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## **Vitamin A Deficiency during the Perinatal Period and First Weeks of Life Modifies Vitamin A and Lipid Postprandial Metabolism in Both Female and Male Young Rats**

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1 **Vitamin A deficiency during the perinatal period and first weeks of life modifies vitamin**  
2 **A and lipid postprandial metabolism in both female and male young rats.**

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15 **Short running head:** Vitamin A deficiency and postprandial metabolism of vitamin A and  
16 lipids

17 Data described in the manuscript will be made available upon request pending application and  
18 approval.

19

20 **Abbreviations:** AUC : Area Under the Curve, GC-C-IRMS : Gas Chromatography-  
21 Combustion-Isotope Ratio Mass Spectrometry, HPLC: High-Performance Liquid  
22 Chromatography, RBP: Retinol Binding Protein.

23

24 Key words: retinol; retinyl esters; [1-<sup>13</sup>C]-oleate; bioavailability; intestine, liver, adipose  
25 tissue.

26 **Abstract**

27

28 **Scope:** We addressed the effect of vitamin A deficiency on vitamin A and lipid postprandial  
29 metabolism in young rats, considering the effect of sex. **Methods and results:** Sprague-  
30 Dawley rats were fed either 400 UI/Kg vitamin A diet (vitamin A-deficient diet: VAD diet) or  
31 2300 UI/Kg vitamin A (control diet), before being mated. Mothers received the same VAD or  
32 control diet during gestation and lactation. Offspring received the same diet than mothers until  
33 8 weeks of age. VAD diet-fed female and male offspring displayed a severe vitamin A  
34 deficiency with no body weight or glucose tolerance defects. Fasting plasma triglyceride  
35 concentrations were decreased in VAD diet-fed animals compared to controls ( $p<0.05$ ).  
36 Retinyl ester postprandial responses after vitamin A gavage, expressed as area under the  
37 curves, were not different in VAD diet-fed and control animals, although retinyl ester  
38 postprandial peak was significantly delayed ( $p<0.05$ ) in VAD diet-fed rats. Lipids also  
39 accumulated in the distal part of the intestine after gavage and [1-<sup>13</sup>C]-oleate postprandial  
40 response was decreased in VAD diet-fed males. **Conclusion:** Vitamin A deficiency modulates  
41 both vitamin A absorption rate and lipid postprandial metabolism, which can partly explain  
42 the altered fasting lipid status observed in VAD diet-fed offspring.

43

44

## 45        **1. Introduction**

46

47 Vitamin A refers to a group of unsaturated organic compounds that includes retinol, retinyl  
48 esters and retinal. In the human diet, vitamin A is found in animal-based foods as retinyl  
49 esters (mainly retinyl palmitate). In plant-based food, it occurs as provitamin A carotenoids  
50 (mainly  $\beta$ -carotene,  $\alpha$ -carotene and  $\beta$ -cryptoxanthin), which can be cleaved and metabolized  
51 into retinol after absorption by the intestinal cells <sup>[1]</sup>.

52 Vitamin A is essential in many physiological processes including embryo development,  
53 immune system differentiation and function, vision and energy metabolism <sup>[2]</sup>.

54 Vitamin A deficiency is a serious and widespread public health problem <sup>[3]</sup>. The requirements  
55 of vitamin A are increased during pregnancy and a daily intake of 800  $\mu$ g retinol equivalents  
56 is recommended as a safe level of vitamin A for pregnant women <sup>[4]</sup>. According to the World  
57 Health Organization (WHO), about 8% of pregnant women in Africa display vitamin A  
58 deficiency symptoms (i.e. night blindness) and about 15% present low serum retinol  
59 concentrations <sup>[5]</sup>. Vitamin A deficiency during pregnancy is associated with increased mother  
60 and infant mortality rates <sup>[6]</sup>. After birth, vitamin A deficiency affects 1 in every 2 children in  
61 sub-Saharan Africa and south Asia <sup>[7]</sup>, and is the leading cause of preventable blindness in  
62 children <sup>[3]</sup>. Vitamin A deficiency also increases susceptibility to infections due to impaired  
63 immune function <sup>[8]</sup>.

64 The transcriptionally active form of vitamin A is retinoic acid. Retinoic acid modulates the  
65 expression of numerous genes via retinoic acid receptor (RAR) and retinoid receptor (RXR)  
66 binding. Among these genes, several play essential roles in energy and lipid metabolism <sup>[9]</sup>.

67 The aim of the present study was thus to characterize the effect of a vitamin A deficiency  
68 during pregnancy, lactation and the first weeks of life on both vitamin A and lipid absorption  
69 and postprandial metabolism after an oral gavage in rats.

## 70        2. Material and methods

### 71        2.1. Animal study (Flowchart, Figure 1)

#### 72        *Animals*

73        All experimental procedures were approved by the ethics committee of Marseille (agreement  
74        number D 13-055-20). Two groups of 10-week-old Sprague Dawley rats (Janvier, Le Genest  
75        St Isle, France, n = 8 males and n = 12 females) were housed under standard conditions of  
76        light (12-h light/dark cycle; lights on at 08h00) and temperature (22-24°C) with free access to  
77        tap water and breeding pellet diet. Diet was supplemented either with 2300 IU/kg of vitamin  
78        A (control diet) or 400 IU/kg vitamin A (vitamin A-deficient [VAD] diet). These diets were  
79        developed by Test Diet Limited (London, UK). After 8 weeks virgin females were mated. In  
80        order to avoid a diet-induced post-natal programming <sup>[10]</sup> litters were culled to 10-12 pups at  
81        postnatal day 3 (P3). Rats were weaned at day 21 and fed as follows:

82        - offspring rats from control breeders were fed control diet (control group, n = 14 males and  
83        13 females)

84        - offspring rats from VAD diet-fed breeders were fed VAD diet (VAD diet-fed group, n = 12  
85        males and 11 females).

