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7 **FIRST-PASS METABOLISM OF CHLOROPHYLLS IN MICE**

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23 List of abbreviations:

24 ABC: ATP-binding cassette; ADME: absorption, distribution, metabolism and excretion; AMF:
25 aqueous micellar fraction; APCI: atmospheric pressure chemical ionization; BHT: butylated

26 hydroxytoluene; BLT1: blocks lipid transport 1; EIC: extracted ion chromatogram; hrTOF: high
27 resolution TOF; PDT: photodynamic therapy; Qq: quadrupole-quadrupole; SR-BI: scavenger
28 receptor B type I; UHR: ultra-high resolution
29 Keywords: chlorophylls; absorption; liver; pheophorbide; SR-BI.

30 **Abstract**

31 Scope: The dietary intake of chlorophylls is estimated to be approximately 50 mg/day. However,
32 their first pass metabolism and systemic assimilation is not well characterized.

33 Methods and results

34 A group of 30 mice were fed a diet rich in chlorophylls, while 10 mice received a standard diet
35 without chlorophylls (control group). Liver extracts were analyzed every 15 days by HPLC-
36 ESI(+)/APCI(+)-hrTOF- MS/MS to measure the accretion of specific chlorophyll metabolites. The
37 chlorophyll profile found in the livers of mice fed a chlorophyll-rich diet showed that the formation
38 and/or absorption of pheophorbides, pyro-derivatives and phytol-chlorin *e*₆ require the occurrence
39 of a precise first-pass metabolism. In addition, the apical absorption of pheophorbide *a*-rich
40 micelles was significantly inhibited in Caco-2 cells pre-incubated with BLT1.

41 Conclusion

42 Pheophorbide *a* absorption is, at least partly, protein-mediated through SR-BI. This active
43 absorption process could explain the specific accumulation of pheophorbide *a* in the livers of
44 animals fed a chlorophyll-rich diet. A complementary mechanism could be the de-esterification of
45 pheophytin *a* in the liver, yielding pheophorbide *a* and phytol, which would explain the origin of
46 phytol in the liver. Hence, our results suggest two molecular mechanisms responsible for the
47 accumulation of the health-promoting compounds pheophorbide and phytol.

48 **1. Introduction**

49 Spirulina is commercialized as a “health food” recognized by the WHO and the FAO.[1] A rich
50 source of biologically active ingredients and nutrients – proteins, lipids, phycobilins and carotenoids
51 – spirulina also contains a relatively high amount of chlorophylls. This group of pigments is the
52 most ubiquitous in nature as they play a key role in photosynthesis. Significant functions of
53 chlorophylls have also been evidenced in animals. Indeed, it can be anticipated that after millions of
54 years of close interaction through diet, the animal body has found some way to take advantage of
55 the physicochemical characteristics of chlorophylls. It is known that consumption of fruits and
56 vegetables is associated with a lower risk of cancer and that they may be protective against the
57 development of several other chronic diseases [2]. In particular, numerous scientific studies have
58 shown that chlorophylls and their derivatives from foods develop benefits to human health when
59 consumed [3], through antioxidant, antimutagenic [4] and antigenotoxic activities [5].
60 Specifically, research has focused on the chemo-preventive effects in humans [6], with evidence
61 from numerous *in vivo* [7] and *in vitro* [8] studies.

62 Despite the extensive literature pointing to the health benefits derived from the intake of a diet rich
63 in chlorophyll pigments, our understanding of the first-pass metabolism and subsequent systemic
64 assimilation of chlorophylls is scarce [8]. This is particularly remarkable considering that green
65 vegetables are present in our daily dietary patterns and their intake is actively promoted by
66 international health agencies through campaigns like 5 A Day [3]. This lack of understanding about
67 the biochemical changes of chlorophyll pigments that take place during digestion, and their
68 bioaccessibility and metabolism has restrained the interest in chlorophylls as bioactive compounds.

69 Preliminary research on the assimilation of chlorophylls was based on the quantification of
70 chlorophylls derivatives in human and animal feces, assuming that they were not absorbed in the
71 organism [9] as almost all the ingested chlorophylls were excreted. More recently, Motta-
72 Fernandes, Gomes, and Lanfer-Márquez,[10] applied the same balance mass (determining ingested

73 against excreted) chlorophylls and estimated the apparent absorption of 3.4% of chlorophylls from
74 spinach. Recently, it has been published [11] an organ-specific distribution of chlorophyll-related
75 compounds from dietary spinach in rabbits. But the uncertain chlorophyll identification, the lack of
76 control samples and the presence of chlorophyll derivatives at time 0 due to an existing “green diet”
77 before the beginning of the experiment meant that conclusions were ambiguous. Indeed, to date, the
78 only convincing *in vivo* evidence of the absorption and accumulation of chlorophyll structures in
79 mammals is related to copper-chlorophyllins [12]. This work described the presence of copper-
80 chlorin *a* ethyl ester in the sera of individuals participating in a chemoprevention trial after the
81 ingestion of copper-chlorophyllins. Moreover, a later study estimated that dietary sodium copper-
82 chlorophyllins accumulated in different organs of Wistar rats [13]. However, these compounds arise
83 from a chemical process (chemical hydrolysis plus addition of copper salt) applied to natural
84 chlorophyll extracts. Consequently, copper-chlorophyllins should not be denoted as natural pigments
85 [14] as they are not found in raw fruits, vegetables or seaweeds. Sodium copper-chlorophyllins are
86 synthetic compounds that are commercially available as food colorants, and their
87 absorption/accumulation is unlikely to represent the *in vivo* absorption/accumulation of the natural
88 chlorophylls present in fruit and vegetables.

