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RESEARCH ARTICLE

Comparative Studies on Phospholipase A2 as a Marker for Gut Microbiota-liver-brain Axis in a rodent Model of Autism

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Abstract: ***Background*:** Lipid homeostasis and gut flora can be related to many metabolic diseases, especially autism. Lipid metabolism in the brain can control neuronal structure and function and can also take part in signal transduction pathways to control metabolism in peripheral tissues, especially in the liver. Impaired phospholipid metabolism promotes oxidative stress and neuroinflammation and is, therefore, directly related to autism.

***Objective*:** The effect of propionic acid (PPA) toxicity on lipid homeostasis in the gut-liver-brain axis was evaluated to understand their inter-connection. Cytosolic phospholipase A2 (cPLA2) concentration and activity was measured in autistic model and protective role of omega-3 (ω-3) and vitamin B12 was evaluated.

***Methods*:** Animals were divided into five groups: Group I (control group); Group II (autistic model treated with neurotoxic dose of PPA); Group III (treated with vitamin B12 (16.7 mg/kg/day) for 30 days post PPA treatment); Group IV (treated with ω-3 (200 mg/kg body weight/day) for 30 days post PPA treatment; Group V (combined dose of ω-3 and Vitamin B12, for 30 days post PPA treatment). Phospholipase A2 activity and protein expression level in the liver homogenate of all the groups was analyzed by western blotting and was compared to brain cPLA2.

***Results*:** PPA increased the levels of liver and brain cPLA2. However, independent or combined treatment with ω-3 and vitamin B12 was effective in neutralizing its effect. Moreover, PPA-induced dysbiosis, which was ameliorated with the above treatments.

***Conclusion*:** This study showed the role of cPLA2 as a lipid metabolism marker, related to PPA-induced inflammation through a highly interactive gut-liver-brain axis.

Keywords: Autism, phospholipase A2, western blotting, gut microbiota, gut-liver-brain axis, metabolism.

1. INTRODUCTION

The gut microbiota forms a complex bacterial community that has recently shown to have a notable impact on human well-being [1, 2]. There are more than 100 trillion microbes in the human gut that display high metabolic activities and are in unceasing interaction with the host immune system [3]. Furthermore, the gut microbiota is an important source of metabolites, hormones, and neuro-mediators that directly control gut function and modulate the function of organs, such as the liver, and the brain [4]. Previous studies have shown that well-balanced gut flora is crucial for overall good health [5], indicating that human health strongly depends on the composition and function of the gut microbiota. Interestingly, each human being has his own discrete characteristic configuration of gut microbiota, defined as the "microbial fingerprint" [6].

Altered gut microbiota disturbs host physiology and is an important contributor to the development of multiple diseases. Gut and liver are highly interconnected through the portal vein and the biliary system. Hence, the liver is continually exposed to gut-derived bacterial metabolites, including propionic acid (PPA). The intestinal barrier is important for maintaining physical and functional separation between in-

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testinal bacteria and the host tissues [7]. Disruption of the barrier integrity can lead to the translocation of bacteria and their metabolites. Liver diseases have been associated with dysbiotic changes in the gut microbiota and disrupted gut barrier [7, 8].

Previous studies suggest that a bidirectional pathway exists between the brain and the gut, with the microbiota being a critical constituent of this gut-brain axis [9-12].

Among the different mechanisms, through which the gut microbiota can affect brain development, is the production of various bacterial metabolites. Short chain fatty acids (SCFAs) such as PPA, butyric, and acetate, are the most common products [10, 13]. These acids circulate in the blood and enter the brain through definite transporters or by crossing the Blood Brain Barrier (BBB). High levels of PPA in animal brains may induce autistic features, which can be restored by butyrate supplementation in Germ-Free (GF) mice. This highlights the critical importance of SCFAs and the influence of gut microbiota in brain development [14-16].

