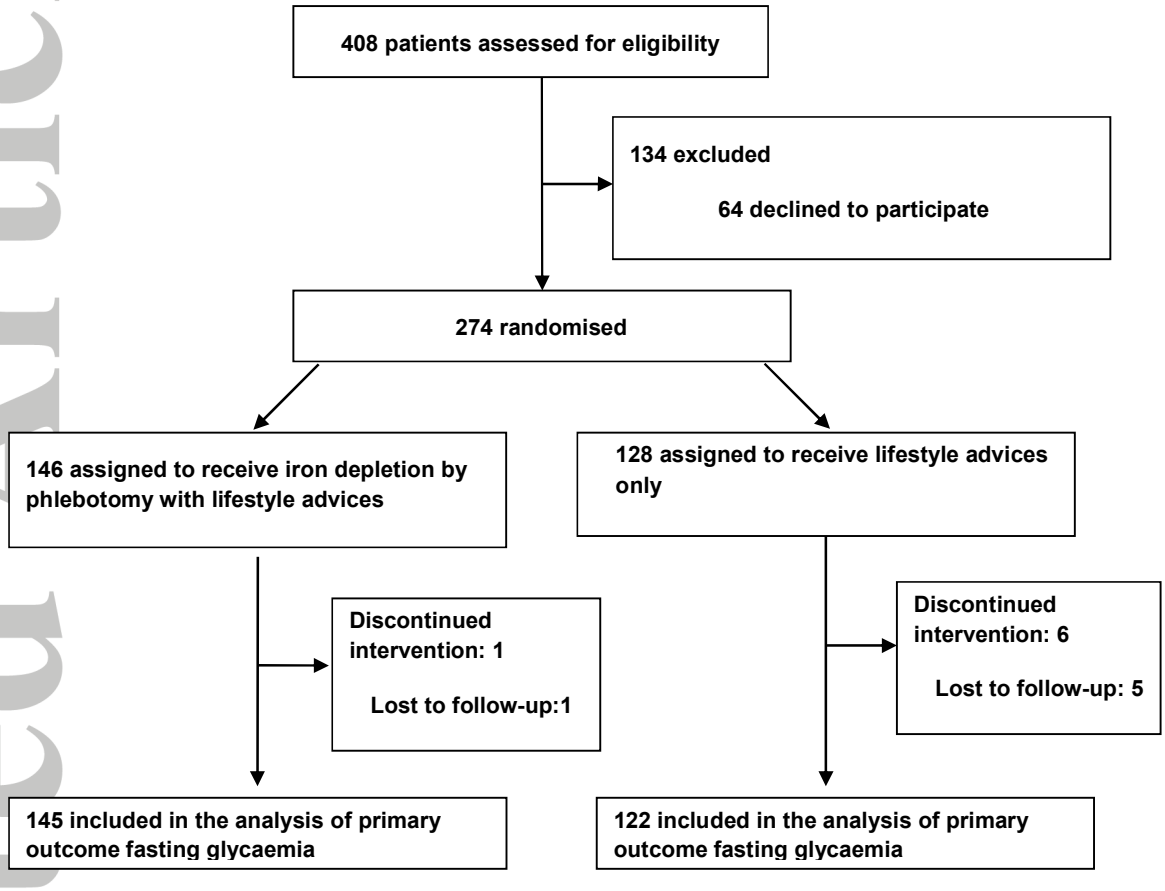
Table 3: Comparison at end-of-treatment of insulin and insulin resistance index between iron-depleted and control groups after adjustment on weight variation (left column) and after adjustment on weight variation and baseline insulin value (right column).

	Adjustment on weight variation			Adjustment on weight variation and baseline insulin value		
	Iron-depleted	Controls	p value	Iron-depleted	Controls	p value
Insulin (µUI/mL)	13.38	9.62	<0.01	12.57	10.64	<0.01
HOMA*	3.33	2.44	<0.01	3.11	2.72	0.07
Insulinogenic index†	24.82	17.14	0.13	24.54	17.48	0.17
Modified Matsuda index‡	74.62	85.84	0.10	77.98	80.49	0.66

*HOMA: homeostasis model assessment. † Insulinogenic index was calculated as as the ratio of the increment of the insulin concentration over the first 30 minutes of the OGTT to the increment in blood glucose over the same period (insulin T0-T30/glucose T0-T30). ‡ Modified Matsuda index was calculated as: $1000/\sqrt{[(\text{glucose T0 x insulin T0}) \text{ x } (\text{glucose T30 x insulin T30})]}$

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Figure 1: Flow chart



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Table 4 : Comparison of variations from baseline to end of study of biological parameters in the subgroup of patients who lost 1 kg or more of body weight.

	Iron depleted (n=31)	Controls (n=32)	*P value*
Glucose (mmol/L)	-0.25 (0.38) - p=0.0008	-0.21 (0.38) – p=0.0025	0.70
Insulin	-0.85 (6.6) – p=0.23	-2.17 (4.8) – p=0.03	0.37
HOMA*	-0.32 (1.6) – p=0.11	-0.64 (1.32) – p=0.009	0.40
HDL-cholesterol (mmol/L)	0.12 (0.16) – p=0.0001	0.03 (0.17) – p=0.46	0.02
LDL-cholesterol (mmol/L)	-0.15 (0.54) – p=018	-0.58 (0.61) – p<0.0001	<0.01
Triglycerides (mmol/L)	-0.38 (0.51) – p=0.0003	-0.14 (0.57) – p=0.14	0.20
Gamma GT (IU/L)	-12 (27) - p =0.0004	-18 (28) – p<0.0001	0.37
ALT (IU/L)	-14 (17) – p<0.0001	-12 (17) – p<0.0001	0.65
Ferritin (µg/L)	-678 (226) – p<0.001	-121 (190) – p=0.0004	< 0.0001

Data are mean (SD). *HOMA=homeostasis model assessment.