**IN VIVO EVALUATION OF THE EFFECT OF ARSENITE ON THE
INTESTINAL EPITHELIUM AND ASSOCIATED MICROBIOTA IN MICE**

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Highlights:

In vivo intestinal toxicity of As(III) was evaluated using female BALB/c mice.

As(III) increases oxidative stress and the pro-inflammatory response in large intestine.

As(III) produces down-regulation of *Muc2*, the main mucin of the intestinal mucus.

As(III) alters the barrier function and produces changes in the intestinal microbiota.

This is the first study that shows the intestinal toxicity of As(III) in subchronic exposure.

Abstract

Chronic exposure to inorganic arsenic (As) [As(III) + As(V)], which affects millions of people, increases the incidence of some kinds of cancer and other non-carcinogenic pathologies. Although the oral pathway is the main form of exposure, *in vivo* studies have not been conducted to verify the intestinal toxicity of this metalloid.

The aim of this study is to perform an *in vivo* evaluation of the intestinal toxicity of inorganic As, using female BALB/c mice exposed through drinking water to various concentrations of As(III) (20, 50, and 80 mg/L) for 2 months. An increase was observed in oxygen and/or nitrogen reactive species, and in gene and protein expression of pro-inflammatory cytokines (IL-1 β , IL-2, IL-6) at concentrations equal to or greater than 50 mg/L. These changes were accompanied by a profound remodeling of the intestinal microbial profile in terms of diversity and global composition, which could be at the basis or exacerbate As(III) toxic effects. The histological study showed that there was moderate inflammation of the mucosa and submucosa, accompanied by hyperplasia of crypts at the highest administered dose. In addition, all the treatments with As(III) resulted in a decreased expression of *Muc2*, which encodes one of the main components of the intestinal layer of mucus. The effects described are compatible with the increased intestinal permeability observed at concentrations equal to or greater than 50 mg/L, indicative of loss of barrier function.

Introduction

Arsenic (As) is a metalloid widely distributed in the environment. Of all the forms of arsenic present in nature, inorganic As [arsenite, As(III), and arsenate, As(V)] and the trivalent metabolites monomethylarsonous acid [MMA(III)] and dimethylarsinous acid [DMA(III)] are the most toxic species.

The main dietary sources of inorganic As vary depending on which region of the world is considered. In some areas of Asia and Latin America drinking water contains As concentrations considerably above the maximum value recommended by the World Health Organization (0.01 mg/L) (Bundschuh et al. 2012; George et al. 2014; Yunus et al. 2016). Therefore, water and food cooked with that water are the main sources of exposure in those areas. In countries where drinking water is not contaminated the main dietary source are food products such as cereals and seafood (EFSA, 2009).

Oral intake is the main way of inorganic As exposure; however, there are few studies on the effect of inorganic As on the gastrointestinal tract. In populations chronically exposed to inorganic As through drinking water there have been reports of symptoms of dyspepsia and gastroenteritis (Borgoño et al. 1977; Majumdar et al. 2009). *In vitro* studies using colon epithelial cells have shown that acute exposure to inorganic As, mainly to the trivalent form, generates oxidative stress, a pro-inflammatory response, and also the redistribution of the proteins that form tight junctions (Calatayud et al. 2013; 2014; Chiocchetti et al. 2018). Subchronic exposure of intestinal cells to inorganic As produces a prolonged pro-inflammatory response, which may be the cause of the loss of barrier function of intestinal monolayers and dysregulation of the cell renewal process (Chiocchetti et al. 2018).

The *in vivo* evaluation of the intestinal toxicity of inorganic As is limited. Exposure of rodents to monomethylarsonic acid [MMA(V)] for prolonged periods produced

enlargement of the gastrointestinal wall, edema, hemorrhages, necrosis, and ulceration or perforation of the mucosa of the large intestine; and also a significant increase in the incidence of squamous metaplasia of absorptive epithelial cells in the colon and rectum (Arnold et al., 2003). As(III) exposure has been reported to result in significant changes in the composition (Chi et al. 2017) and functionality (Lu et al. 2014) of the intestinal microbiota of mice, which results in a disrupted metabolome profile in feces, serum and urine (Lu et al. 2014). Moreover, it has been reported that intestinal microbiota can metabolize inorganic arsenic species mainly by methylation (Yin et al. 2015; Van de Wiele et al. 2010), which could affect As intestinal absorption and toxicity.

The results mentioned above suggest that inorganic As could exert toxicity in the intestine. The aim of the present study is to evaluate whether exposure to As(III) affects the structure and functionality of the intestinal epithelium and its associated microbiota in *in vivo* studies in animals subchronically exposed.

Materials and Methods

Animals. Female BALB/c mice were used, obtained from Envigo (n = 36). They were acquired at the age of 6 weeks and with a weight that varied between 15 and 20 g. During the study the animals were kept in controlled environmental conditions (cycles of 12 hours of light and dark, room temperature of 22 °C and humidity of 75%). The mice were fed *ad libitum* with standard rodent maintenance feed which, according to our analyses, contained a small concentration of As (50 ng/g). The protocols applied to the animals were designed in conformity with the Spanish regulations for the use of experimental animals (Real Decreto 53/2013) and were approved by the Ethical Committee for Use of Laboratory Animals of the University of Valencia (Spain) and the Agriculture, Fisheries, and Food Council of the Generalitat Valenciana (Spain).

