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## Transgenerational effects on intestinal inflammation status in mice perinatally exposed to bisphenol S

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27 **Abstract**

28 Increasing evidence has highlighted the critical role of early life environment in shaping the  
29 future health outcomes of an individual in subsequent generations. Bisphenol S (BPS) has  
30 been widely used as a substitute for various plastic materials due to the limited application of  
31 **Bisphenol A (BPA)**, an endocrine disruptor. However, the lack of **efficient** evaluation of BPS  
32 leaves doubts about the **relevant** substitute of BPA. Few studies of transgenerational  
33 inheritance have examined the effects of environmental exposures **to endocrine disruptors** on  
34 the immune system. In this study, we analysed the transgenerational effects of BPS on  
35 intestinal inflammation and consequence on metabolism. **In this study, only F0 pregnant mice**  
36 **were exposed to BPS (1.5 µg/kg bw/day) from gestational day 0 until weaning of offspring.** In  
37 this work, both F1 and F2 male offspring developed inflammatory response in ileum and  
38 colon at adulthood after BPS exposure to F0 mothers, which disappeared in F3. This  
39 inflammatory response in F1 male offspring is associated with a significant decrease of blood  
40 cholesterol without modification of metabolic status. **On the contrary, in F3 offspring male,**  
41 **the decrease of gut inflammatory response is associated with the decrease of fat weight and**  
42 **with an increase of blood glucose and cholesterol level.** A sex-specific profile is observed in  
43 female offspring. Then, we observed that early life exposure to BPS was associated with  
44 strong abnormal intestinal immune status. The study presented here demonstrates that the  
45 immune system, like other organ systems, is vulnerable to transgenerational effects caused by  
46 environmental exposures.

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50 **Keywords:** Bisphenol S, intestine, **perinatal exposure**, inflammation, metabolism

51

52 **Highlights:**

- 53 • Perinatal BPS exposure induces sex-dependent effects on intestinal inflammation;
- 54 • Intestinal inflammation induced by BPS exhibits multigenerational and  
55 transgenerational pattern;
- 56 • Intestinal inflammation induced by BPS is associated with biochemical plasma  
57 changes.

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## 84 **1. Introduction**

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86 Epidemiological and animal studies have demonstrated that the early life environment plays a  
87 critical role in adult metabolic health (Gluckman et al., 2008). Indeed, the Developmental  
88 Origins of Health and Disease (DOHAD) hypothesis suggests that early life experiences can  
89 influence health outcomes later in life (Barker, 2007). A growing research interest within the  
90 DOHAD field is the multi- and transgenerational inheritance of an abnormal phenotype.

91 Multi- and transgenerational exposures refer to observed acquired traits in subsequent  
92 generations that are the result of direct environmental exposure of parents (F0 generation). In  
93 the case of maternal exposure, a multigenerational effect **can be observed** for F1 and F2  
94 generation (exposure *in utero* and *via* F1 gametes, respectively) and a transgenerational effect  
95 **can be observed** only from F3 (first unexposed generation). In the case of paternal exposure,  
96 an effect can be described as transgenerational since the F2 generation (**Brehm and Flaws,**  
97 **2019; Nilsson et al., 2018; Skvortsova et al., 2018**).

98 Recent reports of trans-generationally inherited adverse health effects of environmental  
99 exposures underscore the importance of this phenomenon to human health and disease (Ferey  
100 et al., 2019; Gillette et al., 2018; Klukovich et al., 2019). In contrast, research on  
101 transgenerational inheritance rarely includes assessment of whether maternal exposures  
102 impinge on the function of the immune system. Yet, a properly functioning immune system is  
103 fundamentally important to individual and public health. Even slight alterations can reduce  
104 defenses against infections or diminish vaccine efficacy **were immune reserves considered**  
105 (Dallaire et al., 2006; Winans et al., 2011). Thus, the consequences of maternal and early life  
106 exposures that alter the function of the immune system are broad reaching. Moreover, when it  
107 has been examined, developmental exposures to a range of common pollutants as well as  
108 maternal diet have been associated with changes in immune function later in life (Dietert and

109 Zelikoff, 2008). Animal studies reveal parallel events, showing that maternal and early life  
110 exposures durably change immune responses in the offspring (Malaisé et al., 2018; Xu et al.,  
111 2016). Early life exposure to BPA is particularly efficient to generate metabolic disturbances  
112 later in life, such as obesity and diabetes.

113 Risk factors such as environmental chemicals with endocrine-disrupting activity may promote  
114 chronic inflammation. Among these products, research findings suggest that low levels of  
115 bisphenol A (BPA), a widespread endocrine disruptor, can cause significant health problems.  
116 BPA is used in manufacturing polycarbonate plastics, epoxy resins as well as in thermal  
117 printing papers, making BPA exposure ubiquitous for humans due to these multiple sources  
118 and daily contact. For example, a meta-analysis indicated that BPA exposure is positively  
119 associated with type 2 diabetes mellitus (T2DM) risk in humans (Hwang et al., 2018) whereas  
120 one of the major drivers in diabetes is inflammation.