In their study, Sharon *et al.* [17] suggested that the gut microbiota regulates behavior in mice through the production of neuroactive metabolites, indicating that gut-brain connections contribute to the pathophysiology of Autism Spectrum Disorders (ASD). Altered gut microbiota leads to inappropriate development of the gut immune system and the gut barrier, which can lead to impaired microbiota-gut-brain signaling. Recent studies suggest that multiple cytokines, such as IL-6, TNF-α, IFN-γ, and IL-1β, are significantly elevated in PPA-treated rats, in the rodent model of autism. Through the disrupted tight junctions of the BBB, they could access the brain to impact animal behavior [18, 19].

Activation of PLA2 is one of the most common neurotoxic and hepatotoxic mechanisms downstream of exposure to harmful stimuli. Like phospholipid hydrolases, phospholipases release the FA in the sn-2 position of the phospholipid. FA release is required not only for membrane restoration, but also for making substrates available for the initiation and resolution of the inflammatory signaling [20, 21]. Cytosolic phospholipase A2 (cPLA2) is reported to be the main enzyme to mediate the release of ω-6 arachidonic acid (AA) from the membrane due to its 50-fold preference for phospholipids, containing AA over any other polyunsaturated fatty acid (PUFA) [22].

Studies by Qasem *et al.* [23] showed that diminished phospholipid concentration was concomitant with an elevation of cPLA2 protein level in the plasma of individuals with autism. A positive correlation between cPLA2 levels and sensory abnormalities is a measure of the severity of autism. Their findings suggested that impaired phospholipid led to the activation of cPLA2 and was directly related to oxidative stress and neuroinflammation, the two pathological mechanisms in autism.

Several factors could lead to the proposed activation of PLA2, such as the lack of phosphatidyl choline (PC), changes in the gut environment, or the applied neurotoxic stress in the animals. Finding the right mechanism can help develop a therapeutic strategy for its inhibition [24, 25].

Apart from being important energy resources, SCFAs regulate different host mechanisms, including cytokine production and oxidative stress [26]. They are indirectly or directly involved in regulating lipid and protein metabolism, thereby forming a tripod interconnection between the gut, the liver, and the brain [26, 27]. In the present study, the impact of PPA on the gut microbiota and the activation of cPLA2 was studied. The study aimed to investigate the inter-connection of gut-liver-brain axis and study the effect of alterations in the gut microbiota composition on the phylum and species level on this axis.

2. MATERIALS AND METHOD

2.1. Animals

Thirty-five male western albino rats (with an approximate body weight of 100g) were randomly divided into five groups as follows; Group I, was the control group; Group II was PPA treated group (treated with PPA, 250 mg/kg body weight/day for 3 days), according to the repeatedly used method of El-Ansary *et al.* [15];; Group III was vitamin B12 treated group (treated with 16.7 mg/kg/day of Vitamin B12 for 30 days after PPA treatment) Group IV was ω-3-treated group (treated with ω-3 200 mg/kg body weight/day for 30 days after PPA treatment for 3 days); Group V as ω-3+ Vitamin B12 group (given same dose and duration of ω -3 + Vitamin B12 after PPA treatment for 3 days. All treatments were given orally. The protocol of the present work was approved by the Ethics Committee at the King Saud University and all experiments were performed in accordance with the guidelines of the National Animal Care and Use Committee in accordance to the Helsinki Declaration.

2.2. Liver Sample Collection

The whole liver was collected and properly washed. Tissues were homogenized in RIPA buffer (ten volume/weight). The supernatant was clarified by centrifuging the homogenate at 3000 rpm for 10 min, which was then used for various assays.

2.2.1. Measurement of cPLA2 Enzymatic Activity

The activity of cytosolic Phospholipase A2 (cPLA2) in liver homogenates was investigated on arachidonoyl thio-PC as a substrate using cPLA2 Assay Kit (Item Nº 765021, Cayman, USA) according to the manufacturer's instructions. Free thiol, released after hydrolysis of the arachidonoyl thioester bond at sn-2 position by PLA2, was detected by DTNB assay. The absorbance was then measured at 405 nm using a plate reader.

