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Effect of procyanidin on dietary iron absorption in hereditary hemochromatosis and in dysmetabolic iron overload syndrome: A crossover double-blind randomized controlled trial

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1 Title page

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3 1) Title: Effect of procyanidin on dietary iron absorption in hereditary hemochromatosis and
4 in dysmetabolic iron overload syndrome: a crossover double-blind randomized controlled trial

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22 8) Sources of support: the research was supported by Clermont-Ferrand University Hospital.
23 DRT society provided Oligopin® and placebo capsules free of charge and made a grant to
24 support the research.

25 9) Short running head: Polyphenol and meal-iron absorption in HH and DIOS

26 10) Abbreviations:

27 AUC: Area under the curve.

28 BMI: Body mass index

29 C_{last} : Last observed value

30 C_{max} : Maximum observed value

31 DIOS: Dysmetabolic iron overload syndrome.

32 HH: Hereditary hemochromatosis.

33 11) Clinical Trial Registry: clinicaltrials.gov (NCT 03453918)

34 Data described in the manuscript, code book, and analytic code will be made available on
35 request

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43 Abstract

44 Background & Aims: Type I hereditary hemochromatosis (HH) and dysmetabolic iron
45 overload syndrome (DIOS) are the two most prevalent iron overload diseases. Although many
46 food components, particularly polyphenols, reduce iron bioavailability, there is no clinically
47 validated nutritional strategy to reduce food-iron absorption in patients with these diseases.
48 We aimed to determine whether supplementation with 100 mg of procyanidins during a meal
49 reduces dietary iron absorption in patients with HH or DIOS.

50 Methods: 20 HH and 20 DIOS patients were enrolled in a double-blind three-period crossover
51 randomized study. Basal serum iron level was measured following an overnight fast. Each
52 patient consumed a standardized test iron-rich meal containing 43 mg of iron with two
53 capsules of placebo or procyanidin supplementation. Each period was separated by a 3-day
54 wash-out period. The primary objective was a reduction of dietary iron absorption, assessed
55 by a reduction of serum-iron area under the curve (AUC) corrected for baseline serum iron.

56 Results: all patients completed the study. The meal and the procyanidin supplements were
57 well tolerated. In both HH and DIOS patients, the iron-rich meal induced a significant
58 increase of serum iron compared with baseline at 120, 180, 240 minutes, from 8 to 9.1% ($p =$
59 $0.002, 0.001$ and 0.003 , respectively) in DIOS and from 15.8 to 25.7% ($p < 0.001$) in HH.
60 Iron absorption was 3.5-fold higher in HH than in DIOS ($p < 0.001$). Procyanidin
61 supplementation did not significantly modify iron absorption in DIOS (AUC of added iron
62 332.87 ± 649.55 vs 312.61 ± 678.61 $\mu\text{mol.h/L}$, $p = 0.916$) or in HH (1168.62 ± 652.87 vs
63 1148.54 $\mu\text{mol.h/L} \pm 1290.05$, $p = 0.917$).

64 Conclusions: an iron-rich test meal led to a marked increase in iron absorption in HH but a
65 mild increase in DIOS. Procyanidin supplementation does not significantly reduce dietary
66 iron absorption in either disease.

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68 Keywords: Iron absorption; Polyphenol supplementation; Procyanidin; Iron-overload
69 diseases; Type I hereditary hemochromatosis; Dysmetabolic iron-overload syndrome.

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84 Text

85 INTRODUCTION

86 Iron is involved in multiple functions mediated by iron-containing proteins (1). Owing to its
87 capacity to exchange electrons, non-transferrin-bound iron induces the Fenton reaction,
88 producing reactive oxygen species involved in cellular and DNA damage (2). Whole-body
89 iron stores are tightly regulated. Dietary iron absorption in mature enterocytes balances
90 uncontrolled iron losses (about 1–2 mg/day) (3). Iron fluxes are regulated by hepcidin, a 25-
91 amino-acid peptide synthesized by hepatocytes in response to high iron stores (4).

92 The prevalence of type I hereditary hemochromatosis (HH) is estimated to be 0.4% in the
93 European population (5) and 0.44% in non-Hispanic whites in North America (6). Type I HH
94 is caused by hypohepcidinemia due to p.Cys282Tyr homozygous mutation on the *HFE*-gene,
95 that lead to iron accumulation through an increase of dietary iron absorption (7).
96 Dysmetabolic iron overload syndrome (DIOS) (8) prevalence is 14.5% in patients with
97 metabolic syndrome (9). This syndrome combines various components of the metabolic
98 syndrome with a mild increase in body and hepatic stores of iron. The pathophysiology
99 remains unclear: although the hepcidin level is consistent with iron storage leading to
100 decreased iron absorption (10), iron accumulation could occur during the phase of increased
101 iron availability at the onset of weight gain, as supported by animal experimentation (11).
102 Therapeutic phlebotomy is the main treatment to prevent organ damage due to iron excess in
103 HH (12), whereas in DIOS, phlebotomy is not recommended because it does not improve
104 metabolic and hepatic features (13,14). Improvement of diet and lifestyle remains the
105 mainstay of therapeutic management of DIOS. Surprisingly, nutritional studies focusing on
106 the modulation of dietary iron absorption in iron overload diseases are scarce. Low dietary

107 iron intake was not associated to lower ferritin levels (15), and chelation therapy using
108 deferoxamine is not recommended in HH.