121 Perinatal exposure to BPA in rats has been shown to deeply affect homeostasis of the gut  
122 immune system (Xu et al., 2019), and has been also regarded as a risk factor of developing  
123 pro-inflammatory conditions in adult life (Menard et al., 2014). Recently, we demonstrated  
124 the link between perinatal exposure to BPA, inflammation and the development of obesity  
125 later in life (Malaisé et al., 2017). More precisely, we showed that perinatal exposure to BPA  
126 induced intestinal and systemic immune imbalance contributing to alter glucose sensitivity  
127 and dysbiosis in offspring mice in aging.

128 Since growing evidence showed the negative effects of BPA in human health, it has been  
129 prompted to be removed from consumer products (EFSA Panel on Food Contact Materials,  
130 Enzymes, Flavourings and Processing Aids (CEF), 2015). Major alternatives to BPA are  
131 bisphenol S (BPS) which have few restrictions to date (Björnsdotter et al., 2017; Goldinger et  
132 al., 2015; Sogorb et al., 2019). BPS is now widely used as an alternative to BPA (Björnsdotter  
133 et al., 2017; Liao et al., 2012), that can be explained by similar properties of BPS and its

134 higher thermal stability (Lotti et al., 2011). In Europe, the annual production of BPS is  
135 comprised between 1000 and 10000 t, and growing steadily (Ivry Del Moral et al., 2016). The  
136 increased frequency of BPS detection in urine samples collected between 2000 and 2014  
137 (n=616) in U.S. adult volunteers reflects the reality of substituting BPA with BPS (Ye et al.,  
138 2015).

139 *In vitro* studies demonstrated that even though BPS has a similar molecular size and structure  
140 to BPA, it has a lower affinity to human nuclear Estrogen Receptor (ER)  $\alpha$  and  $\beta$  (Molina-  
141 Molina et al., 2013). BPS can cross the human placenta and, as such, represent a risk for fetal  
142 development (Cabaton et al., 2013; Corbel et al., 2014; Gayrard et al., 2019). In recent years,  
143 BPA regulations have been tightened, particularly to protect against exposure during the fetal  
144 and neonatal period. Indeed, emerging evidences from animal studies suggest that endocrine  
145 disruptor components (EDCs) exposure during the critical developmental stages of pregnancy  
146 and lactation could adversely affect the developing immune system in the offspring, leading  
147 to health defects later in life (Kabir et al., 2015). With the increasing use of BPS, there are  
148 many indications that BPS has become a “regrettable substitution”. At present, the toxicology  
149 of BPS has not been fully explored.

150 Few studies of transgenerational inheritance have examined the effects of environmental  
151 exposures to BPS on the immune system, and no prior studies of developmental exposure to  
152 BPS have examined transgenerational effects on intestinal inflammation. The work presented  
153 here demonstrates that the immune system, like other organ systems, is vulnerable to  
154 transgenerational effects caused by environmental exposures to BPS.

155

## 156 **2. Materials and methods**

157

### 158 **2.1 Animals and materials**

159 Pregnant C57Bl/6J mice were purchased from Charles Rivers (L'Arbresle, France). Bisphenol  
160 S (BPS) was provided by Sigma-Aldrich (Saint Quentin Fallavier, France). The standard diet  
161 (SD) was based on the 4RF21 diet (Mucedola, Milano, Italia). **This diet is certified as**  
162 **estrogen free and accurately tested for the detection of estrogenic activities. In the diet, the**  
163 **percentage of phytoestrogens is certified to be less than 4 ppb (parts per billion) according to**  
164 **international standards (U.S. Food and Drug Administration National Center for**  
165 **Toxicological Research Standard No. 2, September 5, 1973).** Triglyceride FS™, Cholesterol  
166 FS™ and No Esterified Fatty Acids (NEFA) FS™ kits were purchased from DiaSys  
167 (Condom, France). Ultrasensitive™ Mouse Insulin and plasma estradiol and testosterone  
168 ELISA kits were provided from Mercodia France SAS (Paris, France) and R&D Systems  
169 (Lille, France), respectively. Blood glucose test strips (Accu-Check®, Roche Diagnostics)  
170 and glucometers (Accu-Check® Aviva, Roche Diagnostics) were purchased from the  
171 pharmacy of the University (Dijon, France).

172

## 173 **2.2 Experimental design**

174 Mice were housed in a 12h light-dark cycle at a temperature of 22°C in one conventional  
175 animal house and allowed free access to food and water. Cages and bottles were made of  
176 polypropylene (bisphenol-free). Thirty-seven F0 pregnant C57Bl/6J mice were individually  
177 housed. They were divided into two groups and exposed to BPS (20 females) or not (17  
178 females) in their drinking water at concentration of either 0 or 8.5 ng/ml from the first day of  
179 gestation (GD0) to post natal day 21 (PND21) of F1 offspring.