2.2.2. Measurement of cPLA2 Protein using Western Blot

The total protein in the liver was assayed by the Bradford method. 60 µg protein was separated on SDS-PAGE, electro-transferred to nitrocellulose membranes and blocked in 5% skimmed milk in TBS Tween 20. The blocked mem-

branes were probed with rabbit anti- cPLA2 and mouse anti--GAPDH primary antibodies (Santa Cruz Biotechnology-454, & mAbcam 9482, respectively). After washing, the membranes were incubated with the secondary antibodies and developed using an enhanced chemiluminescence kit (Bio-Rad, USA). The blots were scanned, and intensities of the relevant bands were quantified using the Image J software (NIH, USA). Results were normalized to GAPDH and presented as percent of control.

2.3. Fecal Microbial Analysis

2.3.1. Fecal Sample Collection and Processing

Animal fecal pellets were collected in sterile test tubes, every morning at the beginning and end of the experiment and were immediately stored at -80˚C for further bacterial screening. Fecal suspensions from each stool sample were prepared in sterile phosphate buffered saline (PBS, pH 7) (1:10 w/v) [28]. The suspensions were mixed by vortexing for 1 min, followed by centrifugation at 4000 rpm for 3 min at 4˚C [29]. Serial dilutions (ranging from 10-1 to 10-4) of each fecal suspension were prepared in sterile PBS. 100µl of each dilution was spotted on 5 different selective media plates: nutrient agar (NA, Oxoid, MacConkey (MAC, Oxoid) for the identification of coliforms, Blood agar (Oxoid), Cycloserine- Cefoxitine Fructose Agar (CCFA, Oxoid) particularly for the isolation of Clostridium sp. and Bacteroides Bile Esculin Agar (BBE, Oxoid) for the identification of Bacteroides sp. specifically. Plates, particularly NA, Blood, and Mac Conkey, were incubated at 37˚C for 18-24 hours aerobically. CCFA and BBE plates were incubated for 3 days at 37˚C under anaerobic conditions (5% CO2) [29]. All experiments were performed in triplicates and data were presented as histograms.

2.3.2. Bacterial Enumeration and Microscopic Identification

Single colonies obtained in different culture media plates following incubation were studied macroscopically, numbered, and then microscopically identified by gram staining technique. This was performed for both preliminary identification and screening of the alteration in the gut microbiota composition in response to treatments applied in this study.

2.4. Statistical Analysis

The results of the present study were expressed as mean ± SD. All statistical comparisons between the control group and the four studied groups were performed using one-way analysis of variance (ANOVA) tests with Dunnett's test for multiple comparisons. Package for the Social Sciences (SPSS, Chicago, IL, USA) was used. Values were considered to be significant if $P < 0.05$.

3. RESULTS

Table **1** and Fig. (**1**) demonstrate the mean ± SD and the percentage change of liver cPLA2 activity (nmoles/ minute/ mg protein) in PPA-treated rats. PPA treatment induced 87.9% increase in the enzymatic activity.

Table 1. Mean ± SD of cPLA2 activity (nmoles/ minute/ mg protein), and percentage change in the four studied groups relative to the control (*P* value <0.05).

Groups	Mean ± SD	% change
Control	11.17±1.37	100
PPA	21.00 ±1.28**	187.9
PPA+B12	13.43±1.05	120.2
PPA+ ω-3	12.05±0.79	107.9
PPA+B12+ ω-3	11.31±0.52	101.2

Both ω-3 and vitamin B12 were effective in countering the neurotoxic effect of PPA. The three groups treated with ω − 3, vitamin B12, or a combination of both were not significantly different from the control group, but were found to be significantly different from the PPA-treated groups. Fig. (**2**) illustrates the overexpression of cPLA2 and the marked attenuation of its expression in PPA–treated rats together with the ameliorative effects of ω − 3, vitamin B12 either independently or in combination.