89 In this sense, only *in vitro* experiments have been performed to obtain information on the digestion,
90 absorption, and metabolism of natural chlorophyll pigments from food sources. Ferruzzi, Failla and
91 Schwartz,[15] applied an *in vitro* protocol of digestion and estimated the subsequent uptake of
92 chlorophylls from the micellar fraction by the Caco-2 cellular model. The native chlorophylls from
93 spinach were transformed to Mg-free pheophytin-type derivatives during digestion, and 5–10% of
94 the micellar pheophytin-type derivatives accumulated in the cells, suggesting that chlorophyll
95 derivatives could be absorbed and eventually transported to peripheral tissues. Subsequently, these
96 results were confirmed, applying the same *in vitro* protocol to pea preparations [16], standards of
97 chlorophyll derivatives isolated from natural sources [17], and seaweeds [18-19]. Specifically, it has

98 been shown *in vitro* that de-phytylated chlorophylls (pheophorbides) are preferentially absorbed
99 over phytylated chlorophylls (pheophytins) [17-19]. Through comparative experiments with the
100 Caco-2 cellular model, it has been hypothesized that pheophytin *a* at different concentration levels
101 in the cell culture medium is absorbed by passive diffusion, while pheophorbide is absorbed by a
102 combination of two mechanisms. The authors claimed that the saturation of the absorption rate
103 observed at concentrations below 0.5 μM is compatible with a passive transport mechanism. The
104 linear absorption rate obtained for the 0.5-1.5 μM concentration range points to a simple diffusion
105 mechanism. However, these experiments were developed with the aqueous micellar fraction (AMF)
106 of pheophorbide *a* standard obtained after the application of an *in vitro* digestion protocol; thus the
107 composition and concentration of the AMF is limited by the methodology, which does not allow the
108 pigment to reach physiological concentrations in the AMF. The application of synthetic mixed
109 micelles for related lipophilic compounds such as carotenoids [20-22] has allowed physiological
110 micellar concentrations (7 μM) to be reached. Consequently, it is possible to analyze the intestinal
111 absorption process of chlorophyll derivatives with this technique at a concentration similar to the
112 physiological one (*ca.* 2-5 μM , [12]).

113 In summary *in vivo* evidence of the absorption/accumulation of natural chlorophylls from food
114 sources is lacking. The aim of this study was to determine whether natural chlorophylls from a
115 dietary source are metabolized and accumulated by mammals *in vivo*, as well as examine the
116 possible implication of protein-type membrane transporters of chlorophyll metabolites. Therefore,
117 the diet of a mammalian model (mice) was supplemented with a common source of chlorophyll,
118 spirulina, and the first-pass metabolism of chlorophylls determined.

119 **2. Materials and methods**

120 **2.1 Chemicals and standards**

121 Solvents for liquid chromatography were purchased from Teknokroma (Barcelona, Spain), while
122 PAR grade and LC/MS grade solvents and water were supplied by Panreac (Barcelona, Spain). The

deionized water was obtained from a Milli-Q 50 system (Millipore, Milford, USA). Standard of chlorophyll and pheophorbide *a* were purchased from Wako (Neuss, Germany) and chlorin *e₆* from Frontier Scientific (Logan, USA). The other chlorophyll derivatives were obtained following previously detailed the protocols [23-24]. Commercial Spirulina (NaturGreen) was purchased in a local store. N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), butylated hydroxytoluene (BHT), dimethyl sulfoxide (DMSO, purity > 99.9%), sodium taurocholate, tetrabutylammonium acetate and ammonium acetate (purity > 98%), 2-hexyl-1-cyclopentanone thiosemicarbazone (BLT1), 2-oleoyl-1-palmitoyl-sn-glycero-3-phosphocholine (phosphatidylcholine), 1-palmitoyl-sn-glycero-3-phosphocholine (lysophosphatidylcholine), mono-olein, free cholesterol, oleic acid, sodium taurocholate, trypsin (500 BAEE units porcine trypsin), fetal bovine serum, penicillin, streptomycin, L-glutamine, nonessential amino acids, Dulbecco's modified Eagle's medium (DMEM) containing 4.5 g/L glucose, and phosphate-buffered saline (PBS) were provided by Sigma-Aldrich Chemical Co. (Madrid, Spain).

2.2 Experimental section

Rodent-pellets (Harland Teklad) were provided by ssniff-Spezialdiäten (Soest, Germany). To formulate the feed supplemented with chlorophyll pigments, grinded pellets were mixed with spirulina powder (15% w/w). Water was added to improve the homogenization of the feed and recreate its original pellet-shape. The supplemented pellets were dried in an oven at 30°C for 24 h to reestablish their hardness and avoid spoilage during storage of the feed. After drying, the supplemented pellets were vacuum packed and stored at -20 °C.

2.2.1 Animal model

C57BL/6N mice were obtained from the Animal Production and Experimentation Service (Seville University, Spain). 3 months old male mice (*ca.* 40 g) were housed in conventional cages (3-4 animals per cage). The mice were kept on a standard rodent diet and water *ad libitum* for two weeks. Then, a group of 30 mice received the feed supplemented with Spirulina, while 10 mice

continued with the standard diet (control group). At week 2, 5 mice of the control group and 15 mice from the supplemented feed group were euthanized by cervical dislocation. The livers were excised, weighed, rinsed with 0.9% saline solution and then stored at -80 °C until analysis within 1 week. Fecal samples were collected from each group of mice and stored at -80 °C until analysis within 1 week. At week 4, the same protocol was applied to the rest of the mice in both groups. The use of the animals for this experiment and the protocols were carried out according to the European Union directive (2010/63/EU) for the use of laboratory animals and were approved by the local Ethical Committee (study number 0158-N-15, Junta de Andalucía, Spain).

2.2.2 Culture of Caco-2 cells.

Caco-2 (Caucasian colon adenocarcinoma) cells were purchased from Sigma-Aldrich Chemical Co. (Madrid, Spain) and stocks were maintained in complete medium as described previously [19]. At 70%-80% confluency, the cells were sub-cultured with trypsin and seeded in 75 cm² flasks (Nunclon-treated surface, Nunc A/S), at densities of 3×10^4 cells/cm², following published protocols [19]. All the experiments used highly differentiated monolayers around 11-14 days after reaching confluency. Medium was replaced every 2-3 days, and the last replacement of medium before the experiments was carried out using serum-free medium.