180 The determination of GD0 was done in the morning by vaginal plug detection. **Then, pregnant**  
181 **female mice was housed individually. In order to obtain expected BPS exposure of 1.5 µg/kg**  
182 **body weight/day, suitable BPS concentration in drinking water was firstly determined. For**  
183 **this, we carried out a preliminary study evaluating the water consumption of mice during**  
184 **gestation and calculated the concentration of BPS theoretically necessary for the pregnant**



185 mice to consume about 1.5 µg/kg bw/day. On this basis, we determined a BPS concentration  
186 of 8.5 ng/ml in drinking water. During this protocol, we checked the BPS exposure as  
187 follows: weekly water intake was determined by measuring the difference between the  
188 amount of water placed in the water bottle at the beginning and the amount remaining after  
189 seven days. The levels of BPS consumed each week were determined and consequently  
190 divided by seven (days). The average BPS intake by the mice was 1.78 (± 0.03) µg/kg body  
191 weight/day. BPS was dissolved in absolute ethanol (0.1%). Control group drinking water  
192 contained only 0.1% ethanol. The drinking water was replaced each week and BPS is very  
193 persistent with no biodegradation observed after 60 days in seawater (Danzl et al., 2009).  
194 To generate F2 generation, male and female mice of the F1 generation were mated at the age  
195 of 10 weeks. Under the same conditions, the F2 mice were cross-breed to obtain the  
196 individuals of the F3 generation. For crossbreeding, male and female animals came from  
197 different litters and one male was only mated with one female.  
198 Mice were divided into 10 mice per batch (except F2 females where the mortality rate was  
199 unexpected and too high. The females were preferably used for F3.  
200 At weaning, a maximum of five mice were housed in each cage according to the BPS  
201 exposure status of their mother, grandmother or great-grandmother. The litters were mixed  
202 randomly after weaning in order to minimize a possible litter effect. For each generation, the  
203 numbers of male and female mice according to their BPS exposure are reported in Suppl  
204 Table 1. These mice fed a standard diet from weaning to 23-weeks of age. All mice (fasted 4h  
205 before) were sacrificed at 23-weeks old. Before sacrifice, the body weight was measured. At  
206 the sacrifice, ileum and colon were immediately frozen in liquid nitrogen and stored at -80°C.  
207 Experimental protocol (#11422) was approved by the ministry and the University of  
208 Burgundy's ethic committee. Animal experiments have been carried out in accordance with  
209 EU Directive 2010/63/EU for animal experiments.

210

### 211 **2.3 Body weight monitoring and corporal composition measurement**

212 Just before (4h fasted) sacrifice, body weight, fat and lean masses of 23-weeks old mice were  
213 measured. The fat and lean masses of each mouse were determined individually using a  
214 quantitative EchoMRI 500T™ (EchoMRI, Houston, USA). Before each measurement,  
215 calibration was performed in compliance with the manufacturer's guidelines.

216

### 217 **2.4 Food intake measurement**

218 For each generation (F1; F2; F3), these experiments were performed in 14-week old mice.  
219 The mice were not individualized in order not to generate stress, which could modify the  
220 animal's feeding behavior. **The food intake was determined as follows: On the first day, the**  
221 **amount of food placed in the cage was weighed. Then, the remaining food not consumed was**  
222 **also weighed after 24h. The difference between these two weights was calculated. This**  
223 **experiment was repeated over four consecutive days. Measurements were done for each cage**  
224 **and then normalized to the number of mice per cage and per 24-hour period. Spilled food was**  
225 **weighed and subtracted from the measurements when necessary.**

226

### 227 **2.5 Biochemical analysis**

228 Just before sacrifice (*i.e.* fasted 4h before), mouse blood samples were collected by an  
229 intracardiac puncture using heparinized syringe. After a centrifugation of 10 min at 2000g and  
230 4°C, the level of total plasma cholesterol, triacylglycerol (TG) were measured using  
231 respective kits mentioned above. Assays were performed according to the manufacturer's  
232 instructions. The day of sacrifice, blood glucose levels were measured, using a glucometer,  
233 from a blood sample collected at the animal's tail and before anaesthesia. The homeostatic  
234 model assessment of insulin resistance (HOMA-IR) was calculated by a formula adapted to a  
235 previous method in human [1]. For mice, reference values were calculated using average

236 fasting glucose (4.16 mmol/l) and plasma insulin (26.11 mU/l) concentrations from the  
237 control mice fed with standard diet [2]. The homeostatic model assessment (HOMA) adapted  
238 to mice was calculated as ( $[\text{glucose (mmol/l)}] \times [\text{insulin (mUI/l)}] / 108.6$ ) and used as a  
239 surrogate measure of whole-body insulin sensitivity.

240

## 241 **2.6 Cytokines measurement**

242 Cytokines were measured in supernatant of jejunal, colonic fragments or feces suspended in  
243 RIPA buffer (0.5% deoxycholate, 0.1% SDS and 1% Igepal in TBS) containing complete anti  
244 protease cocktail (Roche). Jejunal, colonic or fecal protein concentrations were measured  
245 using BCA uptima kit (Interchim). IL1- $\beta$ , TNF- $\alpha$ , IFN- $\gamma$  and lipocalin were assayed using  
246 commercial ELISA kits (R&D Systems), following manufacturer's instructions. **Fecal**  
247 **lipocalin-2 (LCN2) levels provide a sensitive and broadly dynamic method to monitor**  
248 **inflammation, specifically for low levels of inflammation (Chassaing et al., 2012). Data are**  
249 **expressed as picograms of cytokine per milligram of protein in jejunal, colon or feces.**

250

## 251 **2.7 Statistical analysis**

252 All data were expressed as mean  $\pm$  standard error of mean (SEM). To determine the  
253 statistically significant difference between two groups, a Student's t test was used **after prior**  
254 **Shapiro-Wilk Normality test and F-test to compare variances** (two-tailed, paired samples for  
255 means, and equal variance). Statistical tests were performed with GraphPad Prism7®  
256 software. Results were considered statistically significant at  $p < 0.05$ .