A comparison of PPA induced liver toxicity through the increase of cPLA2 protein concentration showed that the liver was more affected than the brain (Fig. **3**), presented from Alfawaz *et al.* [30, 31] with permission). cPLA2 was more affected in the liver compared to the brain with PPA toxicity. Liver cPLA2 was increased by 13-fold as compared to a 2.5-fold increase of cPLA2 in the brain of PPA-treated rats.

Results from the present study indicated that the presence of *S. aureus* in the gut was greatly affected by the PPA intake (30-colony count/plate); they were significantly reduced at day 30 compared to the control group and the other groups in the study, but remained the most dominant bacterial type present throughout the experiment (Fig. **4**). Enterobacteriaceae as well as *Clostridium* sp. both were low or absent in all tested groups at Day 30, this could be attributed to the overgrowth of *Staphylococcus aureus*.

4. DISCUSSION

Under normal physiological conditions, cPLA2 releases phospholipid arachidonic acid (AA) as a substrate for COX-2, an enzyme catalyzing the production of inflammatory prostaglandin. PPA-induced activation of brain cPLA2 could lead to a remarkable increase in membrane phospholipid breakdown [30]. In the present study, Table 1 and Fig. **(1)** demonstrate significant activation of liver cPLA2 in response to the toxic effect of PPA. Previous work showed that PPA-treated rodent model of autism has significantly higher levels of lipid peroxides and 5-lipoxygenase (5-LOX) and significantly lower levels of glutathione (GSH), glutathione S-transferase (GST), and cyclooxygenase 2 (COX2), when compared to healthy control animals [31, 32]. Moreover, a remarkable amelioration of most of the impaired markers was observed when treated with oral supplementation of ω-3 and vitamin B12, either alone or in combination. The results show that impairment at various steps of the lipid metabolic pathways contributes to the development

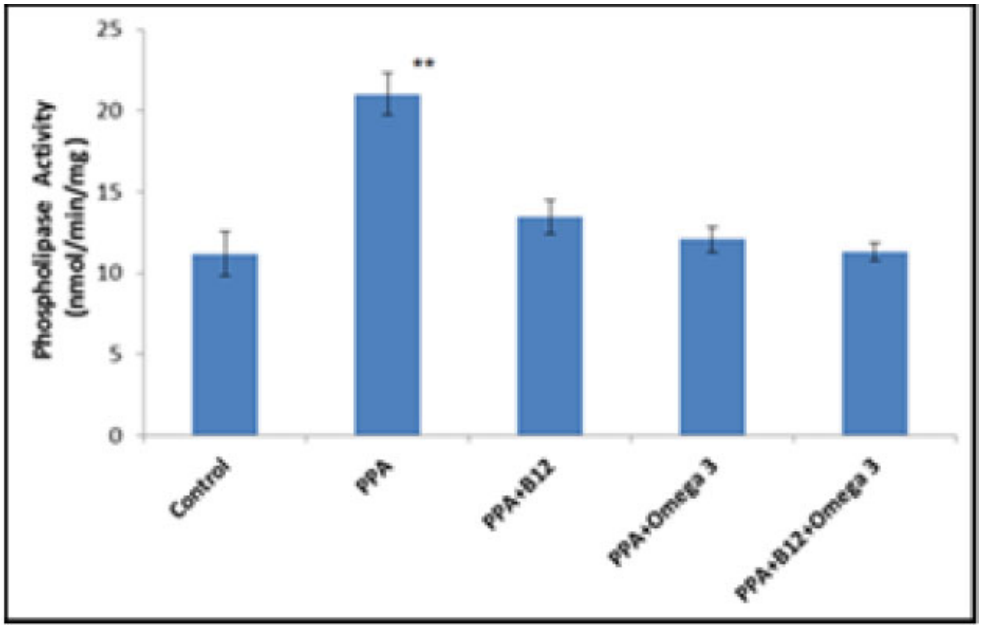

Fig. (1). cPLA2 enzymatic activity in the four studied groups relative to the control (P-value <0.05).