2.2.3 Preparation of pheophorbide *a*-rich micelles

For the delivery of pheophorbide *a* to cells, mixed micelles were prepared as described before. [20] to mimic the physiological micellar concentration with slight modifications. Appropriate volumes of the micellar compounds were transferred to glass bottles to obtain the following final concentrations: 0.04 mM phosphatidylcholine, 0.16 mM lysophosphatidylcholine, 0.3 mM mono-olein, 0.1 mM free cholesterol, 0.5 mM oleic acid. Stock solution solvents were carefully evaporated under nitrogen. The dried residue was dissolved in DMEM containing 5 mM taurocholate and pheophorbide *a* (2.5 µM), and vigorously mixed by sonication at 720W for 3 min. Pheophorbide *a* was dissolved in acetone and the addition of acetone volume is 1% (v/v). The

173 mixtures obtained were sterilized and filtered by passing them through a sterilized 0.20 μm filter
174 (Acrodisc HT Tuffryn membrane). HPLC analysis showed a few amount of 13²-OH pheophorbide
175 (less than 5%) yielded after this process, and the pigment in micelles presented the normal
176 absorption spectrum as before. Micelles were freshly prepared before the incubation with Caco-2
177 cells, as well as the measurement of the pheophorbide *a* concentration in the micellar preparations.
178 The size of micelles was evaluated with Malvern Zetasizer (Malvern, UK).

179 2.2.4 Cytotoxicity assay of organic solvent and BLT1

180 Cytotoxicity assay was carried out on a 96-well plate according to the methods of Reboul et al. [20]
181 to control the addition of exogenous chemicals including acetone in the preparation of mixed
182 micelles, DMSO and BLT1 in the micelles for inhibitory assay. Cytotoxicity was assessed by a
183 CellTiter 96 Aqueous One Solution assay (Promega, Madison, USA). Cells were seeded at a density
184 of 10000 cells per well, and grown for 5 days. Then cells received either acetone ranging from 0.1%
185 to 2% (v/v), or DMSO, 0.1% to 0.5% (v/v) in DMEM for 3 h and the results showed acetone and
186 DMSO were not toxic in the tested range for Caco-2 cells. Latter, the same experiment was
187 conducted to assay BLT1 cytotoxicity (0 ~ 25 μM , in 0.2% DMSO) and results showed BLT1 was
188 not toxic up to 15 μM for Caco-2 cells.

189 2.2.5 Delivery of pheophorbide *a* rich micelles to Caco-2 cells

190 The analytical conditions used have been described previously [25]. Before the incubation
191 experiment, cell monolayers were washed twice with 155 mL of PBS to eliminate interfering
192 compounds. Cell monolayers were incubated at 37 °C for 5 hours with 15 mL of the micelles. After
193 the incubation period, media from apical side of the membrane were harvested and monolayers
194 were washed twice with 10 mL of ice-cold PBS containing 10 mM sodium taurocholate to eliminate
195 adhered pigments, then scraped and collected in 6 mL of PBS containing 10% ethanol (v/v). All the
196 samples were stored at -23°C under nitrogen before extraction and HPLC analysis. Aliquots (20 μL)
197 of cell samples were used to estimate protein concentrations.

198 2.2.6 Protein determination

199 The amount of protein in cell suspension was determined by bicinchoninic acid (BCA) assay
200 (Sigma-Aldrich Chemical Co., Madrid, Spain). Cell lysates and protein standards (bovine serum
201 albumin) were separately mixed with 200 μ L mixture of BCA solution and 4% CuSO₄ and heated
202 at 60 °C for 15 min. The absorbance at 562 nm was measured with a 96-well plates reader (Imark,
203 Bio-Rad).

204 2.2.7 Pheophorbide *a* transport inhibition

205 Methods were described previously [20]. Briefly, cell monolayers were washed twice with 10 mL
206 of PBS and the effect of inhibitor (BLT1) on pheophorbide *a* transport was evaluated by first
207 monolayer pretreatment with 15 mL of either DMSO (control, 0.2%, v/v) or BLT1 at different
208 concentrations ranging from 0.1 to 10 μ M for 1 h, and then cells received pheophorbide *a* micelles
209 with BLT1 at the pre-incubated concentration.

210 2.2.8 Pigment extraction

211 All procedures were performed under dimmed green light to avoid any photooxidative process of
212 the chlorophyll derivatives. Pellets (both standard and supplemented ones), livers, or feces were
213 homogenized with 25 mL of N, N - dimethylformamide saturated with MgCO₃ [24] and vacuum
214 filtered. The solid was collected and extracted twice with the same conditions. The filtrates were
215 combined in a decanting funnel and mixed with 100 mL of diethyl ether and 100 mL of NaCl
216 solution 10% (w/v). The mixture was stirred and allowed to stand until complete separation of the
217 organic layer. The lower phase was discarded and the organic layer was washed twice with 100 mL
218 of Na₂SO₄ solution 2% (w/v), and finally filtered through a solid bed of 50 g anhydrous Na₂SO₄.
219 The filtrate was concentrated in a vacuum rotary evaporator to dryness at 30° C and the dry residue
220 was solved in 0.5 mL (liver sample) or 1 mL (pellet or fecal sample) of acetone. The protocol for
221 chlorophyll extraction from Caco-2 cells has been detailed previously [19] and briefly consists on
222 successive extractions with ethanol (0.1% BHT), acetone (0.2% BHT), diethyl ether and 10% NaCl.

223 Extraction process was repeated for at least three times as above and the dried residue after
224 evaporation was dissolved in 0.25 mL of acetone. All the samples were filtered through a 0.22 μ m
225 nylon filter and stored at -20°C until analysis within 1 week.