93

94 **Treatment and collection of samples.** The mice were divided into four groups of nine
95 animals, which were housed in groups of three. The first group was used as a control
96 and various doses of As(III) were administered to the other three groups for two months
97 through drinking water (20, 50, and 80 mg/L, approximately equivalent to 3, 7.5, and
98 12 mg/kg body weight/day). The doses were selected on the basis of the toxicological
99 data reported for mice in subchronic exposures (ATSDR, 2000). The water was changed
100 every 3 days to reduce the possibility of oxidation of As(III). Throughout the
101 experiment the body weight, quantity of water ingested, and physical and behavioral
102 aspects of the mice were monitored.

103 After anesthesia by inhalation of isoflurane, euthanasia was performed by cervical
104 dislocation. The intestine was immediately recovered, washed abundantly with 0.9%
105 NaCl (w/v), separated into various fragments, and then stored at -80 °C for subsequent
106 analysis. Some of the fragments required specific storage or preparation techniques,
107 which are detailed in each section.

108

109 **Determination of ROS and/or RNS generation.** Samples were homogenized with a
110 TissueRuptor II (QIAGEN), using 3 cycles of 30 s at 4 °C, in modified RIPA buffer
111 [150 mM NaCl (Panreac), 50 mM Tris-HCl pH 8 buffer (Sigma), 5 mM EDTA (Sigma),
112 1% v/v Triton X-100 (Sigma), 0.5% w/v sodium deoxycholate (Sigma), 10% w/v
113 protease inhibitor (Roche)] at a proportion of 1:20 (w/v). The supernatant obtained after
114 centrifugation (5700 ×g, 5 min) was used for the determination of ROS/RNS, using the
115 technique of oxidation of dihydrodichlorofluorescein diacetate (DHCF-DA) to
116 dichlorofluorescein (DCF) (Wang and Joseph 1999). Five µL of a solution of 10 µM
117 DHCF-DA (Sigma) in PBS were added to aliquots of supernatants (100 µL) and

incubated for 30 min. Fluorescence was subsequently determined in a CLARIOstar microplate reader (BMG-Labtech; excitation/emission λ = 488/530 nm). The fluorescence values obtained were normalized per gram of tissue.

Transcriptional analysis of pro-inflammatory cytokines, mucin, and tight junction proteins. The expression of genes encoding mucin 2 (*Muc2*), macrophage inflammatory protein (*Mip2*), inflammatory cytokines (*Tnfa*, *Il-1 β* , *Il-2*, and *Il-6*), different claudins (*Cldn-1*, *Cldn-2*, *Cldn-4*), and zonula occludens protein 1 (*Zo-1*) were evaluated by RT-qPCR.

The tissue obtained after euthanasia was immersed in a solution of RNAlater (Qiagen) for 24 h at 4 °C and then, after elimination of the RNAlater, it was stored at -80 °C until analysis. For the extraction of RNA, approximately 10 mg of tissue were homogenized using the TissueRuptor II and the NucleoSpin RNA II kit (Macherey-Nagel). The extracted RNA was quantified spectroscopically using a NanoDrop ND-1000 system (NanoDrop Technologies). First strand complementary DNA (cDNA) was obtained from 200 ng of total RNA using the SuperScript VILO cDNA Synthesis kit (Invitrogen), following the manufacturer's specifications.

qPCR was performed using the LightCycler 480 Real-Time PCR system (Roche Diagnostics). Reactions were carried out in a final volume of 10 μ L containing 5 μ L of LightCycler 480 SYBR Green I Master Mix (2 $\times$, Roche), 2.5 μ L of cDNA (20 ng/ μ L), 1 μ L of each forward and reverse primer (10 μ M; Biolegio) (Supplementary Table 1), and nuclease-free water. *Rn18S* and *Rplp0* were employed as reference genes. PCR efficiency curves were calculated for each gene, using triplicates of a 10-fold dilution curve.

The qPCR conditions were 95 °C for 5 min, followed by 40 cycles: 10 s denaturation at 95 °C, 10 s annealing at 55 °C, and 20 s elongation at 72 °C. The melting curve of each sample was analyzed to confirm PCR product specificity. The data were analyzed with the Relative Expression Software Tool (REST 2009, Qiagen).

Quantification of pro-inflammatory cytokines. Samples of intestine were homogenized as described in section 2.3.1. The cleared supernatants were used to determine the pro-inflammatory cytokines IL-2, IL-6 and IL-1 β using specific ELISA kits (ELISA Mouse Platinum, eBioscience), following the manufacturer's instructions. The results were normalized per gram of tissue.