109 Polyphenols are well-known iron chelators that are present in a wide variety of meals and
110 beverages. *In-vitro* iron-chelating ability of grape seed proanthocyanidin extract was shown to
111 be equivalent to 30 μM of deferoxamine to chelate iron in a 50 μM solution of ferrous sulfate
112 (16) Several studies investigated the effect of nutritional polyphenols on iron absorption in
113 healthy or iron-deficient humans using radiolabeled iron (17–19) and showed a dose-
114 dependent inhibitory effect on non-heme iron absorption (20). Tea consumption providing 90
115 mg of polyphenols was evaluated in HH (21): radiolabeled test showed reduced iron
116 absorption from 22.1 to 6.9% (70% of reduction) and regular consumption three times a day
117 was shown to be associated with a significantly reduced frequency of phlebotomy.
118 Furthermore, the potential anti-oxidant activity of polyphenols could be of interest in
119 counteracting the pro-oxidant effect of high iron stores (22). Finally, dietary supplementation
120 with polyphenols to prevent iron absorption could be promising after the removal of iron by
121 phlebotomy. Indeed, bloodletting may induce erythropoiesis, enhancing erythroferrone
122 production, leading to a reduction of hepcidin and in turn enhancing iron absorption (23).

123 We conducted a crossover double-blind randomized trial versus placebo to investigate the
124 effects of procyanidin supplementation (Oligopin®) on dietary iron absorption in the two
125 most common iron overload diseases: HH and DIOS.

126

127 **MATERIALS AND METHODS**

128 **Trial design**

129 Three-period randomized double-blind crossover study to assess the effect of procyanidin
130 supplementation versus placebo on iron absorption during a test iron-rich meal. In line with

131 French regulations, ethical approval was obtained from the “Comité de Protection des
132 Personnes Sud-Méditerranée I” on 20 February 2018 (reference 18-10). The study was
133 conducted in accordance with the Declaration of Helsinki and the Good Clinical Practice
134 recommendations. Written informed consent was obtained from each participant. The study
135 was registered on clinicaltrials.gov (NCT 03453918) and on the European Clinical Trials
136 Database (EudraCT 2017-A01955-48). Patients were screened for participation during their
137 normal medical follow-up in the Internal Medicine Department of Clermont-Ferrand
138 University Hospital, France. The sequence of administration was randomized through a
139 computer generator using block randomization with stratification by disease. Patients,
140 experimenters, care-providers and statisticians were blinded to the sequence of administration.

141 **Patients**

142 The inclusion criterion for type I HH was the presence of a p.Cys.282Tyr homozygous
143 mutation on the *HFE* gene. The inclusion criteria for DIOS were: (1) high hepatic iron content
144 assessed by magnetic resonance imaging of the liver, at ≥ 50 $\mu\text{mol/g}$ dry weight, associated
145 with a high ferritin level of ≥ 450 $\mu\text{g/L}$ measured at least twice; (2) at least one of the
146 following metabolic features: body mass index (BMI) ≥ 25 kg/m^2 , abdominal obesity defined
147 by waist circumference (≥ 80 cm for women, ≥ 94 cm for men), blood pressure $\geq 130/85$
148 mmHg or anti-hypertensive therapy, high serum triglyceride level (≥ 1.7 mmol/L), low level
149 of high-density lipoprotein (≤ 1.29 mmol/L for women, ≤ 1.03 for men), high level of fasting
150 blood glucose (≥ 5.6 mmol/L) or type 2 diabetes mellitus. The exclusion criteria were: age $<$
151 18 years; concomitant or use in the last 2 months of drugs or dietary supplements that could
152 affect iron absorption (e.g. ascorbic acid, iron chelators, iron supplements, herbal tea);
153 intestinal malabsorption of any cause; anemia (hemoglobin ≤ 9 g/dL); lactating, pregnant or
154 planning pregnancy; alcohol consumption ≥ 20 g/day for women, ≥ 30 g/day for men;

155 inability to understand and comply with the instructions. Therapeutic phlebotomies before and
156 during the study were not an exclusion criterion

