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Dietary zinc deficiency or supplementation during gestation increases breast cancer susceptibility in adult female mice offspring following a J-shaped pattern and through distinct mechanisms

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ABSTRACT

Zinc is required for fetal development and is involved in key processes associated with breast carcinogenesis. We evaluated whether maternal zinc deficiency or supplementation during gestation influences female offspring susceptibility to breast cancer in adulthood. C57BL/6 mice consumed during gestation control (30 p.p.m. zinc), zinc-deficient (8 p.p.m) or zinc-supplemented (45 p.p.m.) diets. Maternal zinc supplementation increased in female mice offspring the incidence of chemically-induced mammary adenocarcinomas that were heavier, compared to control group. This was accompanied by a decreased number of terminal end buds, increased cell proliferation and apoptosis, and increased tumor suppressors p21, p53 and *Rassf1*, *Zfp382* and *Stat3* expression in mammary glands, as well as increased zinc status. Although maternal zinc deficiency did not alter the incidence of these lesions, it also induced heavier mammary adenocarcinomas, compared to control group. These effects were accompanied by a decreased number of terminal end buds, increased proto-oncogenes *c-Myc* and *Lmo4* expression and H3K9Me3 and H4K20Me3 epigenetic marks in mammary glands of offspring, and decreased zinc status and increased levels of oxidative marker malondialdehyde. The data suggest that both maternal zinc deficiency and supplementation during gestation programmed increased breast cancer susceptibility in adult mice offspring following a J-shaped pattern through distinct mechanisms.

1. Introduction

Zinc (Zn) is an essential micronutrient that presents structural functions in zinc finger proteins (Maret, 2013). This trace-element is required for more than 300 cellular processes such as DNA repair, gene expression, enzyme activity and intracellular signaling (Kelleher et al., 2011). There is increasing interest in the role of Zn on breast cancer development (Alam and Kelleher, 2012; Riesop et al., 2015). Because Zn modulates diverse processes (i.e. oxidative stress, cell proliferation and apoptosis) central to multistage carcinogenesis, altered Zn status could affect breast cancer development and prognosis (Grattan and Freake, 2012).

Accumulating evidence indicates that breast cancer may have a fetal origin (Hilakivi-Clarke and de Assis, 2006; Troisi et al., 2007). Early life represents a vulnerable window for breast cancer risk programming due to environmental factors, particularly inadequate nutrition (Hilakivi-Clarke and de Assis, 2006). Zn is a trace element required in early life for proper growth and development (Terrin et al., 2015). Dietary Zn deficiency during fetal life, lactation and/or post-weaning increased cardiovascular disease risk and renal dysfunction (Tomat et al., 2011; Juriol et al., 2018). Fetal epigenetic modulation has been implicated in Zn deficiency metabolic programming effects (Maret and Sandstead, 2008). Zn is also required for several processes involved in mammary gland development during puberty and reproduction (McCormick et al., 2014). Chronic dietary Zn deficiency (15 p.p.m., for ~20 weeks) in adult mice altered mammary gland architecture and promoted increased oxidative stress that could increase breast cancer risk (Bostanci et al., 2015). However, only one recent study in rats (Da Silva, et al.,

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2017) evaluated the role of Zn nutritional status from the fetal stage until the juvenile phase on later breast cancer risk. In this study, Zn supplementation (180 p.p.m.) but not deficiency (3 p.p.m.) increased mammary tumor development in adult females when compared to the Zn-sufficient counterparts (35 p.p.m.) (Da Silva, et al., 2017).

We designed this study to investigate in mice whether gestational zinc deficiency or supplementation could influence female offspring susceptibility to breast cancer in adulthood. We also investigated the underlying cellular and molecular mechanisms. We fed C57BL/6 mice during gestation with control (CO; 30 p.p.m. zinc), zinc-deficient (ZnD; 8 p.p.m) or zinc-supplemented (ZnS; 45 p.p.m.) diets. Female offspring (6 week-old) were submitted to the development of adenocarcinomas induced by a classical carcinogen 7,12-dimethylbenz[*a*]anthracene (DMBA). Other female offspring (7 week-old and non-DMBA-treated) were investigated for Zn (flame atomic absorption) and malondialdehyde (MDA; reverse phase high-performance liquid chromatography with fluorescence detection) blood and hepatic levels, as well as mammary gland morphology [number of terminal end buds (TEBs) and epithelial elongation)], cell proliferation (Ki67 immunohistochemistry), apoptosis (hematoxylin and eosin), gene (Real-Time PCR) and protein (Western Blot) expression and post-translational histone modifications (immunohistochemistry).

