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Abstract

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

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

Highlights:

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

1. Introduction

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

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

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

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

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

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

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

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

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

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

2. Materials and methods

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

mice to consume about 1.5 µg/kg bw/day. On this basis, we determined a BPS concentration of 8.5 ng/ml in drinking water. During this protocol, we checked the BPS exposure as follows: weekly water intake was determined by measuring the difference between the amount of water placed in the water bottle at the beginning and the amount remaining after seven days. The levels of BPS consumed each week were determined and consequently divided by seven (days). The average BPS intake by the mice was 1.78 (± 0.03) µg/kg body weight/day. BPS was dissolved in absolute ethanol (0.1%). Control group drinking water contained only 0.1% ethanol. The drinking water was replaced each week and BPS is very persistent with no biodegradation observed after 60 days in seawater (Danzl et al., 2009).

To generate F2 generation, male and female mice of the F1 generation were mated at the age of 10 weeks. Under the same conditions, the F2 mice were cross-breed to obtain the individuals of the F3 generation. For crossbreeding, male and female animals came from different litters and one male was only mated with one female.

Mice were divided into 10 mice per batch (except F2 females where the mortality rate was unexpected and too high. The females were preferably used for F3.

At weaning, a maximum of five mice were housed in each cage according to the BPS exposure status of their mother, grandmother or great-grandmother. The litters were mixed randomly after weaning in order to minimize a possible litter effect. For each generation, the numbers of male and female mice according to their BPS exposure are reported in Suppl Table 1. These mice fed a standard diet from weaning to 23-weeks of age. All mice (fasted 4h before) were sacrificed at 23-weeks old. Before sacrifice, the body weight was measured. At the sacrifice, ileum and colon were immediately frozen in liquid nitrogen and stored at -80°C. Experimental protocol (#11422) was approved by the ministry and the University of Burgundy's ethic committee. Animal experiments have been carried out in accordance with EU Directive 2010/63/EU for animal experiments.

2.3 Body weight monitoring and corporal composition measurement

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

2.4 Food intake measurement

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

2.5 Biochemical analysis

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

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

2.6 Cytokines measurement

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

2.7 Statistical analysis

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

3. Results

3.1 BPS induces transgenerational changes with inflammation in ileum of offspring male

In male offspring, we observed a significant increase of inflammatory markers such as TNF-α

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

Figure 1.