Histopathological study of the intestine. Fractions of the small or large intestine (~1 cm) were fixed with 4% paraformaldehyde for 12–24 h and then embedded in paraffin. Sections of paraffin (1–2 μ m) were cut, dried, dewaxed, rehydrated and then histochemically stained with hematoxylin/eosin (Merck). For the detection of goblet cells, periodic acid–Schiff staining (PAS) (Sigma) was performed in accordance with standard methods. Images were obtained using an AxioImager Z1 microscope (Carl Zeiss MicroImaging). The samples were evaluated in accordance with the methodology described by Erben et al. (2014). In order to visualize the distribution of the tight junction protein CLDN-1 in the large intestine, tissue sections were cut, deparaffinized and subjected to a heat-induced epitope retrieval step at pH 8. After blocking endogenous peroxidase, sections were incubated with primary antibody (polyclonal rabbit-anti-mouse claudin-1, ThermoScientific). For detection, EnVision+ System- HRP Labelled Polymer Anti-Rabbit (Agilent Technologies) was employed. Nuclei were counterstained with hematoxylin (Merck) and sections coverslipped with gelatin

(Merck). Negative controls were performed by omitting the primary antibody. Images were acquired using the AxioImager Z1 microscope. All evaluations were performed in a blinded manner.

Evaluation of intestinal permeability. Intestinal permeability was evaluated by determining the concentration of albumin in feces. Feces (~50 mg) were homogenized in 0.5 mL of modified RIPA buffer using a bead beater (FastPrep-24 5G Instrument, MP Biomedical) with glass beads (0.5 g; 0.1 mm diameter). The samples were subjected to three cycles of beating for 60 s at a speed of 6 m/s, during which the samples and 60 s in ice. The homogenate was centrifuged (5700 $\times$ g, 5 min, 4 °C) and the concentration of fecal albumin was determined in the supernatant using the Mouse Albumin ELISA kit (Cusabio), following the manufacturer's instructions. The results were normalized per 100 mg of feces.

Evaluation of changes in the intestinal microbiota composition. DNA was extracted from mice fecal pellets obtained at the end of the experimental period using the Master PureTM kit (Epicentre) including a bead beater treatment with 0.5 g of 0.1 mm glass beads in the lysis step, followed by a further purification of the DNA samples with mi-PCR Purification kit (Metabion). Isolated DNA was quantified with a Qubit 2.0 fluorometer (Invitrogen). Bar-coded amplicons of the 16S rDNA V3-V4 region were subjected to 2 $\times$ 300 bp paired-end run in a MiSeq-Illumina platform (FISABIO sequencing service, Valencia, Spain). Quality assessment and sequence joining was performed with PrinSeq-lite (Schmieder et al. 2011) and FLASH (Magoc et al. 2011), respectively. A total of non-chimera 2334516 reads were obtained with a mean of 70742 sequences per sample. Taxonomical assignments were performed with QIIME v1.9.1

(Caporaso et al. 2010) using the Greengenes database of 16S rDNA version 13.8 at 97% identity. Datasets were rarefied to the minimum library size (52509 reads) and normalized by total-sum scaling for the statistical analyses, which were carried out with Calypso 8.84 (<http://cgenome.net/calypso/>) and MicrobiomeAnalyst (<https://www.microbiomeanalyst.ca/>; Dhariwal et al., 2017) software pipelines. Predictive functional analysis was performed using PICRUSt (Langille et al. 2013) with KEGG pathways and statistically analyzed with STAMP v2.1.3 (Parks et al. 2014). The raw sequencing fastq files have been deposited in the RAST metagenomics server (<https://www.mg-rast.org/>) under project ID mgp87488.

Results

Two animals of the group exposed to the highest dose (80 mg/L) were euthanized before the end of the assay, specifically in the 4th week, as they had experienced a weight loss over 20% and presented symptoms of abdominal pain. The other animals did not present notable physical changes, and the weights in all the groups were similar throughout the study.

Evaluation of oxidative stress in the intestine. Fig.1 shows the formation of DCF in samples of the large intestine of control animals and animals treated with various doses of As(III) for 2 months. No significant differences were observed in samples of small intestine (data not shown). In the tissue from the large intestine, however, an increase in generation of ROS and/or RNS was observed at the higher doses (medians: control, 205 UAF/mg tissue; 20 mg/L, 202 UAF/mg tissue; 50 mg/L, 350 UAF/mg tissue; 80 mg/L, 376 UAF/mg tissue). These data are indicative of oxidative stress in the large intestine as a result of subchronic exposure to As(III).

Evaluation of the intestinal pro-inflammatory response.

Gene expression of cytokines. Only the relative expression of the genes of interest in the large intestine was evaluated because the RNA obtained from the small intestine showed signs of degradation, according to data obtained by electrophoresis. Heumüller-Klug et al. (2015) showed the difficulty of extracting RNA from the small intestine of humans and rats, indicating that it degrades more rapidly than RNA from the colon.

Table 1 shows the relative expression of *Il-1 β* , *Il-6*, *Il-2*, *Mip-2*, and *Tnfa* in tissues obtained from the large intestine. The results indicate overexpression of *Mip-2* and *Il-2* at all the doses assayed, and of *Il-1 β* and *Il-6* at the higher doses.

Protein expression of cytokines. The protein concentration of selected cytokines with up-regulated expression was estimated in samples from both sections of the intestine. Cytokine concentrations in samples of small intestine were below the limit of detection of the methodology employed (data not shown).