226 2.2.9 Sample analysis by HPLC-ESI/APCI-hrTOF-MSⁿ

227 The liquid chromatograph was Dionex Ultimate 3000RS U-HPLC (Thermo Fisher Scientific,
228 Waltham, MA, USA). Chromatographic separation was performed as described before [23-24]. A
229 stainless steel column (200×4.6 mm, 3 μ m particle size) packed with Mediterranea Sea18
230 (Teknokroma, Barcelona, Spain) was used. The injection volume was 30 μ L and the flow rate was 1
231 mL/min, and a split post-column of 0.4 mL/min was introduced directly on the mass spectrometer
232 ion source. Mass spectrometry was performed using a micrOTOF-QIITM High Resolution Time-of-
233 Flight mass spectrometer (UHR-qTOF) with Qq-TOF geometry (Bruker Daltonics, Bremen,
234 Germany) equipped with an ESI and APCI source. The instrument was operated in positive ion
235 mode using a scan range of m/z 50-1200 Da. Mass spectra were acquired through broad-band
236 collision induced dissociation mode, providing simultaneously MS and MS/MS spectra. The
237 instrument control was performed using Bruker Daltonics HyStar 3.2. Data evaluation was
238 performed with Bruker Daltonics DataAnalysis 4.1 as described previously [23-24]. From the
239 HPLC/qTOF-MS acquisition data, an automated peak detection on the EICs expected for the
240 $[M+H]^+$ ion of each compound in the database was performed with Bruker Daltonics
241 TargetAnalysisTM 1.2 software. The software performed the identification automatically according
242 to mass accuracy and in combination with the isotopic pattern in the SigmaFitTM algorithm [26].
243 Only those hits with mass accuracy and SigmaFit values within the tolerance limits, which were set
244 at 5 ppm and 50, respectively are considered as positive matches. The interpretation of the MS/MS
245 spectra was performed using the SmartFormula3DTM module included in the DataAnalysis software
246 [26].

247 2.2.10 Pigment quantification by HPLC-UV-Visible

248 The identified chlorophyll derivatives were quantified by reversed-phase HPLC using a Hewlett-
249 Packard HP 1100 liquid chromatograph. A Mediterranea Sea18 column (200×4.6 mm, 3 µm particle
250 size) was used (Teknokroma, Barcelona, Spain) protected by a guard column (10×4.6 mm) packed
251 with the same material. Separation was attained using the elution gradient described before [23].
252 The on-line UV-Visible spectra were recorded from 350 to 800 nm with the photodiode–array
253 detector, and sequential detection was performed at 410, 430, 450 and 666 nm. Data were collected
254 and processed with a LC HP ChemStation (Rev.A.05.04). Quantification of pigments was achieved
255 with the corresponding calibration curves (amount versus integrated peak area). The calibration
256 equations were obtained by least-squares linear regression analysis over a concentration range
257 according to the observed levels of these pigments in the samples. Triplicate injections were made
258 for five different volumes of each standard solution.

259 2.2.11 Statistical analysis

260 The aim of the study was to observe accumulation of chlorophyll derivatives in the group of
261 supplemented feed of mice. Although the standard rodent-diet does not contain any trace of
262 chlorophyll derivatives, and diet is the single origin of these compounds in animals, a control group
263 was included to take into account whether the supplemented feed introduced any change in the
264 dietary habits and physical performance of the mice. Thus, it was observed that the supplemented
265 feed group of mice did not reject their feed and they followed their normal habits and behavior
266 patterns with similar consumption rates to the control group. In any case, samples were obtained
267 from the control group to clearly show that they did not accumulate any trace of chlorophyll
268 derivatives. The size of the supplemented feed group of mice was calculated by power analysis
269 considering the statistical test to be applied for comparison of data (t-test), the significance level
270 that was set at $P < 0.05$ with a statistical power of 0.90, and the standard deviation values from
271 similar studies [27]. The principles of the 3Rs were applied in the experimental design and the
272 protocols applied in this study.

Each absorption experiment was repeated at least three times and the absorption rate of pheophorbide *a* was expressed as μmol of pigment absorbed $\times \text{min}^{-1}$ (mg of protein) $^{-1}$. For the calculation of absorption percentage in inhibitor experiment, absorption percentage means nanograms of pheophorbide *a* absorbed in pretreated monolayers $\times 100 /$ ngrams absorbed in the control (without inhibitor treatment).

Differences were compared by one-way analysis of variance (ANOVA) using Statistica for Windows (version 6, StatSoft, Inc., 2001) with student's t-test as *post hoc* comparison test. Differences were considered significant at $P < 0.05$

3. Results

3.1 Identification of chlorophyll pigments

Figure S1, Supporting Information depicts the structures identified in the samples analyzed in this study. Only *a* series chlorophyll pigments (methyl group at C7) were observed in the samples analyzed in this study, in accordance with the typical profile of the chlorophyll source used for supplementation (spirulina) that belongs to the cyanobacteria phylum. In addition to chlorophyll *a* (the main parent compound with a characteristic Mg^{+2} central ion and the C17³ position esterified with phytol chain, R₃,) some derivatives were identified in the diet material, including chlorophyllide (derivative preserving the Mg^{+2} , but de-esterified at the C17³ position), pheophytin (the de-metallo-chlorophyll derivative featuring 2H^{+} in the core of the tetrapyrrolic assembling, but retaining the esterification with a phytol chain) and pheophorbide (de-metallo-chlorophyll derivative, also de-esterified at the C17³ position). Allomerized products (the 13²-hydroxy- and the 15¹-hydroxy-lactone-derivatives) were also observed, arising from oxidative reactions at the fifth isocyclic ring of the before mentioned compounds. Finally, the pyro-derivatives which are produced after the decarboxymethylation reaction at C13² position were also identified. These products were identified according to their chromatographic behavior (retention time), UV-Visible spectra, and MS high-accuracy measurements, including mass error, isotopic pattern and the

298 featured product ions. These characteristic data, presented in Table S1, Supporting Information,
299 were compared with those of pure standards and data available in the literature [15, 23-24].
300 However, the identification of phytol-chlorin e_6 was supported by the chromatographic behavior and
301 features of the UV-visible spectrum in comparison with its de-esterified counterpart chlorin e_6
302 because this is the commercial standard available [28]. The MS measurements described for phytol-
303 chlorin e_6 are the first experimental data reported for this chlorophyll derivative.

