UNIVERSITY OF SÃO PAULO
Faculty of Pharmaceutical Sciences
Graduate Program in Food Sciences
Area of Experimental Nutrition

Influence of paternal consumption of high-fat/high-sugar diet and/or orange juice during preconception on C57BL/6 female mice offspring breast cancer risk

Lívia Beatriz Aparecida Ribeiro Silva

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Influence of paternal consumption of high-fat/high-sugar diet and/or orange juice during preconception on C57BL/6 female mice offspring breast cancer risk

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Dedication

To my wonderful mother Maria Lúcia Ribeiro who gave me all the support and encouragement during those years.

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RESUMO

SILVA, L. B. A. R. Influência do consumo paterno de uma dieta hiperlipídica/hiperglicídica e/ou suco de laranja durante a preconcepção no risco de câncer de mama na prole feminina de camundongos C57BL/6. 2019. 90 f. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo.

O câncer de mama é o mais frequente entre as mulheres em todo o mundo. Foi demonstrado que o consumo paterno de uma dieta hiperlipídica aumenta o risco de câncer de mama nas filhas. O suco de laranja é amplamente consumido e é conhecido pelo seu conteúdo de compostos bioativos que podem ter um papel na regulação dos processos epigenéticos. Portanto, o objetivo do presente estudo foi avaliar os efeitos da obesidade paterna e do consumo de suco de laranja na suscetibilidade da prole feminina à carcinogênese mamária quimicamente induzida. Camundongos C57BL/6 machos com três semanas de idade foram distribuídos nos grupos controle (CO), controle de suco de laranja (CJ), obeso (OB) e obeso suco de laranja (OJ), alimentados com ração controle ou com ração hiperlipídica e hiperglicídica (45% das calorias proveniente de lipídeos, a base de banha de porco) suplementada com leite condensado, com água ou suco de laranja, durante 11 semanas antes do acasalamento. A prole feminina foi desmamada e recebeu ração controle até 7 semanas de idade e, então, foi iniciada com 7,12-dimetil-benzo[a]antraceno para induzir tumores mamários. A prole feminina CJ apresentou maior multiplicidade de tumores mamários ($p \leq 0.05$) em relação a prole feminina CO. A prole feminina OB apresentou maior latência tumoral ($p \leq 0.05$), menor incidência tumoral ($p \leq 0.05$), maior multiplicidade de tumores ($p \leq 0.05$), menor proliferação celular (Ki67) nos ductos mamários ($p \leq 0.05$) e menores níveis globais de H3K27me3 na glândula mamária ($p \leq 0.05$), quando comparada a prole feminina CO. Não foram observadas diferenças ($p \geq 0,05$) entre a prole feminina do OB e OJ em relação a esses parâmetros. O consumo de suco de laranja por pais não obesos durante o período pré-concepcional aumentou a susceptibilidade da prole feminina à carcinogênese mamária. Embora o consumo paterno de uma dieta hiperlipídica e hiperglicídica durante a preconcepção tenha diminuído a incidência e aumentado a latência, a multiplicidade dos tumores mamários aumentou. Ainda, os resultados indicam que a resposta ao consumo de suco de laranja depende do contexto metabólico paterno.

Palavras-chave: paterno; obesidade; suco de laranja; início da vida; câncer de mama; epigenética.

ABSTRACT

SILVA, L. B. A. R. Influence of paternal consumption of a high-fat/high-sugar diet and/or orange juice during preconception on C57BL/6 female mice offspring breast cancer risk. 2019. 90 p. Thesis (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo.

Breast cancer is the most frequent cancer in women worldwide. Paternal consumption of a high-fat diet has been shown to program breast cancer risk in female offspring. Orange juice is widely consumed and is known for its content of bioactive compounds that may have a role in regulating epigenetic processes. Therefore, the aim of the present study was to evaluate the effects of paternal obesity and orange juice consumption on female offspring susceptibility to chemically-induced breast carcinogenesis. Three-week-old C57BL/6 male mice were distributed in control (CO), control-orange juice (CJ), obese (OB) and obese-orange juice (OJ) groups, fed either a standard chow or a high-fat/high-sugar diet (45% lard-based diet supplemented with sweetened condensed milk), with water or orange juice, for 11 weeks before mating. Female offspring were weaned onto standard chow until 7 weeks of age and then were initiated with 7,12-dimethyl-benzo[a]anthracene to induce mammary tumors. CJ female offspring presented higher multiplicity of mammary tumors ($p \leq 0.05$) compared to CO offspring. Female offspring from OB group showed higher tumor latency ($p \leq 0.05$), lower tumor incidence ($p \leq 0.05$), higher multiplicity of tumors ($p \leq 0.05$), lower cell proliferation (Ki67) in the mammary ducts ($p \leq 0.05$) and lower global levels of H3K27me3 in the mammary gland ($p \leq 0.05$) when compared to CO offspring. No differences ($p \geq 0,05$) were observed between OB and OJ female offspring regarding these parameters. Consumption of orange juice by non-obese fathers during preconception increased susceptibility of female offspring to mammary carcinogenesis. Although paternal consumption of a high-fat/high-sugar diet during preconception decreased incidence and increased latency of tumors, the multiplicity of lesions increased. In addition, the data indicates that the response to orange juice consumption depends of the paternal metabolic context.

Keywords: paternal; obesity; orange juice; early life; breast cancer; epigenetics.

List of Figures

Figure 1. Experimental design. 28
Figure 2. Evaluation of the antioxidant capacity of orange juice by the DPPH method during the period that orange juice was exposed in the experiment (0h, 24h, 48h). 40
Figure 3 A-B. HPLC-DAD chromatogram (280 nm) of supernatant fraction (A) and insoluble (precipitated) fraction (B) of orange juice. 41
Figure 4. Estimated daily intake of control and high-fat diets of male mice (fathers). 44
Figure 5 A-B. Estimated daily intake of liquids and flavonoids by male mice (fathers). 45
Figure 6. Estimated daily consumption of sweetened condensed milk of male mice (fathers). 45
Figure 7. Estimated daily energetic intake of male mice (fathers). 48
Figure 8. Estimated daily macronutrient consumption of male mice (fathers). 48
Figure 9 A-B. Estimated daily intake of fatty acids from male mice (fathers). 49
Figure 10 A-B. Body weight gain of male mice (fathers) from groups CO, CJ, OB, OJ after 11 weeks treatment. 50
Figure 11. Body composition images of X-ray computed tomography of the male mice (fathers) from CO, CJ, OB, OJ groups, after 11 weeks of treatment. 51
Figure 12 A-B. Serum glucose concentration of male mice (fathers) (A) and incremental serum glucose area (B) of groups CO, CJ, OB, OJ after 11 weeks treatment. 51
Figure 13. Plasma fatty acid profile of male mice (fathers). 53
Figure 14. Liver fatty acid profile of male mice (fathers). 53
Figure 15. Epididymal adipose tissue fatty acid profile of male mice (fathers) 54
Figure 16. Liver MDA concentration of male mice (fathers) 54
Figure 17 A-C. Activity of superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and catalase (CAT) (C) in liver and plasma of male mice (fathers). 55
Figure 18. Expression of inflammation-related genes evaluated in epididymal adipose tissue of 14-week-old male mice (fathers). 56
Figure 19 A-B. Weight at weaning (A) and weight gain (B) during the first 7-weeks of life of female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) 58
Figure 20. Body composition images of X-ray computed tomography of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). 59

Figure 21 A-B. Serum glucose concentration of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) (A) and area under the curve (AUC) of serum glucose (B). 60
Figure 22. Liver MDA concentration of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father) and orange juice). 61
Figure 23. Activity of superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and catalase (CAT) (C), in liver and plasma of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) 62
Figure 24. Expression of inflammation-related genes evaluated in retroperitoneal adipose tissue of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). 63
Figure 25 A-B. Epithelial growth: distance from the lymph node to the end of epithelial tree (A) and distance from the tip of the epithelial tree to the end of fat pad (B) from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). 64
Figure 26 A-B. Terminal end buds (TEBs), indicated by arrows (A); and Number of TEBs (B) of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 65
Figure 27 A-B. Cell proliferation in the mammary gland ducts and lobules of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). (A). Photomicrograph (×40 original magnification) of Ki-67 immunostaining (cells indicated by arrows) (B). 65
Figure 28 A-B. Quantification of apoptosis in the mammary gland ducts and lobules of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) (A). Photomicrograph (×40 original magnification) of apoptotic cells (indicated by arrows) (B). 66
Figure 29 A-B. Mammary gland histone modification (A) and miRNA expression (B) of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 67
Figure 30 A-B. Weight (A) and volume (B) of mammary tumors of female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 68

Figure 31 A-B. Representative images of mammary tumors of female offspring, submitted to breast carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 70

List of Tables

Table 1. Macronutrient distribution of control AIN-93G (CO) and high-fat (HF) diets. 27
Table 2. Composition of control AIN-93G (CO) and high-fat 45% (HF) diets. 28
Table 3. Sequence of primers used to evaluate gene expression in epididymal adipose tissue of male mice (fathers) and retroperitoneal adipose tissue of 7-week-old female offspring..... 36
Table 4. List of qPCR primers used in miRNA analysis. 38
Table 5. Evaluation of the antioxidant capacity of orange juice measured by DPPH method. 40
Table 6. Chemical and flavonoid composition of orange juice. 42
Table 7. Fatty acid profile of control (CO) and high-fat (HF) diets, evaluated by gas chromatography and presented in g/100g of the lipid fraction. 43
Table 8. Fatty acid profile of sweetened condensed milk (SCM), evaluated by gas mass chromatography and presented in g/100g of the lipid fraction. 43
Table 9. Estimated intake of mineral content from diets, sweetened condensed milk, added to the sweetened condensed milk and orange juice. Data are presented as average of daily consumption per animal/per group/per day during the 11 weeks of treatment. 46
Table 10. Estimated intake of vitamins from diets, sweetened condensed milk, added to the sweetened condensed milk and orange juice. Data are presented as estimated average intake per animal, per group, per day during the 11 weeks of treatment. 47
Table 11. Fat mass percentage, lean and bone mass, estimated by computed tomography of the male mice (fathers) from CO, CJ, OB, OJ groups, after 11 weeks of treatment..... 50
Table 12. Relative organ weight of male mice (fathers) from groups CO, CJ, OB, OJ after 11 weeks treatment. 52
Table 13. Paternal reproductive parameters 57
Table 14. Fat mass percentage, lean and bone mass of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). 59
Table 15. Relative organ weight of 7 weeks old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father), and OJ (obese father and orange juice). 60
Table 16. Development of mammary tumors in female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 68

Table 17. Histopathological classification of mammary tumors of female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice)..... 69

Table of Contents

1. Introduction 16

2. Literature Review 18

2.1 Breast Cancer 18

2.2 Breast carcinogenesis 18

2.3 Fetal origin of breast cancer 19

2.4 Terminal end buds (TEBs) and the experimental model of breast carcinogenesis 20

2.5 Paternal programming 21

2.6 Paternal obesity and metabolic and breast cancer programming 22

2.7 Orange juice and its bioactive compounds 23

3. Objectives 26

3.1 General Objectives 26

3.2 Specific objectives 26

4. Material and methods 27

4.1 Experimental design and animal manipulation 27

4.2 Calculation of vitamin and mineral mix 29

4.3 Orange juice analyses 29

4.3.1 Antioxidant activity of orange juice 29

4.3.2 Identification and quantification of flavonoids in orange juice 30

4.3.3 Quantification of total phenolics in orange juice 31

4.3.4 Identification and quantification of organic acids in orange juice 31

4.3.5 Identification and quantification of sugars in orange juice 32

4.4 Profile of lipids in diets and tissues of male mice (fathers) and 7-week-old female offspring 32

4.4.1 Extraction of lipids from diets and tissues 32

4.4.2 Determination of the lipid profile by gas chromatography 32

4.5 Body composition of male mice (fathers) and 7-week-old female offspring 33

4.6 Intraperitoneal glucose tolerance test (IGTT) of male mice (fathers) and 7-week-old female offspring 33

4.7 Determination of the relative weight of the organs of male (fathers) and 7-week-old female offspring 34

4.8 MDA concentration in liver of male mice (fathers) and 7-week-old female offspring 34

4.9 Antioxidant activity of enzymes in liver and plasma of male mice (fathers) and 7-week-old female offspring 34

4.9.1 Superoxide Dismutase (SOD) activity 34

4.9.2 Glutathione Peroxidase (GPx) activity 35

4.9.3 Catalase (CAT) activity 35

4.10 Determination of inflammation's gene expression in male mice epididyimal adipose tissue (fathers) and in 7-week-old female offspring retroperitoneal adipose tissue 35