86        At week 6, young rats were weighted and an intraperitoneal glucose tolerance test was  
87        performed at week 7 (1.5 g glucose/ kg) as previously published <sup>[10,11]</sup>

#### 88        *Organ collection at fast*

89        At week 8, a group of offspring rats (control group, n = 7 males and 6 females and VAD diet-  
90        fed group n = 6 males and 5 females) were fasted overnight before a plasma sample was taken  
91        at the tail under light isoflurane anesthesia. Animals were then euthanized, and organs  
92        including intestine, liver, white (omental) and brown adipose tissues were promptly harvested.  
93        Intestines were carefully rinsed with ice-cold PBS before being cut in fragments. All samples  
94        were stored in liquid nitrogen until lipid/vitamin extraction.

## 95 *Postprandial experiments*

96 At week 8, another group of offspring rats (control group, n = 7 males and 7 females and  
97 VAD diet-fed group n = 6 males and 6 females) were fasted overnight and each animal was  
98 forced-fed with 1 mL of an oily emulsion (triolein:water, 1:2, v:v) containing 3 mg of retinyl  
99 palmitate and 2 mg of [1,1,1- <sup>13</sup>C<sub>3</sub>]-triolein (Sigma Aldrich, Saint-Quentin-Fallavier, France).  
100 The mixture was vigorously mixed in ice-cold water in a sonication bath (Branson 3510,  
101 Branson, Danbury, CO, USA) for 15 min and used for force-feeding within 10 min. Blood  
102 sample were obtained before force feeding and every 90 min for 6 h from a tail nick under  
103 light isoflurane anesthesia and collected into tubes containing 50 µL of a 0.109 M sodium  
104 citrate solution. Blood was immediately centrifuged at 1400 g for 20 min at 4°C and the  
105 resulting plasma was immediately frozen at – 80°C.

106 At t = 6 h , animals were euthanized, and their intestine and organs (liver, white and brown  
107 adipose tissues) were promptly harvested. Intestines were carefully rinsed with ice-cold PBS  
108 before being cut in different fragments. All samples were stored in liquid nitrogen until  
109 lipid/vitamin extraction.

110

## 111 **2.2. Tissue lipid content**

112 Tissue samples (~100 mg) obtained at fast were homogenized in 800 µL of PBS with two 3  
113 mm-diameter stainless steel balls in 2 ml Eppendorf tubes using a MM301 ball mill (Retsch,  
114 Haan, Germany). Lipids were first extracted following the Bligh and Dyer method <sup>[12]</sup>, and  
115 samples were resuspended in isopropanol. Total cholesterol, phospholipid and triglyceride  
116 plasma and tissue concentrations were then assessed in triplicate using kits from Biolabo  
117 (Maisy, France) according to the manufacturer's instructions.

118

### 119 2.3. Vitamin A extraction and analysis

120 Vitamin A (in the form of retinol or retinyl esters) from plasma (100  $\mu$ L) and organ samples  
121 (~ 20 mg for the liver and 40 mg for the intestine in suspension in PBS) were extracted from  
122 aqueous phases as follows (when necessary, distilled water was added to samples to reach a  
123 final volume of 500  $\mu$ L): internal standard (5-apo-8'-carotenal) was added to the samples in  
124 500  $\mu$ L of ethanol, and the mixture was extracted twice with two volume of hexane. The  
125 hexane phase obtained after centrifugation (1200 x g, 10 min, 4 °C) was evaporated to  
126 dryness under nitrogen. All dried extracts were dissolved in 200  $\mu$ L acetonitrile:  
127 dichloromethane: methanol (70:20:10; v:v:v). A volume of 50–180  $\mu$ L was used for HPLC  
128 analysis.

129 All the HPLC analyses were realized using a 250 x 4.6 nm RP C18, 5  $\mu$ m Zorbax column  
130 (Agilent) maintained at a constant temperature (35°C) and a guard column. Analysis was  
131 performed with a 70% acetonitrile, 20% dichloromethane and 10% methanol mobile phase  
132 (flow rate = 1.8 mL/min). The HPLC system comprised a Shimadzu separation module (LC-  
133 20ADSP HPLC Pumps and SIL-20CHT Autosampler; Shimadzu, Champs-sur-Marne,  
134 France), and a SPD-M20A Shimadzu photodiode array detector (PDA, vitamin A detection at  
135 325 nm and 5-apo-8'-carotenal detection at 450 nm). Retinol and retinyl palmitate were  
136 identified by retention time compared with pure (> 95%) standards. Quantification was  
137 performed using Chromeleon software (version 6.8) comparing peak area with standard  
138 reference curves. Retinyl linoleate, retinyl oleate, retinyl stearate were identified by retention  
139 time and spectral analysis and quantified regarding their molecular extinction coefficient ratio  
140 compared to retinyl palmitate.

141 Vitamin A postprandial response was expressed as area under the curve (AUC) of retinyl ester  
142 plasma concentrations.

143

144 **2.4. [1-<sup>13</sup>C]-oleate analysis by Gas Chromatography-Combustion-Isotope Ratio Mass**  
145 **Spectrometry (GC-C-IRMS)**

146 Glyceryl triheptadecanoate (C17:0, Sigma Aldrich) was added to the samples as an internal  
147 standard to quantify the oleic acid occurring in total lipids of plasma (250 ng of C17:0 /  $\mu$ L)  
148 and tissues (5  $\mu$ g for liver and 5  $\mu$ g for duodenum, 0.5 to 12  $\mu$ g for ileum and 5 to 11  $\mu$ g / mg  
149 of tissue for jejunum).