257

## 258 **3. Results**

259

### 260 **3.1 BPS induces transgenerational changes with inflammation in ileum of offspring** 261 **male**

262 In male offspring, we observed a significant increase of inflammatory markers such as TNF- $\alpha$

263 and lipocalin in ileum of F1 generation after BPS exposure (Fig 1B and 1C). This increase is  
264 accentuated for TNF- $\alpha$  in F2 and the difference with control group became significant for  
265 IL1- $\beta$ . (Fig 1B and 1A). Interestingly, drastic changes were observed in the male F3  
266 generation with a decrease of inflammatory markers such as IL1- $\beta$ , TNF- $\alpha$  and lipocalin (fig  
267 1A, B and C). In contrast, only IFN- $\gamma$  levels was higher in BPS group in comparison to  
268 control in the male F3 generation, the same effect was observed in female offspring (Fig1D,  
269 suppl, Fig 1C). On the contrary, in the female F3 generation, IL1- $\beta$  concentration was  
270 increased in BPS group demonstrating a sex-specific effect of BPS on this parameter (suppl  
271 fig 1A).

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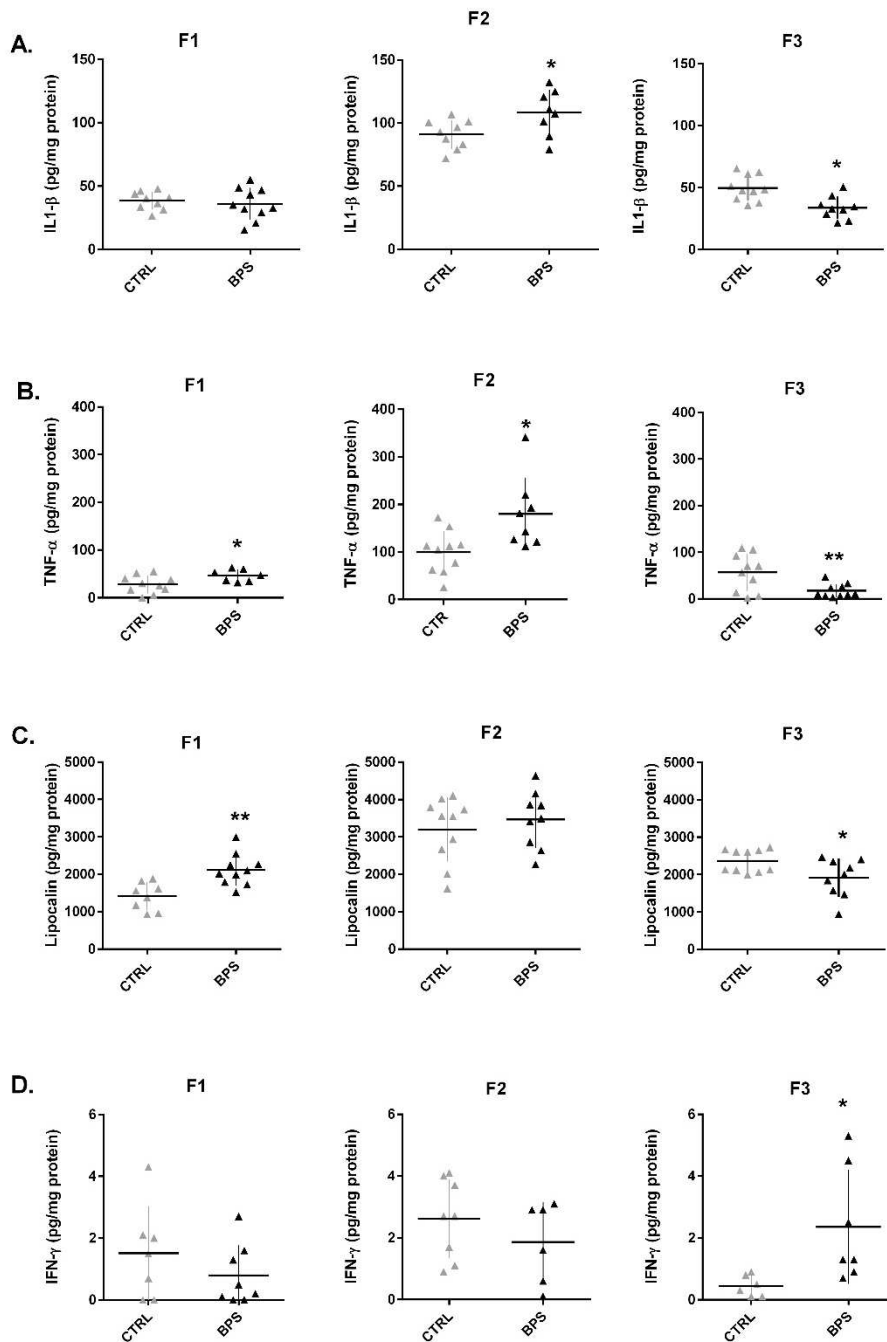
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Figure 1.