2. Materials and method

2.1. Experimental design

Male and female C57BL/6 mice were provided by the Animal Facility of the School of Pharmaceutical Sciences, University of São Paulo, where they were maintained on controlled conditions (55% ± 10 °C relative humidity; 22 ± °C room temperature; 12-h light/dark cycle; lights on at 7 a.m.). 6-week-old females were randomly distributed into three groups that received 5 days before mating until offspring birth the following diets: CO diet (CO group; 30 p.p.m. of Zn; n = 28); ZnD diet (ZnD group; 8 p.p.m. of Zn; n = 30); and ZnS diet (ZnS group; 45 p.p.m. of Zn; n = 32). All diets were based on AIN-93G (Reeves et al., 1993) and the quantity of $ZnSO_4$ was adjusted to reach the required concentrations. Zn concentrations in CO and ZnD diets were based on AIN-93G (Reeves et al., 1993) and a previous study by Tomat et al. (2010), respectively. Because excess Zn supplementation during rat pregnancy induced reproductive and developmental toxicities, such as reduced number of live pups/litter and decreased live birth index, as well as an increased mortality and fetal resorption (Johnson et al., 2011), ZnS diet presented a moderate (50%) increase in Zn concentration as compared to CO diet. To reduce dietary Zn background, casein was replaced with egg white solids in all 3 diets. Diets were provided by Harlan Laboratories (USA). Mating was performed by housing one 10-week-old male mice with two 7-week-old female mice per cage. All dams and offspring received during lactation or after weaning AIN-93G control diet, that was changed to AIN-93M control maintenance diet (30 p.p.m. of Zn) in the case of offspring when they reached 10 weeks of age. Body weight and food intake were recorded 3 times per week. Part of the female offspring was submitted to the DMBA-induced model of mammary carcinogenesis when they reached the age of 6 weeks. The remaining female offspring was euthanized at the age of 7 weeks and analyzed for Zn and MDA blood and hepatic levels, and mammary gland morphology, cell proliferation and apoptosis, gene expression and post-translational histone modifications. This experiment was approved by the Ethics Committee on Animal Experiments of the School of Pharmaceutical Sciences, University of São Paulo (Protocol #407) and the experiments were performed following the National Institutes of Health guidelines for the proper and humane use of animals in biomedical research.

2.2. Mammary tumor induction in female offspring

Mammary tumors were induced by administration of a subcutaneous injection of 15mg/100 μL of medroxyprogesterone acetate (Depo-Provera, Pfizer, Brazil) to 6-week-old female offspring (n = 28–32 animals/group), followed by oral gavage of 1 mg of DMBA (Sigma, USA) dissolved in corn oil, once a week for 4 weeks (de Assis et al., 2011). Tumors were detected by palpation of mammary glands twice a week. Latency of 1st tumor appearance, the number of animals with tumors (tumor incidence), the number of tumors per animal (tumor multiplicity) and tumor weight were evaluated. Animals in which tumor burden reached approximately 10% of total body weight were euthanized (Fontelles et al., 2016). All other animals were euthanized at 20 weeks after the last dose of DMBA administration. Mammary tumors were classified by a pathologist using parameters established in the literature (Ward et al., 1988).

2.3. Measurement of Zn and MDA concentration in plasma, erythrocyte and/or liver tissue samples of 7-week-old female offspring

Blood and liver tissue samples from CO, ZnD and ZnS groups non-DMBA-treated 7-week-old female offspring were collected and kept at −80 °C until use. All materials were washed with nitric acid solution (20%, v/v) for 12 h and rinsed 10 times with minerals-free deionized water. Samples were digested with a 65% nitric acid solution (Merck Millipore, USA) and 30% hydrogen peroxide solution (Merck Millipore, USA) at 100 °C. Zn concentration was analyzed by using flame atomic absorption spectrometer (F AAS, model –AS52/Analytic Jena AG, Germany). The calibration curve solutions were prepared from a Zn stock standard (Tritizol, Merck Millipore, USA) diluted with 0.1% HNO_3. The accuracy of the method was established by analysis of the National Institute of Standards and Technology Standard Reference 1577b Bovine Liver. The accuracy of the method used for Zn determination in this experiment was established by the observation that the obtained value for Zn concentration in bovine liver (124.41 μg/g) was in agreement with the certified value (127 μg/g).

MDA concentration in plasma and liver homogenate of female offspring was quantified by reverse phase high-performance liquid chromatography with fluorescence detection (HPLC-FLD) as previously described (Guido et al., 2016).

2.4. Analysis of mammary gland morphology, cell proliferation and apoptosis in 7-week-old female offspring

Whole-mount preparations of right fourth abdominal mammary gland were obtained from non-DMBA-treated 7-week-old female offspring, as previously described (de Assis et al., 2011). The number of terminal end buds (TEBs) and epithelial elongation were determined according to Fontelles et al. (2016).

Cell proliferation was evaluated in the left fourth abdominal mammary gland obtained from non-DMBA-treated 7-week-old female offspring by Ki-67 immunohistochemistry as previously described (de Oliveira Andrade et al., 2014). After harvesting, mammary glands were fixed at 10% buffered formalin, embedded in paraffin and sectioned. Nonspecific protein binding was blocked with 5% non-fat dry milk for 30 min at room temperature. Tissue sections were incubated overnight with the primary monoclonal anti-rabbit Ki67 antibody (Abcam, UK) at a 1:50 dilution. Cell proliferation labeling index was determined by calculating the percentage of Ki67-positive epithelial cells among 1,000 cells per mammary structure (lobule or duct). Slides were evaluated with Image J software (NIH, USA).

Cell apoptosis analysis was conducted in mammary glands from non-DMBA-treated 7-week-old female offspring. Mammary glands were fixed in neutral buffered 10% formalin and stained with hematoxylin and eosin. Epithelial cells presenting loss of adhesion between adjacent cells, cytoplasmic condensation and formation of apoptotic bodies were

considered in apoptosis (Elmore et al., 2016). The apoptotic index was determined by calculating the number of apoptotic bodies per mammary structure. Slides were evaluated with Image J software (NIH, USA).