157 **Intervention**

158 The patients attended for three visits, at the same hour each time, after a minimum of 6 hours
159 fasting overnight. They were asked not to modify their nutritional habits during the 5 days
160 before each visit. Clinical features were measured by the same physician during each visit:
161 height, weight, BMI, blood pressure, and waist circumference. During the first visit (V0),
162 under fasting conditions, serum iron concentrations were measured six times (0 min = T0, 30
163 min = T1, 60 min = T2, 120 min = T3, 180 min = T4, 240 min = T5) to record the basal
164 intrinsic variation in fasting serum iron. At 0 min, we also measured ferritin, C-reactive
165 protein, glucose, lactate dehydrogenase, haptoglobin, total bilirubin, alanine transaminase and
166 aspartate transaminase. During the first visit, water intake was limited to 100 mL distributed
167 regularly throughout the duration of the visit. At the following visits (V1 and V2), the patients
168 were randomly assigned to receive polyphenols (Oligopin®) or placebo as capsules during a
169 test meal containing 43 mg of iron (**Table 1**). Oligopin® is a French Maritime Pine Bark
170 extract rich in procyanidins, available in a readily-administrated form. Procyanidins are
171 catechin and epicatechin oligomers, which are major polyphenols found in black tea infusion,
172 which consumption showed reduced iron absorption in HH (21). Assumed the high
173 antioxidant properties of procyanidin (24), Oligopin® could also be of interest to prevent
174 oxidative stress due to the high iron-stores.

175 To ensure adequate iron intake to achieve a significant elevation of serum iron level, we
176 elaborated an iron-rich meal based on the average nutritional composition of the most
177 commonly consumed food in France (25). The test meal comprised two courses: 50 g poultry
178 liver and 40 g green salad as a starter, 120 g black pudding and 100 g pasta as the main

179 course, and 30 g cheese with 100 g soft bread. Each ingredient was weighed in grams to
180 ensure reproducibility; each participant was asked to eat the full meal in no more than 20
181 minutes. Immediately after the first course and before the second, two capsules of procyanidin
182 (50 mg/capsule, Oligopin®, Dérivés Résiniques et Terpéniques, DRT laboratory) or placebo
183 were administered with a glass of water (**Table 2**); polyphenols and placebo capsules were
184 visually identical. Iron capsules content was not measured. Serum samples were collected at
185 the same time: 0 (just before the meal), and 30, 60, 120, 180 and 240 minutes after the
186 beginning of the meal. During V1 and V2, water intake was limited to 500 mL distributed
187 regularly throughout the visit. The patients were asked to stay in a restful position with
188 minimal physical activity during the entire visit. Between V1 and V2, the wash-out period
189 was 3 days. Clinical tolerance was assessed at the end of each visit.

190 **Outcome assessment**

191 Blood samples were collected through a catheter inserted in the median cubital vein at the
192 beginning of each visit (before T0). To reduce blood hemolysis, samples were analyzed in the
193 usual laboratory flow. All biochemical parameters were measured using an automatic
194 biochemical analyzer (Dimension-Vista, Siemens®). Hemoglobin was measured by a
195 hematological analyzer (XN 9000, Sysmex®).

196 **Statistical analysis**

197 The primary outcome was the iron area under the curve (AUC) 4 hours after the meal,
198 corrected for the basal value in the fasting condition, and the secondary outcome was the
199 immediate tolerance of procyanidin supplementation. Due to insufficient resource funding,
200 oxylipin analyses were not performed during the first phase of the study; blood samples were
201 frozen and stored to be tested in a second phase as ancillary study.

202 For a two-sided type I error at 5%, a statistical power > 80%, and with an intra-individual
203 correlation coefficient = 0.5 (owing to the crossover design and no carryover effect assumed),
204 20 patients for each disease (DIOS and HH) were needed to highlight a difference of at least
205 30% in the primary outcome, for a standard deviation (SD) of 43% and 10% drop out. The
206 assumption of a 30% difference was based to be clinically relevant in iron-overloaded
207 population.

208 Statistical analysis was performed using SAS® version 9.4 software (Cary, NC, USA);
209 hypothesis testing was two-sided with significance interpreted as $p < 0.05$. The AUC, C_{\max}
210 (maximum observed value of serum iron concentration) and C_{last} (last observed value of
211 serum iron concentration) were calculated using Phoenix 64® Winnolin V7 (Certara
212 Princeton NJ, USA). The AUCs were calculated using the linear trapezoidal rule. For
213 continuous parameters, mean \pm SD or median and interquartile range were calculated,
214 according to the statistical distribution. The Shapiro–Wilk test was used to investigate the
215 assumption of normality for continuous data.

216 The primary endpoint was compared between the groups using analysis of variance for
217 correlated data including sequence, visit, treatment and time fixed effects in addition to the
218 patient effect (as a random effect). When appropriate, the pathology was added to the model
219 as a fixed effect. Type III sum of squares and least squares (LS) means were used. We used
220 the Bonferroni correction for multiple comparisons. The normality of residuals was studied
221 using the Shapiro–Wilk test. Owing to negative AUC values no logarithmic transformation
222 was proposed.