150 Plasma samples (50  $\mu$ L) were submitted to direct methylation as described previously <sup>[13]</sup>.

151 Briefly, plasma was dissolved in 2 mL of a solvent mixture of methanol:hexane (4:1, v:v) and  
152 200  $\mu$ L of acetyl chloride were added. The tubes were tightly closed and subjected to  
153 methanolysis at 100 °C for 1 h. After cooling the tubes in ice, 640  $\mu$ L of a solution of K<sub>2</sub>CO<sub>3</sub>  
154 11% solution was added and the phases were mixed by vortexing. Then 1.25 mL of hexane  
155 were added and after separation the organic layer was isolated. The Fatty Acid Methyl Esters  
156 (FAMES) of the total lipids were then dried and reconstituted in hexane.

157 Aliquots (50 to 300 mg) of homogenized different tissue samples (duodenum, jejunum, ileum,  
158 liver, adipose tissues) were weighed exactly before proceeding to the total lipid extraction  
159 using 6 to 7 mL of a mixture of chloroform:methanol (2:1, v:v) according to the Folch method  
160 <sup>[14]</sup>. Particular attention was paid to maintaining the ratio of 20 mL CHCl<sub>3</sub>:MeOH (2:1, v:v)  
161 /mg of tissue. Sometimes it was necessary to add PBS to keep a ratio of 0.2 between aqueous  
162 phase and CHCl<sub>3</sub>: MeOH solvent phase. The total lipids were then submitted after drying to  
163 direct methylation as for the plasma samples.

164 The amounts and isotopic enrichments of oleic acid in plasma, and in different tissues were  
165 determined using GC-C-IRMS (Isoprime Ltd; Elementar) <sup>[13]</sup>. The capillary column was a  
166 SP2380, 30 m  $\times$  0.25 mm  $\times$  0.20  $\mu$ m film thickness (Supelco, Sigma-Aldrich). Injection (1  $\mu$ L)



167 was performed in splitless mode at 240 °C, with a splitless time of 1 min. The FAMES were  
168 separated at constant flow (1.2 mL.min<sup>-1</sup>) with the following oven program: (a) 50°C for 1  
169 min; (b) increase at a rate of 11°C.min<sup>-1</sup> up to 175°C and hold for 5.64 min; (c) increase at a  
170 rate of 5°C.min<sup>-1</sup> up to 220°C. <sup>13</sup>C isotopic enrichment was expressed in δ ‰ against Pee Dee  
171 Belemnite <sup>13</sup>C international standard (PDB).

$$\delta^{13}\text{C}_{\text{sample}} = \left( \frac{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{sample}}}{\left( \frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{PDB}}} - 1 \right) \times 1000$$

172  
173 [1-<sup>13</sup>C]-oleate postprandial response was expressed as area under the curve (AUC) of [1-<sup>13</sup>C]-  
174 oleate plasma concentrations.

175

## 176 **2.5.Statistical analysis**

177 Results are expressed as means ± SEM (standard error of the mean). The trapezoidal  
178 approximation method was used to calculate the AUC of the postprandial plasma retinyl ester  
179 and lipid concentration over 6 h. Differences in the postprandial concentrations over time  
180 were tested by repeated-measures ANOVA. Differences between only two groups were  
181 analysed by Student's t test. Values of p < 0.05 were considered significant. All statistical  
182 analyses were performed using Prism (GraphPad Prism version 8.4.3).

183

### 184 3. Results

185

#### 186 3.1. Characteristics of rat offspring

187 VAD diet-fed young rats display a body weight non-significantly different from that of  
188 control young rats (**Figure 2A and 2E**). However, heart and liver were smaller ( $p < 0.05$ ) in  
189 VAD diet-fed females than in control females, while kidney and heart were smaller ( $p < 0.05$ )  
190 in VAD diet-fed males than in control males (**Table 1**).

191 In VAD diet-fed rats, fasting glycemia was lower than in control animals ( $p < 0.05$ ; **Figures 2B**  
192 **and 2F**), while glucose tolerance was improved, especially in male rats ( $p < 0.05$ ; **Figures 2C-**  
193 **D and 2G-H**). However, when expressed as delta value with fasting glycemia, glycemia AUC  
194 was significantly lower in VAD-diet-fed males compared to control males only ( $49.2 \pm 8.1$   
195  $\text{mg}\cdot\text{h}/\text{dL}$  vs  $52.7 \pm 9.5 \text{ mg}\cdot\text{h}/\text{dL}$  in control females vs VAD diet-fed females;  $51.5 \pm 6.8$   
196  $\text{mg}\cdot\text{h}/\text{dL}$  vs  $22.1 \pm 9.4 \text{ mg}\cdot\text{h}/\text{dL}$  in control males vs VAD diet-fed males,  $p < 0.05$ ; graphs not  
197 shown).

198

#### 199 3.2. Rat offspring from VAD diet-fed group displays vitamin A deficiency

200 Both female and male young rats from VAD diet-fed group showed a significant decrease in  
201 fasting plasma retinol concentrations compared to control animals ( $-67 \pm 1.8\%$  and  $-77 \pm$   
202  $2.8\%$ , in females and males,  $p < 0.05$ , respectively; **Figure 3A and 3C**).

203 Additionally, both female and male young rats from VAD diet-fed group exhibited almost no  
204 vitamin A stock in the form of retinyl esters at the hepatic level (**Figure 3B and 3D**).

205 Interestingly, in control animals, retinolemia was significantly lower in female than in male  
206 young rats ( $-47 \pm 1.2\%$ ,  $p < 0.05$ ), while retinyl ester content was higher in female liver than  
207 in male liver ( $+156\% \pm 19.8\%$ ,  $p < 0.05$ ).