285

286 **Fig 1. Inflammatory response in ileum of male offspring after perinatal exposure to BPS**

287 IL1-β (A) TNF-α (B), lipocalin (C) and IFN-γ (D) levels in ileum samples from male

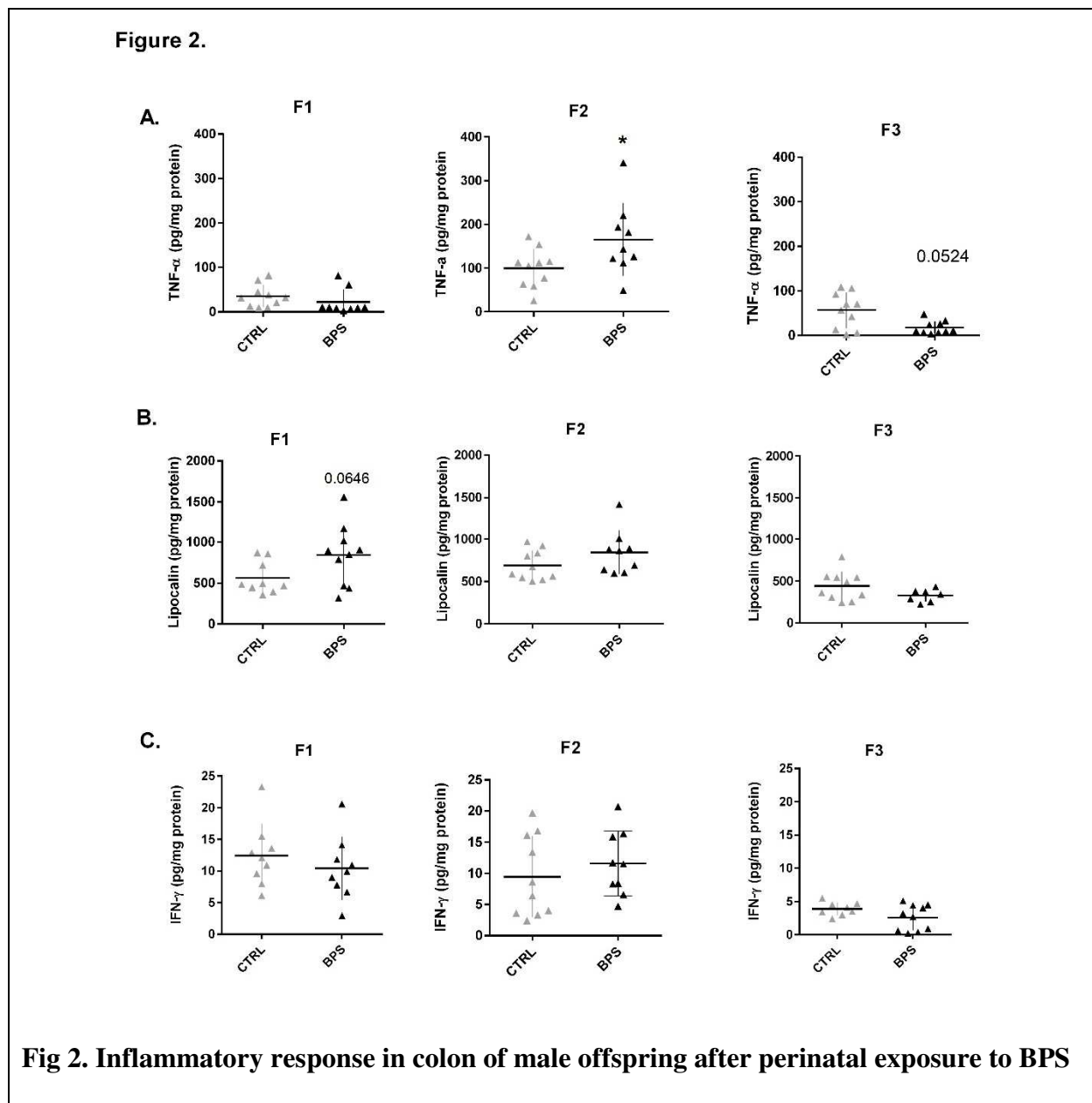
288 offspring mice at 23 weeks old from F1, F2 and F3 generation. \* P < 0.05; \*\* P < 0.01 vs.

289 vehicle group. N = 10 offspring mice per group.

290

291 **3.2 BPS induces transgenerational changes with inflammation in colon of offspring**  
 292 **male**