223 First, analysis of the values observed at T0 was carried out (without correction for missing
224 values) to evaluate within- and between-subject variability; following this, V0 data (under
225 fasting conditions) were analyzed to estimate the difference between the pathologies in the

226 fasting state (basal values), followed by the evaluation of the meal and treatment. To identify
227 the effect of procyanidin supplementation, we used a two-step correction of the mean iron
228 concentrations. First, we corrected the value to the endogenous serum iron level collected
229 during visit V0 under fasting conditions to calculate the added iron due to the meal replacing
230 endogenous basal conditions. In the second step, we corrected the T0 values in order to scale
231 all individual profiles to the same starting point, allowing a valid comparison between only
232 the iron-meal induced variations in serum iron. In cases with missing data at T0 (before meal
233 and treatment), the mean of all values observed for the visit was used because no treatment
234 and no meal were given (mean bias). The other missing data were not replaced and were left
235 missing to avoid any bias.

236 For the non-crossover comparisons, the usual statistical tests were performed for quantitative
237 parameters: an independent Student *t*-test or Mann–Whitney test (when the assumptions of the
238 *t*-test, normality and homoscedasticity, were not met).

239 **RESULTS**

240 Between March and July 2018, 91 patients were screened for participation in Clermont-
241 Ferrand University hospital and 41 were recruited (**Figure 1**). The primary reasons for
242 declining to participate were time and distance constraints. One patient in the DIOS group
243 was excluded at the beginning of V0 because of poor venous access. Finally, 20 patients in
244 each group were randomized; no patient was lost to follow-up, allowing 20 patients to remain
245 in each group for the intention-to-treat analysis. As recruitment was completed, we stopped
246 the study in July 2018.

247 Baseline characteristics are presented in **Table 3**. In the DIOS group, the mean age was $61 \pm$
248 7.5 years, with a male:female ratio of 19:1. In the HH group, the mean age was 55.4 ± 15.3
249 years, with a sex ratio of 1:1. As expected, ferritin levels at inclusion were significantly higher

250 in the DIOS group compared with the HH group (median [IQR]: 508 $\mu\text{g/L}$ [267; 597] vs. 54
251 $\mu\text{g/L}$ [31; 87], $p < 0.001$). Only two participants (one in each group) had a mildly increased C-
252 reactive protein level (6.8 mg/L in DIOS and 7.3 mg/L in HH). No patient in the DIOS group
253 was undergoing therapeutic phlebotomy, whereas 18 (80%) of the HH patients were
254 undergoing phlebotomy. The two remaining HH patients were in the grade 2 preclinical phase
255 of the disease: elevation of serum transferrin saturation and ferritin levels with no impairment
256 of quality of life or life-threatening clinical complications (26). Three patients in the HH
257 group were undergoing phlebotomy induction therapy. The delay between the phlebotomy
258 and each visit for these patients ranged from 1 to 8 days. The 15 remaining HH patients
259 underwent no phlebotomy during the study; the delay between their last phlebotomy and V0
260 ranged from 6 to 107 days (median 43 days \pm 28). In the DIOS group, two patients were
261 regular blood donors but neither made a blood donation during the study.

262 Two patients in the DIOS group had type 2 diabetes mellitus. One patient in the HH and two
263 in the DIOS group received proton pump inhibitors since several years as usual treatment (4
264 and 10 years for DIOS patients, 12 years for HH patient). Only one patient underwent
265 modification of their usual treatment: a woman in the HH group received a single dose of 200
266 mg doxycycline for a tick bite, with no sign of early Lyme's disease, 5 days before the third
267 visit.

268 Altogether, 11 points of serum iron concentration data were missing (1.5% of the data).
269 Noticeably, four of the missing points were for one DIOS patient, who had poor venous
270 access causing hemolysis that led to uninterpretable serum iron concentrations.

271 **Iron value in the fasting condition**

272 We found no significant effect on the variability of iron value in fasting condition at T0
273 regardless of the randomized sequence of polyphenol or placebo administration in either

274 disease ($p = 0.7463$ in the HH group, $p = 0.169$ in the DIOS group). The mean value of the
275 serum iron concentration in the DIOS group at each timepoint was 19.7–21% lower than in
276 the HH group ($p < 0.001$). Interindividual variation was overall 48% (54% in the HH group
277 and 26% in the DIOS group) and was associated with intraindividual variability of around
278 23% in both groups.