208

### 209 **3.3. Rat offspring from VAD diet-fed group displays lipid abnormalities at fast**

210 Female young rats from VAD diet-fed group had lower plasma triglyceride, phospholipid and  
211 cholesterol concentrations than control animals ( $-26 \pm 5.4\%$ ,  $-18 \pm 2.6\%$  and  $-31 \pm 3.6\%$ ,  
212  $p < 0.05$ , respectively; **Figure 4A, 4B and 4C**). Male young rats from VAD diet-fed group had  
213 lower plasma triglyceride concentrations than control animals ( $-34 \pm 5.6\%$ ,  $p < 0.05$ ; **Figure**  
214 **4G**). Male fasting plasma phospholipid and cholesterol concentrations remained unchanged  
215 (**Figure 4H,4I**).

216 Liver triglyceride concentration was reduced in VAD diet-fed female young rats compared to  
217 control females ( $-25 \pm 18\%$ ; **Figure 4D**), but the difference remained non-significant. Female  
218 liver phospholipid and cholesterol concentrations remained unchanged (**Figure 4E, 4F**). Male  
219 young rats from VAD diet-fed group displayed a non-significant ( $p = 0.10$ ) lower triglyceride  
220 concentration compared to control animals ( $-57 \pm 17\%$ ; **Figure 4J**), while phospholipid  
221 concentration was significantly higher ( $+63 \pm 10\%$ ,  $p < 0.05$ ; **Figure 4K**). Male cholesterol  
222 concentration was not modified (**Figure 4L**).

223

### 224 **3.4. Postprandial retinyl ester kinetics of appearance in plasma is modified in VAD diet-** 225 **fed young rats compared to controls**

226 Dietary vitamin A is absorbed by the enterocytes in its free form. Once absorbed, vitamin A is  
227 re-esterified and secreted to bloodstream packed into chylomicrons. Postprandial plasma

228 retinyl ester concentrations after an oral vitamin A load were lower in both VAD diet-fed  
229 male and female young rats compared to controls during the first 3 h ( $-48 \pm 7.9\%$  in females  
230 at  $t=1h30$ ;  $-24 \pm 12.2\%$  in females at 3h;  $-55 \pm 6.8\%$  in males at 1h30,  $-49 \pm 2.4\%$  in males at  
231 3h), although these differences remained non-significant due to interindividual variability.  
232 Then, the postprandial plasma concentrations in retinyl esters increased in VAD diet-fed  
233 young rats compared with control animals 4h30 after gavage ( $+192 \pm 144\%$  in females and  
234  $+158 \pm 40\%$  in males ( $p<0.05$ ), respectively) (**Figure 5A and 5E**). Retinyl ester peak was  
235 delayed in both VAD diet-fed groups (maximum peak at 4h30 in VAD diet-fed groups vs 1.5-  
236 3h in control groups). Overall, no difference in total dietary vitamin A absorption, expressed  
237 as area under the postprandial plasma concentration of retinyl esters, between young rats from  
238 VAD diet-fed group compared to control animals was observed (**Figure 5B and 5F**).

239 After being released in the bloodstream, retinyl esters are taken up by the liver, where they  
240 can be either stored or secreted back to the bloodstream in the form of free-retinol. Retinol  
241 postprandial plasma concentrations were significantly increased in both VAD diet-fed female  
242 and male young rats compared with control animals 4h30 after gavage ( $+116 \pm 54\%$  and  $+303$   
243  $\pm 39\%$ ,  $p<0.05$ , respectively) (**Figure 5C and 5G**), leading to an increase in retinol  
244 postprandial response (expressed as a delta value with fasting retinol concentration, **Figure**  
245 **5D and 5H**).

246 In the ileum, decreases in retinyl ester and total vitamin A concentrations were observed in  
247 VAD diet-fed females compared to controls ( $-70 \pm 8.4\%$  for retinyl esters,  $p<0.05$ ), while  
248 increases in retinyl esters and retinol were observed in VAD diet-fed males compared to  
249 controls ( $+81 \pm 76\%$  for retinyl esters,  $p<0.05$ ; **Figure 6A and 6B**).

250

251 **3.5. VAD diet-fed young rats accumulate lipids in their intestine during the postprandial**  
252 **period compared to controls**

253 Table 2 showed that VAD diet-fed animals display significantly higher lipid concentrations in  
254 their intestines at fast than control animals:  $+474 \pm 32.0$  % ileal triglycerides,  $+182 \pm 37.0$  %  
255 duodenal cholesterol,  $282 \pm 65.2$ % ileal cholesterol,  $+114 \pm 18.4$  % ileal phospholipids in  
256 females;  $+144 \pm 43.9$  % of duodenal triglycerides,  $+243 \pm 21.1$  % jejunal triglycerides in  
257 males ( $p < 0.05$ ). VAD diet-fed males also displayed a significant decrease in duodenal  
258 phospholipids concentration compared to controls ( $-49 \pm 3.4$ %, ( $p < 0.05$ ).

259 This observation was exacerbated during the postprandial period for triglycerides in males.  
260 Indeed, **Figure 7** shows that VAD diet-fed animals also accumulated lipids in their intestine  
261 during the postprandial period, compared to control animals:  $+88 \pm 8.3$ % of ileal triglycerides  
262 (**Figure 7A**),  $+152 \pm 35$ % ileal cholesterol (**Figure 7B**) and  $+73 \pm 7$ % ileal phospholipids in  
263 females (**Figure 7C**);  $+162 \pm 31$ % of jejunal and  $+3614 \pm 558$ % of ileal triglycerides (**Figure**  
264 **7D**),  $+70 \pm 16$ % jejunal and  $+109 \pm 11$ % ileal phospholipids in males (**Figure 7F**) ( $p < 0.05$ ).