Fig. 2 shows the quantities of pro-inflammatory cytokines IL-2 (2A), IL-6 (2B) and IL-1 β (2C) in samples of large intestine of control mice and mice treated with various doses of As(III). At protein level, there were also increments of the cytokines with As(III) treatments. For IL-2, significant increases were observed in the animals exposed to 20 mg/L (35%) and 50 mg/L (136%). IL-6 level only was significantly increased in the animals treated with 50 mg/L (89%). The cytokine IL-1 β presented the highest increments [50 mg/L (558%), 80 mg/L (279 %)]. Unlike what was observed at transcriptional level, the highest dose of As(III) did not produce significant increases in the quantities of IL-2 and IL-6 cytokines. This discrepancy indicates that other regulatory mechanisms (e.g. those controlling translation and/or protein turnover) can have a major contribution to protein levels, specially under high As(III) doses.

Structural studies of the intestine. The histological studies showed that the small intestine did not present notable anomalies in the animals treated with As(III) (data not shown). The observations of tissue from the large intestine showed adverse effects, especially in animals exposed to the highest concentration. Of the seven animals treated with 80 mg/L, five presented moderate inflammation of the mucosa and two of them showed a mild inflammation of the submucosa and hyperplasia of crypts. This hyperplasia was revealed by the presence of crypts elongated as a result of a volume

increase in epithelial cells (Fig. 3). Furthermore, lymphocyte infiltration, characteristic of mucosal inflammation, was also observed in As(III)-treated mice.

On the other hand, the density of goblet cells determined by means of PAS staining did not vary significantly between treatments [PAS⁺ cells/crypt: control (12.6 ± 4.9); 20 mg/L (11.5 ± 4.4); 50 mg/L (12.0 ± 7.4); 80 mg/L (18.0 ± 3.3)]; however, expression of *Muc2* decreased considerably as the exposure dose increased (reductions: 20 mg/L, 2.4 times; 50 mg/L, 5.4 times; 80 mg/L, 11.1 times).

Evaluation of intestinal permeability. Intestinal permeability was determined by analyzing the concentration of fecal albumin. Albumin is a globular protein produced by hepatocytes that occurs in the blood plasma where it represents about 50% of the protein content. An intact epithelial and endothelial barrier prevents the passage of albumin from the blood stream to the intestinal lumen, whereas situations in which the integrity of the intestinal barrier is altered can give rise to an increase in permeability, favoring the passage of albumin to the lumen and its appearance in feces (Nicholson et al. 2000).

Fig. 4 shows the concentration of albumin in the feces of control mice and mice treated with the various doses of As(III). The results show that there were statistically significant increases in fecal albumin in the mice treated with 50 mg/L (median: 0.85 μ g/100 mg feces; percentage increase: 39%) and 80 mg/L (median: 1.30 μ g/100 mg feces; percentage increase: 112%) with respect to the control mice (median: 0.61 μ g/100 mg feces).

Effect on expression and distribution of tight junction proteins. Expression and distribution of tight junction proteins is affected by As exposure in *in vitro* studies, and

it has been indicated as one of the causes of the increased permeability observed in Caco-2 cell monolayers (data not published). The present study evaluated whether the changes in permeability observed in animals were also accompanied by changes in expression of the tight junction *Cldn-1*, *-2*, and *-4*, and *Zo-1* in tissues obtained from the large intestine. The results indicate that there were no significant changes in expression of tight junction proteins irrespective of the As(III) dose, with expression values ranging from 1.18 ± 1.02 to 1.79 ± 0.62 (*Cldn-1*); 0.83 ± 0.77 to 1.81 ± 1.05 (*Cldn-2*); 0.78 ± 0.18 to 0.86 ± 0.29 (*Cldn-4*); 1.22 ± 0.28 to 1.34 ± 0.31 (*Zo-1*) fold changes relative to control samples. However, immunodetection of CLDN-1 (Fig. 5) showed that while this protein was membrane-located in epithelial cells from the large intestine in control samples, it displayed an increased cytoplasmic localization after As(III) treatment.

Effects on the intestinal microbiota composition. Analysis of the intestinal microbiota at OTU level showed that the four experimental groups were separately clustered, highlighting a concentration-dependent effect on the global microbial composition (Fig. 6a). Arsenic exposure resulted in an increased alpha diversity measured by Chao1 index (microbial richness; $p=0.0089$, $F=4.7$, ANOVA), while Shannon indexes did not vary ($p=0.16$, $F=1.8$, ANOVA). After the As(III) treatment multiple differences were observed in the distribution of bacterial taxa, which in most cases followed a dose-dependent evolution. As(III) resulted in enhanced numbers of the filo Bacteroidetes and Proteobacteria and decreased numbers of Firmicutes. The Verrucomicrobia experimented a sharp decrease at 20 mg/L dose, while they were progressively increased with the As(III) dose to levels higher than those observed in the control (Fig. 6b). At the family levels, and especially at 80 mg/L dose, increased numbers of the Lactobacillaceae, S24-7 (Bacteroidales), Lachnospiraceae, F16 (TM7 phylum),