279 **Effect of the meal on serum iron concentration**

280 In the DIOS group, after the meal, mean serum iron was significantly increased ($p < 0.005$)
281 compared with the fasting condition at T120 (20.87 ± 4.39 vs 19.2 ± 4.81 $\mu\text{mol/L}$, 8.7%
282 increase, $p = 0.002$), T180 (21.84 ± 4.61 vs 20.17 ± 4.79 $\mu\text{mol/L}$, 9.1% increase, $p = 0.001$)
283 and T240 (21.91 ± 4.43 vs 20.49 ± 4.67 $\mu\text{mol/L}$; 8% increase, $p = 0.003$) (**Figure 2**). The
284 increase in the mean serum iron was twofold higher in the HH compared with the DIOS group
285 ($p < 0.001$). In the HH group, after the meal, mean serum iron was significantly increased ($p <$
286 0.001) compared with the fasting condition at T120 (28.64 ± 8.7 vs 24.73 ± 9.94 ; 15.8%
287 increase), T180 (31.02 ± 9.09 vs 24.96 ± 10.17 $\mu\text{mol/L}$; 24.2% increase) and T240 ($31.76 \pm$
288 8.06 vs 25.25 ± 10.20 $\mu\text{mol/L}$; 25.7% increase). At T0, when no meal or treatment had been
289 given, there was also a significant difference in mean serum iron (24.83 ± 10.23 vs $26.48 \pm$
290 10.28 $\mu\text{mol/L}$, $p = 0.008$) that led us to proceed to a correction of T0 values as described in
291 “Statistical analysis” paragraph, to assess only the added iron, allowing comparison of the
292 results.

293 **Effect of polyphenol supplementation**

294 Immediate tolerance of the procyanidins was excellent, and no adverse effect was noted
295 during the entire study. As shown in **Figure 3** (corrected values of iron absorption), in the
296 DIOS group, procyanidin supplementation during the test meal did not significantly modify
297 the mean increase in serum iron when compared with placebo (1.46 vs 1.15 $\mu\text{mol/L}$, $p =$

298 0.241). The same results were found in the HH group (mean serum iron increase 4.12 vs 4.02
299 $\mu\text{mol/L}$, $p = 0.706$). These results were confirmed by the corrected serum-iron AUC analysis
300 that reflected the extent of added iron. AUC after the test meal was not significantly different
301 with procyanidin supplementation compared with placebo in the DIOS group ($332.87 \pm$
302 649.55 vs 312.61 ± 678.61 $\mu\text{mol.h/L}$, $p = 0.916$) or in the HH group (1168.63 ± 652.87 vs
303 1148.55 ± 1290.05 $\mu\text{mol.h/L}$, $p = 0.917$) (**Supplemental Figure 1**). The AUC values were
304 3.5-fold higher in the HH than DIOS group, with both procyanidin supplementation and
305 placebo ($p < 0.001$). Other pharmacokinetics parameters (C_{max} and C_{last}) were not
306 significantly modified by the procyanidin supplementation in either group (**Supplemental**
307 **Figures 2 and 3**).

308

309 DISCUSSION

310 This study was designed to investigate the effect of procyanidin supplementation on iron
311 absorption during an iron-rich meal, in 20 patients with DIOS and 20 with HH. To the best of
312 our knowledge, this is the first study to investigate the effect of supplemental polyphenol on
313 iron absorption in iron-overload diseases. Moreover, this is also believed to be the first study
314 to investigate iron absorption by measurement of serum iron kinetics after iron loading by an
315 iron-rich meal. We showed: (i) a significant increase in serum iron after the high-iron meal
316 compared with the fasting condition; and (ii) higher dietary absorption of iron in HH than in
317 DIOS. In answer to the primary objective of the study, dietary polyphenol (procyanidin)
318 supplementation during a meal does not modify dietary-iron absorption in HH or in DIOS.

319 Iron absorption has been investigated using iron radioisotopes (27) or a stable iron isotope
320 (28), but these methods are not available in routine clinical practice. Several alternative
321 methods have been developed using serum iron increase after oral iron loading (29–32), with

322 a good correlation between absorption of radiolabeled iron and serum iron increase. In those
323 studies, a significant increase in serum iron and transferrin saturation was observed 180 min
324 after oral administration of 50–200 mg of diverse iron pharmaceutical salts. In our study, we
325 decided to use a standardized iron-rich test meal to get as close as possible to normal
326 nutritional conditions while providing a significant increase in serum iron. Similar to the
327 recommended methodology to assess the bioequivalence of endogenous substances (33), we
328 used a crossover study versus placebo, with measurement of basal iron serum level in the
329 same patients as part of the main study, to remove the effect of endogenous iron as well as
330 any chronobiological effect, in order to assess only iron absorption linked to the test meal. In
331 both diseases, the iron-rich meal showed good efficacy in increasing serum iron, reflecting
332 iron absorption. Another limitation of our results is the wide range of time between each visit
333 and phlebotomy in HH subjects undergoing maintenance therapy, that could have influenced
334 iron absorption. Variation of hepcidin after blood donation has been barely reported (34):
335 hepcidin level decreases while erythroferrone decreases significantly from 2.5 to 14 days after
336 blood donation; baseline levels are recovered 16 weeks later.