265

266 **3.6. [1-<sup>13</sup>C]-oleate postprandial response is decreased in VAD diet-fed male young rats**  
267 **compared to controls**

268 As triglyceride postprandial accumulation was higher in male intestines than in female  
269 intestines (Figures 7A and 7D), [1-<sup>13</sup>C]-oleate postprandial metabolism was further explored  
270 in male offspring. For both VAD diet-fed young males and control males, the maximum  
271 absorption peak of [1-<sup>13</sup>C]-oleate was at 3h post-ingestion of the emulsion containing the  
272 labelled triacylglycerol [1,1,1-<sup>13</sup>C]-triolein (**Figure 8A**). However, the postprandial plasma  
273 response in [1-<sup>13</sup>C]-oleate was decreased in VAD diet-fed young male rats between 0-6h:  $-27$   
274  $\pm 4.7$ %,  $p < 0.05$ , **Figure 8B**). Concomitantly, [1-<sup>13</sup>C]-oleate concentration in the ileum was

275 higher in VAD diet-fed young rats than in control animals ( $+428 \pm 215\%$ ,  $p < 0.05$ ; **Figure**  
276 **8C**).

277 Interestingly,  $[1-^{13}\text{C}]$ -oleate concentrations in the liver and in the white adipose tissues at  $t =$   
278 6h post gavage were also higher in VAD diet-fed young rats than in control animals (**Figure**  
279 **8D and 8E**). Conversely,  $[1-^{13}\text{C}]$ -oleate concentrations in the brown adipose tissues at  $t = 6\text{h}$   
280 post gavage remained unchanged (**Figure 8F**).

281

## 282 Discussion

283

284 Our aim was to study the effect of vitamin A deficiency during pregnancy, lactation and the  
285 first weeks of life in young rats on vitamin A and lipid postprandial metabolism. This period  
286 corresponds to the perinatal and childhood/beginning of adolescence period in humans <sup>[15]</sup>,  
287 which is critical for both a healthy development and a future healthy life <sup>[16]</sup>.

288 Real vitamin A deficiency is usually difficult to reach when performing animal study. Indeed,  
289 vitamin A is stored in high amounts in rodent liver <sup>[17]</sup>, which allows the organism to go  
290 through mid- to long-term vitamin A deprivation without phenotypic incidence. In this study,  
291 we fed rats with either a VAD diet or a control diet 6 weeks before mating them. The females  
292 also received these diets during the gestation/ lactation period. Vitamin A amount in the  
293 deficient diet was chosen according to previous work performed in rats <sup>[18]</sup>, and can be  
294 considered as moderate compared to other studies <sup>[19]</sup>. As expected, the offspring born from  
295 mothers fed the VAD diet showed a significant vitamin A deficiency proven by the lack of  
296 retinyl esters in the liver associated with a low retinolemia as compared to control rats. It is  
297 noteworthy that liver was almost totally deprived of vitamin A, likely to maintain the  
298 circulating vitamin A concentration as high as possible. Interestingly, retinolemia was lower  
299 in female than in male young rats, which is in agreement with data obtained in humans <sup>[20]</sup>. As  
300 females stored higher amounts of vitamin A in their liver compared to males, this may be a  
301 different regulation of hepatic vitamin A mobilization between females and males.

302 Food intake was not monitored in this study, but no major body weight or brain weight  
303 alterations were measured in offspring. Liver and/or kidney and/or heart could be smaller in  
304 the VAD diet-fed groups, as previously observed <sup>[21]</sup>. The reduction in fasting glycemia and  
305 increase in glucose tolerance in both female and male VAD diet-fed rats indicated an

306 improvement of glucose homeostasis. We hypothesize that the VAD diet-induced decrease in  
307 triglyceride concentrations in plasma and liver (see below) explains this improved glucose  
308 homeostasis. Indeed, excess of circulating triglycerides is known to result in their  
309 accumulation in peripheral tissues, including liver and skeletal muscle. Both tissues are  
310 implicated in glucose homeostasis and an increase in triglyceride concentration in these  
311 tissues renders them less sensitive to insulin, which results in an increased production of  
312 glucose by the liver and a reduced uptake of glucose by muscle, both contributing to a  
313 deterioration of glucose homeostasis <sup>[22]</sup>. In our study, the decrease in fasting triglyceride  
314 concentrations in plasma and liver (and possibly also in muscle) in VAD diet-fed rats  
315 probably led to an improvement of insulin action. However, this improvement is likely  
316 transient, as adult VAD diet-fed rodents have been reported to display glucose homeostasis  
317 impairment <sup>[23]</sup> together with increased adiposity <sup>[24]</sup> compared with control animals.

318 Both VAD diet-fed female and male young rats displayed plasma lipid abnormalities  
319 compared to control animals. In particular, fasting plasma triglycerides were lower in VAD  
320 diet-fed animals compared to controls. Fasting cholesterol and phospholipid levels in plasma  
321 of VAD diet-fed females were also lower than in control females. These data are consistent  
322 with a recent study showing that retinoic acid reduced hepatic lipoprotein secretion <sup>[25]</sup>. These  
323 data also confirm an alteration of lipid metabolism in rats as a symptom of vitamin A  
324 deficiency, in agreement with previous published data <sup>[26,27]</sup>. In particular, Oliveros and  
325 colleagues showed that vitamin A deficiency induced a hypolipidemic effect by decreasing  
326 both serum triglyceride and cholesterol levels in male rats <sup>[27]</sup>. Authors also observed a  
327 significant decrease in phospholipid hepatic contents, while our results highlighted an  
328 increase in total phospholipid contents. Further investigations are needed to explain this  
329 discrepancy. However, authors showed that the *de novo* synthesis of fatty acids was decreased  
330 in the liver of VAD diet-fed rats compared to controls, which may explain the non-significant



331 - but relatively important - decrease in hepatic triglyceride contents observed in our study (-  
332 25% in females and -57% in males).