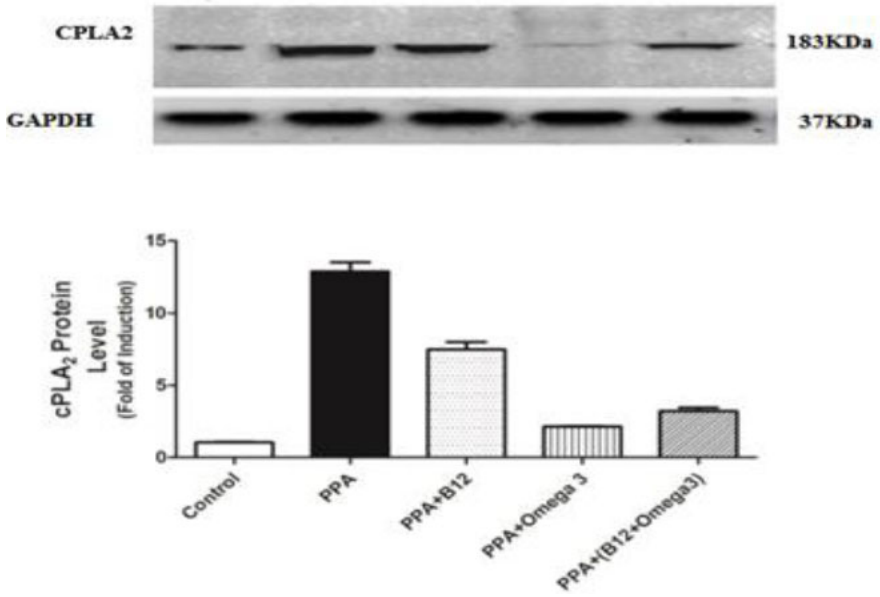

Fig. (2). Western blot analysis of whole liver homogenates of control, PPA-treated and B12 and ω-3- treated groups.

of autism. However, supplementation with ω-3 and vitamin B12 can result in a positive therapeutic effect.

The remarkable increase of protein and activity levels of cPLA2 (Table **1**, Fig. **(1)** and Fig. **(2)**) in PPA-treated rat liver and brain, and their amelioration with ω-3 PUFA can easily be supported by the findings of Rapoport *et al.* [33]. This study reported that ω-3 PUFA-deficient diet increased brain mRNA, protein and activity levels of AA-selective cPLA2, secretory sPLA2, and COX-2 [34], enzymes that directly contribute to brain AA metabolism [35, 36]. Based on the fact that ω-3 - deficient diet up-regulated brain ω -6 PUFA metabolisms, suggest that dietary ω-3 PUFA supplementation may have a reverse effect. Excess AA metabolism can contribute to neuronal damage through glutamate excitotoxicity and neuroinflammation as two etiological mechanisms of autism either clinically or in PPA- induced rodent