4.11 Reproductive parameters of male mice (fathers) 36

4.12 Mammary gland harvesting of 7-week-old female offspring 37

4.13 Analysis of mammary gland morphology and development in 7-week-old female offspring 37

4.14 Analysis of mammary gland cell proliferation, H3K27me and apoptosis in 7-week-old female offspring 37
4.15 miRNA expression profile in mammary glands of 7-week-old female offspring 38
4.16 Mammary tumor induction 38
4.17 Histopathological analysis of mammary tumors 39
4.18 Statistical analysis 39
5. Results 40
5.1 Orange juice analyses 40
5.1.1 Antioxidant activity of orange juice 40
5.1.2. Identification of flavonoids in orange juice 40
5.1.3 Chemical and flavonoid composition of orange juice 41
5.2 Fatty acid profile of lipid fractions in control diet, high-fat diet and sweetened condensed milk 42
5.3 Fathers' dietary and health parameters 44
5.3.1 Dietary consumption of male mice (fathers) 44
5.3.2 Weight gain and body composition of male mice (fathers) 49
5.3.3 Intraperitoneal glucose tolerance test (IGTT) of male mice (fathers) 51
5.3.4 Relative organ weight of male mice (fathers) 52
5.3.5 Plasma, liver and epididymal adipose tissue fatty acid profile of male mice (fathers) 52
5.3.6 MDA concentration in liver homogenate of male mice (fathers) 54
5.3.7 Antioxidant activity of enzymes in the liver and plasma of male mice (fathers) 55
5.3.8 Expression of inflammatory genes in epididymal adipose tissue of male mice (fathers) 56
5.3.9 Reproductive parameters of male mice (fathers) 57
5.4 Female offspring health and metabolic parameters 58
5.4.1 Weight at weaning and weight gain of 7-week-old female offspring 58
5.4.2 Body composition of 7-week-old female offspring 58
5.4.3 Intraperitoneal glucose tolerance test in 7-week-old female offspring 59
5.4.4 Relative weight of organs of 7-week-old female offspring 60
5.4.5 MDA concentration in liver homogenate of 7-week-old female offspring 61
5.4.6 Activity of antioxidant enzymes in liver tissue and plasma of 7-week-old female offspring 61
5.4.7 Expression of inflammatory genes in retroperitoneal adipose tissue of 7-week-old female offspring 62
5.5 Female offspring mammary gland development 64
5.5.1 Mammary gland morphology of 7-week-old female offspring 64
5.5.2 Mammary gland cell proliferation and apoptosis of 7-week-old female offspring 65
5.5.3 Mammary gland histone modification and miRNA expression of 7-week-old female offspring female offspring 66
5.6 Female offspring mammary carcinogenesis 67
5.6.1 Incidence, latency and multiplicity of tumors 67
5.6.2 Tumor weight and volume 68
5.6.3 Histopathological classification of tumors 69
6. Discussion 72
6.1 Dietary intervention 72
6.1 Paternal dietary intervention and reproductive parameters 73
6.2 Programming effects of paternal obesity in female offspring metabolic health 74
6.3 Mammary gland development and susceptibility to carcinogenesis in programmed female offspring 75
7. Conclusions 77
References 78

1. Introduction

Breast cancer is a public health problem (BARRIOS; REINERT; WERUTSKY, 2018) and the number one cause of cancer death among women (IARC, 2018). This disease is heterogeneous (TURASHVILI; BROGI, 2017) and its etiology has not been completely clarified. However, some of the risk factors for breast cancer are well known, such as genetic, hormonal and environmental exposures, which includes nutrition (MOMENIMOVAHED; SALEHINIYA, 2019). An interesting hypothesis was published in 1990 by Trichopoulos, suggesting that breast cancer could have its origin in the intrauterine environment, in response to high levels of endogenous estrogen (TRICHOPOULOS, 1990).

Currently it is recognized that the origin of some diseases occurs during early development, a theory known as Developmental Origins of Health and Disease (DOHaD) (ARIS; FLEISCH; OKEN, 2018). Although this theory has been investigated mostly from a maternal perspective, recent evidence highlights a potential role of fathers in this context (FONTELLES et al., 2018a). It was demonstrated that paternal under or overnutrition can induce metabolic dysfunction (MCPHERSON et al., 2016; MAGNUS et al., 2018; NG et al., 2010) and increase susceptibility to breast cancer development in female offspring (FONTELLES et al., 2016b; DA CRUZ et al., 2018).

The most studied mechanism associated with paternal pre-conceptional nutritional status and diseases risk in the offspring is the heritability of epigenetic information through spermatozoa (DONKIN; BARRÈS, 2018). These epigenetic mechanisms, such as DNA methylation, histone modifications and non-coding RNAs (micro RNAs) have an important role in the development of sperm cells (SU; PATTI, 2019). To form a highly specialized mature sperm and to facilitate the totipotency of the zygote, reprogramming of epigenetic marks to rearrange the chromatin structure is necessary. Thus, spermatogenesis and early embryogenesis are periods of particular vulnerability to environmental exposures that may alter the epigenetic information of sperm cells (GE et al., 2017). Male obesity, for example, is a disturbance that alters both quality and epigenetic marks of the spermatozoa (OU; ZHU; SUN, 2019), changing gene expression in fetal tissues, with long lasting effects on adulthood (LANE et al., 2015).

Interestingly, bioactive compounds, such as polyphenols, which are present in orange juice, can also modify or reverse epigenetic events (MONTGOMERY; SRINIVASAN, 2019). Currently, there are reports showing a positive effect of some classes of bioactive compounds on male reproductive parameters (JIANG et al., 2018; RAHMAN et al., 2018). Overall, there are few studies that have investigated paternal eating behavior and offspring health and only

one study was found on research database regarding paternal preconception consumption of foods containing bioactive compounds and its protective effects in offspring health (MCPHERSON et al., 2016). Although little is known about bioactive compounds' effects on mammary gland development, there are reports of beneficial effects of maternal consumption of bioactive compounds, such as resveratrol and anthocyanins, on breast cancer development in the offspring (WU et al., 2009; PAPOUTSIS et al., 2015).

Therefore, the aim of this study was to evaluate the effects of paternal obesity, induced by a high-fat/high-sugar diet, and/or consumption of orange juice on mammary gland morphology and mammary tumor development in female mice offspring. Our results indicated that consumption of orange juice by non-obese fathers during preconception increased the susceptibility of female offspring to mammary carcinogenesis. In addition, although paternal intake of a high-fat/high-sugar diet during preconception reduced incidence and increased latency of mammary tumors, it increased their multiplicity. In addition, the data indicates that the response to orange juice consumption depends of the paternal metabolic context.

2. Literature Review

2.1 Breast Cancer

Breast cancer is a major public health problem (BARRIOS; REINERT; WERUTSKY, 2018). In 2018, 2.089 million new cases were registered. In addition to being the fifth leading cause of cancer death in the world, this is the most frequent cause of cancer death among women (IARC, 2018). In Brazil, it was estimated 59,700 new cases of breast cancer in 2018, which accounts for 29.5% of new cancer cases (INCA, 2017).

Among the known risk factors associated with the development of this disease, BRCA1 and BRCA2 (genetic factors), early menarche, late menopause, first pregnancy after 30 years, nulliparity, use of oral contraceptives and postmenopausal hormone replacement therapy (hormonal factors), exposure to ionizing radiation, alcohol consumption, overweight and obesity after menopause (environmental factors) can be highlighted (INCA, 2019).

Approximately 3-5% of breast cancer cases are estimated to be a cause of BRCA1 and BRCA2 genes mutations (KEMP et al., 2019). These genes are involved in DNA repair and cell cycle progression. The BRCA cancer-associated mutation decreases the efficiency of DNA repair mechanisms and thus its tumor-suppressive effect (LÓPEZ-URRUTIA et al., 2019). In addition to genetic risk factors, lifestyle characteristics may increase the risk of developing breast cancer (ARTHUR et al., 2018). Because these habits may change, these risk factors are considered modifiable (BROWN; LIGIBEL, 2018). Breast cancer preventive measures include adoption of healthy habits, such as exercise, adequate body weight maintenance, moderation in ethanol consumption and consumption of a healthy diet (ARTHUR et al., 2018).

2.2 Breast carcinogenesis

Breast cancer is a complex, heterogeneous disease (NAGINI, 2017) and its initiation is a consequence of altered (genetic and epigenetic) events in a single cell (POLYAK, 2007). It is suggested that this disease progresses from no-obliged pre-invasive lesions (atypical ductal hyperplasia, ADH), and/or precursor ductal carcinoma in situ (DCIS) to invasive and metastatic disease (PASCULLI; BARBANO; PARRELLA, 2018). In the normal mammary gland, ducts are comprised by an outer myoepithelial cell layer producing the basement membrane (BM) and an inner luminal epithelial cell layer which produces milk during lactation. The ducts are surrounded by the microenvironment composed of extracellular matrix (ECM) and various

stromal cell types (e.g., endothelial cells, fibroblasts, myofibroblasts, and leukocytes), called microenvironment (POLYAK; KALLURI, 2010). Ductal carcinoma *in situ* of the breast occurs when malignant epithelial cells are confined to the ductal system of the breast, without evidence of invasion through the basement membrane into the surrounding stroma (RUSSO, 2016). The transition from *in situ* to invasive ductal carcinoma is defined by the loss of myoepithelial cell layer and basement membrane, which usually results in the spread of tumor cells to distant sites, known as metastasis (PLACE; HUH; POLYAK, 2011).

One common event during the early stages of carcinogenesis, as result of genetic lesions or environmental hits, is the alteration of chromatin structure through DNA methylation and post-translational modifications of histones, affecting cellular plasticity and favoring tumor development and progression (PASCULLI; BARBANO; PARRELLA, 2018). Epigenetic changes can contribute to the development of the disease through a variety of mechanisms, including silencing of tumor suppressors; genomic instability; and blockade of differentiation (LOCKE et al., 2015).

Based on the histological features of tumors they are classified as hormone-receptor-positive, human epidermal growth factor receptor-2 overexpressing (HER2+) and triple-negative breast cancer (TNBC) (NAGINI, 2017). The three subtypes have distinct risk profiles and require specific treatment strategies (WAKS; WINER, 2019). Although there is intense research in the biology and treatment of breast cancer, studies generally focus on its development in already adult women, when a lot of lifestyle-associated risk factors are well established and difficult to modify. Few consider the fetal origin hypothesis as a prevention strategy.

2.3 Fetal origin of breast cancer

The development of the mammary gland is a long process that starts during the fourth week of intrauterine life and progresses throughout puberty and ends after a 1st complete gestation/lactation. During these critical developmental stages mammary gland is highly plastic and prone to environmental disturbances, such as maternal exposures during gestation and lactation (RUSSO, 2016). In 1990, Trichopoulos suggested that breast cancer could have its origin in the intrauterine environment, linking increased estrogen concentrations and possibly other hormones during pregnancy with the likelihood of daughters to develop breast cancer in adulthood (TRICHOPOULOS, 1990).

More recently studies showed that dietary exposures during the intrauterine period may also alter later susceptibility to breast tumors development (DE ASSIS et al., 2012; NGUYEN et al., 2017). Exposure of Sprague-Dawley rats to a high-fat diet during pregnancy, for example, resulted in increased breast cancer risk in two generations of female offspring (daughters and grandchildren) (DE ASSIS et al., 2012). However, dietary modulations in the intrauterine period may also result in reduced susceptibility to breast cancer. In the study of de Oliveira Andrade et al. (2014), female offspring of rats consuming a lard-based high-fat diet during pregnancy, or pregnancy and lactation, presented lower incidence, multiplicity and lower weight of the tumors compared to control offspring.

In this context, it is proposed that persistent epigenetic alterations caused by maternal diet or exposure to certain environmental factors are inherited by somatic cells of the daughters and maintained throughout life (GOYAL; LIMESAND; GOYAL, 2019). Such epigenetic modifications may lead to changes in mammary gland development, affecting highly vulnerable epithelial targets, such as terminal end buds (TEBs) in rat mammary gland or in terminal ductal-lobule units (TDLU) in women breast, that are subject to malignant transformation when exposed to an initiating agent, resulting in a higher risk of tumor development (HILAKIVI-CLARKE; DE ASSIS, 2006).

2.4 Terminal end buds (TEBs) and the experimental model of breast carcinogenesis

In the rat mammary gland, it was demonstrated that cancer starts in TEBs of young virgin rats (RUSSO, 2016). Therefore, it has been proposed that increased number of TEBs are associated with higher breast cancer risk, whereas decreased number of TEBs are associated with lower breast cancer risk (HILAKIVI-CLARKE, 2007). This concept, however, has been challenged by findings indicating that some early-life dietary modifications, despite reducing number of TEBs, increased susceptibility to breast tumorigenesis (OLIVO; HILAKIVI-CLARKE, 2005).

TEBs are structures with an outer layer of undifferentiated cap cells and inner layers of luminal epithelial cells. Both layers of the end bud display high rates of mitosis (HINCK; SILBERSTEIN, 2005). When cap cells are sensitized by ovarian hormones, for the expansion of the mammary parenchyma through branching and lobular formation, cell proliferation rates increase (RUSSO, 2016). At 50 days of age, female rats and mice present the highest susceptibility to carcinogen initiation due to the high number of TEBs present in the mammary epithelial tree (RUSSO, 2015).