333

334 Previous studies have evaluated the effect of vitamin A status on provitamin A carotenoid  
335 intestinal absorption and cleavage efficiency <sup>[28-30]</sup>. However, there was no study dedicated to  
336 assessing whether vitamin A status also modulates the intestinal absorption efficiency of  
337 preformed vitamin A so far. We thus explored the ability of the young rats to absorb retinyl  
338 palmitate from an oil-in-water emulsion.

339 After ingestion, retinyl palmitate is hydrolyzed into retinol in the lumen under the action of  
340 pancreatic lipases. Retinol is incorporated into mixed micelles to be taken up by enterocytes.  
341 Once in the cytosol of the intestinal cells, the majority of the retinol is re-esterified into retinyl  
342 palmitate, retinyl oleate, retinyl stearate and retinyl linoleate. These esters are packed into  
343 chylomicrons and then secreted in the bloodstream (see for review <sup>[1]</sup>). Finally, these esters  
344 are taken up by the tissues, and mainly by the liver. The liver then secreted retinol back to the  
345 bloodstream associated with its carrier proteins RBP (retinol binding protein) and  
346 transthyretin <sup>[31]</sup>. Postprandial retinyl ester response, i.e. AUC, to a vitamin A load thus  
347 constitutes a good marker of vitamin A secretion to the blood. Overall, postprandial plasma  
348 retinyl ester responses did not differ between VAD diet-fed and control groups. However, a  
349 close look at the plasma retinyl ester kinetics showed marked differences between VAD diet-  
350 fed and control animals. Indeed, in both females and males, plasma retinyl ester  
351 concentrations were lower during the first 3 h after force-feeding, and a sudden increase of  
352 retinyl esters occurred at 4.5 h, compensating the initial reduced concentrations. The  
353 mechanism underlying such compensation is not known but may be linked to either a delay  
354 of lipid intestinal absorption/secretion, or a modification of retinyl ester clearance (see next  
355 paragraph). Interestingly, our data also highlight an increase in postprandial plasma retinol

356 concentration in the VAD diet-fed groups compared to controls at 4.5 h post force-feeding.  
357 Such increase in retinolemia after an oral charge in vitamin A is expected in VAD diet-fed  
358 individuals. It actually corresponds to the relative dose response (RDR) test that was  
359 developed to evaluate vitamin A status in humans suspected to be vitamin A deficient. Serum  
360 retinol concentrations are determined before and 5 hours after an oral dose of vitamin A. In  
361 VAD diet-fed adults and children, the retinolemia is expected to increase significantly at 5 h  
362 as compared to the fasting value due to the mobilization of a stock of apo-RBP accumulated  
363 in the liver <sup>[32,33]</sup>. Our results are consistent with these data, and further highlight that our  
364 VAD diet-fed groups present a real vitamin A deficiency. Besides this release in hepatic RBP,  
365 we suggest that another unknown intestinal mechanism leading to a concomitant release of  
366 retinyl esters also occur, leading to a simultaneous peak of retinyl esters.

367 At the intestinal level, our data showed a decrease of vitamin A in female ileum, while  
368 vitamin A ileal content was increased in males. This difference may be due to difference in  
369 either the rate or the efficiency of vitamin A secretion to the bloodstream. The effect of sex on  
370 chylomicron secretion rate has not been specifically assessed yet. However, it is  
371 acknowledged that vitamin A response is often lower in women than in men, due to a more  
372 efficient chylomicron-remnant clearance <sup>[34]</sup>.

373 Interestingly, lipids (triglycerides and cholesterol for females, triglycerides only for males)  
374 were shown to accumulate in the distal intestine of VAD diet-fed animals compared to control  
375 during the postprandial period. This was confirmed by the analysis of the fatty acid [1-<sup>13</sup>C]-  
376 oleate coming from the triacylglycerol [1,1,1-<sup>13</sup>C] triolein incorporated into the oil-in-water  
377 emulsion used for the force-feeding. Indeed, [1-<sup>13</sup>C] oleate also accumulated in the ileum at  
378 6h in male intestine. Such accumulation can be either due to an increased absorption by ileal  
379 enterocytes, or due to a defect in secretion from ileal enterocytes to the bloodstream. This  
380 accumulation of [1-<sup>13</sup>C]-oleate was accompanied by a decrease in [1-<sup>13</sup>C]-oleate postprandial

381 response in the VAD diet-fed group compared to the control group. A first hypothesis is thus  
382 that VAD-diet-fed animals display abnormal lipid secretion to the bloodstream compared to  
383 control animal. This may explain the alteration of plasma and hepatic lipids measured at fast  
384 in the VAD groups. It may also explain the decrease of retinyl ester appearance in plasma  
385 during the initial phase of the postprandial period. However, as the intestine is able to store  
386 fat-soluble compounds from a first meal to secrete them to the bloodstream following a  
387 subsequent meal <sup>[35]</sup>, further investigations are needed to assess the fate of both vitamin A and  
388 lipids accumulated in VAD diet-fed rat intestine. A second hypothesis that could explain the  
389 decrease lipid postprandial response in VAD diet-fed rats is that these animals display  
390 increased lipid clearance during the postprandial period. To this aim, we measured [1-<sup>13</sup>C]-  
391 oleate accumulation in the liver, white and brown adipose tissues of male young rats. The  
392 uptake of [1-<sup>13</sup>C]-oleate by the liver and the white adipose tissue was significantly increased  
393 in VAD diet-fed rats compared to controls. The reduced postprandial oleate response in VAD  
394 animals can thus also be due to an increased fatty acid uptake by some peripheric tissues, in  
395 relation with an increased chylomicron clearance.