2.5. Analysis of gene expression in the mammary gland of 7-week-old female offspring

Rassf1 (Ras association [RalGDS/AF-6] domain family member 1), *Zfp382* (zinc finger protein 382), *Stat3* (signal transducer and activator of transcription 3), *Lmo4 (*LIM domain only 4) and c-*Myc* (myelocytomatosis oncogene) gene expression was analyzed by Real-Time PCR in mammary glands obtained from non-DMBA-treated 7-week-old female offspring. RNA was extracted with the RNeasy Lipid Tissue Mini Kit (Qiagen, USA), according to manufacturer's protocol. Its concentration and quality were then determined using NanoDrop ND-1000 (Thermo Scientific, USA). Total RNA (1 μg) was used for the synthesis of cDNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystem, USA) following the manufacturer's instructions. The expression levels of target genes were evaluated using a ViiA™ 7 Real-Time PCR System (Applied Biosystem, USA). For that cDNA (2 μL) was mixed with 5 μL of the TaqMan® Gene Expression Master Mix with UNG (Applied Biosystem, USA), 1 μL of the TaqMan® Assay and 2 μL of the ultrapure water (UltraPure™ DNase/RNase-Free Distilled Water) in 384-well plates. The amplification reactions were performed with a thermal cycling method, consisting of two-initial cycles of 2 min at 50 °C and 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The expression levels of target genes were normalized to the reference gene GAPDH. The variation in the expression of target genes among experimental groups was analyzed using delta Ct (Livak and Schmittgen, 2001).

2.6. Analysis of p53 and p21 protein expression in the mammary gland of 7-week-old female offspring

p53 and p21 protein expression was evaluated by Western Blot in total thoracic (left) mammary gland of non-DMBA-treated 7-week-old female offspring. Briefly, mammary glands were homogenized with lysis buffer [Sodium chloride (150 Mm); NP-40 1.0%; Tris (50 mM) pH 8.0; phenylmethanesulfonyl fluoride (PMSF) (1 mM)], incubated in agitation at 4 °C for 2 h, centrifuged at 12,000 rpm at 4 °C for 20 min and the total protein was collected from the supernatant. Protein concentration was measured using Bradford Assay (Bio-Rad, USA). Approximately 50 μg of protein was resolved in 4–12% gel Mini-Protean TGX (Bio-Rad, USA), and transferred to nitrocellulose membrane using Trans-Blot® Turbo Transfer System (Bio Rad, USA). Protein blocking was performed with blocking reagents (Bio-Rad, USA) for 1 h at room temperature, followed by incubation with primary antibodies anti-p53 and anti-p21 (1:1000; Santa Cruz Biotechnology, USA) and anti- β-actin (1:10,000; Cell Signaling, USA) overnight at 4 °C. After several washes with PBST [PBS with 10% Tween-20 (Sigma, USA)], the membrane was incubated with horseradish peroxidase-conjugated secondary antibody (1:5000; GE Healthcare Life Sciences, USA) at room temperature for 1 h. Membranes were developed using the ECL enhanced chemiluminescence method. The optical density of the bands was quantified using Quantity-One software (Bio-Rad, USA) and normalized for the β-actin signal.

2.7. Analysis of post-translational histone modification in mammary gland of 7-week-old female offspring

Tri-methylation of histone marks H3K9 and H4K20 was assessed by immunohistochemistry in the fourth abdominal (right) mammary gland obtained from non-DMBA-treated 7-week-old female offspring. The mammary gland was fixed at 10% buffered formalin, embedded in paraffin and sectioned. Sections were then deparaffinized in xylene and hydrated through graded alcohols. Antigen retrieval was performed in Tris-EDTA buffer at pH 6.0 (0.5% Tween-20) in a pressure cooker (Dako Cytomation, Denmark) for 10 min at 120 °C. Tissues were incubated with a 2N HCL solution for 30 min at 60 °C. Endogenous peroxidase and nonspecific protein binding were blocked for 10 min with 3% H_2O_2 and 30 min with 5% non-fat dry milk at room temperature, respectively. Tissue sections were incubated overnight with the primary monoclonal anti-rabbit H3K9 or H4K20 antibodies (Cell Signaling, USA) at 1:50 dilution. After several washes, sections were incubated with secondary biotinylated link Streptavidin-horseradish peroxidase for 1 h at room temperature. The sections were washed and stained with 3.3′-diaminobenzidine tetrahydrochloride in chromogen solution (Sigma Aldrich Co, USA) for 5 min and then washed and counterstained for 45 s with Harris Hematoxylin. The histone expression level was determined by calculating the mean cell staining intensity per structure (lobule or duct). The slides were analyzed using Image J software (NIH, USA). Optical density (OD), which reflects the histone level expression present in the epithelial cells, within 1000 cells per mammary structure (lobule or duct).

2.8. Statistical analysis

The results are expressed as the mean ± standard error of the mean (SEM). Statistical analyses were conducted with STATISTIC 8.0 (Statsoft, USA). Multiple-group comparisons were based on one-way analysis of variance (ANOVA) followed by Duncan's post hoc test or Kruskal-Wallis followed by Dunn's multiple comparison test. For caloric intake and tumor incidence, repeated-measures ANOVA test and Kaplan-Meier curve followed by log-rank test, were applied, respectively. For all data analysis, $p \leq 0.05$ was applied as the threshold for statistical significance.

3. Results

3.1. Maternal and litter parameters

There were no differences ($p > 0.05$) among CO, ZnD and ZnS groups regarding the maternal caloric intake (Supplementary Fig. 1) and body weight gain (Table 1) during pregnancy, as well as the number of total pups/litter, number of female pups/litter and female offspring's weight at weaning (Table 1).

3.2. Female offspring mammary carcinogenesis

All mammary tumors were classified as adenocarcinomas showing different phenotypes (Supplementary Fig. 2). Compared to the CO group, female offspring from ZnS group presented higher ($p \leq 0.05$) incidence of mammary adenocarcinomas (Fig. 1). There was no difference ($p > 0.05$) between female offspring from CO and ZnD groups regarding this parameter (Fig. 1). Compared to the CO group, female

Table 1
Effects of maternal Zn deficiency or supplementation on gestation and pups' parameters.