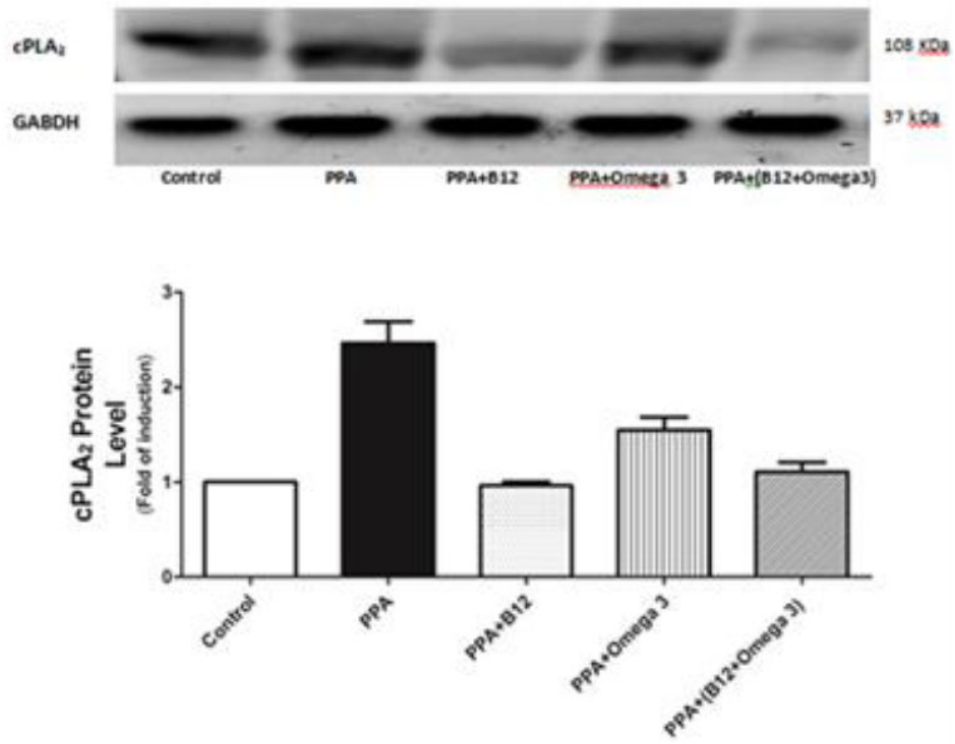

Fig. (3). Western blot analysis of the whole brain homogenates of control, PPA-intoxicated and B12 and ω-3- treated groups (Re-presented from: Alfawaz *et al.* [30, 31], after online permission through: joseph.hasan@biomedcentral.com.

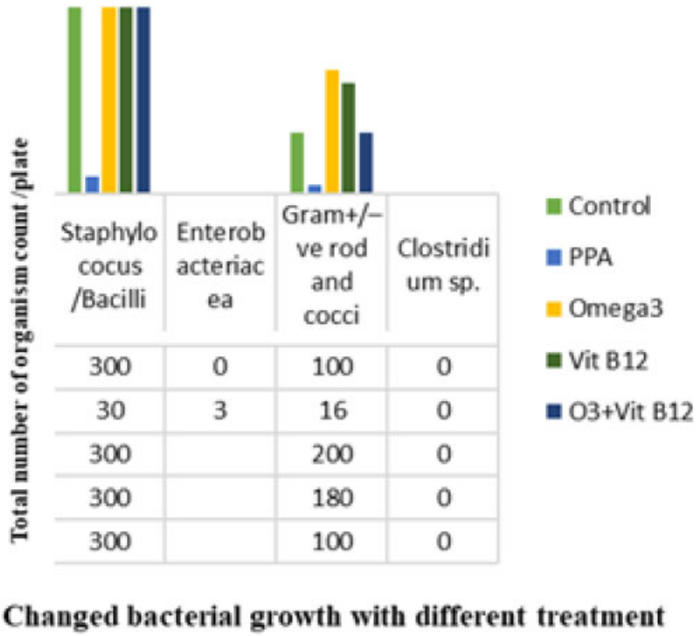

Fig. (4). Total bacterial plate count at day 30 showing *Staphylococcus aureus* suppression in PPA group compared to the control and other groups in the study. The abundance of *S. aureus* in all treated groups including control was noted to overcome the presence of other bacterial species.

models [15, 35-37]. The findings that an increase in cPLA2 (activity and concentration) is inter-related with the induced autistic features in PPA rodent model can be further supported by the findings presented in the clinical study by Bell *et al*. [38]. They showed that a dramatic loss of PUFA from red blood cells (RBC) membranes in cold storage (-20 C°) could be attributed to the increased phospholipid breakdown due to elevated PLA2 activity and/or concentration in samples isolated from autism patients but not in controls.