Rikenellaceae, Prevotellaceae, Paraprevotellaceae and Verrucomicrobiaceae (attributed to OTUs belonging to *Akkermansia muciniphila* species (Fig. 7), a common inhabitant of the gastrointestinal tract) were observed. On the contrary, members of the Turicibacteraceae and Enterococcaceae were diminished (Supplementary Fig. 1). Feces of As(III)-treated animals were enriched in members of the genus *Prevotella* and *Lactobacillus*, while *Turicibacter*, *Coprobacillus* and *Enterococcus* were markedly reduced (Fig. 7). The dose-dependency in the changes was evidenced by Pearson's correlation analyses, which showed that the abundances of several taxa such as unclassified bacteria from S24-7 ($R=0.5$; $p<0.01$), *Prevotella* ($R=0.48$; $p<0.01$) and *Lactobacillus* ($R=0.48$; $p<0.01$) were positively correlated to As(III) concentrations, while *Turicibacter* ($R=-0.66$; $p<0.001$) and *Enterococcus* ($R=-0.47$; $p<0.01$) decreased with the As(III) doses (Supplementary Fig. 2). Of note, a clear positive correlation with As(III) dose was found for OTUs belonging to *Helicobacter*, which were almost absent in the control group ($R=0.7$; $p<0.001$).

The study of the metabolic capacities inferred from the microbiota composition of the fecal samples evidenced several functional categories that were enriched after As(III) treatments in a dose-dependent manner. These included glycan biosynthesis and metabolism ($p=0.0056$), pores and ion channels ($p=0.0066$), cell motility and secretion ($p=0.017$), folding and degradation ($p=0.018$) and membrane structural molecules ($p=0.027$), while sporulation was decreased ($p=0.035$). Lipopolysaccharide (LPS) synthesis and beta-lactam resistance, two categories which were shown to be increased in a previous work (Chi et al. 2017), were also increased in our study (Supplementary Fig. 3).

Discussion

The data obtained in the present study show an increase in production of pro-inflammatory cytokines (IL-1 β , IL-2, and IL-6) in the large intestine of the animals treated with As(III) concentrations equal to or greater than 50 mg/L in drinking water (Table 1, Fig. 2). This is the first *in vivo* evidence that this inflammatory process can establish in mice exposed to As(III), confirming the data obtained *in vitro* (Calatayud et al. 2014; Chiocchetti et al. 2018).

The greater gene and protein expression of pro-inflammatory cytokines observed in the intestine of animals treated with As(III) may be a consequence of the production of ROS/RNS, which, as shown, increased in the large intestine of animals treated with concentrations equal to or greater than 50 mg/L (Fig. 1). It is known that redox signaling tightly modulates the inflammatory response. Increased production of ROS/RNS can activate signal transduction cascades and also induce changes in transcription factors, such as NF- κ B, leading to subsequent expression of inflammatory cytokines, chemokines, and growth factors, which act by further recruiting inflammatory cells to the site of inflammation and thus lead to production of more reactive species (Morgan and Liu 2011; Lei et al. 2015).

An increase in expression of pro-inflammatory cytokines as a result of both stimuli (ROS and inorganic As) has been demonstrated in populations exposed through drinking water. Ahmed et al. (2011) evaluated the cause of the increase in inflammation markers in the placenta of women exposed during pregnancy and showed that up-regulation of expression of TNF α and IL-6 is only associated with oxidative stress; however, both As and oxidative stress seemed to play an important role in placental pro-inflammatory IL-1 β production.

Just as occurred *in vitro*, the pro-oxidant and pro-inflammatory response was accompanied by structural and functional changes in the epithelium. Histology showed

hyperplasia of crypts in some of the tissues of animals treated with the highest dose of As(III), and inflammation of the mucosa and, in some cases, the submucosa (Fig. 3). Furthermore, reduced expression of *Muc2* was observed in the animals treated with As(III). In physiological conditions, goblet cells constantly produce mucins to maintain the layer of mucus that surrounds the intestinal epithelium, the first line of defense in the gastrointestinal tract. However, the function of the goblet cell can be altered by various factors, including pro-inflammatory cytokines (Enss et al. 2000). If there is no mucus layer, as in the case of *Muc2*^{-/-} mice, colonization of enteric pathogens occurs to a greater extent and more easily than in wild type animals (Bergstrom et al. 2010). The fact that the animals treated with As(III) presented a lower expression of *Muc2* might imply a loss of integrity in the mucus layer, and therefore a reduction in the effectiveness of this first line of defense. Unfortunately, the structure of the mucus layer could not be characterized in this study, so that additional experimental evidence will be required to support this hypothesis.

The second component of the intestinal defense system is the epithelial cells that form a continuous cellular monolayer interconnected by tight junctions, which are responsible for maintaining the structure and polarization of the intestinal epithelium, and which provide it with its selective permeability. The present study showed an increase in permeability in the animals treated with concentrations equal to or greater than 50 mg/L (Fig. 4). This increase in intestinal permeability with As(III) was demonstrated earlier in *in vitro* studies, where it was accompanied by alterations in expression (down-regulation of *Cldn-1*) and localization (redistribution of ZO-1) of some tight junction proteins (Chiocchetti et al. 2018). Unlike what happened *in vitro*, in the *in vivo* exposure there was no reduction in expression of these proteins. However, a redistribution of the tight junction protein CLDN-1 was detected in our study. Changes

in the distribution of tight junction proteins often accompany inflammatory conditions (Capaldo and Nusrat 2009; Han et al. 2003). Therefore, changes in permeability upon As(III) exposure might be due to a modification in the distribution pattern of these proteins.