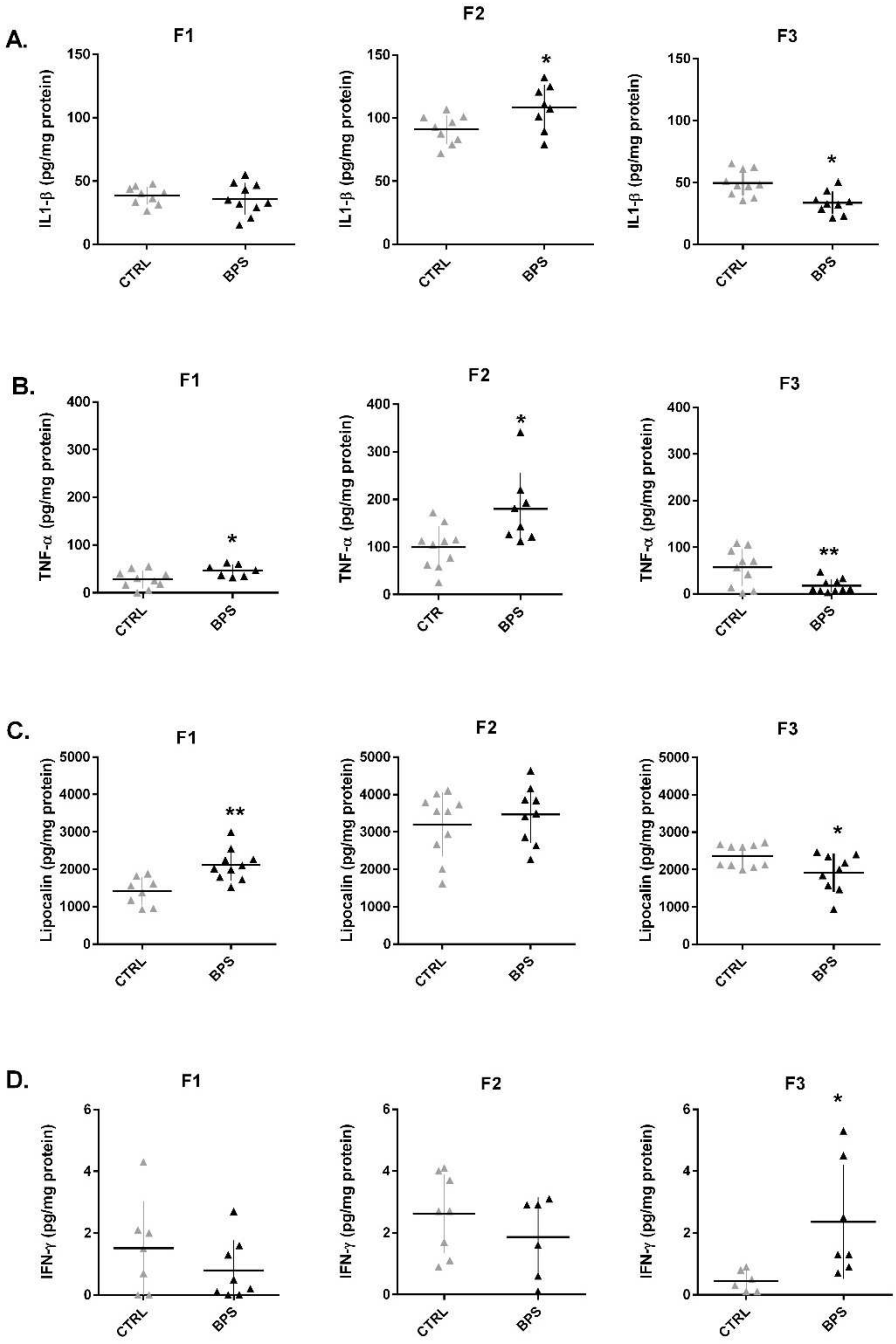

Fig 1. Inflammatory response in ileum of male offspring after perinatal exposure to BPS

IL1-β (A) TNF-α (B), lipocalin (C) and IFN-γ (D) levels in ileum samples from male offspring mice at 23 weeks old from F1, F2 and F3 generation**.** * P<0.05; ** P<0.01 vs. vehicle group. N = 10 offspring mice per group.

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

On the contrary to small intestine, we noticed only a weak increase of inflammatory parameters such as lipocalin in male F1 generation after BPS exposure (fig 2B). Then, a significant rise of TNF-α was observed after BPS exposure in male F2 generation (fig 2A). Comparable to small intestine observation, a strong decrease of TNF-a level were observed in male F3 generation exposed to BPS in comparison to control group.

No change was noticed in IFN-y level in male colon (Fig 2C) whereas a significant increase was observed in female demonstrating a sex-specific effect of BPS (suppl Fig1A).

Figure 2.