7,12-Dimethylbenz[*a*]anthracene (DMBA) or N-Nitroso-N-methylurea (MNU)-induced rat tumors reproduce the pathological features of most common breast adenocarcinomas in women (RUSSO, 2016). DMBA binds to DNA from mammary gland parenchyma cells between 1 and 16h after *in vivo* oral administration, and the binding of DMBA to DNA, RNA, and tissue proteins is associated with the cell proliferation rate (RUSSO et al., 1982). Although there are some differences in the mouse mammary gland and human breast, the mouse model for human breast cancer is widely used and has greatly improved our understanding of the molecular mechanisms involved in breast cancer development and progression (MCNALLY; STEIN, 2017).

2.5 Paternal programming

The concept that the mother's nutritional, metabolic, and hormonal environment can permanently program the structure and physiology of her offspring is not new (BARKER, 1994). David Barker and Nicholas Hales began using the term "Fetal Origins of Adult Disease" based on studies that showed a relationship between low birth weight and later development of cardiovascular disease and impaired glucose tolerance (HALES & BARKER, 1992). The term “programming” was proposed to describe the process where an insult or stimulus during critical or sensitive periods of development may have long-term effects, changing structure or function of the organism (LUCAS, 1998). This was later explained by the modifications of the epigenetic machinery, which influences gene expression and are inherited from one cell generation to the next, causing long lasting changes in gene expression and in organ structure (GOYAL; LIMESAND; GOYAL, 2019). Because key developmental stages such as preconception, lactation, early childhood and puberty are important in influencing long-term phenotypes (EL-HEIS et al., 2018; ALMEIDA et al., 2019), the term fetal programming has been substituted to Developmental Origins of Health and Disease (DOHaD) (NYIRENDA; BYASS, 2019).

The programming of chronic disease through maternal exposure to dietary and environmental factors in their offspring has been well documented (HE et al., 2017; BERENDS et al., 2018; ROSEBOOM, 2019). Lately, this view has been expanded, as a growing number of studies also link paternal environmental conditions such as eating habits before conception with their offspring's phenotypes (SOUBRY, 2018). The potential underlying mechanism for paternal programming is the epigenetic information carried by the spermatozoa, that can be influenced by dietary or/and environmental factors (DONKIN; BARRÈS, 2018). Epigenetic modifications are important regulators of male spermatogenesis. Packaging of sperm DNA with

protamines (exchange of 85%–95% of the histones by protamines), for example, protects the sperm genome from oxidation, allows nuclear compaction, is essential for sperm motility, and also protects sperm DNA from detrimental molecules within the female reproductive tract (GUNES et al., 2018).

There are specific windows of susceptibility to epigenetic changes by environmental exposures during sperm development, including the intrauterine period, prepuberty, and the reproductive and zygotic phases of the man (SOUBRY et al., 2014; FONTELLES et al., 2018a). A likely factor contributing to the epigenetic integrity of sperm is paternal age. Advanced paternal age is a well-established risk factor for childhood morbidity and it is plausible that the ability to reprogram the epigenome diminishes with advancing years, as do other cellular processes (RANDO; CHANG, 2012). In this context, considering the DOHaD hypothesis, it is essential to analyze how a paternal transient stimulus (eg, high-fat diet, a current public health problem) can give rise to lasting phenotypic consequences that persists for many cell generations (RANDO, 2015).

2.6 Paternal obesity and metabolic and breast cancer programming

In the last four decades the prevalence of obesity has nearly tripled and it has been pointed out as the fifth leading modifiable risk factor for global deaths, remaining a current public health problem (GBD Obesity Collaborators, 2017). It is estimated that 1.9 billion adults worldwide are overweight (body mass index (BMI) greater than 25kg/m^2) and at least 650 million has a BMI exceeding 30kg/m^2, classified as obese (WHO, 2016). In Brazil, 19.8% of adults are obese and it is estimated that almost 20% of the male population men are obese (MS, 2018). The consequences of obesity for an individual are well evidenced and involve higher risks for the development of chronic diseases, such as type 2 diabetes, cardiovascular disease and cancer (CHOBOT et al., 2018; COLDITZ; PETERSON, 2018; KOLIAKI; LIATIS; KOKKINOS, 2019). Like obesity, the etiology of most human diseases involves complex interactions of genetics and various environmental factors, which is initially generated early in human life, during the embryogenesis and fetal development, for example (EL-HEIS et al., 2018).

Obesity can alter the structure and molecular composition of male germ cells and change sperm epigenetic marks (DNA methylation, histone modification and miRNAs) that alters access to transcription and translation of paternal genes during embryogenesis (MCPHERSON et al, 2014). Experimental and epidemiological evidence have shown that paternal overweight

in preconception can impair offspring metabolism (DE CASTRO BARBOSA et al., 2016; MAGNUS et al., 2018). Ng et al. (2010) showed that obese rats that mated with control females had female offspring with glucose intolerance, reduced insulin secretion and altered gene expression in pancreatic islets, potentially associated with reduced methylation of the alpha-2 interleukin 13 receptor (*IL13ra2*). De Castro Barbosa et al. (2016) also observed metabolic changes in offspring of high-fat fed male rats. Body weight and glucose metabolism were altered in two generations of offspring of high-fat fed rats. These results were associated with altered expression miRNA let-7c, found in metabolic tissues of the offspring, which induced a transcriptomic shift of the let-7c predicted targets (DE CASTRO BARBOSA et al., 2016). More recently, one of the world's largest pregnancy cohorts revealed a positive association between paternal obesity in preconception and childhood-onset of type 1 diabetes (MAGNUS et al., 2018).

Regarding breast cancer programming, the study by Fontelles et al. (2016a) showed that a consumption of high-fat diet during preconception epigenetically reprogrammed male mice germ cells and modulated birth weight of female offspring, as well as the susceptibility of female offspring to develop breast cancer. Additionally, the authors observed that a lard-based high fat diet can impair sperm quality and function of obese fathers, which was associated with metabolic programming disorders and increased breast cancer risk in rats (FONTELLES et al., 2016b). Contributing to the idea that breast cancer can be programmed by paternal factors, one meta-analysis showed that higher paternal age is associated with increased susceptibility of breast cancer in adult daughters (XUE & MICHELS, 2007).

These studies indicate that paternal overweight or obesity, especially during gametogenesis, may play an important role in offspring health. However, little is known about paternal consumption of bioactive compounds during preconception and its influence on mammary gland differentiation.

2.7 Orange juice and its bioactive compounds

Orange juice is one of the most consumed fruit juices in the world, with Brazil and United States producing around 50% of the world's total supply (FRANKE et al., 2013). This juice is a dietary source of bioactive compounds, including vitamin C, flavonoids such as hesperidin and narirutin, carotenoids, sugars, and fibers that contribute to general health status (BRASILI et al., 2019). The main flavonoid class found in citrus juice are flavanones, presented in the glycoside or aglycone forms. In orange juice, hesperidin and narirutin are present in the

glycoside form, presenting a flavanone and a disaccharide residue (rutinose), which are tasteless (KHAN; ZILL-E-HUMA; DANGLES, 2014). The composition of orange juice and its bioactivity can be influenced by different forms of cultures, climates, extraction methods, storage temperatures, packaging and shelf life, among other factors (ERCISLI; OHRAN, 2007). Previous studies have shown that orange juice has anti-tumor effects, and antioxidant and anti-inflammatory properties that may have protective effects against chronic disease (FRANKE et al., 2013; YE, 2017).

In obese C57BL6 mice, Moro orange juice showed an anti-obesity effect, resulting in less accumulation of fat (TITTA et al, 2010). In addition to limited weight gain, mice that drank Moro orange juice presented increased insulin sensitivity and decreased triglycerides and total cholesterol levels, possibly due to increased expression of peroxisome proliferator-activated receptor (PPAR-α) and its target gene acylCoA-oxidase, which is a key enzyme of lipid oxidation (SALAMONE et al, 2007). A randomized, double-blind, crossover study showed that consuming orange juice with at least 300mg of flavanones/day over a period of 12 weeks protected against DNA damage and lipid peroxidation, enhanced the antioxidant defense system, and lowered blood pressure in overweight or obese non-smokers adults (RANGEL-HUERTA et al, 2015). In another randomized controlled trial with obese patients showed, orange juice intake (500 mL/day) was associated with a reduced-calorie diet, ameliorated insulin sensitivity, lipid profile and inflammatory status (RIBEIRO; DOURADO; CESAR, 2017).

Polyphenols have also been shown to influence fertility and sexual development; fetal health and the bioavailability of nutrients (LY, et al 2015). Some classes of polyphenols such as green tea catechins have been shown to have antioxidant properties, which improved fertility parameters, such as sperm concentration, motility, morphology, DNA damage, fertility rate, and gamete quality (RAHMAN et al., 2018). Also, cyanidin-3-O-glucoside at low doses improved the number and motility of the sperms, alleviating the seminiferous tubule injury caused by 3-chloro-1,2-propanediol (3-MCPD) (JIANG et al., 2018).

There are limited studies in the literature on polyphenols in the context of paternal programming (SILVA et al., 2019). McPherson et al., (2016) evaluated in a rat model whether programming effects of paternal undernutrition could be prevented by supplementation with a mixture of green tea extract, lycopene and micronutrients (vitamin C, vitamin E, folate, zinc and selenium). The authors observed that paternal supplementation with the BFC/micronutrient mix was able to reduce oxidative damage to DNA and restored global methylation patterns in paternal sperm, preventing fat accumulation and dyslipidemia in offspring (MCPHERSON et

al., 2016). As, some bioactive compounds, such as polyphenols, are capable of altering the expression of innumerable genes through epigenetic modifications (MONTGOMERY; SRINIVASAN, 2019), it would be important to investigate its effects on male gametogenesis. Even though little is known about the action of bioactive compounds of foods in modification of mammary gland development, some bioactive compounds, such as resveratrol and anthocyanins, have already shown potential benefits for breast cancer risk reduction through maternal nutrition in rodents (WU et al., 2009; PAPOUTSIS et al., 2015).

Therefore, we hypothesized that paternal obesity during preconception could increase the susceptibility to breast cancer in their female offspring. Furthermore, consumption of orange juice and its bioactive compounds, in the presence of paternal obesity, could have a beneficial effect in the context of breast cancer programming of female offspring.

3. Objectives

3.1 General Objectives

To evaluate the effect of paternal obesity in breast cancer risk of female offspring and to assess the role of paternal consumption of orange juice, in the presence or absence of obesity, in breast cancer risk of female offspring.

3.2 Specific objectives

To evaluate paternal dietary interventions in:

- reproductive parameters of male mice (fathers);
- morphology, epithelial growth, cellular proliferation and apoptosis in 7-week-old female offspring mammary glands;
- global pattern of histone modification in 7-week-old female offspring mammary glands;
- expression of miRNAs associated with breast cancer in in 7-week-old female offspring mammary glands;
- development of mammary tumors in female offspring submitted to chemical induction of mammary carcinogenesis by DMBA (7,12-dimethylbenz[a]anthracene).

4. Material and methods

4.1 Experimental design and animal manipulation

This study was approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil (Protocol CEUA/FCF/USP/504). Three-week old male (n = 48) and female (n = 96) mice C57BL/6J, were obtained from the Colony of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil and maintained at 22°C ± 2°C, in an atmosphere of 55% ± 10% relative humidity in a 12 hours light/dark cycle. Male mice were randomly divided into 4 groups (n = 10/group), that received experimental diets (Table 1 and 2) for 11 weeks from weaning (week 4 to 14 of age): control (C) (AIN-93G + water *ad libitum*), obese (O) (high-fat/high-sugar diet; 45% lard-based), supplemented with sweetened condensed milk which was fortified with mineral mix and vitamin mix + water *ad libitum*), control-orange juice (CJ) (AIN-93G + orange juice *ad libitum*) and obese-orange juice (OJ) (high-fat/high-sugar diet + orange juice *ad libitum*) (SAMUELSSON et al., 2008). Mating was performed by housing one male with two females per cage. In each litter were maintained 6 pups with their respective dams. Control diet AIN-93G was offered to female mice during pregnancy and lactation and to female offspring until 7 weeks of age when the diet was changed to AIN-93M until the end of the experiment (Figure 1). Body mass and food intake were recorded 3 times a week for male mice receiving the experimental diets and once a week for female offspring. At the conclusion of the study period, the animals were anesthetized and exsanguinated by cardiac puncture and plasma was stored at −80°C until processed. The tissues were flash frozen in liquid nitrogen and stored at −80°C until processed.