396 The molecular mechanisms underlying such modulation of lipid postprandial metabolism are  
397 not known yet. However, several hypotheses can be done. First, the effect of vitamin A  
398 deficiency, via retinoid signaling, on intestinal lipid transporter such as SR-BI has previously  
399 been described <sup>[29]</sup>, although lipid intestinal absorption was not fully characterized in this  
400 work. The authors showed that i) retinoic acid via retinoic acid receptors induced expression  
401 of the intestinal transcription factor ISX and ii) ISX then repressed the expression of SR-BI.  
402 In another study, Vega and colleagues explored the effect of dietary vitamin A deprivation on  
403 lipid composition and mRNA expression of regulatory enzymes involved in rat heart  
404 energetic lipid metabolism. Authors showed that lipid metabolism was significantly modified  
405 in heart of vitamin A-deficient rats, and that alteration of mitochondrial energetic processes

406 was associated with a high PPAR $\alpha$  and  $\beta$  expression induced by vitamin A deprivation <sup>[36]</sup>. At  
407 the intestinal level, the activation of PPAR $\alpha$  has been associated with a decrease in  
408 hyperlipidemia in mice <sup>[37]</sup>, and more specifically with a decrease in cholesterol absorption in  
409 mice <sup>[38]</sup> and a decrease in lipid uptake and secretion to the basolateral medium in Caco-2  
410 cells <sup>[39]</sup>. Intestinal PPAR $\alpha$  activation in our VAD diet-fed group may have contributed to the  
411 decrease in lipid absorption in these animals compared to controls.

412 Finally, another study have showed that vitamin A deficiency altered host and gut microbial  
413 metabolism leading to altered energy homeostasis in mice <sup>[40]</sup>. As modifications of gut  
414 microbiota are likely linked to modulations of intestinal lipid metabolism <sup>[41]</sup>, vitamin A  
415 deficiency may have indirectly impacted lipid absorption in the VAD diet-fed groups.  
416 Possible interactions between lipid and vitamin A postprandial metabolism should be  
417 addressed in a specific postprandial study, using a control condition with an oil-in-water  
418 emulsion without retinyl palmitate.

419

420 Overall, we showed for the first time that in rats, vitamin A deficiency delays the peak of  
421 retinyl esters in plasma after an oral vitamin A load, although total vitamin A postprandial  
422 response remains unchanged compared to control animals. We also showed that vitamin A  
423 deficiency modulates lipid postprandial metabolism, which can partly explain the altered lipid  
424 status in the plasma and the liver of VAD diet-fed rats observed at fast. Further research is  
425 needed to evaluate whether such modifications in vitamin A and lipid postprandial  
426 metabolism are due to differential gene/protein regulations and/or to gut microbiota  
427 modulation. Furthermore, in connection to vitamin A deficiency, the impact of  
428 supplementation studies is currently under investigation in our laboratory.

429

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436

437 **Author's contribution**

438 ER, PB and MG designed the study. PB acquired funding. MG and PG conducted the study.

439 ER, CH, MN and TG performed tissue sampling. TA, CH and MN performed vitamin A and  
440 lipid analyses. LM and VS performed [1-<sup>13</sup>C]-oleate analysis. TA did the statistical analyses.

441 ER and TA interpreted the data with PB and RG; ER and TA drafted the manuscript; all

442 authors have read and approved the final manuscript; ER has primary responsibility for the

443 final content of the manuscript.

444

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**Table****Table 1. Organ weights**

	Diet	n	Brain (g)	Liver (g)	Kidney (g)	Heart (g)
Females	Control	7	1.88 ± 0.03	8.19 ± 0.26	1.05 ± 0.07	0.93 ± 0.02
	VAD	6	1.89 ± 0.02	7.06 ± 0.21*	0.90 ± 0.03	0.86 ± 0.02*
Males	Control	6	1.96 ± 0.02	10.66 ± 0.79	1.25 ± 0.03	1.24 ± 0.03
	VAD	5	1.95 ± 0.03	8.84 ± 0.24	0.99 ± 0.02*	1.00 ± 0.04*

n= 5 to 6.

**Table 2. Fasting lipid concentrations in the intestine (mg/ g of tissue)**

		Diet	Duodenum	Jejunum	Ileum
Triglycerides	Females	Control	4.50 ± 1.35	13.42 ± 4.76	3.14 ± 2.67
		VAD	5.26 ± 1.46	19.75 ± 3.28	<b>18.03 ± 1.00 *</b>
	Males	Control	7.81 ± 1.22	8.33 ± 2.62	15.00 ± 8.36
		VAD	<b>19.07 ± 3.43 *</b>	<b>28.61 ± 1.76 *</b>	24.15 ± 7.73
Cholesterol	Females	Control	0.62 ± 0.081	1.04 ± 0.36	0.32 ± 0.05
		VAD	<b>1.74 ± 0.23 *</b>	1.41 ± 0.13	<b>1.22 ± 0.21 *</b>
	Males	Control	3.48 ± 1.04	2.82 ± 0.84	3.22 ± 0.38
		VAD	1.74 ± 0.33	3.05 ± 0.31	3.70 ± 0.50
Phospholipids	Females	Control	0.56 ± 0.14	0.85 ± 0.12	0.33 ± 0.03
		VAD	0.93 ± 0.037	0.92 ± 0.14	<b>0.70 ± 0.06 *</b>
	Males	Control	7.04 ± 0.43	3.78 ± 0.84	2.64 ± 0.41
		VAD	<b>3.57 ± 0.24 *</b>	3.65 ± 0.15	2.66 ± 30.31

n= 6 to 7.

An asterisk indicates a significant difference between the VAD and the control group ( $p < 0.05$ ).