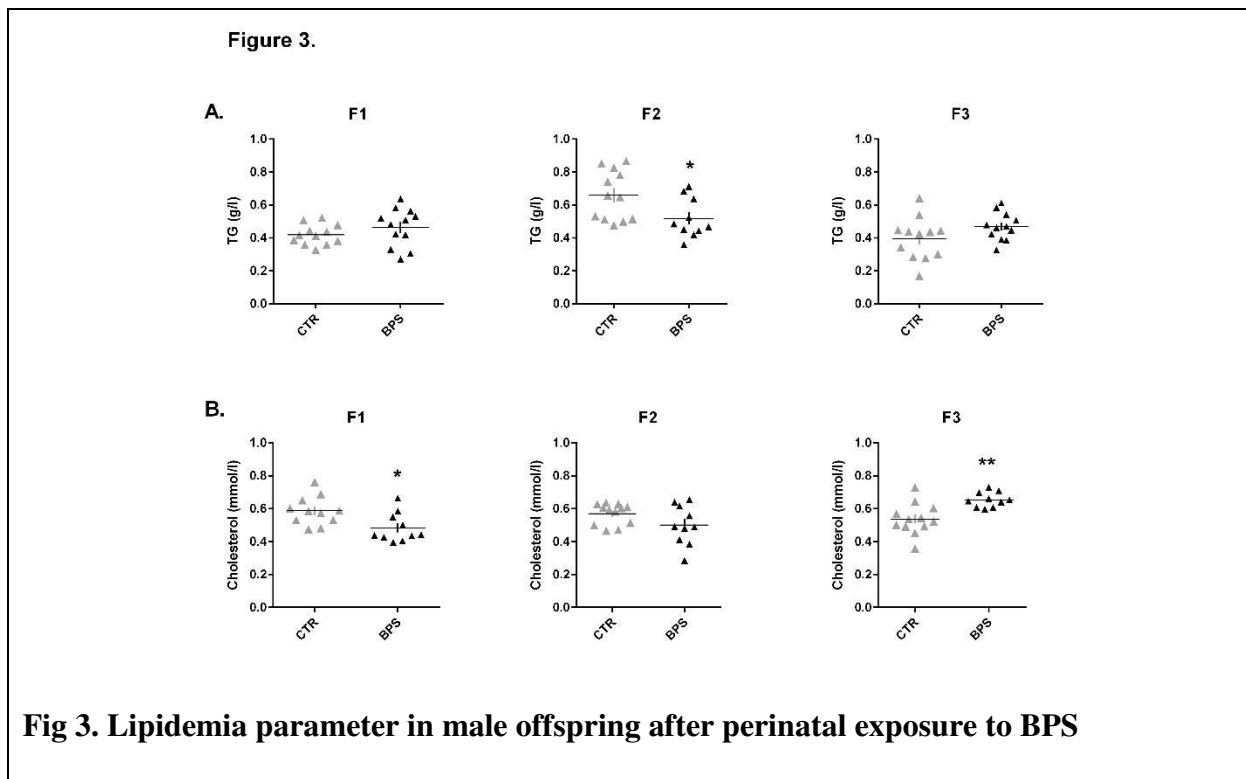
293 On the contrary to small intestine, we noticed only a weak increase of inflammatory  
 294 parameters such as lipocalin in male F1 generation after BPS exposure (fig 2B). Then, a  
 295 significant rise of TNF- $\alpha$  was observed after BPS exposure in male F2 generation (fig 2A).  
 296 Comparable to small intestine observation, a strong decrease of TNF-a level were observed in  
 297 male F3 generation exposed to BPS in comparison to control group.  
 298 No change was noticed in IFN- $\gamma$  level in male colon (Fig 2C) whereas a significant increase  
 299 was observed in female demonstrating a sex-specific effect of BPS (suppl Fig1A).



302 TNF- $\alpha$  (A) lipocalin (B) and IFN- $\gamma$  (C) levels in colon samples from male offspring mice at  
303 23 weeks old from F1, F2 and F3 generation. \* P<0.05; \*\* P<0.01 vs. vehicle group. N = 10  
304 offspring mice per group.

305  
306 **3.3 Transgenerational changes in intestinal inflammation were associated with**  
307 **lipidemia parameter modifications**

308 No difference in body weight was observed in male mice exposed to BPS in comparison with  
309 unexposed male mice whatever the generation (suppl Fig 2A). However, in comparison to  
310 control group, a significant decrease of body fat was observed in male F3 generation after  
311 exposure to BPS (suppl Fig 2B). In male mice, blood cholesterol level was decreased  
312 significantly in male F2 generation and increase in F3 generation exposed to BPS (Fig 3A).  
313 On the contrary, plasmatic triglyceride level was decreased significantly in male F2  
314 generation (Fig 3B).