The present study also evidences that As(III) exposure altered the microbiome diversity and its bacterial taxa profiles (Fig. 6). The intestinal microbiota plays a pivotal role in multiple metabolic and immune functions in the host and alterations in its composition (dysbiosis) are evident for certain pathologies possessing an inflammatory component (e.g.: inflammatory bowel diseases, obesity, type II diabetes) (Turnbaugh et al. 2006; Man et al. 2011; Tilg and Moschen 2014). The microbiome alterations may be attributed to the intrinsic As(III) toxicity on bacteria or to altered mucosal characteristics which impacted the bacterial profile. As observed in our study, decreased *Muc2* expression is generally linked to inflammatory processes. Furthermore, mucin 2 have been shown to play a regulatory role on the expression of intestinal β -defensin 2, for which changes in *Muc2* expression indirectly impact the microbial intestinal composition (Cobo et al. 2015).

The microbial changes observed in our study were consistent with previous findings during As dosage in mice (Chi et al. 2016, 2017). Similar to our results, decreased Firmicutes have been reported after As(III) exposure in mice (0.1 mg/L As(III) in drinking water in C57BL/6 mice for 13 weeks; Chi et al. 2017). However, contrarily to these previous studies, an increase in bacterial richness (based on the number of different OTUs; Chao1) was observed in our experiments. This probably reflects that in our case a high negative impact of As in some bacterial taxa leads to niche occupation by other bacterial groups that are not so severely affected by the metalloid. However, the fact that bacterial diversity (Shannon) was not changed after As treatment, probably

indicates that the higher richness caused by As rely on OTUs with low abundance and therefore the evenness in the distribution of the population was not impacted. The qualitative and quantitative differences between our study and previous works clearly indicates that the experimental settings (i.e.: mice strains, As administration procedures, doses and treatment duration) impact the results. As an example, *A. muciniphila* levels, a bacterium which has been linked to health status in humans (Ottman et al. 2017) were increased after As treatment in the previous studies (10 mg/L As(III) in drinking water in C57BL/6 female mice for 4 weeks, changes were not observed in male mice; Chi et al. 2016, or 0.1 mg/L As(III) in drinking water in C57BL/6 female mice for 13 weeks; Chi et al. 2017). This was also corroborated in our study at the higher (80 mg/L) dose, while 20 mg/L dose resulted in a clear drop in *A. muciniphila* numbers. This bacterium feeds on host mucins (Ottman et al. 2017) and, while decreased *Muc2* expression might account for its low numbers at the 20 mg/L As(III) dose, the increased numbers found at higher doses must respond to a different cause. As discussed earlier, determination of the mucus layer status under As(III) exposure will be crucial to ascertain this point.

Whether changes in the microbiota are just a side effect of As(III) exposure or they contribute to As(III) intestinal damage by exacerbating or triggering some of the observed toxic effects needs to be ascertained. In this respect, it has been suggested that bacterial components and the profiles of metabolites derived from them could play a role in the outcomes of As(III) toxicity (Lu et al. 2014). The perturbations in the Firmicutes/Bacteroidetes ratio or the elevated Proteobacteria observed in our study have been previously linked to human inflammatory diseases (Turnbaugh et al. 2006; Man et al. 2011; Litvak et al. 2017). We have also observed that the microbiota from As(III)-treated mice was enriched in pathways related to the synthesis of the well-known inflammatory bacterial component LPS (as a results of increased numbers of the Gram-

negative Proteobacteria and Bacteroidetes). The reduction of *Muc2* expression in mice treated with As(III) may also have a bacterial contribution. Previous studies have evidenced an influence of members of the microbiota on the intestinal mucins expression (Graziani et al. 2016; Ren et al. 2017). It is also worth mentioning that certain bacteria are able to modify the tight junction's proteins distribution (Zakostelska et al. 2011; Simeoli et al. 2015). Thus, a direct role of the changes in the microbiota in altered permeability is also feasible.

Our data suggest that in chronic exposure to inorganic As, an intestinal inflammatory process might be established, possibly giving rise to chronic pathologies in the intestine or even at systemic level (Arrieta et al. 2006), partly owing to an increase in intestinal permeability. An altered microbiota was also evidenced which could contribute to enhance the toxic burden. Loss or reduction of the barrier function is considered a critical decisive factor in predisposition to the presentation of intestinal inflammatory processes, such as Crohn's disease, ulcerative colitis, and food allergies, and even in functional disorders of the intestine such as irritable bowel syndrome (Camilleri et al. 2012). There are no population studies in which the existence of pathologies of this kind were evaluated in detail. Moreover, an increase in permeability seems to be associated with other pathologies, such as type 2 diabetes (Cox et al. 2017) and some cardiovascular diseases (Rogler and Rosano 2014; Li et al. 2016), which are known to present a greater incidence in populations chronically exposed to inorganic As.