304 3.2 Quantitative profile of chlorophyll derivatives in the diet

305 The diet of mice in the control group did not contain any chlorophyll pigments. Thus, it is evident
306 that the control group did not ingest any chlorophyll derivatives. On the contrary, fresh prepared
307 spirulina pellets mainly contain chlorophyll a and its epimer (peaks 12 and 13, Figure 1a), a
308 chlorophyll profile typical of cyanobacteria. However, during the slow drying process (30° C for 24
309 h), different chlorophyll derivatives were produced (Figure 1b). The drying process was necessary
310 to obtain the required consistency of the spirulina and feed mix, to avoid spoilage of the final
311 product, and produce a feed with the representative pattern of frequently ingested chlorophylls [29].
312 Thus, the mice in the experimental group were fed a dried chlorophyll-supplemented feed with a
313 chlorophyll profile consisting of 19% intact chlorophyll a , *ca.* 7% of pheophorbide-type derivatives
314 and 74% of pheophytin-type derivatives (Table 1). The allomerized products reached 4% of the
315 total chlorophyll profile and the pyro-derivatives 17%. The total amount of chlorophyll pigments in
316 the dry mixture of spirulina-supplemented feed was 1.5 g/kg, an amount predicted to be close to the
317 quantity obtained from the regular dietary intake of vegetables [30]. Spinach, broccoli, rucula, kale,
318 lettuce, green pepper, avocado and peas [14] can provide even higher amounts of chlorophylls in
319 the Western diet.

320 3.3 Accumulation of chlorophyll derivatives in the liver

321 The livers of mice fed with the spirulina-supplemented diet accumulated chlorophyll derivatives
322 (Figure 2a). In contrast, the livers of mice fed the control diet did not accumulate any chlorophyll

323 derivatives (Figure 2a, insert). Mice were fed for 1 month with the feed supplemented with
324 Spirulina as the source of chlorophyll derivatives. After two weeks of supplementation, the total
325 content of chlorophyll derivatives in the tissues reached a maximum value and then plateaued until
326 the end of the experiment (Table 1), with no significant differences observed between 2 and 4
327 weeks ($P>0.05$). The chlorophyll *a* (and chlorophyllide *a* to a lesser extent) present in the spirulina
328 feed was not detected in the liver. Chlorophyll *a* is prone to transformation processes, including de-
329 metallation and allomerization, as observed in simulated *in vitro* digestion [15], yielding the
330 corresponding pheophytin, 13²-OH and 15¹-OH-lactone-type derivatives. While the pheophytin-
331 type derivatives continued as the major contributors to the pigment profile in the livers of spirulina-
332 fed mice (71% of the total chlorophyll content), pyro-derivatives, allomerized products, and
333 pheophorbide-type derivatives increased their presence in the chlorophyll pattern accumulated in
334 that tissue. Thus, pyro-derivatives increased from a 17% representation in the supplemented feed
335 to *ca.* 43% of the total chlorophyll derivatives present in the liver. Likewise, pheophorbide-types
336 increased from *ca.* 7% in the supplemented feed to almost 28% of the total chlorophyll pigments
337 detected in the liver ($P<0.05$), and allomerized products increased significantly from 4% in the feed
338 to 22% in the liver.

339 The identification of phytyl-chlorin *e*₆ in the livers of the supplemented group of mice is of
340 particular note because no other chlorophyll derivatives observed in the tissue lack the integrity of
341 the macrocycle assembly (structure C, Figure S1, Supporting Information). This study reports for
342 the first time the MS characteristics of phytyl-chlorin *e*₆ (Table S1, Supporting Information) and its
343 presence in an animal tissue after dietary supplementation with a chlorophyll source. Phytyl-chlorin
344 *e*₆ presents a main product ion (m/z 597.2710 Da) corresponding to the loss of the phytyl chain, the
345 principal fragmentation in phytylated chlorophylls [23].

346 3.4 Effect of SR-BI inhibitor on pheophorbide *a* uptake by Caco-2 monolayers

Livers from animals with a chlorophyll-supplemented diet had a significant accumulation of pheophorbide *a*. Pheophorbide *a* could be transported into the liver through a protein-type carrier, although the molecular nature of the possible transporters implied in chlorophyll absorption are currently unknown. Carotenoids, similar lipophilic compounds, are taken up by enterocytes through several apical protein-type membrane transporters, which have been shown to facilitate carotenoid uptake [20-22]. Although few proteins have been proposed as mediators in the cell absorption of carotenoids, the scavenger receptor B type I (SR-BI/SCARB) is involved in the transport of different carotenoids (lutein, β -carotene, α -carotene, β -cryptoxanthin and lutein) in diverse animal tissues (intestinal and retinal) [20-22]. Most of these results were obtained from experiments employing blocks lipid transport 1 (BLT1), a specific inhibition of transport by SR-BI. We used a similar strategy to check the possible implication of SR-BI in the intestinal absorption of pheophorbide *a*. We report here for the first time the production of pheophorbide *a*-rich micelles, which were delivered to the apical medium of Caco-2 culture at an absorption rate of 2.16 $\mu\text{mol}/\text{min}/\text{mg}$ protein (Table 2). Pre-incubations of the cell monolayers with different amounts of BLT1 significantly inhibited ($P<0.05$) the absorption of pheophorbide *a*.

3.5 Quantification and distribution of chlorophyll derivatives in feces

The chlorophyll profile of the feces of the supplemented group of mice was similar to the chlorophyll pattern observed in the feed they ingested, with the exception of chlorophyll *a* and chlorophyllide-type derivatives that were almost completely transformed during digestion into other chlorophyll derivatives (Table 1). Surprisingly, some intact chlorophyll *a* was detected in the feces. The percentage value of the pheophorbide-type derivatives in the feces (8%) correlates well with the value observed in the supplemented feed (*ca.* 7%). Indeed, the percentage value of allomerized derivatives in the feces (6%) also resembles that of the supplemented feed (4%). This steady trend was not observed for the content of pheophytin-type and pyro-derivatives, which had an increased

371 presence in the chlorophyll profile of the feces, reaching 91% and 42% of the total chlorophylls ,
372 compared to starting values of 74% and 17% in the supplemented feed, respectively,.