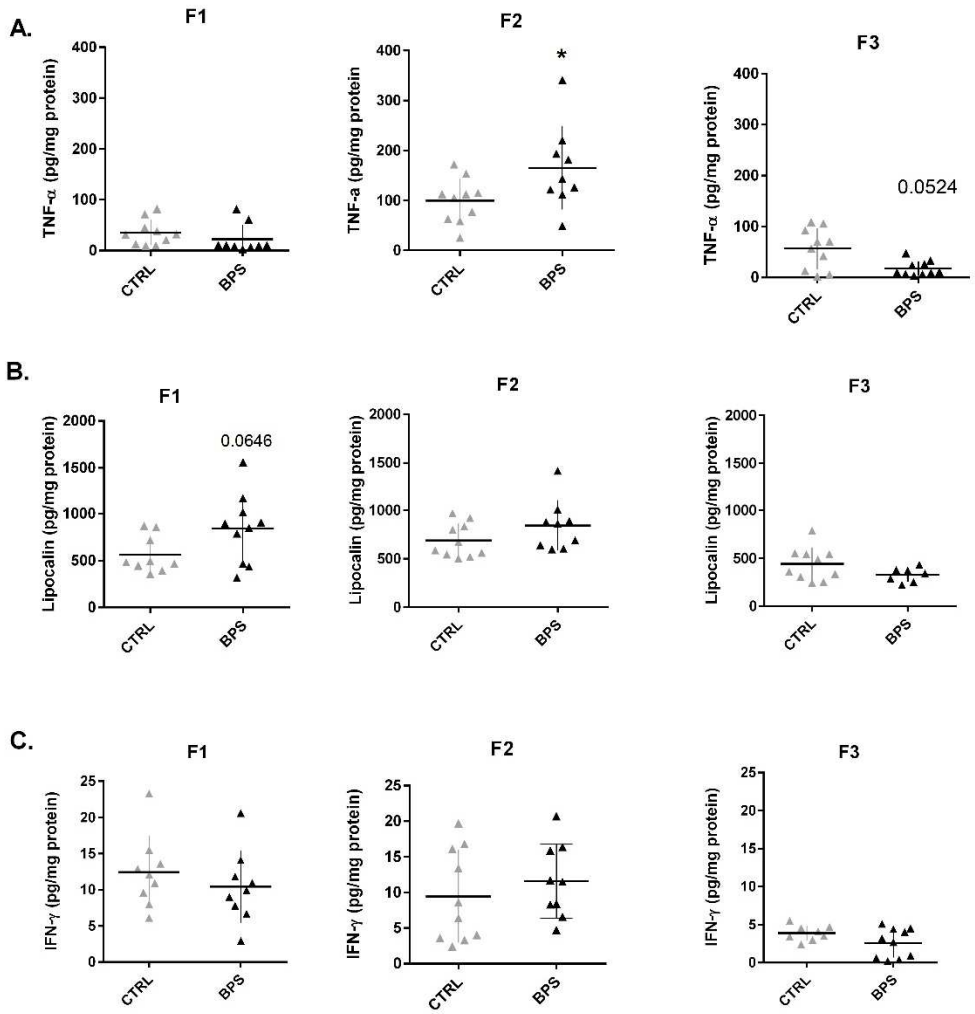

Fig 2. Inflammatory response in colon of male offspring after perinatal exposure to BPS

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

3.3 Transgenerational changes in intestinal inflammation were associated with lipidemia parameter modifications

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

Figure 3.

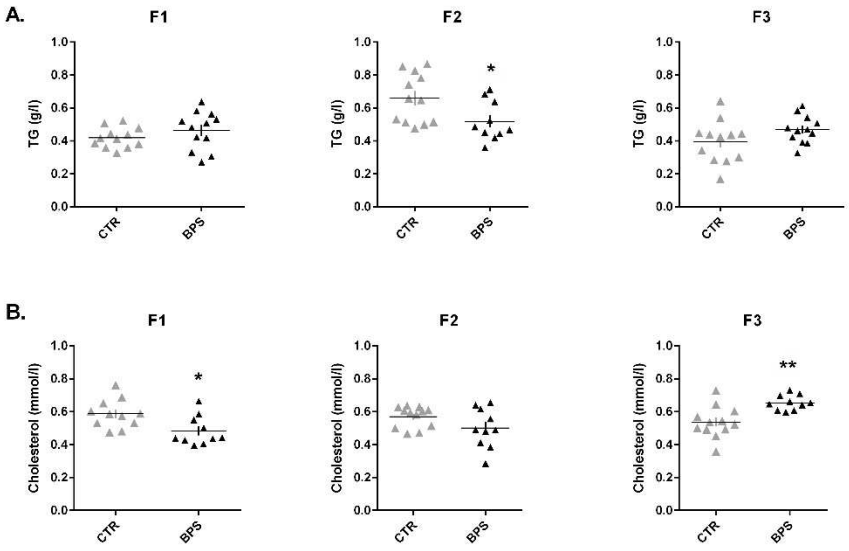

Fig 3. Lipidemia parameter in male offspring after perinatal exposure to BPS

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

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

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

Figure 4.