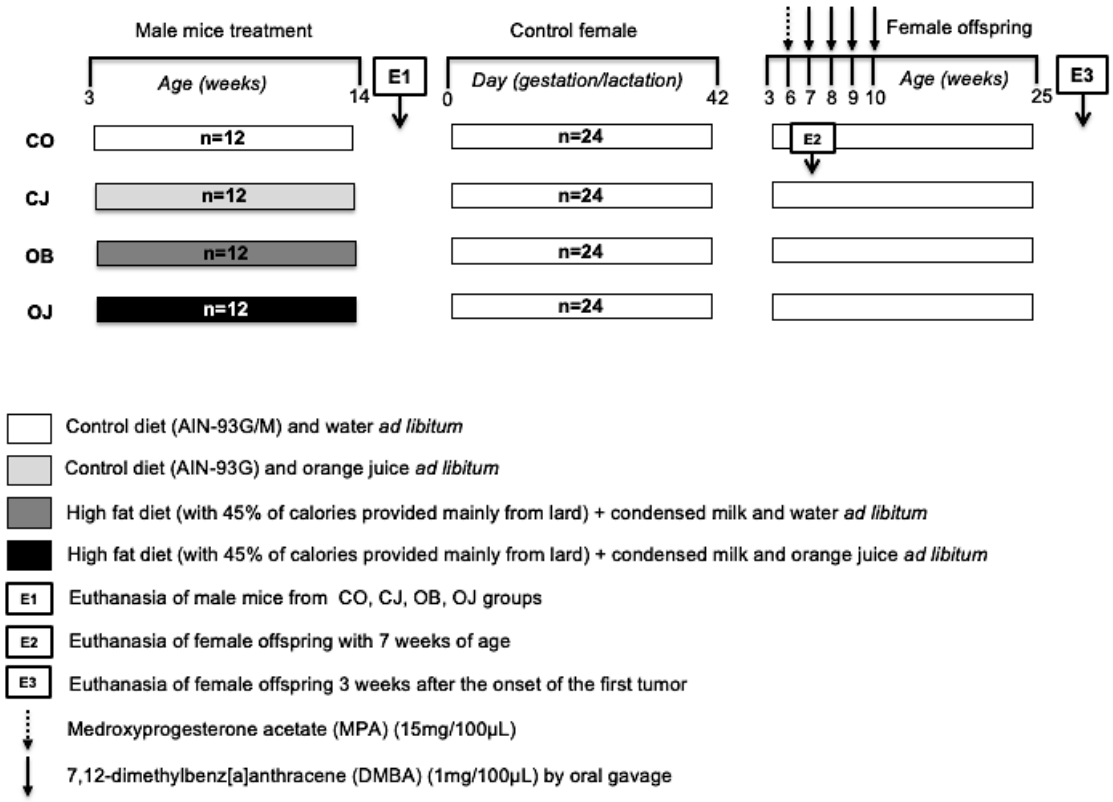
Table 1. Macronutrient distribution of control AIN-93G (CO) and high-fat (HF) diets.

	CO		**HF**	
Macronutrients	% per weight	% of calories	% per weight	% of calories
Carbohydrate	72	64	50	35
Protein	21	20	25	20
Lipids	7	16	25	45
kcal/g	**3,96**		**4,71**	

Table 2. Composition of control AIN-93G (CO) and high-fat 45% (HF) diets.

g/100g	**CO**	**HF**
Casein	20,00	23,52
L-cysteine	0,30	0,35
Starch	39,75	25,98
Maltodextrin	13,20	8,63
Sucrose	10,00	6,54
Cellulose	5,00	5,88
Lard	0,00	15,28
Soy oil	7,00	8,23
Mineral mix	3,50	4,12
Vitamin mix	1,00	1,18
Choline bitartrate	0,25	0,30
Energy	**3,96Kcal/g**	**4,71Kcal/g**

Figure 1. Experimental design.



4.2 Calculation of vitamin and mineral mix

The vitamin and mineral mix were supplemented weekly according to the daily consumption of the experimental diets of each week, calculation is shown below:

Mixvit = (MixvitCO - MixvitHF) / CSCM
Mixmin = (MixminCO - MixminHF) / CSCM

Where: MixCO: vitamin or mineral mix of the CO diet; MixHF: vitamin or mineral mix of the HF diet; CSCM: consumption of sweetened condensed milk; expressed in g of mix/g of sweetened condensed milk.

4.3 Orange juice analyses

4.3.1 Antioxidant activity of orange juice

4.3.1.1 Antioxidant activity (DPPH)

The antioxidant activity of orange juice was determined according to Brand-Williams et al., (1995), via free radical DPPH*. The orange juice samples were homogenized and centrifuged (10min, 8000rpm, 4ºC) for the separation of supernatant and precipitate. The supernatant fraction was filtered and the insoluble (precipitated) fraction was added with 20mL of DMSO, the sample was homogenized in Turrax and the extraction was performed for 18h under agitation. After this period the samples were centrifuged under the same conditions previously described, filtered and analyzed. For this, 25 µl of each sample, in triplicate, was mixed with the DPPH* solution and incubated for 30 min in the dark. Absorbance reading was performed at 515nm in a microplate reader (Synergy HT, BioTek Instruments, Inc., USA). Results were expressed as µmoL Trolox equivalent/L.

4.3.1.2 Antioxidant Activity (ORAC)

The antioxidant activity of orange juice determined by the ORAC (Oxygen Radical Absorption Activity) method was performed as proposed by Huang et al., (2002). The separation conditions of the samples were the same as those used for DPPH analyses (item 4.3.1.1). Samples were pipetted in quadruplicate and read in microplate reader (Synergy HT, BioTek Instruments, Inc., USA). For this, the equipment injected 150µL of fluorescein with 30

min of incubation. Then 25µl of the AAPH 2,2'-azobis (2-amidinopropane) dihydrochloride solution was added. After shaking the plate, the reading was taken at 1 min intervals for 60 min at one length of 485nm excitation and 528nm emission. Antioxidant capacity was calculated from the area under the curve (AUC), using the Trolox calibration curve, and the results were expressed as µmoL Trolox equivalent/L.

4.3.2 Identification and quantification of flavonoids in orange juice

4.3.2.1 Flavonoid Extraction

For extraction of soluble flavonoids, 10mL of homogenized orange juice were centrifuged (10min, 8000rpm, 4ºC), the supernatant fraction was filtered and analyzed by HPLC-DAD as described in item 4.3.2.2. To the insoluble fraction (precipitate) was added 20mL of DMSO, the sample was homogenized in Turrax and the extraction was performed for 18h under agitation. After this period the samples were centrifuged under the same conditions previously described, filtered and analyzed by HPLC-DAD.

4.3.2.2 High performance liquid chromatography-diode array detector (HPLC-DAD)

Identification and quantification of phenolic compounds was performed using a 1260 Infinity Quaternary LC System (Agilent Technologies, USA), with an autosampler and a quaternary pump coupled to a diode array detector (DAD). Prodigy 5µ ODS3 (250x4.60mm) (Phenomenex Ltd., United Kingdom) column was used at a flow rate of 1mL/min, 25°C and elution was performed with solvent gradient consisting of A: 0.5% formic acid in water and B: acetonitrile. The solvent gradient was applied as follows: 10% B at the beginning, 10% in 5 min, 20% in 15 min, 25% in 25 min, 35% in 33 min, 50% in 38 min, 90% in 43-44 min and 10% in 45 min. The runs were monitored at 280nm and peaks were identified by retention time and by similarity of absorption spectra with commercial standards, in addition to mass spectra. Flavonoid and phenolic acid standards were used to compose the calibration curve (Extrasynthese, Genay, France and Sigma-Aldrich) (Hassimoto et al., 2008).

4.3.2.3 High performance liquid chromatography coupled to mass/mass spectrometer

Flavonoids identification was carried using a Prominence Liquid Chromatograph linked to an ion trap Esquires-LC mass spectrometer with an electrospray ionization interface. The

solvent gradient conditions were described for HPLC-DAD (item 4.3.2.2). After DAD process, the flow rate was changed to 0.2 ml/min for application to the mass spectrometer. The mass spectrometer operated as follows: collision energies of 4500V were used for the positive mode, 4000V for the negative mode and capillary temperature was 275 °C. The analysis was conducted using a full scan from *m/z* 100 to 1500. The compounds were identified according to comparison with the retention times of authentic standards, as well as by absorption spectrum similarity, mass spectral characteristics and by comparison with the literature data (TEIXEIRA et al., 2015).

4.3.3 Quantification of total phenolics in orange juice

The quantification of total phenolics was performed by the Folin-Ciocalteau spectrophotometric method (SWAIN; HILLIS, 1959), based on the reduction of the Folin-Ciocalteau reagent (phosphomolibdate-phosphotungstate complex) by the phenolic compounds to blue staining product (molybdenum and tungsten) in alkaline medium. Samples of orange juice supernatant, in appropriate dilution, was added to 25 μL Folin Reagent and 200 mL deionized water. Subsequently, 25 μL of a saturated sodium carbonate solution was added and samples were incubated in room temperature for 1 hour. The absorbance at 725 nm was then read on a plate reader (Multi-Detection microplate reader; Synergy – BioTek integrated with Gen 5 software). The total phenolic content was expressed as gallic acid equivalent (GAE) using a calibration curve. All analyzes were performed in triplicate and the results were expressed as mg GAE equivalent/L.

4.3.4 Identification and quantification of organic acids in orange juice

For the determination of organic acids, the orange juice supernatant was filtered with a 0.45 μm Millipore® membrane and diluted with metaphosphoric acid. The samples were analyzed by high performance liquid chromatography (Hewlett Packard Series 1100) using SUPELCOGEL C-610H column (30 cm X 7.8 mm) and isocratic mobile phase composed of 0.1% phosphoric acid (w/v), with a flow rate of 0.5mL/min and detection at 210 and 240nm. Quantification and identification of organic acids were performed from the calibration curves of ascorbic, citric and malic acids. Results were expressed as mg/mL of orange juice.

4.3.5 Identification and quantification of sugars in orange juice

To determine the soluble sugars of orange juice, it was used the methodology described by Cordenunsi, Shiga, & Lajolo (2008). For this, the orange juice supernatant was filtered through a 0.45 μm Millipore® membrane and diluted in deionized water. Sugars were detected by high efficiency anion exchange chromography (HPAEC) (Dionex, DX500) with pulsed amperometric detection (PAD) using PA1 column (Dionex). The mobile phase consisted of 100% 20mM NaOH with a constant flow of 1.0mL/min at 30ºC. Quantification was performed from glucose, fructose and sucrose calibration curves and the results expressed in g/L of orange juice.

4.4 Profile of lipids in diets and tissues of male mice (fathers) and 7-week-old female offspring

4.4.1 Extraction of lipids from diets and tissues

The extraction of lipids from control and high-fat diet, sweetened condensed milk, liver, plasma and epididymal adipose tissue was carried out by the Bligh and Dyer, (1959) methodology according to Iverson et al., (2001). Briefly samples were homogenized with a mixture containing chloroform (125 μL) and methanol (200 μL). The solution was again homogenized with chloroform (125 μL) and 125 μL of a saline solution (0.88% NaCL). The final emulsion was centrifuged at 13,000 x g for 5 min at 25ºC. Upper phase was removed and lower phase was evaporated under nitrogen stream. Fat samples were esterified as described by Shirai, et al. (2005). Extracted fat aliquots were added in tubes containing: Tricosanoic Acid Methyl Ester, 0.5% BHT and 0.5 M methanolic NaOH. The solution was heated in water bath at 100ºC/5 min. Samples were mixed with 14% BF_3 in methanol and heated in water bath at 100ºC/5 min. Next, isooctane was added to the tubes that were shaken vigorously. Lastly, saturated solution of NaCl were added in tubes and solution was homogenized slowly. After centrifugation, the organic phase was transferred to a new vessel and dried under nitrogen stream. The recovered lipids were reconstituted in isooctane (NOGUEIRA et al., 2019).

4.4.2 Determination of the lipid profile by gas chromatography

Fatty acids quantification was determined by gas chromatograph (GC) equipped with a G3243A MS detector (Agilent 7890 A GC System). A fused silica capillary column (J&W DB-

23 Agilent 122-236) was used for injection of samples. High-purity helium was used as the carrier gas at a flow rate of 1.3 ml/min with a split injection of 50:1. The oven temperature was programmed from 80ºC to 175ºC at a rate of 5º C/min, followed by another gradient of 3ºC/min to 230ºC, and kept at this temperature for 5 min. The temperature of GC inlet and transfer line were 250ºC and 280ºC, respectively. GC-MS was performed using 70 eV EI in scan acquisition and quantified by TIC. The fatty acids were identified comparing the retention times with standard mixture of fatty acid methyl esters. All mass spectra were obtained over the m/z range of 40-500. The results were expressed as g of each fatty acid/100g of oil (NOGUEIRA et al., 2019).

4.5 Body composition of male mice (fathers) and 7-week-old female offspring

Determination of body composition of 14-week-old male mice (n=3/group) and 7-week-old female offspring (n=4/group) was performed using the Albira PET-SPECT-CT imaging station (Bruker Biospin Corp., Woodbridge, CT, USA). Animals were anesthetized with 3% isoflourane and placed in the animal bed within the instrument, where anesthesia was maintained, during all X-ray computed tomography (CT) imaging. CT scans were acquired at 45 kV with a 400 μA current. Imaging was performed with either a 118.5 mm or 65.0 mm axial FOV. After reconstruction with CT software, the data was analyzed with PMOD v3.17 (PMOD Technologies Ltd., Zurich, Switzerland) (WATHEN et al., 2013).