373 **4. Discussion**

374 Identification of chlorophyll derivatives in the samples was performed with the analysis of the
375 chromatographic behavior, features of the UV-visible spectrum and characteristics of the mass
376 spectra, including isotopic mass values, mass error and isotope pattern of both the protonated ion
377 $[M+H]^+$ and the corresponding product ions obtained after MS-fragmentation reactions of the
378 former. Acquisition of mass spectra was made with an ESI source for the de-phytylated chlorophyll
379 derivatives while the ionization of the phytylated ones was made with an APCI source. The use of
380 different ionization sources for polar and apolar chlorophyll derivatives was applied to obtain
381 higher signal records and to increase the resolution of the mass spectra, as it has been shown before
382 [23-24]. A considerable change of the chlorophyll profile was observed from the raw material
383 (spirulina, Figure 1a) to the processed supplemented feed (Figure 1b). This difference should be
384 noted as the common transformation course that the parent chlorophyll structure follows during the
385 *in vivo* metabolism of photosynthetic tissues [31], or to the storage time or temperature applied for
386 processing [32]. The parent chlorophyll is prone to transformation reactions [33], and thus
387 chlorophyll metabolites/derivatives, such as pheophytin, pheophorbide and their allomerized
388 products, are observed in fresh green vegetables and seaweeds, and their presence increases during
389 cooking, microwaving, pasteurization of canned vegetables, production of vegetable oils, and
390 through storage [29]. Indeed, most of the evidence supporting the health benefits of chlorophyll
391 derivatives has been examined with pheophorbide- and pheophytin-type derivatives because they
392 are the chlorophyll derivatives present in our regular diet. When fresh chlorophyll sources are
393 ingested, the parent chlorophyll structure is completely transformed into pheophytin-types
394 derivatives during digestion as it will be shown below. Pyro-derivatives are also commonly present
395 in our diet [29], and they are produced following the application of higher temperature regimes, like

396 those applied for the refining of vegetable oils, or a longer storage period, both of which influence
397 the progress of the decarboxymethylation reaction [34].

398 The pH conditions reached in the stomach and the enzymatic reactions taking place there and later
399 in the small intestine conform the perfect environment to transform the original chlorophyll into
400 pheophytin-type derivatives, as observed in *in vitro* studies [15-18]. Almost all the native
401 chlorophyll content is transformed in the stomach through pheophytinization, although a small
402 fraction of intact chlorophyll *a* was still present in the feces of mice. Additionally, the
403 corresponding allomerized products of pheophytin increased their presence through enzymatic
404 reactions [16] and chemical processes [17] that take place during digestion. However, there seems
405 to be no reaction yielding pheophorbide-type derivatives or pyro-derivatives. Actually, the
406 standards of pheophytin, pheophorbide and its pyro-derivatives are stable during *in vitro* digestion
407 and no transformation was observed in their structures during that process [17]. The subsequent
408 absorption of chlorophyll derivatives has been studied *in vitro* using the Caco-2 cell model [15-17,
409 19]. Using this model, which simulates the absorption processes of the intestinal epithelium, the
410 uptake of *in vitro* digested chlorophyll seems to be selective for pheophorbide *a* and
411 pyropheophorbide *a*, showing similar absorption rates. However, an *in vivo* observation of this
412 selective absorption had not been presented until now. Our results confirm that the majority of
413 parent chlorophyll *a* does not reach the intestinal epithelium, considering the pH conditions of the
414 stomach during digestion and the straightforward progress of the pheophytinization reaction. The
415 initial chlorophyll *a* fraction was transformed into pheophytin *a* and its allomerized products
416 (Figure S1, Supporting Information). Surprisingly, a small amount of chlorophyll *a* maintained its
417 structure during digestion and was eliminated intact in the feces. Consequently, the significance of
418 pheophytin-type derivatives in the chlorophyll profile after digestion, and potentially available for
419 absorption, is even higher than the 74% value observed in the supplemented feed (Table 1).

420 Assuming that the complete chlorophyll *a* content in the supplemented feed is transformed into

421 pheophytin-type derivatives, their percentage value increased to 92%, a number close to the one
422 observed in the feces (91%, Table 1). Therefore, we consider that pheophytin-type derivatives
423 represent the chlorophyll profile available for absorption in the intestinal epithelium. However,
424 although pheophytin-type derivatives were the major chlorophylls present in livers, their value
425 declined significantly in comparison to the initial amount in the feed, while the pyro-derivatives and
426 the pheophorbide-types, as well as the allomerized products significantly increased their presence in
427 the distribution pattern of chlorophyll derivatives of the livers. It is worth restating that no exchange
428 of pheophytin-type derivatives to pyro-derivatives or pheophorbide-type derivatives takes place
429 during digestion, taking into account the results observed from *in vitro* studies. Only the conversion
430 of pheophytin *a* into its allomerized products has been shown to take place to some extent during
431 digestion. In any case, the total chlorophyll derivatives content in the liver reached a plateau after
432 two weeks of supplementation. In humans, the steady state of copper chlorophyllins was reached at
433 8 weeks [12].