Variables	Experimental groups		
	CO	ZnD	ZnS
Body weight gain during pregnancy (g)	12.4 ± 2.8	13.1 ± 2.6	14.1 ± 3.0
Number of total pups/litter	5.7 ± 2.0	5.6 ± 2.1	5.9 ± 1.9
Number of female pups/litter	2.5 ± 1.9	2.6 ± 1.1	3.2 ± 2.0
Body weight female offspring at weaning (g)	10.2 ± 1.1	10.3 ± 1.1	10.6 ± 1.1

No statistically significant difference ($p > 0.05$) among groups according to Kruskal-Wallis followed by Dunn's multiple comparison test. The data are expressed as mean ± SEM. n = 23 (CO and ZnS groups) and 26 (ZnD group).

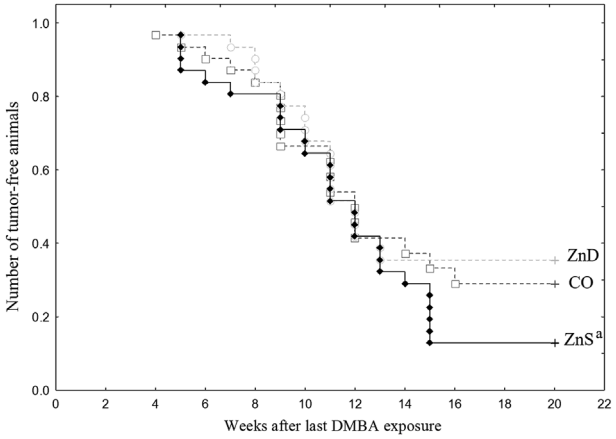

Fig. 1. Mammary adenocarcinoma incidence in female offspring from CO, ZnD and ZnS groups. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnS groups. Kaplan–Meier analysis and log-rank test. n = 31 (CO and ZnD groups) and 33 (ZnS group).

Table 2
Effects of maternal zinc deficiency or supplementation on mammary carcinogenesis in the female offspring.

Parameters	Experimental groups		
	CO	ZnD	ZnS
First tumor latency (weeks)	9.9 ± 0.7	10.1 ± 0.4	9.3 ± 0.6
Multiplicity (number of tumors/mouse)	1.4 ± 0.7	1.6 ± 0.8	1.9 ± 0.9
Tumor weight distribution (%)			
0.01–0.30 g	59	44	30
0.30–0.60 g	14	17	18
> 0.60 g	27	39[a]	52[a,b]

"a" indicates statistical difference ($p \leq 0.05$) between CO and ZnD groups or between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. Fisher's exact test. The data are expressed as mean ± SEM. n = 31 (CO and ZnD groups) and 33 (ZnS group).

offspring from both ZnS and ZnD groups presented higher ($p \leq 0.05$) proportion of heavier mammary adenocarcinomas (Table 2). Compared to the ZnD group, female offspring from ZnS group presented higher ($p \leq 0.05$) proportion of heavier mammary adenocarcinomas (Table 2). There was no difference ($p > 0.05$) among female offspring from CO, ZnD and ZnS groups regarding mammary adenocarcinoma latency and multiplicity (Table 2).

3.3. Female offspring body weight gain and Zn and MDA plasma, erythrocytes and/or hepatic levels

Body weight gain and plasma Zn concentration were not different ($p > 0.05$) among non-DMBA-treated 7-week-old female offspring from CO, ZnD and ZnS groups (Table 3). However, female offspring from ZnD group presented lower ($p \leq 0.05$) erythrocyte Zn concentration compared to the female offspring from CO and ZnS groups. There was no difference ($p > 0.05$) between female offspring from CO and ZnS groups regarding erythrocyte Zn concentration. Female offspring from ZnS group presented higher hepatic Zn concentration compared to the female offspring from CO ($p = 0.07$) and ZnD ($p \leq 0.05$) groups. There was no difference ($p > 0.05$) between female offspring from CO and ZnD groups regarding hepatic Zn concentration (Table 3).

Female offspring from ZnD group presented a tendency of increased ($p = 0.08$) plasma MDA concentration compared to the ZnS group, and increased ($p \leq 0.05$) hepatic MDA concentration compared to the CO and ZnS groups (Table 3). There were no differences ($p > 0.05$) between female offspring from CO and ZnS groups regarding these parameters.

Table 3
Body weight gain and zinc and malondialdehyde (MDA) plasma, erythrocytes and hepatic levels in 7-week-old female offspring from CO, ZnD and ZnS groups.

Variables	Experimental groups		
	CO	ZnD	ZnS
Body weight gain (g)	13.4 ± 2.1	13.2 ± 2.7	13.0 ± 2.6
Zinc plasma concentration (μg/mL)	1.6 ± 0.8	1.4 ± 0.6	1.2 ± 0.4
Zinc erythrocytes concentration (μg/mL)	7.5 ± 2.8	4.4 ± 2.0[a,b]	8.8 ± 2.2
Zinc liver concentration (μg/g)	50.9 ± 7.0	48.8 ± 2.8	58.4 ± 6.7[a,b]
Plasma MDA concentration (ηmol/mg protein)	1.7 ± 0.1	1.9 ± 0.3[b]	1.6 ± 0.3
Hepatic MDA concentration (ηmol/mg protein)	37.8 ± 21.9	75.4 ± 15.6[a,b]	35.4 ± 15.7

"a" indicates statistical difference ($p \leq 0.05$) between CO and ZnD groups or between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. Kruskal-Wallis test followed by Dunn's multiple comparison test. The data are expressed as mean ± SEM. n = 28 (CO and ZnD group) and 33 (ZnS group) (body weight gain), 10 (zinc levels) and 5 (MDA levels).