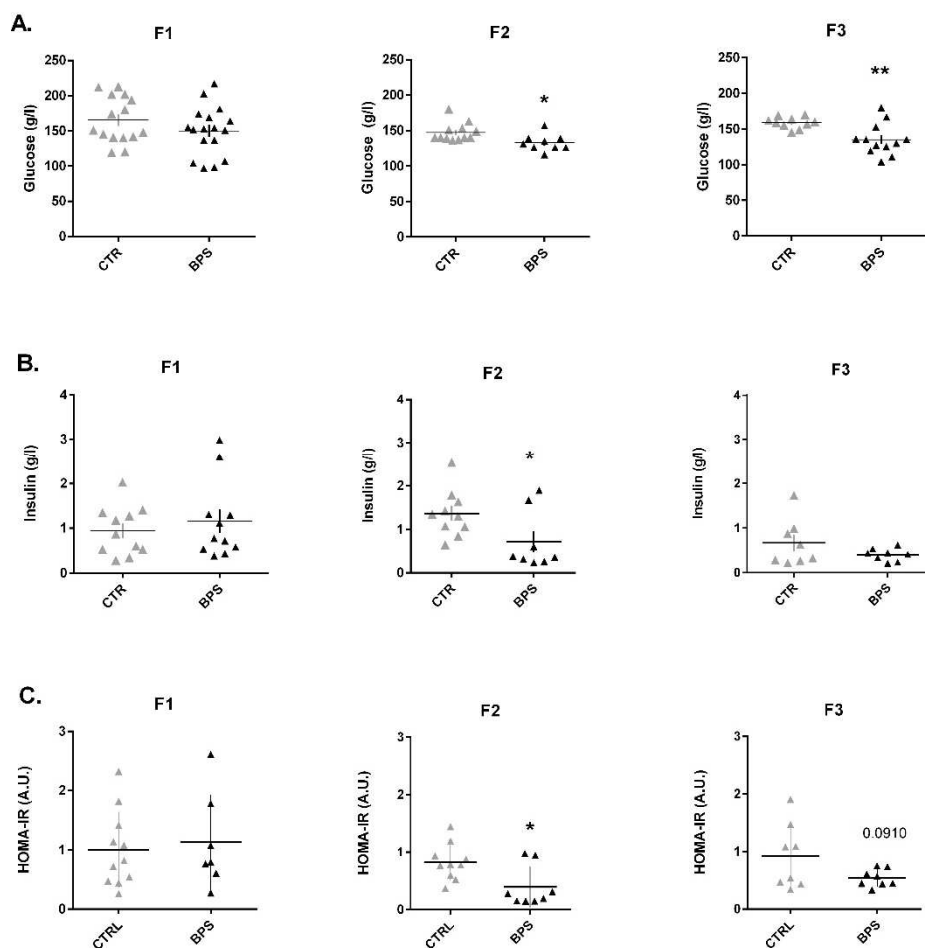


317 Triglycerides (A) and cholesterol (B) levels in blood from male offspring mice at 23 weeks  
318 old from F1, F2 and F3 generation. \* P<0.05 vs. vehicle group. N = 10 offspring mice per  
319 group.

### 320 3.4 Exposure to BPS contributes to dysregulation of glucose metabolism through 321 generation in male mice

322 After BPS exposition, male offspring developed no significant increase of blood glucose level  
323 (Fig 4). However, in male F2 generation exposed to BPS, we noticed a significant decrease of  
324 blood glucose and insulin level, which was also observed in male F3 generation for glucose  
325 level.

Figure 4.



326

327 **Fig 4. Perinatal exposure to BPS provokes metabolic disorders in offspring mice**



328 Glucose (A) and insulin (B) level in blood before sacrifice (i.e. fasted 4h before) from male  
329 offspring mice at 23 weeks old from F1, F2 and F3 generation. (C) The homeostatic model  
330 assessment (HOMA) adapted to mice was calculated as  $([\text{glucose (mmol/l)}] \times [\text{insulin}$   
331  $(\text{mUI/l})]) / 108.6$  and used as a surrogate measure of whole-body insulin sensitivity. \* P<0.05;  
332 \*\* P<0.01 vs. vehicle group. N = 10 offspring mice per group.

333  
334 The HOMA-IR index was decreased in male exposed BPS compared to control group, in F2  
335 and F3 generation (Fig 4C). However, no significant differences on liver weight were detected  
336 between the two groups regardless of generation of mice (suppl Fig 2D and 2F).

337

#### 338 **4. Discussion**

339

340 Increasing evidence has suggested that the early life environment can have a significant  
341 impact on future health of offspring. Laboratory animal work has provided conclusive  
342 evidence that early-life exposure to BPA is particularly effective to generate metabolic  
343 disturbances later in life, such as obesity and diabetes. BPA were removed from consumer  
344 products and replaced by chemical substitutes such as BPS questioning its impact multi- and  
345 transgenerational.

346 In this work, we observed that early life exposure to BPS was associated with strong  
347 abnormal intestinal immune status following multigenerational (F1 and F2 exposure) or  
348 transgenerational pattern (F3 exposure).

349 Both F1 and F2 male offspring developed inflammatory response in ileum and colon at  
350 adulthood after BPS exposure to mothers. These results are in accordance with our previous  
351 studies on perinatal exposure to BPA, showing dysregulations in the maturation of gut barrier  
352 functions and the development of both intestinal and systemic immune homeostasis of male

353 offspring mice (Malaisé et al., 2018).

354 The increase of intestinal inflammatory response in F1 male offspring was associated with a  
355 significant decrease of blood cholesterol without modification of metabolic status (no change  
356 of glucose or insulin level in blood relative to control group). Interestingly, this  
357 hypocholesterolemia was associated with intestinal inflammation in F1 and  
358 hypercholesterolemia with a less inflammatory phenotype in F3 male mice. Cytokines  
359 produced during the inflammation, such as interleukin (IL-1 $\beta$  and TNF- $\alpha$ ), may be sensed by  
360 hepatocytes to trigger changes to the serum concentrations of their secreted products. An  
361 emerging hypothesis showed that intestinal low-grade inflammation may be associated with  
362 low-density lipoprotein-cholesterol (LDL-C) plasma level increase contrary to systemic  
363 inflammation which tends to reduced total cholesterol level (Herbert and Erridge, 2018). A  
364 significant association was recently demonstrated between elevated gut permeability and  
365 elevated serum HDL cholesterol (Robertson et al., 2018). Then, the role of perinatal exposure  
366 of BPS on gut permeability and its relationship with cholesterol level in male offspring will be  
367 explored to better understand the different response observed between bisphenol analogues.