337 Although our study was not designed to compare iron absorption between the two diseases,
338 iron absorption was significantly lower in DIOS than in HH. To the best of our knowledge,
339 this is the first study assessing dietary iron absorption in both diseases with dietary iron
340 tolerance test. This result is in line with those obtained by a stable isotope method (10) and an
341 oral iron tolerance test using 105 mg ferrous sulfate (35) and consistent with the mild increase
342 of iron stores in DIOS compared to the marked increase in HH. As such, the underlying
343 mechanisms resulting in iron overload in DIOS remain challenging. As hepcidin is
344 synthesized in the liver, we can hypothesize that transient impaired hepatic function could
345 induce decreased hepcidin levels leading to transient enhanced iron absorption. Another
346 hypothesis is a transient decreased hepcidin production due to enhanced erythroferrone during

347 the phase of weight gain to increase erythropoiesis. In our study, procyanidin supplementation
348 did not reduce iron absorption in DIOS or HH. However, several limitations of our study can
349 be raised. First, the duration of the kinetics study (4 hours) could have been too short to show
350 a difference in AUC; however, most of the oral iron tolerance tests established previously
351 were performed for up to 4 hours. In addition, the availability of polyphenol to chelate iron
352 could have been counteracted by the proportion of heme-iron in our meal. Indeed, formation
353 of a polyphenol–iron complex in the intestinal tract is believed to limit iron absorption, which
354 may be reduced with heme-iron. Moreover, the dosage of 100 mg procyanidin could have
355 been too low to make an impact on the amount of iron absorbed. Indeed, Wu and colleagues
356 (16) showed *in vitro* that procyanidin at 300 $\mu\text{g/mL}$ chelate 30% of a 50 μM solution of
357 ferrous sulfate. Therefore, 100 mg procyanidin could have bind about 1% of the iron content
358 of the meal. However, *in vitro* experimental conditions are quite different from normal
359 digestion and as we do not have *in vivo* polyphenol-to-iron molar ratio, we are unable to
360 establish a relevant comparison. These parameters could explain the contradictory results of
361 human studies. Furthermore, iron content of the capsules was not measured, however iron
362 content of a very similar product (Pycnogenol®, extract from the bark of *Pinus maritima*) is
363 very low (65 $\mu\text{g/g}$ of dry product) (36). Finally, the enhanced iron absorption in patients with
364 HH could have exceeded the inhibitory iron absorption of the polyphenols.

365 Delimont and colleagues reviewed several studies of the impact of tannin consumption on
366 iron bioavailability (37): they emphasized the use of tannic acid in most of the studies that
367 showed a reduction of iron absorption but these compounds are virtually absent in our diet.
368 Furthermore most of the studies using condensed tannins (flavonoid oligomers, of which
369 procyanidin are a subgroup) did not support their ability to reduce iron absorption (38–40).
370 Although our work was designed to assess only the acute effect of procyanidin

371 supplementation on dietary iron absorption, our results does not support the relevance of a
372 long-term supplementation study.

373 Low-grade inflammation is usually associated with insulin-resistance in patients with DIOS
374 (41), and iron overload is associated with oxidative stress and DNA damage (42). In line with
375 these data, another potential use of polyphenols in DIOS or HH could be to reduce or
376 modulate the oxidative stress and inflammation caused by iron overload. Our previous studies
377 have shown an antioxidant action of the tested procyanidins on LDL oxidation *in vitro* (43)
378 and in non-human animals *in vivo* (44). Although C-reactive protein did not differ between
379 the groups in the current study, it would be interesting to assess the inflammatory and
380 oxidative status of DIOS and HH patients using lipidomic profiling of oxylipins, which would
381 allow subtle and integrative characterization of inflammation and oxidative stress (45).

382 In summary, acute nutritional supplementation with procyanidins in patients with DIOS or
383 HH does not significantly reduce dietary iron absorption during an iron-rich meal, in a
384 double-blind randomized controlled study. This standardized-meal method could be used to
385 assess the efficacy of other nutritional and pharmacological iron-absorption modulators.

386

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Statement of Authorship,

The authors' responsibilities in this work are as follows: MR and HL proposed the work; MR, HL, BP, JMC, AM and CG designed the protocol trial; CD and HL contributed to data collection; HL and JMC designed the figures; HL wrote the manuscript. All authors contributed to data interpretation, reviewed and approved the final manuscript.

Conflict interest Statement and funding sources.