434 Therefore, the increases observed for pheophorbide-type derivatives in the chlorophyll pattern of
435 the livers could be attributed to a preferential absorption of these derivatives or their metabolic
436 conversion from pheophytins in the liver. The former mechanism is supported by the results
437 obtained with the specific inhibitor of SR-BI, BLT1. BLT1 was selected because it inhibits lipid
438 transport by SR-BI in nanomolar concentrations and it does not cross-react with other protein-type
439 transporters [20]. The finding that the absorption of pheophorbide *a* was significantly decreased
440 when cells were treated with BLT1 strongly suggests that SR-BI is involved in the transport of
441 pheophorbide *a*. The fact that increasing amounts of BLT1 were unable to completely inhibit the
442 absorption of pheophorbide *a* could suggest that additional absorption routes might be involved.
443 This is characteristic in carotenoid absorption, for which inhibition with specific inhibitors or
444 antibodies is not complete [20-22]. Thus, we consider that the preferential absorption of
445 pheophorbide *a* is the most plausible mechanism explaining this result. To the best of our

446 knowledge, there is no research related with the identification of transporters implicated in
447 chlorophyll compounds absorption. Only Jonker et al.,[5], indirectly and *in vitro*, identified the
448 breast resistance protein, an intestine ABC transporter, as a reasonable candidate for a pheophorbide
449 *a* efflux-transporter. Nevertheless, we cannot exclude the possibility that an additional mechanism
450 could help in part in the significant *in vivo* accumulation of pheophorbide *a* in the liver, such as the
451 de-esterification of the phytol chain from accumulated pheophytin. Several esterase-type enzymes
452 have been described in liver [35] that may perform this reaction. Indeed, phytol metabolites present
453 in animal tissues are exclusively derived from the phytol side chain of chlorophylls. As a
454 consequence of the consumption of large quantities of grass, ruminant animals contain high
455 amounts of phytol derivatives. In the organism, phytol can be metabolized in the liver to phytenic
456 acid, phytanic acid and pristanic acid [36]. However, the intriguing question is how the phytol
457 reaches the liver. The possibility that pheophytin could be effectively de-esterified in the liver
458 would explain the molecular mechanism of the origin of the phytol present in the liver.
459 Independently of the active mechanism, the *in vivo* accumulation of pheophorbide *a* in the liver is a
460 hint at the existence of a specific mechanism of chlorophyll metabolism in animals.
461 The significant increase of pyro-derivatives in the pool of chlorophyll derivatives accumulated in
462 the livers cannot be explained by enzymatic hydrolysis from their parent chlorophyll derivatives as
463 no decarboxymethylase activity has been described in the liver to date. Gandul-Rojas et al.[17]
464 reported the preferential *in vitro* absorption of pyropheophorbide *a* but not of pyropheophytin *a*.
465 Therefore, it would be interesting to investigate the role of the protein-type transporters involved in
466 the accumulation of pyro-derivatives in future studies. On the other hand, the increase of pyro-
467 derivatives observed in the feces raises further questions. As previously noted, no transformation
468 process of pheophytin or pheophorbide into pyro-derivatives exists, at least in the small intestine.
469 Thus the increased proportion of pyro-derivatives in the feces compared to the chlorophyll profile
470 of the supplemented feed could be attributed to conversion processes specifically available in the

colon. Ferruzzi et al.,[15] showed that intestinal microflora might be involved in the interconversion of chlorophyll derivatives in the gut. Another plausible alternative is the activity of efflux transporters in the colon that excrete the accumulated pyro-derivatives since we observed similar values of pyro-derivatives in the liver and in the feces. The protein-type transporters mentioned above, breast cancer resistance protein and bilitranslocase-like protein could be candidates for the excretion of accumulated pyro-derivatives. In any case, it is important to highlight that a chlorophyll structure similar to pyro-derivatives obtained from the diet can be metabolized and utilized as a visual pigment by the dragon fish [37]. Finally, the interest in pyro-derivative structures has been recently expanded due to their application as photosensitizers in photodynamic therapy (PDT) [38]. Therefore, our results showing the accumulation of these compounds in the liver may point to specific treatment of cancer by PDT in that tissue. Up to now, treatment with photosensitizers is administered intravenously. The results obtained in the present work emphasize the importance of the diet during cancer treatment, as enhancers of a specific therapy or an alternative for photosensitizer administration.

The identification of phytyl-chlorin e_6 in the livers of chlorophyll-supplemented mice introduces a divergence from the results of *in vitro* studies (which lack the portal vein and hepatic system) on the digestion and intestinal absorption of chlorophyll pigments in animals. Thus, it is assumed that the structural modifications and interconversions of chlorophyll derivatives exclusively affect the peripheral positions leaving the macrocycle intact. Phytyl-chlorin e_6 lacks the isocyclic ring (structure C, Figure S1, Supporting Information), while two carboxylic acid groups appear at the C13 and C15¹ positions. The opening of the isocyclic ring to originate chlorins, rhodins or purpurins is traditionally associated with chemical reactions such as saponification [39]. Although the accumulation of this compound in livers was secondary in comparison with the rest of chlorophyll derivatives, its presence indicates the existence of alternative metabolic pathways that modify the macrocycle structure. The detection of the phytylated form of chlorin e_6 points to

pheophytin-type derivatives as its precursors, because pheophorbides are dephytylated structures. Consequently, this kind of metabolic transformation probably increases the polarity of the parent compound, facilitating further metabolism and/or excretion. The fate of these kinds of metabolites is unknown so far, although some information exists regarding the use of chlorin *e*₆ derivatives in photodynamic therapy. For example, several studies related to the development of “second generation” photosensitizers based on the chlorin *e*₆ structure [40] have dealt with their tissue distribution. These studies implied the intravenous administration of chlorin *e*₆ photosensitizers and the posterior localization of these compounds in the liver and other tissues [41]. In addition, the presence of chlorin *e*₆ in the retina of several animals have been shown to enhance the red vision [42-43]. However, our data reveal Table 1) that a chlorin *e*₆ counterpart is accumulated in liver because of the metabolism of parent derivatives through processes that modify the macrocycle, rather than through intravenous administration, an unprecedented result in the literature.