The liver-brain axis is inter-related through the capacity of both organs to synthesize docosahexaenoic acid (DHA) ω-3 fatty acid from alpha-linolenic acid (α-LNA). However, the brains capacity for DHA synthesis is relatively low. During ω-3 fatty acid deficiency, the liver increases its coefficients for DHA synthesis by up-regulating activities of relevant desaturases and elongases. Moreover, lower levels of brain-derived neurotrophic factor (BDNF) and cAMP response element-binding protein (CREB) in ω-3 diet deficient rats, likely render their brain more vulnerable to neuropathological insults [33].

With the increase in interest in the microbiota and the gut-brain axis, it is not surprising that researchers are investigating beyond the common phenotypes, such as depression or anxiety, and studying other neurological and developmental disorders. A study in the rodent model showed that the composition of gut microbiota in animals with autism-like behavior showed maximum alterations in *Clostridium* and *Bacteroides* species [39-41]. In the current study, while *Clostridium sp.* was undetectable in all groups, *S. aureus* demonstrated much lower abundance in PPA-treated rats as an autistic model compared to control, and ω-3 and vitamin B12, either alone or in combination (Fig. **(4)**). This can find support through considering two phenomena, first is the suppression of *S. aureus* growth with the bactericidal effect of PPA-induced cPLA2 activity, and second, is the production of endogenous PLA2 enzyme through the expression of S. *aureus* bacterial messenger RNA (mRNA) [42]. All of these may lead to alteration in the gut microbial composition with PPA as well as in response to other treatment groups indicating the lowest *Staphylococcus* abundance in individuals with autism [43, 44]. It is not unusual for one bacterial species to overcome or even eliminate another species. In this study, changed microbiota was observed mainly dominated by *S. aureus*. In agreement with the findings, previous studies suggested that colonization of the intestine by *S. aureus* can occur. However, this phenomenon has not been intensively studied and could be of clinical importance for limiting or eliminating the invasion of other pathogenic bacteria [45, 46]. The mechanism of action or the metabolic pathways between the gut-liver-brain axes is still not clear. To date, no suitable *in vivo* experiments have demonstrated the survival of *S. aureus* in the human intestine. Consequently, highly present *S. aureus* in this study could be explained by a reciprocal influence of increased level of SFACs on the *S. aureus* growth and vice versa [46] and by de novo synthesis of cPLA2, as reported by Foreman-Wykert [47] in agreement with the findings, assuming as such that there is an inter-connection between the gut-brain and the liver.

Another study has reported a significant difference in the fatty acid composition of phospholipids in children with autism and normal children. Autistic children showed an increased level of most of the saturated fatty acids, except for PPA acid, and a decreased level of ω-3 PUFA [38]. They concluded that a lower level of PPA in the plasma is due to its influx from blood to the brain. This change in the composition of fatty acids can lead to abnormalities in oxidative stress or cause mitochondrial dysfunction that might play a role in the pathogenesis of ASD [48].

Recent evidence suggests that certain bacterial pathogens have acquired genes encoding secreted phospholipase A2 enzymes. *Pseudomonas aeruginosa* and group A Streptococcus are among the PLA2 producers [49]. These enzymes modulate the host inflammatory response, increase the severity of disease, and otherwise, alter host-pathogen interactions. In an attempt to correlate the PPA-induced dysbiosis (Fig. **4**) to the remarkably activated liver cPLA2, it was interesting to know that the mitogen-activated protein kinases (MAP kinases), extracellular signal-regulated kinase (ERK) and p38, have both been related to endothelial survival or death [50], and when activated, lead to the triggering of highly active cPLA2. Stimulation of these proteins with selected bacteria caused AA release and enhanced *in vitro* cPLA2 activity of cell lysate by 1.5-fold and 1.7-fold, respectively [51].