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Legends for figures

Figure 1: Flowchart. In the dysmetabolic iron overload syndrome (DIOS) group, five patients were excluded: two because of abdominal surgery that could cause malabsorption, and three because of excessive alcohol consumption. In the type I hereditary hemochromatosis (HH) group, two patients were excluded because they were receiving chemotherapy for hematological malignancies.

Figure 2: Variation in mean \pm standard deviation (SD) serum iron ($\mu\text{mol/L}$) in the fasting condition and after the test meal, by time and by disease. \circ and small dotted line show mean \pm SD serum iron values in fasting condition. Δ and large dotted line show mean \pm SD serum iron values after the iron-rich meal associated with placebo.

* shows significant difference between fasting and after-meal mean serum iron concentration at each time. In DIOS, at T120 ($p = 0.002$), T180 ($p = 0.001$) and T240 ($p = 0.003$). In HH, $p < 0.001$ at T120, T180 and T240.

Figure 3: Variation in corrected mean \pm standard deviation (SD) serum iron concentration ($\mu\text{mol/L}$) after iron-rich meal, by disease and by time. \square and solid line show corrected mean \pm SD serum iron values after iron-rich meal associated with polyphenol. Δ and dotted line show corrected mean \pm SD serum iron values after iron-rich meal associated with placebo. \circ shows mean corrected serum iron values in the fasting condition (equal to 0 owing to the correction for basal endogenous serum iron).

No significant difference was found in mean corrected serum iron when the iron-rich meal was associated with placebo or polyphenol, in DIOS ($p = 0.241$) or in HH ($p = 0.706$).

Supplemental Figure 1: Effect of procyanidin supplementation on mean corrected iron concentration area under the curve (AUC, $\mu\text{mol}\cdot\text{h}/\text{L}$), by disease. Upper whiskers boundary shows 95th and lower whiskers 5th percentile. Triangles denote outliers.

No significant difference was found between placebo and polyphenol in DIOS ($p = 0.916$) or in HH ($p = 0.917$). The AUC was significantly greater in the HH group than in the DIOS group, with both placebo and polyphenol ($p < 0.001$).

Supplemental Figure 2: Effect of procyanidin supplementation on mean corrected iron concentration *C-max* ($\mu\text{mol}/\text{L}$), by disease. Upper whiskers boundary shows 95th and lower whiskers 5th percentile. Triangle and square denote outliers. No significant difference between placebo and procyanidin supplementation was found in DIOS ($p = 0.608$) or in HH ($p = 0.266$).

Supplemental Figure 3: Effect of procyanidin supplementation on corrected iron concentration *C-last* ($\mu\text{mol}/\text{L}$), by disease. Upper whiskers boundary shows 95th and lower whiskers 5th percentile. Triangle and square denote outliers. No significant difference between placebo and procyanidin supplementation was found in DIOS ($p = 0.597$) or in HH ($p = 0.249$).

Tables

Table 1: Standardized meal

Meal	Weight (g)	Total iron content (mg)	Non-heme iron (mg)	Heme-iron (mg)
Green salad	40	0.25	0.25	-
Poultry liver	50	5.3	3.18	2.12
Black pudding	120	27.36	16.4	10.96
Pasta	100	0.34	0.34	-
Cheese	30	0.08	0.08	-
Soft bread	100	10	10	-
Total	-	43.3	30.25	13.08