3.4. Female offspring mammary gland morphology

Non-DMBA-treated 7-week-old female offspring from ZnD group presented lower ($p \leq 0.05$) number of TEBs compared to the female offspring from CO and ZnS groups (Fig. 2C). Female offspring from ZnS group showed lower ($p \leq 0.05$) number of TEBs compared to the female offspring from CO (Fig. 2C). There was no difference ($p > 0.05$) among female offspring from CO, ZnD and ZnS groups regarding epithelial tree elongation (Fig. 2D).

3.5. Female offspring mammary gland cell proliferation, apoptosis and p21 and p53 protein levels

Compared to the CO and ZnD groups, non-DMBA-treated 7-week-old female offspring from ZnS group presented higher ($p \leq 0.05$) number of proliferating epithelial cells in both ducts and lobules (Fig. 3B). Compared to the CO group, ZnS group offspring presented higher ($p \leq 0.05$) number of apoptotic epithelial cells (Fig. 4B). In addition, female offspring from ZnS group tended to present increased protein levels of p21 ($p = 0.07$) and p53 ($p = 0.06$) in the mammary gland compared to the CO and ZnD groups, respectively (Figs. 3D and 4D). There was no statistical difference ($p > 0.05$) between female offspring from CO and ZnD groups regarding all these parameters (Figs. 3 and 4).

3.6. Female offspring mammary gland Rassf1, Zfp382, Stat3, c-Myc and Lmo4 gene expression

Non-DMBA-treated 7-week-old female offspring from ZnS group presented increased ($p \leq 0.05$) mammary gland expression of *Rassf1* compared to the CO and ZnD groups and of *Zfp382* ($p \leq 0.05$) and *Stat3* ($p = 0.07$) compared to the ZnD group (Fig. 5). There was no statistical difference ($p > 0.05$) between female offspring from CO and ZnD groups regarding expression of these target genes (Fig. 5). Female offspring from ZnD group tended ($p = 0.07$) to present increased mammary gland expression of *c-Myc* and *Lmo4* compared to the CO group. There was no statistical difference ($p > 0.05$) between female offspring from CO and ZnS groups regarding expression of these target genes (Fig. 5).

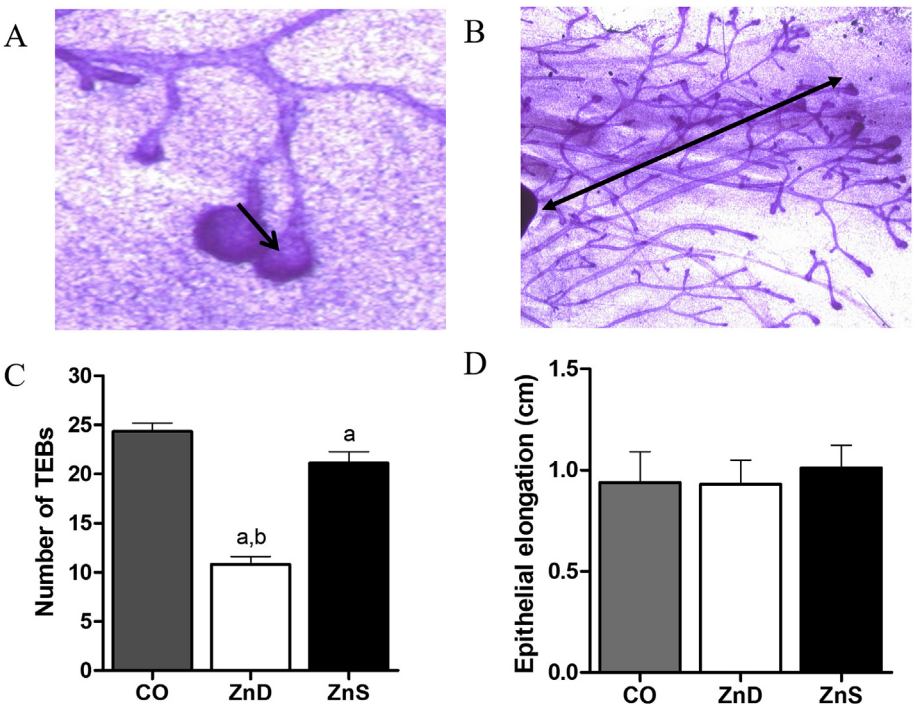

Fig. 2. Mammary gland morphology of 7-week-old female offspring from CO, ZnD and ZnS groups. A. Representative photomicrography of terminal end buds (TEBs), indicated by arrow. B. Distance between lymph node and the end of the mammary epithelial tree, as shown by the bar, indicates epithelial elongation. C. Number of TEBs. D. Epithelial elongation. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnD groups or between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. ANOVA followed Duncan post-hoc. The data are expressed as mean ± SEM. n = 5/group.

3.7. Female offspring mammary gland H3K9Me3 and H4K20Me3 levels

Compared to the CO and ZnS groups, non-DMBA-treated 7-week-old female offspring from ZnD group showed increased ($p \leq 0.05$) levels of H3K9me3 in both ducts and lobules, as well as increased ($p \leq 0.05$) levels of H4K20me3 in ducts and a tendency of increased ($p = 0.08$) levels of this epigenetic mark in lobules (Fig. 6). There was no difference ($p > 0.05$) between female offspring from CO and ZnS groups regarding these epigenetic marks.