368 In this study, we did not investigate systemic inflammation, but in male mice, the  
369 inflammatory properties of BPS have already been shown in liver and adipose tissue in F1  
370 (Meng et al., 2019) and now in the intestine with this study. **In previous study, we observed a  
371 significant increase of IFN $\gamma$  production by splenocytes after perinatal exposure to BPA in F1  
372 offspring male (Malaisé et al., 2017). We can assume to obtain comparable results with BPS,  
373 but this needs to be further investigated.** Therefore, it cannot be excluded that it has an effect  
374 on systemic inflammation in exposed mice.

375 A sex-specific profile was observed in female offspring with a decrease of inflammatory  
376 response in F1 generation and the opposite in F3 generation. Bansal et al. (2017) observed  
377 also that maternal (F0) exposure to BPA has multigenerational sex-specific effects, such that

378 the first (F1) and second generation (F2) adult female offspring were unaffected, but adult F1  
379 and F2 male offspring had increased percent body fat and reduced glucose stimulated insulin  
380 secretion (Bansal et al., 2017).

381 In F2 generation male offspring, we observed a significant decrease of triglycerides associated  
382 with a drop of glucose and insulin levels in blood in BPS exposed animal relative to non-  
383 exposed control mice without modification of body weight at adulthood. A recent work has  
384 shown that inflammation can lead to the inhibition of fatty acid absorption in intestine (Liu et  
385 al., 2019). Indeed, these authors demonstrated that lipopolysaccharide (LPS) as one of the  
386 main pathogenic components did not suppress fatty acid absorption directly in the intestine,  
387 but may work on macrophages that secrete cytokines, such as TNF- $\alpha$ , inducing caspase-3  
388 activation and finally leading to the inhibition of fatty acid absorption in intestine (Liu et al.,  
389 2019). A gut dysbiosis provoked by BPS exposure of offspring may cause an increase of LPS  
390 leading to intestinal low-grade inflammation that we observed in this study.

391 In the present study, in F3 offspring male, we observed a decrease of gut inflammatory  
392 response, an increase of blood glucose level associated with decrease of fat weight and to an  
393 important increase of cholesterol level. It was now well described that the intestine could  
394 contribute to about 20-25% of total endogenous glucose production during fasting (Mithieux,  
395 2018a, p. 20). More importantly, intestinal gluconeogenesis is capable of regulating energy  
396 homeostasis through a communication with the brain. In response, the brain appropriately  
397 regulates many peripheral functions involved in energy homeostasis, such storage of lipids in  
398 adipose tissue (Mithieux, 2018b). This process of intestinal gluconeogenesis could be  
399 developed after transgenerational exposure to BPS as compensation mechanism in F3  
400 offspring mice.

401 Because the third-generation offspring were not exposed to BPS, the persistence of the  
402 metabolic abnormalities in the third generation suggested that epigenetic modifications may

403 be involved in the transmission of the effects associated with BPS exposure across three  
404 generations. The ability to directly expose a germ cell to induce effects in the offspring (*i.e.*  
405 multigenerational exposure) are important, but the ability to produce a permanent epigenetic  
406 alteration in the germ cells which is maintained in the absence of the continued environmental  
407 exposure suggests a novel form of inheritance which could have a much greater impact on  
408 biology, disease etiology, and evolution. A wide variety of environmental factors from  
409 nutrition to toxicants have now been shown to promote the epigenetic transgenerational  
410 inheritance of disease or phenotypic variation such as BPA or BPS (Brulport et al., 2020;  
411 Manikkam et al., 2013; Wolstenholme et al., 2012). Our results described for the first time,  
412 important transgenerational effects of BPS in male offspring, which can explain the decrease  
413 of intestinal inflammation observed only in F3 offspring mice. Epigenetic control of intestinal  
414 barrier function and inflammation has been recently described showing that the loss of the  
415 maintenance DNA methylation regulator *uhrfl* can lead to hypomethylation of the *tnfa*  
416 promoter (Marjoram et al., 2015).

417 Epigenetic remodeling of chromatin via DNA methylation regulates gene expression. The  
418 mammalian DNA methylation process is composed of two components. The first components  
419 are the DNMTs, which are concerned with DNA methylation patterns, and the second are the  
420 methyl-CpG binding proteins, which are involved in reading methylation signatures. DNMT1  
421 is required to maintain DNA methylation at the IFN $\gamma$  locus in undifferentiated CD4<sup>+</sup> T cells.  
422 By contrast, DNMT3a catalyzes DNA methylation of the IFN $\gamma$  promoter in response to Th2  
423 and Th17 differentiation signals to sustain IFN $\gamma$  silencing (Gonsky et al., 2009). Then, the  
424 epigenetic methylation status of IFN $\gamma$  may play a mechanistic role in the modulation of  
425 cytokine secretion in the mucosa. **This process could explain the increase of IFN $\gamma$  secretion in  
426 ileum of male offspring observed in F3 generation after perinatal exposure to BPS.** Epigenetic  
427 regulation and dysregulation of Th cells are involved in the maintenance of intestinal

428 homeostasis (Hagihara et al., 2019). This process could explain the decreased of intestinal  
429 inflammation observed after transgenerational exposure to BPS.

430 This work showed that maternal exposure to industrial pollution can harm the immune system  
431 of offspring and that injury is passed along to subsequent generations, weakening the body's  
432 defenses (Post et al., 2019).

433

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439

#### 440 **CRedit authorship contribution statement**

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447

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