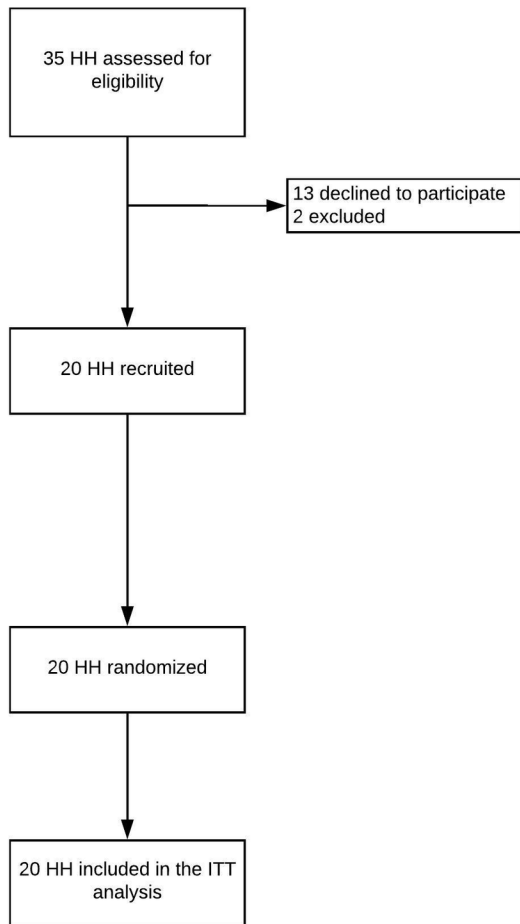
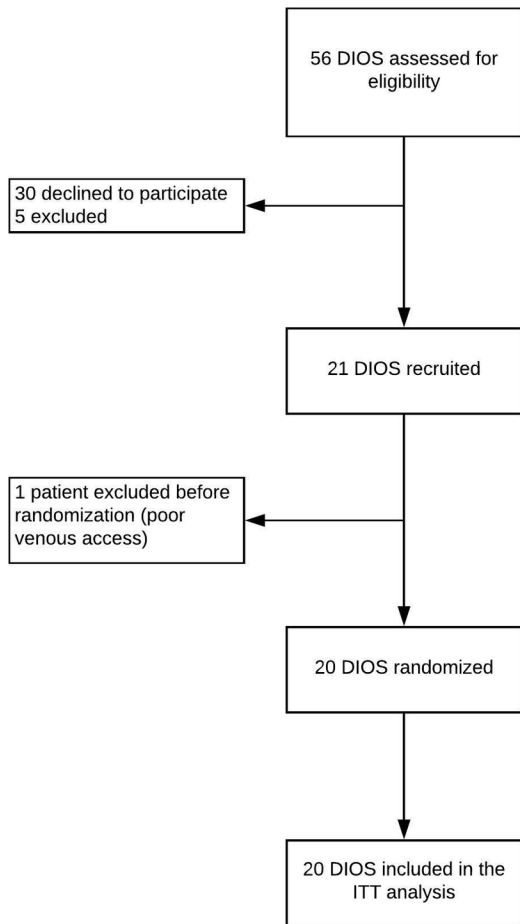
Table 2: Capsule composition of polyphenols and placebo

Component	Oligopin®	Placebo
Proanthocyanidin	50 mg	-
- catechin	4–10%	
- taxifoliol	0.5–4%	
- taxifoliol glucoside	3–8%	
- ferrulate glucoside	4–10%	
- gallic acid	0.1–1%	
- protocatechic acid	0.5–3%	
- caffeic acid	0.5–3%	
- p-coumaric acid	0.3–2%	
- ferulic acid	1–5%	
Wheat maltodextrin	150 mg	218.9 mg
Magnesium stearate	1.1 mg	1.1 mg
Iron	Not available	Not available

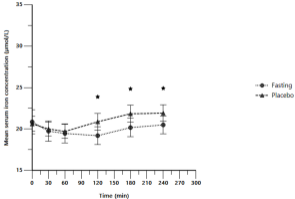
Table 3: Clinical and demographic baseline characteristics of the patients

	HH n = 20	DIOS n = 20
Age (years)	55.4 ± 15.3	61 ± 7.5
Sex male, n (%)	10 (50)	19 (95)
BMI (kg/m²)	24.9 ± 3.5	27.8 ± 3.0
Hepatic iron content (µmol/g)	NA	83 [63;92]
Number of metabolic syndrome criteria, n (%)	NA	
1		1 (5%)
2		2 (10%)
3		4 (20%)
4		8 (40%)
5		5 (25%)
Ferritin (µg/L)	54 [31;87]	508 [267;597]
Hemoglobin (g/L)	14.8 ± 1.0	15.6 ± 1.3
CRP (mg/L)	3.3 ± 1.1	3.2 ± 0.9
Venous glycemia (µmol/L)	4.8 ± 0.6	5.5 ± 2.2
Haptoglobin (g/L)	1.2 ± 0.4	1.2 ± 0.5
LDH (IU/L)	224.7 ± 41.1	221.6 ± 36.2
AST (IU/L)	22.4 ± 6.8	25.3 ± 6.5
ALT (IU/L)	29.1 ± 12.9	42.4 ± 15.4
GGT (IU/L)	33.1 ± 21.3	44.2 ± 17.5
ALP (IU/L)	61.2 ± 14.0	68.0 ± 19.1
Total bilirubin (µmol/L)	9.9 ± 5.3	10.5 ± 3.6

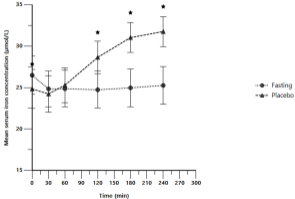
Data are presented as mean ± standard deviation; ferritin and LDH are presented as median and inter-quartile range. BMI: body mass index. CRP: C-reactive protein; LDH: lactate dehydrogenase; AST: aspartate transaminase; ALT: alanine transaminase; GGT: gamma-glutamyl transpeptidase; ALP: alkaline phosphatase.



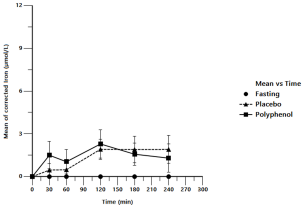
DIOS



HH



DIOS



HH