4. Discussion

Compared to other essential micronutrients such as selenium (Davis and Finley, 2003), the role of Zn in breast carcinogenesis has been less frequently investigated (Hoang et al., 2016). Consumption of a Zn deficient diet (3 p.p.m., for 18 weeks) by juvenile/adult female rats suppressed chemically-induced breast carcinogenesis in a rat model by reducing tumor incidence (46% vs. 84% and 80%) and multiplicity compared to the adequate (12 p.p.m.) and pair-feed controls (Lee et al., 2004). In another study, Zn supplementation (6.9 mg/mL, $ZnSO_4.7H_2O$ in aqueous suspension) during adulthood did not affect DMBA-induced breast carcinogenesis in rats (Bobrowska-Korczak et al., 2012). However, when Zn supplementation (180 p.p.m. in diet) started from in *utero* life until juvenile phase, an increase in breast cancer susceptibility was observed in the DMBA-treated female offspring rats (da Silva et al., 2017). This indicates that timing of exposure and dietary Zn

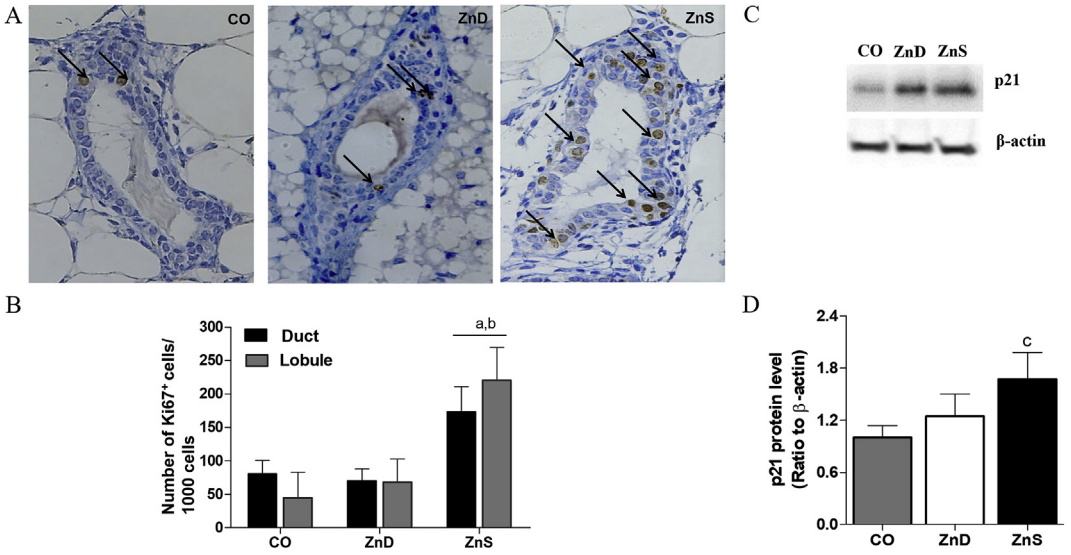

Fig. 3. Representative photomicrographys of Ki67 staining (A) and quantification of cell proliferation (B) in mammary gland of 7-week-old female offspring from CO, ZnD and ZnS groups. Western Blot (C) analysis and quantification of p21 protein expression (D) in mammary gland of 7-week-old female offspring from CO, ZnD and ZnS groups. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. ANOVA test followed Duncan post-hoc test. "c" indicates statistical difference ($p \leq 0.05$) between CO and ZnS groups. Student's *t*-test. The data are expressed as mean ± SEM. n = 5/group.

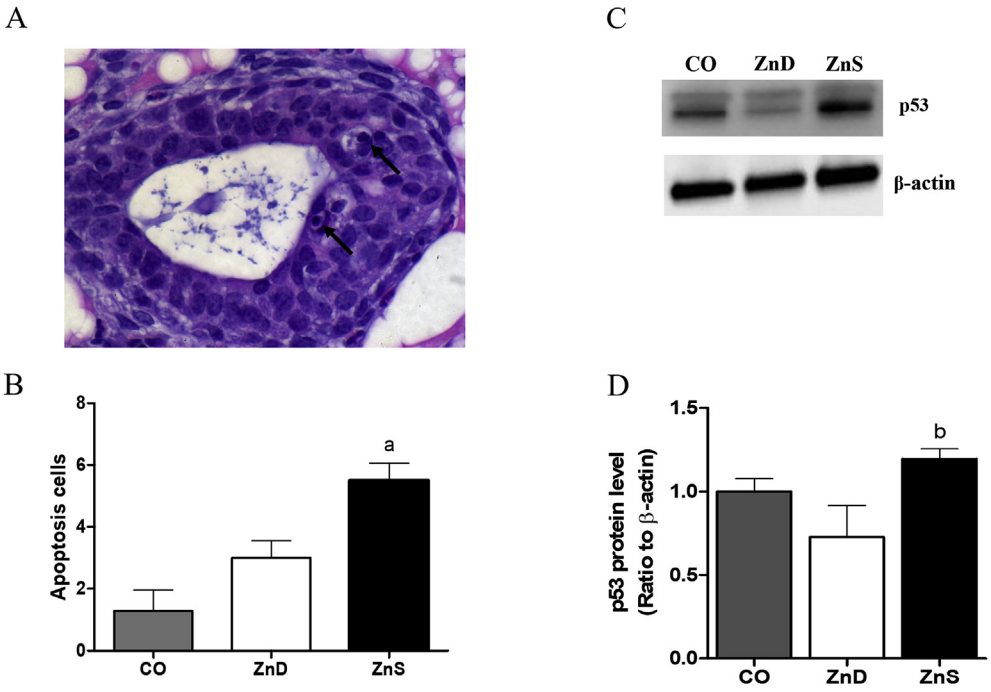

Fig. 4. Representative photomicrography showing apoptotic cells indicated by arrows (A) and quantification of apoptosis (B) in mammary gland of 7-week-old female offspring from CO, ZnD and ZnS groups. Western Blot (C) analysis and quantification of p53 protein expression (D) in mammary gland of seven-week-old female offspring from CO, ZnD and ZnS groups. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. ANOVA test followed Duncan post-hoc. The data are expressed as mean ± SEM. n = 5–7/group.

concentration are key factors influencing its effects on mammary carcinogenesis. However, because in this study (da Silva et al., 2017) Zn supplementation was continuously promoted from the *in utero*/neonatal life until the juvenile phase (51 days of age), it is not clear which specific developmental stage would be implied in such adverse effects.