4.6 Intraperitoneal glucose tolerance test (IGTT) of male mice (fathers) and 7-week-old female offspring

Part of male mice from groups CO, CJ, OB, OJ, at 11 weeks of age (n=8/group) and female offspring at 7 weeks of age (n=6/group), were submitted to the tolerance test to intraperitoneal glucose. For this, the animals remained in an 8-hour fasting period, prior to the experiment. After collection of fasting glucose in glucometer (AccuCheck Performa Nano), the animals were submitted to intraperitoneal injection of dextrose solution (2g/kg body weight), and the glucose was evaluated by caudal puncture at time 15, 30, 60, 90 and 120 minutes after injection. From the data obtained the area under the curve (AUC) was calculated (TAKADA et al., 2008).

4.7 Determination of the relative weight of the organs of male (fathers) and 7-week-old female offspring

Absolute weights of testicle, epididymis, kidney, liver, heart, epididymal adipose tissue (EAT), retroperitoneal adipose tissue (RAT), inguinal subcutaneous adipose tissue (ISAT) of 14-week-old male and ovarian, kidney, liver, heart and RAT of 7-week-old female offspring were determined by analytical balance. In the case of even organs, such as testicle, epididymis, ovaries and kidneys, the mean between left and right side was used. The relative weight of the organs was considered.

4.8 MDA concentration in liver of male mice (fathers) and 7-week-old female offspring

The malondialdehyde (MDA) concentration in liver of 14-week-old male mice and 7-week-old female offspring was determined by reverse-phase HPLC, following a previously described protocol (HONG et al., 2000), with modifications. Liver homogenate samples (n=5/group) (0.05 ml) were mixed with 12.5 µl of 0.2% BHT and 6.25 µl of 10 N NaOH. About 20 µl of the derivative conjugate were injected in the HPLC (Agilent Technologies 1200 Series) in a Phenomenex reverse-phase C18 analytical column with an LC8-D8 pre-column and MDA was fluorometrically quantified at an excitation of 515 nm and emission of 553 nm. The HPLC pump delivered the isocratic mobile phase: 60% potassium phosphate buffer (PBS) (10 mM, pH 7.4) + 40% methanol at a flow rate of 1 ml/min. A standard curve was prepared using TEP (0.5-15 µmol). Samples were analyzed in triplicate and results are expressed as ηmol MDA/mg protein (NOGUEIRA et al., 2019).

4.9 Antioxidant activity of enzymes in liver and plasma of male mice (fathers) and 7-week-old female offspring

4.9.1 Superoxide Dismutase (SOD) activity

SOD activity was determined according to a previously described procedure (EWING; JANERO, 1995). Liver homogenate and plasma samples (n=5/group) were added to the wells of a microplate containing freshly prepared 0.1 mM EDTA, 62 µM nitrotetrazolium blue chloride (NBT) and 98 µM NADH. The reaction was initiated with the addition of freshly prepared 33µM phenazine methosulphate (PMS) (pH 7.4) containing 0.1 mM EDTA.

Absorbance at 560 nm was monitored over 5 min as an index of NBT reduction. A standard curve was prepared using SOD enzyme (CHASSOT et al., 2018).

4.9.2 Glutathione Peroxidase (GPx) activity

GPx activity was determined according to a previously described procedure (FLOHÉ; GÜNZLER, 1984). In brief, liver homogenate and plasma samples (n=5/group) were incubated at 37°C for 5 min with 0.1 M PBS pH 7.4 with 1 mM EDTA, freshly prepared 0.08 M GSH and freshly prepared GR (9.6 U). Next, 1.2 mM NADPH and 0.46% tert-butylhydroquinone (TBHQ) were added in each well. Absorbance at 340 nm was monitored over 4 min at 37°C. A standard curve was prepared using GPx enzyme (CHASSOT et al., 2018).

4.9.3 Catalase (CAT) activity

CAT activity in the liver and plasma was determined as previously described (BONAVENTURA, SCHROEDER, & FANG, 1972; NABAVI et al., 2012). Liver homogenate and plasma samples (n=5/group) were added to the wells of a UV microplate containing 0.1 M PBS pH 7.4 with 1 mM EDTA. Lastly, 30 mM hydrogen peroxide solution were added in each well. The reading was monitored for 8 min, at 240 nm and 30°C. The calibration curve was prepared with CAT enzyme (CHASSOT et al., 2018).

4.10 Determination of inflammation's gene expression in male mice epididymal adipose tissue (fathers) and in 7-week-old female offspring retroperitoneal adipose tissue

The expression of genes involved in inflammation (F4/80, IL-1β, IL-6, TNF-α, TLR-4, PPARγ, leptin and adiponectin) was evaluated in epididymal adipose tissue of 14-week-old male mice with and in retroperitoneal adipose tissue of 7-week-old female offspring (n=10/group). About 100 mg of adipose tissue was sprayed in liquid nitrogen and homogenized in TRIZOL reagent for total RNA extraction (CHOMZYNSKI; SACCHI, 1987). The RNA was quantified by reading in the Nano Drop 2000 apparatus (Thermo Scientic, Uniscience, São Paulo, Brazil) and the degree of purity determined by the ratio 260/280nm. cDNA was synthesized from 1µg of total RNA extracted using reverse transcriptase.

Gene expression was quantified by real-time PCR (KUBISTA et al., 2006) using Corbett Research's ROTOR GENE 3000 apparatus (Mortlake, NSW, Australia) and SYBER

Green (Invitrogen, Life Technologies) as a fluorescent marker, per primers of interest specified in Table 3. The quantification of gene expression was performed using the comparative Ct method (Ct = threshold cycle; number of cycles at which the PCR product reaches the detection threshold), with 18s expression as control of the epididymal and retroperitoneal adipose tissue.

Table 3. Sequence of primers used to evaluate gene expression in epididymal adipose tissue of male mice (fathers) and retroperitoneal adipose tissue of 7-week-old female offspring.

Gene	**RefSeq**	***Primer* Sequence**
Adiponectin	NM_009605.4	FW 5' – TCTTAATCCTGCCCAGTCATGC – 3' RV 5' – TCCAACATCTCCTGTCTCACCC – 3'
F4/80	NM_010130.4	FW 5' – CCTGAACATGCAACCTGCCAC – 3' RV 5' – GGGCATGAGCAGBCTGTAGGATC – 3'
IL-6	NM_001314054.1	FW 5' – GGTAGCATCCATCATTTCTTTG – 3' RV 5' – CGGAGAGGAGACTTCACAAGAG – 3'
IL-1β	NM_008361.4	FW 5' – GGCAGCTACCTGTGTCTTTCCC – 3' RV 5'– ATATGGGTCCGACAGCACGAG – 3'
PPARγ	NM_001127330.2	FW 5' – GTCTCGGTTGAGGGGACG – 3' RV 5' – GTGTCAACCATGGTAATTTCAGT – 3'
Leptin	NM_008493.3	FW 5' – TCACACACGCAGTCGGTATCC – 3' RV 5' – ATGGAGGAGGTCTCGGAGATT – 3'
TLR-4	NM_021297.3	FW 5' – TTCAGAACTTCAGTGGCTGG – 3' RV 5' – TGTTAGTCCAGAGAAACTTCCTG – 3'
TNF-α	NM_001278601.1	FW 5' – TCTTCTCATTCCTGCTTGTGGC – 3' RV 5' – CACTTGGTGGTTTGCTACGACG – 3'
18s	NM_030720.1	FW 5' – CGCTACACTGACTGGCTCAG – 3' RV 5' – CAGGGACTTAATCAACGCAAG – 3'

F4/80 is a macrophage marker. Abbreviations: IL - interleukin, TNF-α - tumor necrosis factor alpha, TLR - toll like receptor; PPAR - peroxisome proliferator activated receptor; 18S - Ribosomal RNA.

4.11 Reproductive parameters of male mice (fathers)

Testicles were collected and conserved at -20°C until determination of the daily sperm production (n=6/group) according to Thayer et al., (2001). After homogenization of the tissue in STM solution (0.9% NaCl and 0.05% Triton X-100), spermatids were counted in stages 14 to 16 of spermatogenesis in a hemocytometric chamber. For sperm morphology analyses the collected epididymis underwent incision and were immersed in phosphate-buffered saline (PBS). The solution was placed on slides for light microscope examination. Two hundred sperm cells per animal were morphologically analyzed at 40x magnification (n=6/group) (GUIDO et

al., 2016). For testicular histomorphometric analysis the percentage of interstitial and tubular area of the testis was determined by the measurement of the area occupied by seminiferous tubules and interstitium in 15 images per animal randomly selected (DE SOUZA PREDES; DIAMANTE; DOLDER, 2010). The tubular diameter was also analyzed in 30 round tubules per animal randomly selected (DE SOUZA PREDES; DIAMANTE; DOLDER, 2010). These analyzes were performed using the software AxioVision 4.8 associated to the light microscope at 20x magnification (n=6/group).

4.12 Mammary gland harvesting of 7-week-old female offspring

Abdominal mammary glands of female offspring of CO, CJ, OB, OJ (n=6/group) were collected from 7-week-old female offspring and used for preparing mammary whole mounts and miRNA extraction.

4.13 Analysis of mammary gland morphology and development in 7-week-old female offspring

Whole-mount preparations of the fourth abdominal mammary gland from 7-week-old female offspring (n=6/group) were obtained, and the epithelial elongation and number of terminal end buds (TEBs) were determined as described by de Assis et al., (2010).

4.14 Analysis of mammary gland cell proliferation, H3K27me and apoptosis in 7-week-old female offspring

Cell proliferation and H3K27me3 was evaluated in mammary gland from 7-week old female offspring (n=6/group) by immunohistochemistry. Prepared sections of mammary tissue were deparaffinized in xylene and hydrated through graded ethanol. Sections were then immersed in 10 mM citrate buffer pH 6 for 20 min in pressure cooker. Next, 10% H2O2 for 10 min was used for peroxidase blocking and nonspecific binding was blocked for 1h with milk in PBS. Sections were incubated overnight with anti-mouse Ki67 primary antibody (Cell Signaling) at a 1:200 dilution and H3K27me3 primary antibody (Cell Signaling) at a 1:100 dilution. After washes, sections were incubated with the LABS + System – HRP Kit (Dako-Agilent Technologies, USA) and stained with 3,3'-diaminobenzidine in chromogen solution (Dako-Agilent Technologies, USA) for 2 min, washed and counterstained for 1.5 min with hematoxylin. Cell proliferation was quantified by assessing the number of Ki67 positive cells among 1,000 cells per structure (ducts or lobules) (GUIDO et al., 2016). H3K27me3 was

evaluated by the intensity of the histone and quantified by the amount of specific stain present in a virtual slide by evaluating an average intensity of all pixels for subsequent calculation of optical density according to Pogribny et al., (2011). Apoptosis analysis was performed according to Elmore et al., (2016). The results are represented as mean number of apoptotic cells/1,000 cells per structure (ducts or lobules). The slides were evaluated using Image J software (NIH, USA).

4.15 miRNA expression profile in mammary glands of 7-week-old female offspring

miRNA expression profiling (n=7 samples per group) was analyzed by TaqMan miRNA assay (Applied Biosystems). The miRNAs TaqMan assays used were: hsa-miR-199a-3p (assay ID,002304), hsa-miR-15b-5p (assay ID, 000390), and hsa-miR-200c (assay ID, 002300) (Table 4). miRNA expression was normalized to the geomean of the housekeeper snoRNA142 (assay ID, 001231), the expression of which did not change between the experimental groups. These miRNAs were selected after miRNA PCR Array Mouse Breast Cancer was performed in the mammary gland of three female offspring from each group.

Table 4. List of qPCR primers used in miRNA analysis.

TaqMan assay	Sequence
hsa-miR-199a-3p	ACAGUAGUCUGCACAUUGGUUA
hsa-miR-15b-5p	UAGCAGCACAUCAUGGUUUACA
hsa-miR-200c	UAAUACUGCCGGGUAAUGAUGGA
snoRNA142	GTCAGTGCCACGTGTCTGGGCCACTGAGA CCACATGATGGGATTGAGGACCTGAGGAA

4.16 Mammary tumor induction

Mammary tumors were induced in 7-week old female offspring (n=24/group) by administration of medroxyprogesterone acetate (MPA) (15mg/100µL) at 6 weeks of age, followed by four weekly doses of 7,12-dimethylbenz[a]anthracene (DMBA) (1mg/100µL; Sigma, USA) dissolved in corn oil by oral gavage (DE ASSIS et al., 2011; FONTELLES et al., 2018b). Mice were examined for mammary tumors by palpation twice per week, starting three weeks after the last dose of DMBA and continue for a total of 18 weeks. Tumor growth was measured using a caliper and the length (a), height (b) and width (c) of each tumor were recorded. The formula "(1/6*3.14)*(a*b*c)" was used to calculate the tumor volume, as

described by Spang-Thomsen et al., (1989). The end-points for data analysis were (i) latency to tumor appearance, (ii) the number of animals with tumors (tumor incidence), and (iii) the number of tumors per animal (tumor multiplicity). During follow-up, tumor burden animals were sacrificed after 3 weeks of the first tumor appearance.