Conclusions

Subchronic exposure to As(III) in mice had structural and functional impacts on the large intestine whereas the small intestine was apparently slightly affected. As(III) exposure compromised the large intestine barrier function in treated animals as

evidenced by fecal albumin content is due to processes similar to those shown *in vitro*, mainly connected with the establishment of an inflammatory process which may be exacerbated by the experimented profound changes in the microbial profiles at the intestine.

This is the first study that shows the toxicity of inorganic As in the intestine *in vivo*. Further research is required to determine whether toxic effects in the intestine and microbiota changes are also observed as a result of emulating the exposure conditions that exist in areas of endemic chronic arsenicism. If this pattern of toxicity exists in longer exposures, it would also be interesting to determine whether it might be connected with some of the systemic pathologies associated with chronic exposure to inorganic As.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Table 1. Effect of exposure to As(III) on gene expression of pro-inflammatory cytokines in the large intestine of mice. Relative expression of *Il-1 β* , *Il-6*, *Mip-2*, *Il-2*, and *Tnfa* in animals treated with As(III) (20, 50, and 80 mg/L) for 2 months compared to control animals. Values expressed as fold-changes (mean $\pm$ SD, n = 7-9). Asterisks indicate statistically significant increases with respect to the control (p < 0.05).

	<i>Il-1β</i>	<i>Il-6</i>	<i>Mip2</i>	<i>Il-2</i>	<i>Tnfa</i>
20 mg/L	0.93 $\pm$ 0.46	1.42 $\pm$ 0.49	1.50 $\pm$ 0.51*	2.40 $\pm$ 0.64*	1.32 $\pm$ 0.65
50 mg/L	8.77 $\pm$ 3.81*	2.15 $\pm$ 1.16*	1.71 $\pm$ 0.70*	2.50 $\pm$ 0.68*	1.21 $\pm$ 0.63
80 mg/L	19.3 $\pm$ 19.4*	4.18 $\pm$ 2.92*	5.08 $\pm$ 2.95*	4.12 $\pm$ 1.80*	2.03 $\pm$ 1.14

Figure legends

Fig. 1. Formation of DCF in control animals and animals treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as arbitrary units of fluorescence (UAF)/mg tissue (n = 7-9). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to the control mice.

Fig. 2. Concentration of pro-inflammatory cytokines IL-2 (a), IL-6 (b) and IL-1 β (c) in large intestine of control mice and mice treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as pg/mg tissue (n = 7-9). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

Fig. 3.

Histological study of the large intestine of mice treated with As(III). Sections of tissue stained with PAS, obtained from a control animal (a) and from animals treated with 50 mg/L (b) and 80 mg/L As(III) (c) for 2 months are shown. White arrowheads point to lymphocyte infiltrates. Original magnification: $\times 100$ (left column, scale bars 100 μm) and $\times 400$ (right column, scale bars 20 μm).

Fig. 4. Effect of subchronic exposure to As(III) on intestinal permeability in mice. Concentration of albumin in feces of control animals and animals treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as $\mu\text{g}/100$ mg feces (n = 7-9). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to the control mice.

Fig. 5. Immunohistochemical detection of CLDN-1 in mice large intestine. Sections of tissue probed with an anti-CLDN-1 antibody from a control animal (a) and from animals treated with 50 mg/L (b) and 80 mg/L As(III) (c) for 2 months are shown. Original magnification: $\times 100$ (left column, scale bars 100 μm) and $\times 400$ (right column, scale bars 20 μm). Black arrows point to membrane-localized CLDN-1, whereas white arrows indicate cytoplasmic localization of CLDN-1.

Fig. 6. Changes in intestinal bacterial composition depending on As(III) dose (a) Multivariate analysis of global microbial composition in mice feces. A CCA (canonical correspondence analysis) was applied to the four experimental groups; c, control; As20, 20 mg/L; As50, 50 mg/L; As80, 80 mg/L. (b) Relative abundances of bacterial taxa at the phylum and order levels.

Fig. 7. Abundances of different bacterial genera in mice feces after As(III) treatment. Normalized data by total-sum scaling ($\times 10^7$) are presented. c, control; As20, 20 mg/L; As50, 50 mg/L; As80, 80 mg/L. Kruskal-Wallis test with $p < 0.05$ was applied to samples.

Fig. 1

Formation of DCF in the large intestine tissue of control animals and animals treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as arbitrary units of fluorescence (UAF)/mg tissue (n = 7-9). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to the control mice.

Fig. 2
Concentration of pro-inflammatory cytokines IL-2 (a), IL-6 (b) and IL-1 β (c) in large intestine of control mice and mice treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as pg/mg tissue (n = 7–9). Different letters indicate statistically significant differences ($p < 0.05$) between treatments.

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Histological study of the large intestine of mice treated with As(III). Sections of tissue stained with PAS, obtained from a control animal (a) and from animals treated with 50 mg/L (b) and 80 mg/L As(III) (c) for 2 months are shown. White arrowheads point to lymphocyte infiltrates. Original magnification: ×100 (left column, scale bars 100 μm) and ×400 (right column, scale bars 20 μm).