Our feeding study in mice show that Zn-supplementation (45 p.p.m.) specifically during gestation also programmed increased breast cancer susceptibility in the female offspring. Thus, it highlights *in utero* life as a particular vulnerable developmental window when a woman's breast cancer risk can already be influenced by high Zn levels. Interestingly, in our study the consumption of a Zn-deficient diet (8 p.p.m.) during gestation also increased breast cancer susceptibility in the offspring although with lower magnitude as compared to Zn supplementation. This suggests that Zn breast cancer programming effects follow a J-shaped pattern. Similarly, a recent study in humans found a U-shaped association between serum Zn and oral cancer risk (Chen et al., 2018). The pivotal role of Zn on cancer development is therefore rather complex, and potential early life intervention strategies with this micronutrient should take into consideration that it can be both carcinogenic or protective (Gumulec et al., 2011), cancer-dependent (Gumulec et al., 2011; Lee et al., 2004), and that its biological effects do not follow a linear pattern as we observed in this work.

Inadequate maternal nutrition during gestation has been shown to program breast cancer risk through alterations in fetal mammary gland development (Hilakivi-Clarke and de Assis, 2006). In our study, neither female offspring from ZnS group mothers nor from ZnD ones presented increases in mammary epithelial elongation and number of TEBs, parameters normally associated with increased breast cancer susceptibility (Hilakivi-Clarke, 2007). Interestingly, we found that ZnS but not ZnD group non-DMBA treated female offspring presented increased levels of cell proliferation and apoptosis in epithelial cells of lobules and ducts from the mammary glands. These results suggest that although

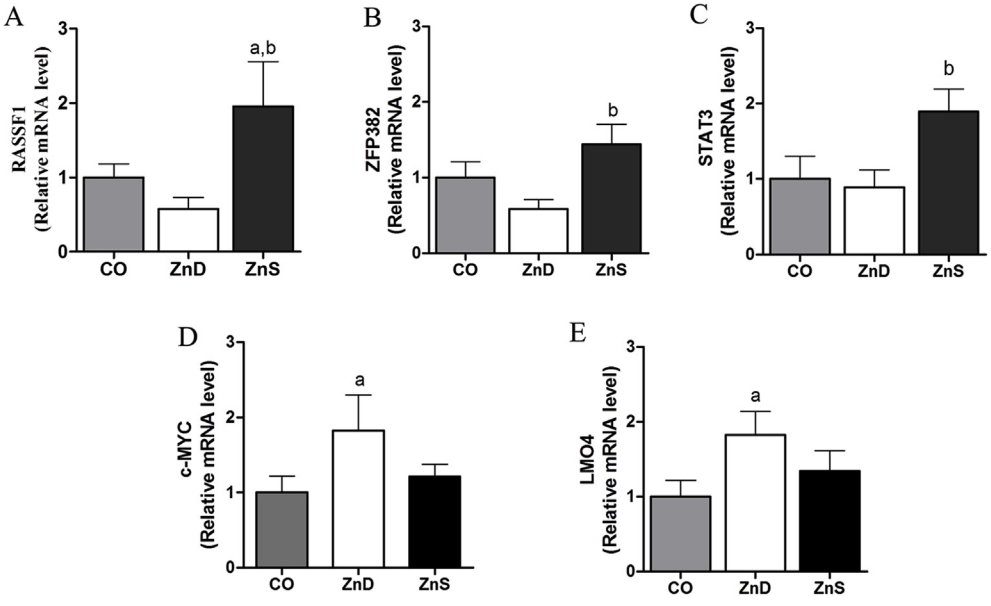

Fig. 5. mRNA levels of *Rassf1* (A), *Zfp382* (B), *Stat3* (C), *c-Myc* (D) and *Lmo4* (E) in the mammary gland of 7-week-old female offspring from CO, ZnD and ZnS groups. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnD groups or between CO and ZnS groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. ANOVA followed Duncan post-hoc. The data are expressed as mean ± SEM. n = 5/group.