Previous studies have shown a remarkable increase of lipid hydroperoxides (PLOOH) as reactive oxygen species concomitant with glutathione depletion in PPA-treated rats. It is, therefore, suggested that these free radicals, in addition to their damaging effects, can be a source of inflammatory lipid mediators in the liver and the brain [14, 15]. Because of their hydrophilic – OOH group, they induce rearrangement in the membrane lipids, which might affect cPLA2 as membrane phospholipid hydrolyzing enzymes with subsequent increased activity of other oxidases.

In the present study, the significant increase of liver cPLA2 in PPA-treated rats can be easily related to glutamate excitotoxicity as a repeatedly reported etiological mechanism in autism. Acute and chronic liver dysfunction elevates the concentration of ammonia in the brain, a condition known as hyperammonemia in patients and rodent model of autism [52]. Hyperammonemia, consequently upsurges the extracellular level of glutamate, since ammonia reduces the glutamate uptake by glutamate transporters and inhibits astrocyte glutamine synthase [53, 54]. This elevated level of extracellular glutamate activates the NMDA type of glutamate receptor in the brain [55]. Activation of the NMDA receptor leads to excitotoxicity and neuronal injury, which is associated with several diseases, including acute or chronic liver failure [56].

An increased liver and brain cPLA2 activities stimulate an abnormal efflux of FFAs, which are substrates of inflammatory enzymes, such as COXs and LOXs, resulting in increased generation of both oxidized lipids and inflammatory eicosanoids. PLA2 activity also influences the level of phospholipids that contain PUFAs on the neuronal cell mem-

brane, and those of lysophospholipids (LPLs), with consequent changes in the phospholipidome of the brain [54].

CONCLUSION

This study demonstrates that altered gut microbiota homeostasis can affect the physiological functions of the liver and the brain. This study provides evidence which links dysbiotic gut microbiota and harmful microbial metabolites, such as PPA and ammonia, with liver inflammation through the increase in protein level and activity of cPLA2. The inability of the liver of dysbiotic rats to clear such products, plays an important role in neuroinflammation, and glutamate excitotoxicity as neurotoxic events related to autism. This study suggests that gut microbiota could be considered to be a target for autism treatment, through the use of prebiotics (*e.g*: ω-3, and vitamin B12), probiotics, and fecal transplantation.

LIST OF ABBREVIATIONS

ASD	=	Autism Spectrum Disorders
α-LNA	=	Alpha-linolenic acid
AA	=	ω-6 arachidonic acid
BDNF	=	Brain derived neurotrophic factor
BBB	=	blood brain barrier
BBE	=	Bile Esculin Agar
cPLA2	=	Cytosolic phospholipase A2
CREB	=	cAMP response element-binding protein
COX2	=	Cyclooxygenase 2
CCFA	=	Cycloserine- Cefoxitine Fructose Agar
DHA	=	Docosahexaenoic acid
GSH	=	Glutathione
GST	=	Glutathione S-transferase
5-LOX	=	5-lipoxygenase
LPLs	=	Lysophospholipids
IFN-γ	=	Interferon gamma
IL-6	=	Interleukin 6
TNF-α	=	Tumor necrosis factor
MAC	=	MacConkey
NA	=	nutrient agar
NMDA	=	N-methyl-d-aspartate
ANOVA	=	One-way analysis of variance
ω -3	=	Omega -3
PPA	=	Propionic acid
PBS	=	Phosphate buffered saline
PC	=	Phosphatidyl choline
RBC	=	Red blood cells
RIPA	=	Radioimmunoprecipitation assay buffer
SCFAs	=	Short chain fatty acids

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Ethics Committee of College of Science, King Saud University, Riyadh, Saudi Arabia.

HUMAN AND ANIMAL RIGHTS

The reported experiments in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

Written informed consent was taken from all participants.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author [Afaf El-Ansary], upon reasonable request.

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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