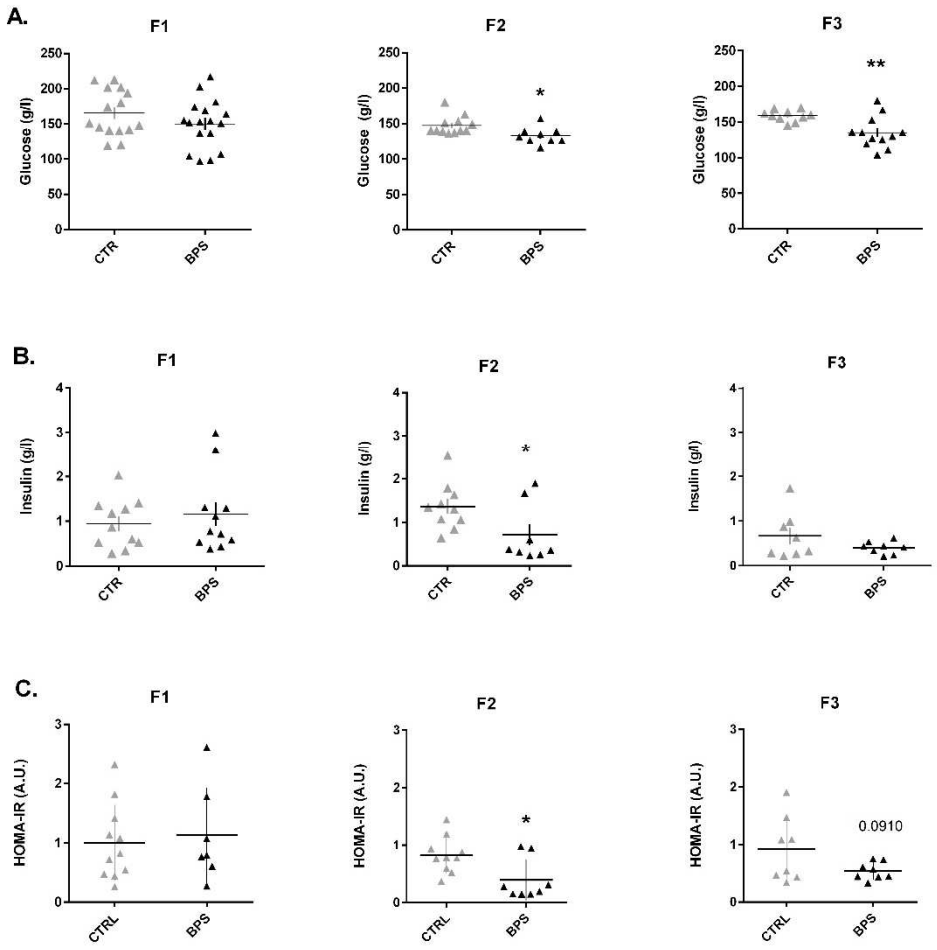

Fig 4. Perinatal exposure to BPS provokes metabolic disorders in offspring mice

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

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

4. Discussion

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

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

Both F1 and F2 male offspring developed inflammatory response in ileum and colon at adulthood after BPS exposure to mothers. These results are in accordance with our previous studies on perinatal exposure to BPA, showing dysregulations in the maturation of gut barrier 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-1β and TNF-α), 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 IFNy 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

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

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

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

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

be involved in the transmission of the effects associated with BPS exposure across three generations. The ability to directly expose a germ cell to induce effects in the offspring (*i.e.* multigenerational exposure) are important, but the ability to produce a permanent epigenetic alteration in the germ cells which is maintained in the absence of the continued environmental exposure suggests a novel form of inheritance which could have a much greater impact on biology, disease etiology, and evolution. A wide variety of environmental factors from nutrition to toxicants have now been shown to promote the epigenetic transgenerational inheritance of disease or phenotypic variation such as BPA or BPS (Brulport et al., 2020; Manikkam et al., 2013; Wolstenholme et al., 2012). Our results described for the first time, important transgenerational effects of BPS in male offspring, which can explain the decrease of intestinal inflammation observed only in F3 offspring mice. Epigenetic control of intestinal barrier function and inflammation has been recently described showing that the loss of the maintenance DNA methylation regulator *uhrf1* can lead to hypomethylation of the *tnfa* promoter (Marjoram et al., 2015).

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

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

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

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CRediT authorship contribution statement

Axelle Brulport: Formal analysis, Investigation, Writing - original draft, Writing – review & editing, **Corinne Lencina:** Formal analysis, Investigation, **Marie-Christine Chagnon:** Conceptualization, Funding acquisition, **Ludovic Le Corre:** Conceptualization, Funding acquisition, Formal analysis, Investigation, Writing – original draft, Writing - review & editing, **Laurence Guzylack-Piriou:** Conceptualization, Funding acquisition, Formal analysis, Investigation, Writing - original draft, Writing – review & editing**.**

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