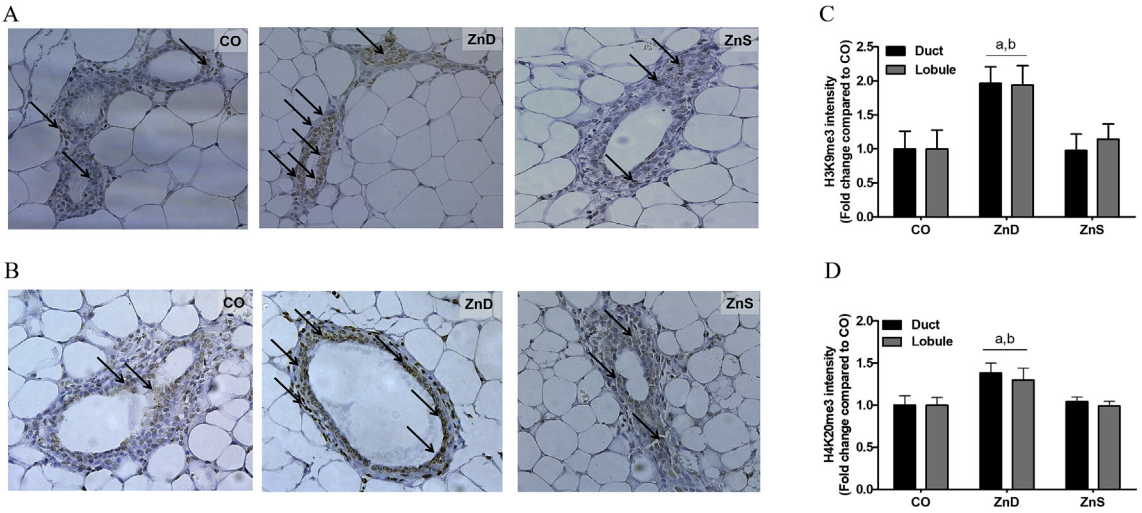

Fig. 6. Representative photomicrographys of H3K9me3 (A) and H4K20me3 (B) staining and quantification of H3K9me3 (C) and H4K20me3 (D) expression in mammary gland of 7-week-old female offspring from CO, ZnD and ZnS groups. "a" indicates statistical difference ($p \leq 0.05$) between CO and ZnD groups. "b" indicates statistical difference ($p \leq 0.05$) between ZnD and ZnS groups. ANOVA followed Duncan post-hoc. The data are expressed as mean ± SEM. n = 5/group.

maternal Zn supplementation programming effects do not involve alterations in offspring mammary gland morphology, excess Zn effects on breast cancer susceptibility could be associated with increased cell proliferation. Zn is an essential nutrient involved in all phases of the cell cycle and high doses of this element could promote cellular division (Beyersmann and Haase, 2001). The ratio of cell proliferation and apoptosis is tightly controlled in the mammary gland (Kutanzi et al., 2010). The increased levels of apoptosis and of the tumor suppressors p21 and p53, *Rassf1*, *Zfp382* and *Stat3* in mammary tissue of ZnS group offspring could represent a feedback mechanism in order to maintain tissue homeostasis. Interestingly, although maternal Zn deficiency did not alter cell proliferation and apoptosis, it tended to increase in the female offspring mammary gland expression of the proto-oncogenes *c-Myc* and the zinc finger protein *Lmo4,* which are associated with increased breast cancer susceptibility (Wade et al., 2015; Sum et al., 2005).

During fetal life the epigenome is especially plastic and prone to environmental stressors such as poor maternal nutrition (Ong and Ozanne, 2015). Alterations at the level of DNA methylation and histone modifications have been proposed as potential breast cancer programming underlying mechanisms (Kanwal et al., 2015). Because Zn is an essential co-factor for different enzymes involved in the epigenetic machinery and is associated with the 1-carbon metabolism pathway, maternal Zn deficiency or excess could program the offspring phenotype through alterations at the level of DNA methylation and histone modifications (Maret and Sandstead, 2008; Vidal et al., 2015). However, few studies have addressed Zn epigenetic modulatory potential. In mice acute Zn deficiency just before ovulation altered oocyte epigenetic patterns comprising reductions in global DNA methylation and H3K4Me3 levels that were associated with disruption of embryonic development (Tian and Diaz, 2013). Interestingly in our study maternal Zn deficiency (8 p.p.m.) increased trimethylation of H3K9 and H4K20, epigenetic marks that have been linked with gene transcription and cell-cycle regulation (Svobodová Kovaříková, 2018; de Miranda et al., 2014), in the mammary gland ducts and lobules of non-DMBA-treated female offspring, an effect that could be associated with increased breast cancer susceptibility. Because we only evaluated global histone marks, it would be important in future studies to evaluate specific genes affected by these epigenetic alterations in order to better elucidate Zn epigenetic effects. In a zebrafish model parental Zn deficiency (12 p.p.m.) altered the expression of DNA methyltransferase and Zn homeostasis genes (MTF-1 and Zn transporters) and decreased Zn levels in the embryos when compared to the Zn adequate group (33 p.p.m.) (Beaver et al., 2017). Consistent with this study and others showing that maternal Zn deficiency is associated with low mineral status in the young offspring (Beaver et al., 2017; Jou et al., 2010), we observed that ZnD group adult offspring presented low Zn levels in the erythrocytes, as well as increased MDA plasma and hepatic levels. These results suggest that disruption of Zn homeostasis in early life could contribute to increased breast cancer risk later in life by programming increased oxidative stress. It would be interesting in further studies to evaluate whether the observed alterations in Zn status could be associated with epigenetically programmed alterations in the Zn transporters ZnT and Zip. Maternal Zn supplementation did not alter any of the analyzed histone marks showing that Zn excess or deficiency breast cancer programming effects operate through distinct mechanisms.

The majority of studies on the fetal programming of chronic diseases including breast cancer have focused on the importance of macronutrients, especially lipids. However, micronutrients are also essential to sustain pregnancy and promote adequate growth of the fetus by controlling its structural and cellular metabolism (Rao et al., 2012). Our study advances the knowledge of the impact of Zn in early life and show that either *in utero* deficiency or excess increased breast cancer susceptibility in adult life following a J-shaped pattern through distinct mechanisms. Importantly, these adverse effects occurred at moderate levels of dietary Zn deficiency or supplementation. This observation is of special concern since the range between the recommended dietary allowance and the reference dose for safe intake of Zn is narrow (Maret and Sandstead, 2006). In addition, women frequently tend to consume excessive amounts of supplements in order to promote good fetal development based on the misconception that "more is necessarily better" (Vanhees et al., 2014). Our results suggest caution when considering supplementing pregnant women with high levels of Zn. Thus, a more prudent approach would be to promote Zn ingestion at the daily-recommended levels during gestation in order to potentially prevent breast cancer in the adult offspring. Furthermore, because paternal diet has been recently shown to also affect the offspring breast cancer risk (Guido et al., 2016; da Cruz et al., 2018), it would be also important to further evaluate Zn relevance in this context since this micronutrient is essential for male reproductive physiology (Fallah et al., 2018).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fct.2019.110813.

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