4.17 Histopathological analysis of mammary tumors

Histopathological analysis of tumors for the characterization and classification of mammary tumors was performed according to Rudmann et al., (2012), using histological slides stained with hematoxylin and eosin.

4.18 Statistical analysis

The statistical analysis was conducted with STATISTICA 8.0 (USA). The normality of the data was evaluated and then variables were compared using ANOVA followed by Tukey or LSD test. Kaplan-Meier and long-rank test were performed to determine differences in tumor incidence. $P \leq 0.05$ was used as threshold for statistical significance and data were presented as mean ± standard error of the mean.

5. Results

5.1 Orange juice analyses

5.1.1 Antioxidant activity of orange juice

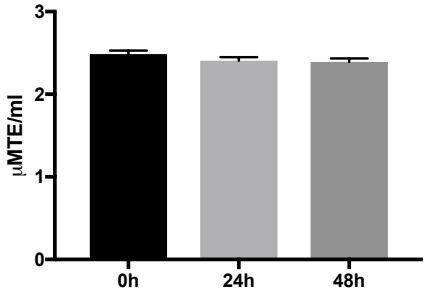
The antioxidant capacity of orange juice evaluated by the DPPH and ORAC method is presented in Table 5. The antioxidant activity estimated by DPPH of the orange juice was not modified by time of exposure (from 0 to 48 hours) at the animal housing, as can be observed in Figure 2.

Table 5. Evaluation of the antioxidant capacity of orange juice measured by DPPH method.

	μmol TE/L
DPPH	2,48 ± 0,10
ORAC	7,53 ± 0,19

Result presented as mean ± standard error of the mean.

Figure 2. Evaluation of the antioxidant capacity of orange juice by the DPPH method during the period that orange juice was exposed in the experiment (0h, 24h, 48h).

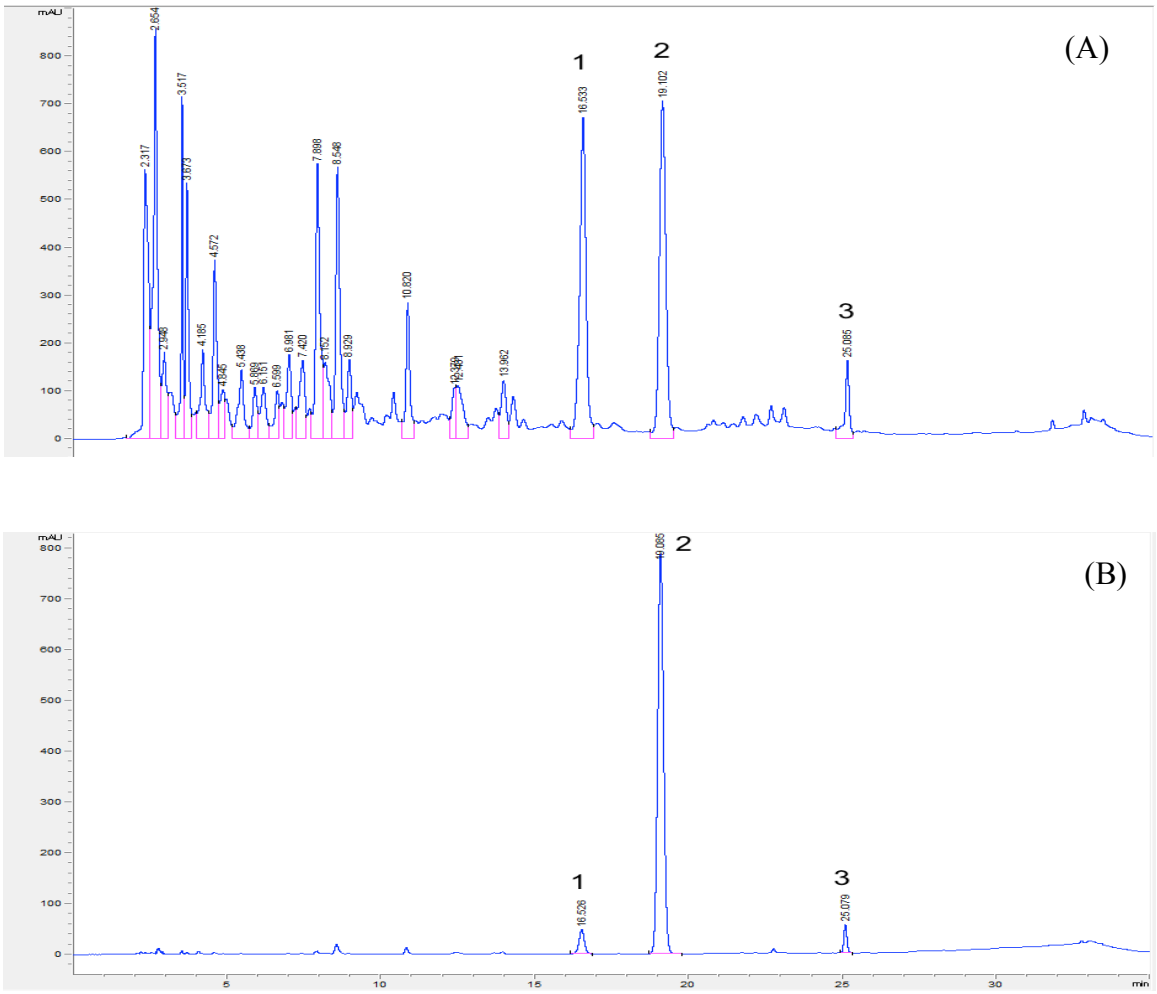


Result presented as mean ± standard error of the mean.

5.1.2. Identification of flavonoids in orange juice

Three major flavonoids (Figure 3 A and B) were identified in the supernatant fraction and in the insoluble (precipitated) fraction. The three identified peaks correspond to narirutin, hesperidin and didimin. Identities were confirmed by co-elution with the respective standard.

Figure 3 A-B. HPLC-DAD chromatogram (280 nm) of supernatant fraction (A) and insoluble (precipitated) fraction (B) of orange juice.



1. Narirutin; 2. Hesperidin; 3. Didimin.

5.1.3 Chemical and flavonoid composition of orange juice

The principal sugars identified in orange juice were glucose, sucrose and fructose corresponding 70,60ug/ml in total sugar of the juice (Table 6). There were two organic acids identified in the juice, citric acid and ascorbic acid (Table 6). The three major flavanones identified in orange juice were hesperidin, narirutin and didimin and their quantification are presented in Table 6.

Table 6. Chemical and flavonoid composition of orange juice.

Compounds	Orange Juice
Soluble Sugars (g/L)	
Glucose	18,60 ± 0,17
Sucrose	20,3 ± 0,33
Fructose	31,7 ± 0,28
Total	70,60 ± 0,78
Organic acids (mg/ml)	
Citric acid	7,16 ± 0,042
Ascorbic acid	0,48 ± 0,005
Total	7,63 ± 0,046
Flavanones (µg/ml)	
Total narirutin	81,42 ± 0,12
Total hesperidin	123,87 ± 0,31
Total didimin	17,97 ± 0,61
Total	223,25 ± 0,76
Total phenolics (mg gallic acid/L)	404,70 ± 2,57

Result presented as mean ± standard error of the mean.

5.2 Fatty acid profile of lipid fractions in control diet, high-fat diet and sweetened condensed milk

The fatty acid profile of the lipid fractions of the control and high-fat diets are presented in Table 7. The control diet has a higher amount of polyunsaturated fatty acids, which are linoleic acid and α-linoleic acid. In relation to the control diet, the high-fat diet had approximately twice the amount of saturated fatty acids, the main ones being palmitic and stearic fatty acids. In addition, a higher amount of monounsaturated fatty acids, mainly oleic acid, were quantified in high-fat diet.

The fatty acid profile of sweetened condensed milk is presented in Table 8. Sweetened condensed milk has approximately 70% of its lipid fraction of saturated fatty acids, 27% of monounsaturated fatty acids and 2% of polyunsaturated fatty acids.

Table 7. Fatty acid profile of control (CO) and high-fat (HF) diets, evaluated by gas chromatography and presented in g/100g of the lipid fraction.

g/100g		CO	HF
Myristic acid	C14	0,00	0,72
Palmitic acid	C16	14,66	23,74
Palmitoleic acid	C16:1	0,00	0,85
Stearic acid	C18	7,23	15,54
Oleic acid	C18:1	24,47	31,87
Linoleic acid	LNA	48,74	25,24
Alpha-linoleic acid	ALA	4,76	2,04
	Monounsaturated	**24,47**	**32,72**
	Polyunsaturated	**53,50**	**27,28**
	Saturated	**21,89**	**40,00**

Table 8. Fatty acid profile of sweetened condensed milk (SCM), evaluated by gas mass chromatography and presented in g/100g of the lipid fraction.

g/100g		SCM
Lauric acid	C12	2,31
Tridecylic acid	C13	0,13
Myristic acid	C14	10,35
Myristoleic acid	C14:1	0,91
Pentadecylic acid	C15	0,55
Palmitic acid	C16	38,56
Palmitoleic acid	C16:1	1,55
Margaric acid	C17	0,39
Stearic acid	C18	17,92
Oleic acid	C18:1	24,94
Linoleic acid	LNA	2,01
Alpha-linoleic acid	ALA	0,21
Arachidic acid	C20	0,21
Behenic acid	C22	0,23
Lignoceric acid	C24	0,15
	Monounsaturated	**27,00**
	Polyunsaturated	**2,20**
	Saturated	**70,80**

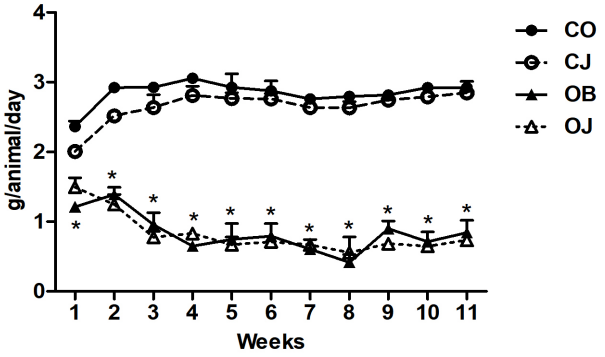
5.3 Fathers' dietary and health parameters

5.3.1 Dietary consumption of male mice (fathers)

5.3.1.1 Food intake of male mice (fathers)

The paternal food intake is shown in Figure 4. As expected, the average intake of the high-fat diet by the group OB was 69% lower ($p \leq 0.05$) than the control diet by the CO group (0.88g/animal/day vs 2.84g/animal/day). No differences were observed in mean chow intake between CJ and CO (2.65g/animal/day vs 2.84g/animal/day, respectively). There were also no differences between intake of the high-fat diet amongst OB and OJ (0.88g/animal/day vs 0.82g/animal/day, respectively) group. Therefore, macronutrient content of orange juice apparently did not affect food consumption between groups.

Figure 4. Estimated daily intake of control and high-fat diets of male mice (fathers).

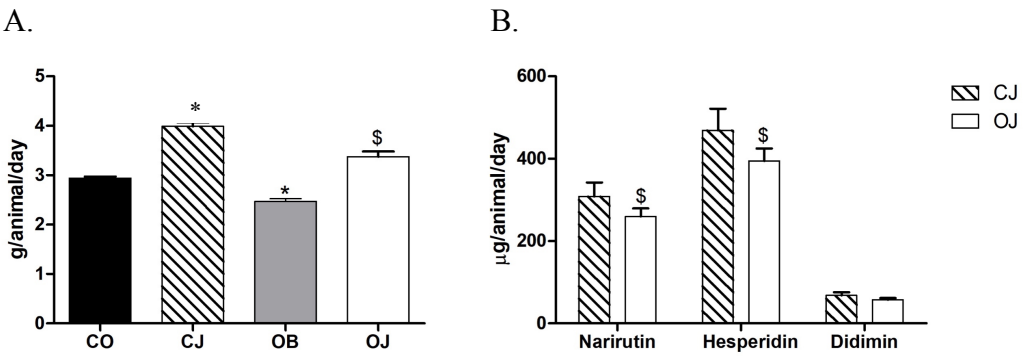


Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean.

5.3.1.2 Liquid and flavonoid consumption of male mice (fathers)

In relation to the CO group, estimated daily water consumption (Figure 5A) of OB group was lower ($p \leq 0.05$) during the experiment. In relation to CJ group, OJ group presented lower intake of orange juice ($p \leq 0.05$, Figure 5A). Figure 5B shows the estimated daily consumption of flavonoids present in orange juice from CJ and OJ groups. The OJ group consumed less ($p \leq 0.05$) narirutin and hesperidin when compared to the CJ group.

Figure 5 A-B. Estimated daily intake of liquids and flavonoids by male mice (fathers).

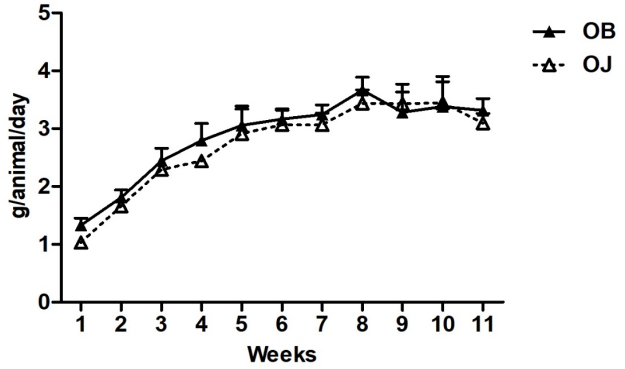


Statistically significant difference ($p \leq 0.05$) compared to *CO or $CJ according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean.