5. Conclusions

Recent discoveries have shown the specific functions of several chlorophyll metabolites of developing in animal tissues, including the production of ATP [44], the regulation of the redox status in plasma [45], implications as visual pigments [37, 42-43], and activation of specific nuclear receptors that could control lipid abnormalities in common diseases such as obesity, diabetes, and hyperlipidemia [46] among others. Evidently, the origin of chlorophyll metabolites present in the animal body is circumscribed to the diet. In humans, the ingestion of chlorophyll derivatives is estimated at around 25-85 mg/day [47], but knowledge on the fate of derivatives in the organism are lacking. Our results show the existence of a first-pass metabolism of chlorophylls. We have demonstrated *in vitro* that the transport of pheophorbide *a* is, at least partly, facilitated by the scavenger receptor SR-BI. Further research is required to elucidate the molecular mechanisms implicated in the absorption and metabolism of chlorophylls in mammals. Our study highlights that apolar chlorophyll derivatives, i.e. those retaining the phytol chain in the structure, are available for

absorption from a dietary source and accumulate in the liver where they can be further metabolized to provide a source of phytol.

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Figure legends

Figure 1: HPLC chromatograms at 660 nm of chlorophyll compounds in fresh *Spirulina* pellets (a) and in dried *Spirulina* pellets (b). Peak numbers as in Table S1, Supporting Information.

Figure 2: HPLC chromatograms at 660 nm of chlorophyll compound in biological samples: liver (a) and feces (b). Inserts correspond to control diets. Peak numbers as in Table S1, Supporting Information.

Author contributions

I.V., K.C., I.B., performed the experiments; I.V., J.R., A.P., M.R. analyzed and interpreted the data; A.P., M.R., conceived the project, designed the study and supervised the research.; I.V., A.P., M.R. wrote the manuscript.

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669 Conflict of interest

670 All the authors declare that they have no conflict of interest.

Table 1: Chlorophyll derivatives identified and quantified in pellets and biological samples of spirulina fed mice (mg/kg d.w. $\pm$ SD). Chlorophylls, pheophorbides, pheophytins and chlorins accounts for the 100% of total chlorophylls. The chemical modification responsible of the pyro-derivatives or allomerized (13^2 -hydroxy and 15^1 -hydroxy-lactone) chlorophylls impacts to pheophorbides and pheophytins amounts (Figure S1, Supporting Information).

Compound	Pellets	Liver 2 weeks	Liver 4 weeks	Feces
Chlorophyllide <i>a</i>	6.90 ± 0.77	-	-	-
15^1 -Hydroxy-lactone pheophorbide	1.16 ± 0.04	0.42 ± 0.03	0.42 ± 0.02	-
13^2 -Hydroxy-pheophorbide <i>a</i>	1.93 ± 0.22	-	-	-
Pheophorbide <i>a</i>	48.52 ± 4.38	1.30 ± 0.16	1.62 ± 0.02	26.22 ± 0.17
Pheophorbide <i>a'</i>	-	0.60 ± 0.02	0.80 ± 0.01	-
13^2 -Hydroxy-pheophorbide <i>a</i>	8.09 ± 0.46	0.44 ± 0.07	0.51 ± 0.08	9.40 ± 0.07
Pyropheophorbide <i>a</i>	33.85 ± 1.79	2.41 ± 2.01	2.86 ± 1.91	77.70 ± 5.42
Pyropheophorbide <i>a'</i>	6.17 ± 0.41	0.47 ± 0.05	0.44 ± 0.05	12.65 ± 0.95
Phytol chlorin <i>e</i> ₆	-	0.30 ± 0.06	0.32 ± 0.01	-
15^1 -Hydroxy-lactone chlorophyll <i>a</i>	6.00 ± 0.75	-	-	-
13^2 -Hydroxy-chlorophyll <i>a</i>	7.38 ± 0.58	-	-	-
Chlorophyll <i>a</i>	233.6 ± 28.78	-	-	3.47 ± 0.01
Chlorophyll <i>a'</i>	21.13 ± 4.39	-	-	-
15^1 -Hydroxy-lactone-pheophytin <i>a</i>	13.31 ± 1.53	1.73 ± 0.38	1.70 ± 0.32	23.82 ± 0.67
15^1 -Hydroxy-lactone-pheophytin <i>a'</i>	-	0.32 ± 0.04	0.30 ± 0.02	-
13^2 -Hydroxy-pheophytin <i>a</i>	16.42 ± 1.87	0.77 ± 0.13	0.79 ± 0.06	26.01 ± 1.75
13^2 -Hydroxy-pheophytin <i>a'</i>	15.08 ± 2.18	0.72 ± 0.08	0.74 ± 0.07	31.22 ± 2.35
Pheophytin <i>a</i>	681.87 ± 99.63	3.82 ± 2.11	3.46 ± 0.84	478.11 ± 26.87
Pheophytin <i>a'</i>	136.41 ± 23.60	1.18 ± 0.46	1.08 ± 0.18	270.89 ± 14.21
Pyropheophytin <i>a</i>	210.58 ± 16.2	5.76 ± 3.17	4.32 ± 2.10	561.16 ± 36.28
Total Chlorophylls	1448.4 ± 187.58	20.24 ± 9.19	19.35 ± 6.67	1520.70 ± 102.64
Chlorophylls (%)	18.98 ± 1.19	-	-	0.22 ± 0.01
Pheophorbides (%)	6.88 ± 0.11	27.86 ± 2.73	34.36 ± 4.57	8.24 ± 0.25
Pheophytins (%)	74.12 ± 2.62	70.65 ± 5.54	64.03 ± 5.30	90.99 ± 4.23
Chlorin (%)	-	1.48 ± 0.17	1.65 ± 0.07	-
Pyro-derivates (%)	17.30 ± 0.60	42.68 ± 7.38	39.37 ± 5.56	42.61 ± 2.13
Allomerized derivatives (%)	4.23 ± 0.06	21.73 ± 0.72	20.36 ± 0.83	5.94 ± 0.27

676 Table 2: Effects of the inhibitor BLT1 on the rate of pheophorbide *a* absorption by differentiated
677 Caco-2 monolayers

Treatment	Absorption rate (pmol/min/mg protein)
Control	2.16±0.68
BLT1 (μM)	Absorption (%)
0.1	56.4±5.02**
1	74.43±3.64*
10	72.66±3.67*

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679 Experiments without treatment of inhibitor were considered as control (100%). Results are means ±
680 S.E.M for three independent experiments. * denotes a significant difference from control, p<0.05
681 and ** p<0.01.

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