## Figure legends

### Figure 1: Study flowchart

### Figure 2: Young rat characteristics

Offspring from control breeders were fed control diet (white symbols/bars) and offspring from VAD diet-fed breeders were fed VAD diet (black symbols/bars).

Young female control and VAD diet-fed rats (6-week-old) were weighted (A) and fasting glycemia was measured (B) before rats were subjected to an intraperitoneal glucose tolerance test (GTT) (C). The area under the curve (AUC) of this GTT was determined (D). Young male control and VAD diet-fed rats (6-week-old) were also weighted (E) and analyzed for fasting glycemia (F), GTT (G) and AUC of the GTT (H). Data represent mean  $\pm$  SEM for control rats (females n = 13; males n = 14) and VAD diet-fed rats (females n = 11; males n = 12).

A difference between control and VAD diet of  $p < 0.05$  was considered significant.

### Figure 3: Young rat vitamin A concentrations in plasma and liver at fast

Plasma sample was taken at fast at the tail of each young rat. After euthanasia, liver was promptly harvested and stored in liquid nitrogen. Retinol and retinyl esters were extracted with two volumes of hexane and analyzed by HPLC.

**A, B:** Females. **C, D:** Males.

Data are expressed as mean  $\pm$  SEM (control rats: white bars, VAD diet-fed rats: black bars).

A difference between control and VAD diet of  $p < 0.05$  was considered significant. ND = Not detectable.

### Figure 4: Young rat lipid concentrations in plasma and liver at fast

Plasma sample was taken at the tail of each young rat fasted overnight. After euthanasia, liver was promptly harvested and stored in liquid nitrogen. Total cholesterol, phospholipid and triglyceride plasma and tissue content were then assessed.

**A-F:** Female rats. **G-L:** Male rats.

Data are expressed as mean  $\pm$  SEM (control rats: white bars, VAD diet-fed rats: black bars).

A difference between control and VAD diet of  $p < 0.05$  was considered significant.

### **Figure 5: Postprandial plasma vitamin A responses in young rats**

Young rats were forced-fed with an oily emulsion containing retinyl palmitate and  $^{13}\text{C}$ -triolein. Blood samples were obtained before force feeding and every 90 min from a tail nick. Retinol and retinyl esters were extracted with two volumes of hexane and analyzed by HPLC.

**A, E:** Postprandial plasma retinyl ester concentrations in females and males, respectively.

**B, F:** Postprandial plasma retinyl ester response expressed as area under the curve (AUC), in females and males, respectively.

**C, G:** Postprandial plasma retinol concentrations in females and males, respectively.

**C, G:** Postprandial plasma retinol response, i.e. area under the curve (AUC) expressed as a delta value with fasting retinol concentration, in females and males, respectively.

Data are expressed as mean  $\pm$  SEM (control rats: white bars, VAD diet-fed rats: black bars).

A difference between control and VAD diet of  $p < 0.05$  was considered significant.

### **Figure 6: Vitamin A concentrations in the intestinal mucosa at the end of the postprandial experiment**

After a force-feeding with an oily emulsion containing retinyl palmitate and  $^{13}\text{C}$ -triolein, young rats were euthanized at 6 h. Duodenal, jejunal and ileal samples were promptly

harvested and stored in liquid nitrogen. Retinol and retinyl esters were extracted with two volumes of hexane and analyzed by HPLC.

**A:** Female rats. **B:** Male rats.

Data are expressed as mean  $\pm$  SEM (control rats: white bars = retinyl esters, light dashed bars = retinol, VAD diet-fed rats: black bars = retinyl esters, bold dashed bars = retinol).

An asterisk (\*) indicates a significant difference in retinyl esters concentrations between control and VAD diet-fed rats. A hash (#) indicates a significant difference in retinol concentrations between control and VAD diet-fed rats. A cross (†) indicates a significant difference in total vitamin A concentrations between control and VAD diet-fed rats. A difference of  $p < 0.05$  was considered significant.

### **Figure 7: Lipid concentrations in the intestinal mucosa at the end of the postprandial experiment**

After a force-feeding with an oily emulsion containing retinyl palmitate and  $^{13}\text{C}$ -triolein, young rats were euthanized at 6 h. Duodenal, jejunal and ileal samples were promptly harvested and stored in liquid nitrogen. Total cholesterol, phospholipid and triglyceride concentrations were then assessed.

**A-C:** Female rats. **D-F:** Male rats.

Data are expressed as mean  $\pm$  SEM (control rats: white bars, VAD diet-fed rats: black bars).

A difference between control and VAD diet of  $p < 0.05$  was considered significant.

### **Figure 8: Postprandial plasma and intestinal mucosa [1- $^{13}\text{C}$ ]-oleate concentrations in male young rats**

Young rats were forced-fed with an oily emulsion containing retinyl palmitate and [1,1,1-<sup>13</sup>C<sub>3</sub>]-triolein. Blood samples were obtained before force feeding and every 90 min from a tail nick. Young rats were euthanized at 6 h min. Duodenal, jejunal and ileal samples were promptly harvested and stored in liquid nitrogen. [1-<sup>13</sup>C]-oleate coming from the labelled triolein ingested was assessed by GC-C-IRMS analysis.

**A:** Postprandial plasma [1-<sup>13</sup>C]-oleate concentrations.

**B:** Postprandial [1-<sup>13</sup>C]-oleate response expressed as area under the curve (AUC).

[1-<sup>13</sup>C]-oleate concentration was measured at the end of the postprandial experiment in the intestinal mucosa (**C**), liver (**D**), white (**E**) and brown (**F**) adipose tissues.

Data are expressed as mean ± SEM (control rats: white bars, VAD diet-fed rats: black bars).

A difference between control and VAD diet of  $p < 0.05$  was considered significant.