5.3.1.3 Consumption of sweetened condensed milk by male mice (fathers)

No differences were observed of sweetened condensed milk consumption by OB and OJ group (2.86g/animal/day vs 2.71g/animal/day, respectively, Figure 6).

Figure 6. Estimated daily consumption of sweetened condensed milk of male mice (fathers)



Values are expressed as mean ± standard error of the mean.

5.3.1.4 Vitamin and mineral consumption by male mice (fathers)

Estimated average daily intake of minerals and vitamins are presented in Table 9 and 10, respectively. No differences were observed between mineral consumption of CJ and CO group. However, intake of calcium, phosphorus, potassium, sodium, magnesium and zinc were higher ($p \leq 0.05$) in the OB when compared to the CO group.

Table 9. Estimated intake of mineral content from diets, sweetened condensed milk, added to the sweetened condensed milk and orange juice. Data are presented as average of daily consumption per animal/per group/per day during the 11 weeks of treatment.

	CO	**CJ**	**OB**	**OJ**
	mg	mg	mg	mg
Calcium	14.16	13.26	21.85*	21.10
Phosphorus	4.42	4.14	9.97*	9.58
Potassium	10.19	9.53	20.07*	19.30
Sodium	2.88	2.70	5.71	5.49
Chlorine	4.45	4.16	4.65	4.53
Sulfate	204.03	190.92	213.38	207.77
Magnesium	1.44	1.34	2.13*	2.06
Iron	0.10	0.09	0.11	0.10
Zinc	0.09	0.08	0.11*	0.11
Manganese	0.04	0.03	0.04	0.04
Copper	0.02	0.02	0.02	0.02
Iodine	0.00059	0.00055	0.00061	0,0006
Selenium	0.00055	0.00052	0.00058	0.00056
Molybdenum	0.00043	0.0004	0.00045	0.00044
Silicon	0.014	0.013	0.015	0.014
Chrome	0.0028	0.0027	0.0030	0.0029
Boron	0.0014	0.0013	0.0015	0.0014
Fluorine	0.0029	0.0027	0.0030	0.0029
Nickel	0.0014	0.0013	0.0015	0.0014
Lithium	0.0003	0.0003	0.0003	0.0003
Vanadium	0.0003	0.0003	0.0003	0.0003

Estimated amount was obtained from: mineral mix (AIN-93G) (REEVES et al., 1993); Sweetened condensed milk (TACO, 2006); Orange Juice (Label). Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test.

Regarding vitamins consumption, vitamin C intake was higher in CJ group than in CO group ($p \leq 0.05$). Animals from OB group presented higher ($p \leq 0.05$) consumption of pyridoxine and riboflavin than animals from CO group. Vitamin C consumption in OJ group was higher ($p \leq 0.05$) compared to animals in OB group.

Table 10. Estimated intake of vitamins from diets, sweetened condensed milk, added to the sweetened condensed milk and orange juice. Data are presented as estimated average intake per animal, per group, per day during the 11 weeks of treatment.

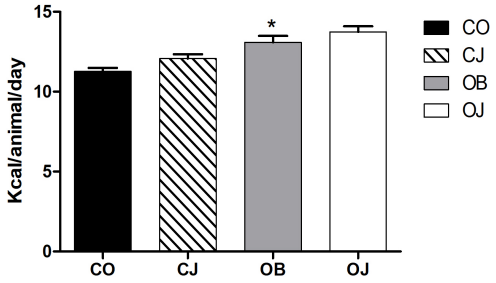
	CO	**CJ**	**OB**	**OJ**
	mg	mg	mg	mg
Nicotinic acid	0.30	0.28	0.31	0.30
Calcium pantoteate	0.16	0.15	0.17	0,.6
Pyridoxine	0.07	0.06	0.09*	0.09
Thiamine	0.06	0.06	0.06	0.06
Riboflavin	0.06	0.06	0.07*	0.07
Folic acid	0.02	0.02	0.02	0.02
D-Biotin	0.002	0.002	0.002	0.002
Vitamin B12	0.25	0.23	0.26	0.25
Vitamin E	1.49	1.39	1.55	1.51
Vitamin A	0.08	0.07	0.08	0.08
Vitamin D3	0.02	0.02	0.03	0.03
Vitamin K	0.007	0.007	0.008	0.008
Vitamin C	0	1.19*	0.06*	0.91$^{\#}$

Estimated amount was obtained from: vitamin and mineral mix (AIN-93G) (REEVES et al., 1993); Sweetened ondensed milk (TACO, 2006); Orange Juice (Label). Statistically significant difference ($p \leq 0.05$) compared to *CO or $^{\#}$OB according to ANOVA followed by Tukey test.

5.3.1.5 Energetic intake by male mice (fathers)

The daily energetic intake of the CJ group was not different from CO group (12.12 kcal/animal/day vs 11.28 kcal/animal/day, respectively; Figure 7). OB group presented higher daily energy intake when compared to animals of CO group ($p \leq 0.05$; 13.42 kcal/ animal/day vs 11.28 kcal/animal/day). No differences were observed between energy intake of OB and OJ group (13.42 kcal/ animal/day vs 14.06 kcal/animal/day).

Figure 7. Estimated daily energetic intake of male mice (fathers).

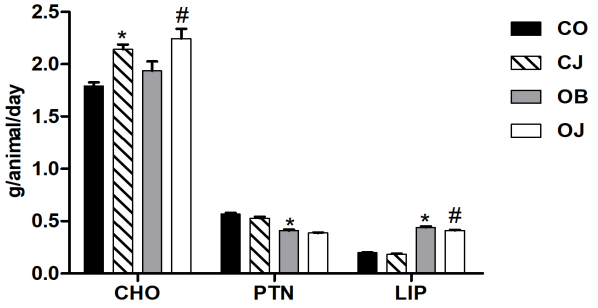


Statistically significant difference (p≤0.05) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean.

5.3.1.6 Macronutrient consumption of male mice (fathers)

CJ group presented higher carbohydrate consumption than CO group animals (p≤0.05; 2.14 vs 1.79 g/animal/day, respectively, Figure 8). The OJ group presented higher carbohydrate consumption than the OB group (p≤0.05; 2.25 vs 1.94 g/animal/day, respectively). Protein consumption of animals from OB group was lower than CO group (p≤0.05; 0.41 g/animal/day vs 0.57 g/animal/day respectively). Lipid consumption of animals from group OB was higher (p≤0.05) than animals from groups CO (0.44 g/animal/day vs 0.20 g/animal/day, respectively). OJ group presented lower lipid consumption when compared to the OB group (p≤0.05; 0.41 g/animal/day vs 0.44 g/animal/day, respectively).

Figure 8. Estimated daily macronutrient consumption of male mice (fathers).



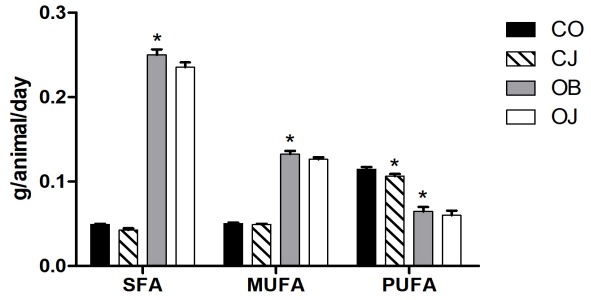
CHO: Carbohydrate, PTN: Protein, LIP: Lipid. Statistically significant difference (p≤0.05) compared to *CO or #OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 12.

5.3.1.7 Profile of fatty acid consumption of male mice (fathers)

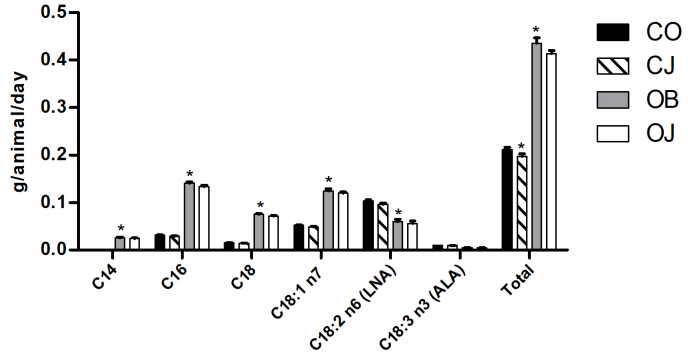
Compared to CO group, male mice that were on high-fat and high-sugar diet (OB group) consumed more saturated fatty acids (SFA) [p≤0.05; predominantly myristic (C14:0), palmitic (C16:0) and stearic (C18:0) acids], monounsaturated fatty acids (MUFA) [predominantly oleic acid (C18:1n9c)] and less polyunsaturated fatty acids (PUFA) [predominantly linoleic acid (C18:2n6c)] (Figure 9A-B).

Figure 9 A-B. Estimated daily intake of fatty acids from male mice (fathers).

A.



B.



Statistically significant difference (p≤0.05) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=12.

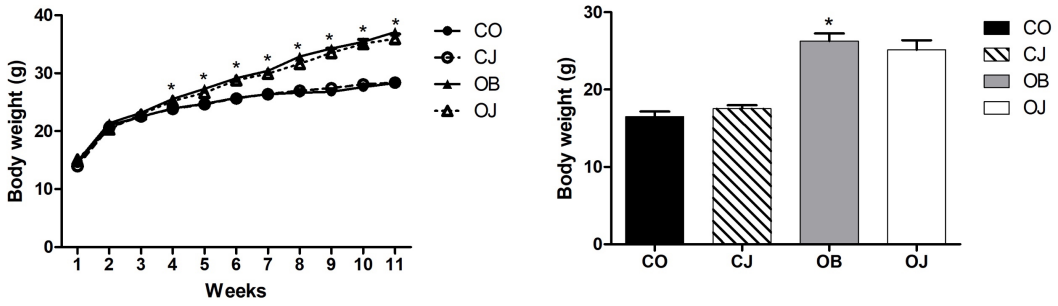
5.3.2 Weight gain and body composition of male mice (fathers)

Weight gain is presented in (Figure 10A-B). No differences in body weight gain were observed between CJ and CO group (17.54 ± 0.44g vs 16.43 ± 0.64g, respectively). Compared to CO group, OB male mice presented higher body weight gain (p≤0.05; 26.23 ± 0.99g vs 16.43 ± 0.64g, respectively). From the 4th week onward of treatment (Figure 10A) the OB group

showed higher weight compared to the CO group. No differences in body weight was observed between OJ and OB group (25.07 ± 1.23g vs 26.23 ± 0.99g, respectively).

Figure 10 A-B. Body weight gain of male mice (fathers) from groups CO, CJ, OB, OJ after 11 weeks treatment.

A. B.



Statistically significant difference (p≤0.05) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=12.

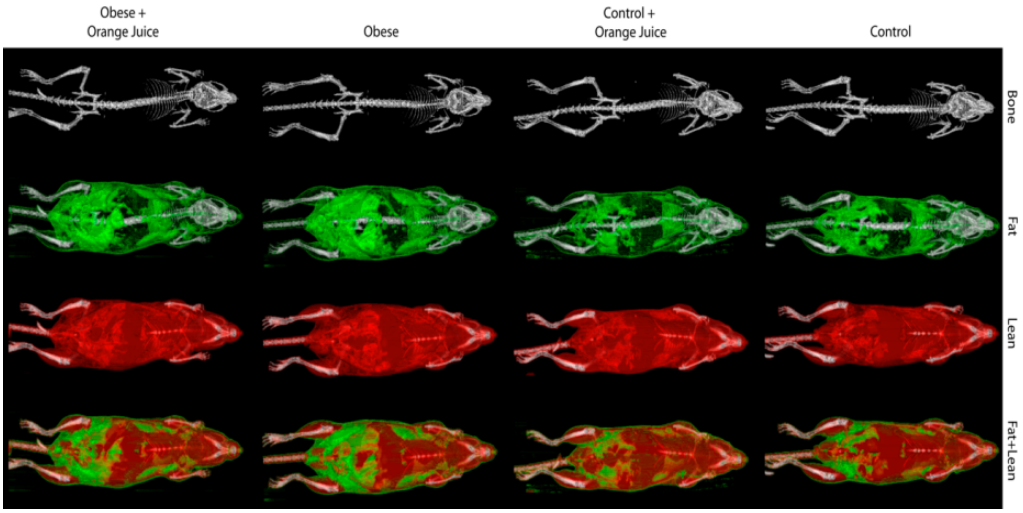
Computed tomography results are presented in Figure 11 and Table 11. The percentage of fat mass in animals treated with high-fat and high-sugar diet, OB group, was higher when compared to CO animals (p≤0.05; 17.11% vs 10.19%, respectively). No differences were observed between the percentage of fat mass from OB and OJ group (17.11% vs 14.31%, respectively). Lean mass percentage was not different between groups. Bone mass percentage was lower in the CJ, OB and OJ groups when compared with CO group (p≤0.05; 5.18%; 4.30%; 4,40% vs 5.57%, respectively).