Fig. 4

Effect of subchronic exposure to As(III) on intestinal permeability in mice. Concentration of albumin in feces of control animals and animals treated with As(III) (20, 50, and 80 mg/L) for 2 months. Values expressed as $\mu\text{g}/100$ mg feces ($n = 7-9$). Asterisks indicate statistically significant differences ($p < 0.05$) with respect to the control mice.

Fig. 5

Immunohistochemical detection of CLDN-1 in mice large intestine. Sections of tissue probed with an anti-CLDN-1 antibody from a control animal (a) and from animals treated with 50 mg/L (b) and 80 mg/L As(III) (c) for 2 months are shown. Original magnification: $\times 100$ (left column, scale bars 100 μm) and $\times 400$ (right column, scale bars 20 μm). Black arrows point to membrane-localized CLDN-1, whereas white arrows indicate cytoplasmic localization of CLDN-1.

Fig. 6

Changes in intestinal bacterial composition depending on As(III) dose (a) Multivariate analysis of global microbial composition in mice feces. A CCA (canonical correspondence analysis) was applied to the four experimental groups; (b) Relative abundances of bacterial taxa at the phylum and order levels. c, control; As20, 20 mg/L; As50, 50 mg/L; As80, 80 mg/L.

Fig. 7

Abundances of different bacterial genera in mice feces after As(III) treatment. Normalized data by total-sum scaling (x10⁷) are presented c, control; As20, 20 mg/L; As50, 50 mg/L; As80, 80 mg/L. Kruskal-Wallis test with p<0.05 was applied to samples.

Supplementary Table 1.

Oligonucleotides used in the differential expression analysis

Gene	Sequence 5'–3'	Amplicon size (bp)	Efficiency
<i>Muc2</i>	F: GCTGACGAGTGGTTGGTGAAT R: GATGAGGTGGCAGACAGGAGA	135	1.98 ± 0.09
<i>Mip2</i>	F: CCAACCACCAGGCTACAGG R: GCGTCACACTCAAGCTCTG	108	2.12 ± 0.04
<i>Il-1β</i>	F: CCAAGCAACGACAAAATACC R: GTTGAAGACAAACCGTTTTTCC	156	1.95 ± 0.06
<i>Il-2</i>	F: AGCAGCACCTGGAGCAGCTG R: GTCCACCACAGTTGGTGACT	320	1.85 ± 0.07
<i>Il-6</i>	F: CTGCAAGAGACTTCCATCCAG R: AGTGGTATAGACAGGTCTGTTGG	131	1.75 ± 0.06
<i>Tnfα</i>	F: CAGGCGGTGCCTATGTCTC R: CGATCACCCCGAAGTTCAGTAG	89	1.82 ± 0.05
<i>Cldn-1</i>	F: AGGTCTGGCGACATTAGTGG R: CGTGGTGTGGGTAAGAGGT	204	2.05 ± 0.03
<i>Cldn-2</i>	F: TATGTTGGTGCCAGCATTGT R: TCATGCCCACCACAGAGATA	205	1.98 ± 0.05
<i>Cldn-4</i>	F: CGCTACTCTTGCCATTACG R: ACTCAGCACACCATGACTTG	71	1.89 ± 0.10
<i>Zo-1</i>	F: GCTAAGAGCACAGCAATGGA R: GCATGTTCAACGTTATCCAT	245	2.11 ± 0.08
<i>Rn18S</i>	F: CGGACAGGATTGACAGATTG R: CAAATCGCTCCACCAACTAA	98	2.03 ± 0.00
<i>Rplp0</i>	F: AGATTCGGGATATGCTGTTGGC R: TCGGGTCCTAGACCAGTGTTTC	109	1.93 ± 0.06

Supplementary Fig. 1

Changes in bacterial composition at the family level depending on As(III) dose (C, control; As20 , 20 mg/L; As50, 50 mg/L; As80, 80 mg/L). Data represents abundances normalized by total-sum scaling (TSS) and square root transformed. *, $p < 0,05$; **, $p < 0,01$; ***, $p < 0,001$

	R(Pearson)	<i>p</i>	FDR	Abundance
<i>Helicobacter</i>	0.7	6.5e-6	0.00015	0.25
<i>Turicibacter</i>	-0.66	2.86e-05	0.00043	4.27
<i>Unclassified.Rikenellaceae</i>	0.55	0.001	0.011	0.47
<i>Catenibacterium</i>	-0.51	0.002	0.022	0.052
<i>Unclassified.S247</i>	0.5	0.003	0.022	1.94
<i>Enterococcus</i>	-0.47	0.005	0.024	0.27
<i>Lactobacillus</i>	0.48	0.005	0.024	5.49
<i>Prevotella</i>	0.48	0.005	0.024	0.56
Unclassified.F16	0.48	0.005	0.024	0.56
Unclassified.RF32	0.46	0.007	0.028	0.2
Unclassified. <i>Ruminococcaceae</i>	0.46	0.008	0.029	0.58

Supplementary Fig. 2

Pearson's correlations for bacterial groups abundance [sqrt(TSS)] and As(III) dose (0, 20, 40 and 80 mg/L). FDR; false discovery rate.

Supplementary Fig. 3

KEGG pathways differently enriched among the distinct As(III) treatments.
c, control; As20 , 20 mg/L; As50, 50 mg/L; As80, 80 mg/L.