Table 11. Fat mass percentage, lean and bone mass, estimated by computed tomography of the male mice (fathers) from CO, CJ, OB, OJ groups, after 11 weeks of treatment.

	CO	**CJ**	**OB**	**OJ**
Fat (%)	10,19±0,67	9,74±0,40	17,11±0,54*	14,31±2,40
Lean mass (%)	81,50±0,64	82,46±0,44	76,43±0,93	79,02±3,82
Bone mass (%)	5,57±0,02	5,18±0,18*	4,30±0,13*	4,40±0,18

Statistically significant difference (p≤0.05) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=3.

Figure 11. Body composition images of X-ray computed tomography of the male mice (fathers) from CO, CJ, OB, OJ groups, after 11 weeks of treatment.

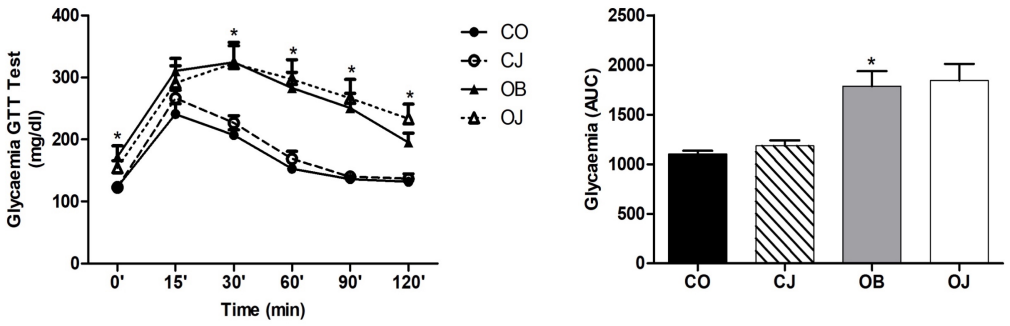


5.3.3 Intraperitoneal glucose tolerance test (IGTT) of male mice (fathers)

No differences were observed between IGTT test of male mice from CJ and CO groups (Figure 12 A-B). Fasting blood glucose of animals from group OB was higher than CO group ($p \leq 0.05$; 172.00mg/dl vs 123.62mg/dl, respectively). From 30 minutes forward to glucose administration, OB group presented higher ($p \leq 0.05$) glucose concentration at all points of the glycemic curve when compared to the CO group.

Figure 12 A-B. Serum glucose concentration of male mice (fathers) (A) and incremental serum glucose area (B) of groups CO, CJ, OB, OJ after 11 weeks treatment.

A. B.



Statistically significant difference ($p \leq 0.05$) compared to *CO or #OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=8.

5.3.4 Relative organ weight of male mice (fathers)

Relative weight of 14-week-old male mice (fathers) organs are presented in Table 12. CJ group presented higher ($p \leq 0.05$) kidney weight compared to CO group. The relative weight of the following organs, kidney, EAT, RAT, ISAT in OB group were higher ($p \leq 0.05$) than CO male mice (Table 12). The relative heart weight of animals from group OB group was lower ($p \leq 0.05$) than CO group.

Table 12. Relative organ weight of male mice (fathers) from groups CO, CJ, OB, OJ after 11 weeks treatment.

	CO	**CJ**	**OB**	**OJ**
Kidney (g/100g b.w.)	0,60 ± 0,03	0,67 ± 0,04*	0,48 ± 0,04*	0,50 ± 0,02
Liver (g/100g b.w.)	4,18 ± 0,08	4,09 ± 0,18	4,42 ± 0,11	4,27 ± 0,15
Heart (g/100g b.w.)	0,53 ± 0,02	0,54 ± 0,04	0,44 ± 0,01*	0,46 ± 0,02
EAT (g/100g b.w.)	2,34 ± 0,17	2,22 ± 0,13	4,15 ± 0,18*	4,23 ± 0,27
RAT (g/100g b.w.)	1,13 ± 0,12	1,06 ± 0,09	2,53 ± 0,17*	2,48 ± 0,17
ISAT (g/100g b.w.)	1,87 ± 0,14	1,57 ± 0,10	3,78 ± 0,19*	3,55 ± 0,17

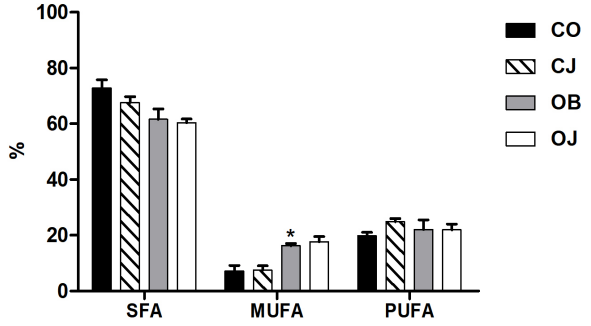
EAT (Epididymal Adipose Tissue), RAT (Retroperitoneal Adipose Tissue), ISAT (Inguinal Subcutaneous Tissue). Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 12.

5.3.5 Plasma, liver and epididymal adipose tissue fatty acid profile of male mice (fathers)

5.3.5.1 Plasma fatty acid profile of male mice (fathers)

No differences in plasma fatty acid profile were observed between CJ and CO groups (Figure 13). The OB group had higher plasma percentages of monounsaturated fatty acids when compared to the CO group ($p \leq 0.05$; 16.33% vs 7.22%, respectively). There were no differences in plasma fatty acid profile between OJ and OB group ($p \geq 0.05$).

Figure 13. Plasma fatty acid profile of male mice (fathers).

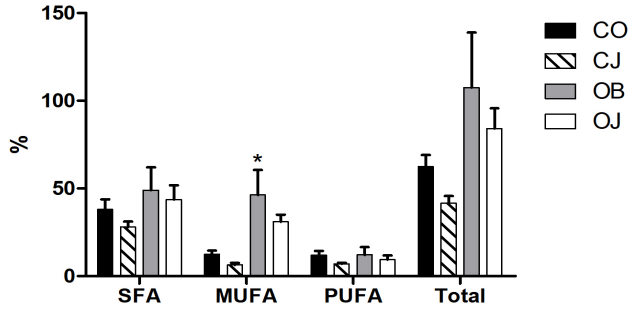


SFA: saturated, MUFA: monounsaturated, PUFA: polyunsaturated. Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 3.

5.3.5.2 Fatty acid profile in liver of male mice (fathers).

No difference was observed between CJ and CO group in liver fatty acid profile of 14-week-old male mice (fathers) (Figure 14). The OB group had higher liver monounsaturated fatty acid percentage when compared to the CO group ($p \leq 0.05$). There was also no difference between liver fatty acid profile of OJ and OB group.

Figure 14. Liver fatty acid profile of male mice (fathers).

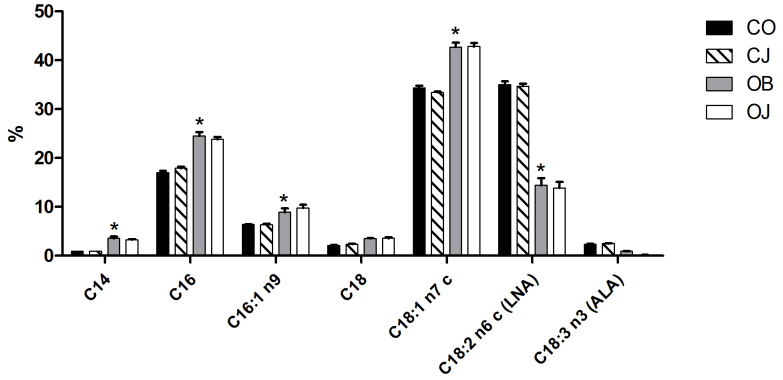


SFA: saturated, MUFA: monounsaturated, PUFA: polyunsaturated. Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 5.

5.3.5.3 Fatty acid profile in epididymal adipose tissue of male mice (fathers)

Compared to CO, 14-week-old OB male mice (fathers) had more ($p \leq 0.05$) saturated fatty acids (SFA) [predominantly myristic (C14:0) and palmitic (C16:0) acids], monounsaturated fatty acids (MUFA) [predominantly oleic acid (C18:1n7c)] and less polyunsaturated fatty acids (PUFA) [predominantly linoleic acid (C18:2n6c)] in epididymal adipose tissue (Figure 15).

Figure 15. Epididymal adipose tissue fatty acid profile of male mice (fathers).

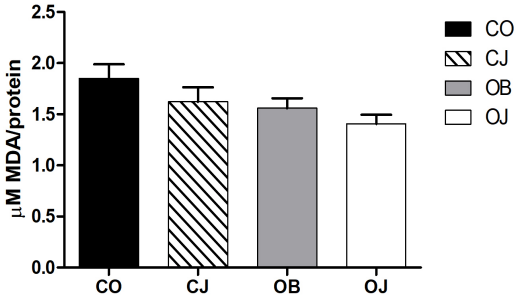


Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 5.

5.3.6 MDA concentration in liver homogenate of male mice (fathers)

There was no difference in liver MDA concentration between the studied groups (Figure 16).

Figure 16. Liver MDA concentration of male mice (fathers).

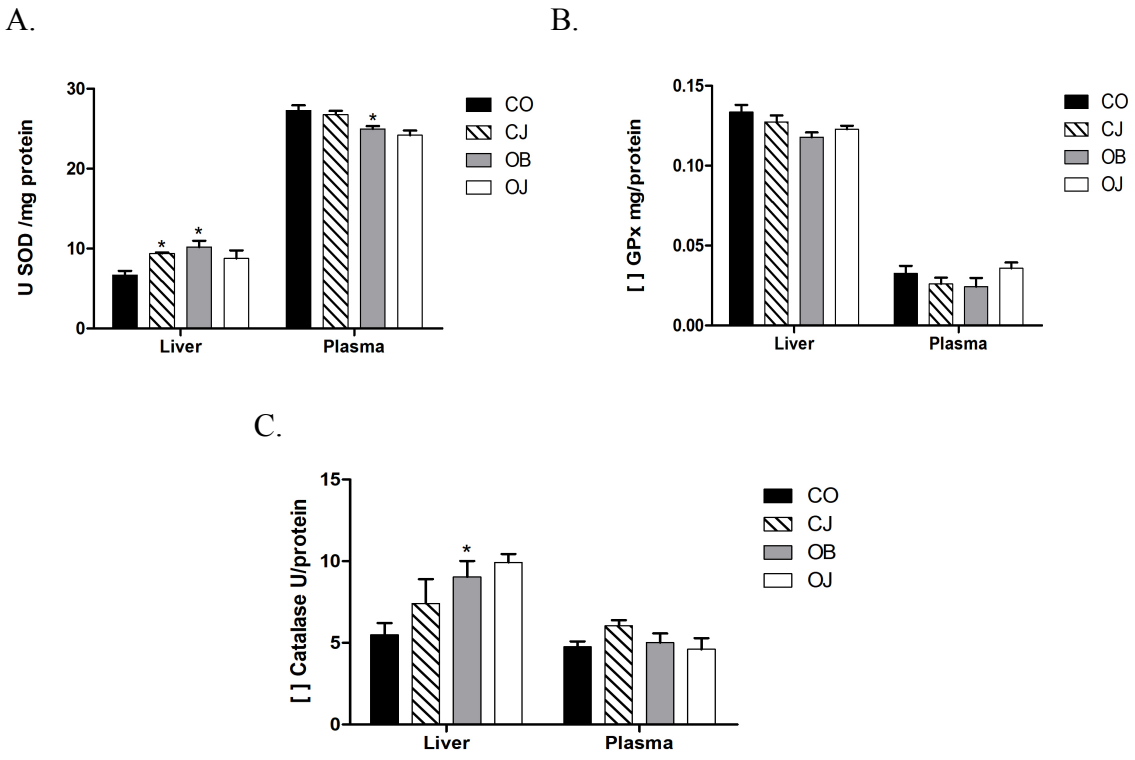


Values are expressed as mean ± standard error of the mean. n = 5.

5.3.7 Antioxidant activity of enzymes in the liver and plasma of male mice (fathers)

Compared to control, CJ fathers had higher SOD activity in liver ($p \leq 0.05$; Figure 17A), possibly indicating an influence of orange juice intake in the absence of the metabolic stress induced by obesity. Even though SOD activity was affected by orange juice intake in control groups, neither increased activities of catalase or GPx were observed in these groups. Compared to control, obese fathers presented higher SOD activity in the liver and lower SOD activity in the plasma ($p \leq 0.05$; Figure 17A) and higher catalase activity in the liver but not in plasma ($p \leq 0.05$; Figure 17C). No differences were observed in liver and plasma of GPx activity between studied groups (Figure 19B). There were also no differences in plasma CAT activity between groups (Figure 17C).

Figure 17 A-C. Activity of superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and catalase (CAT) (C) in liver and plasma of male mice (fathers)

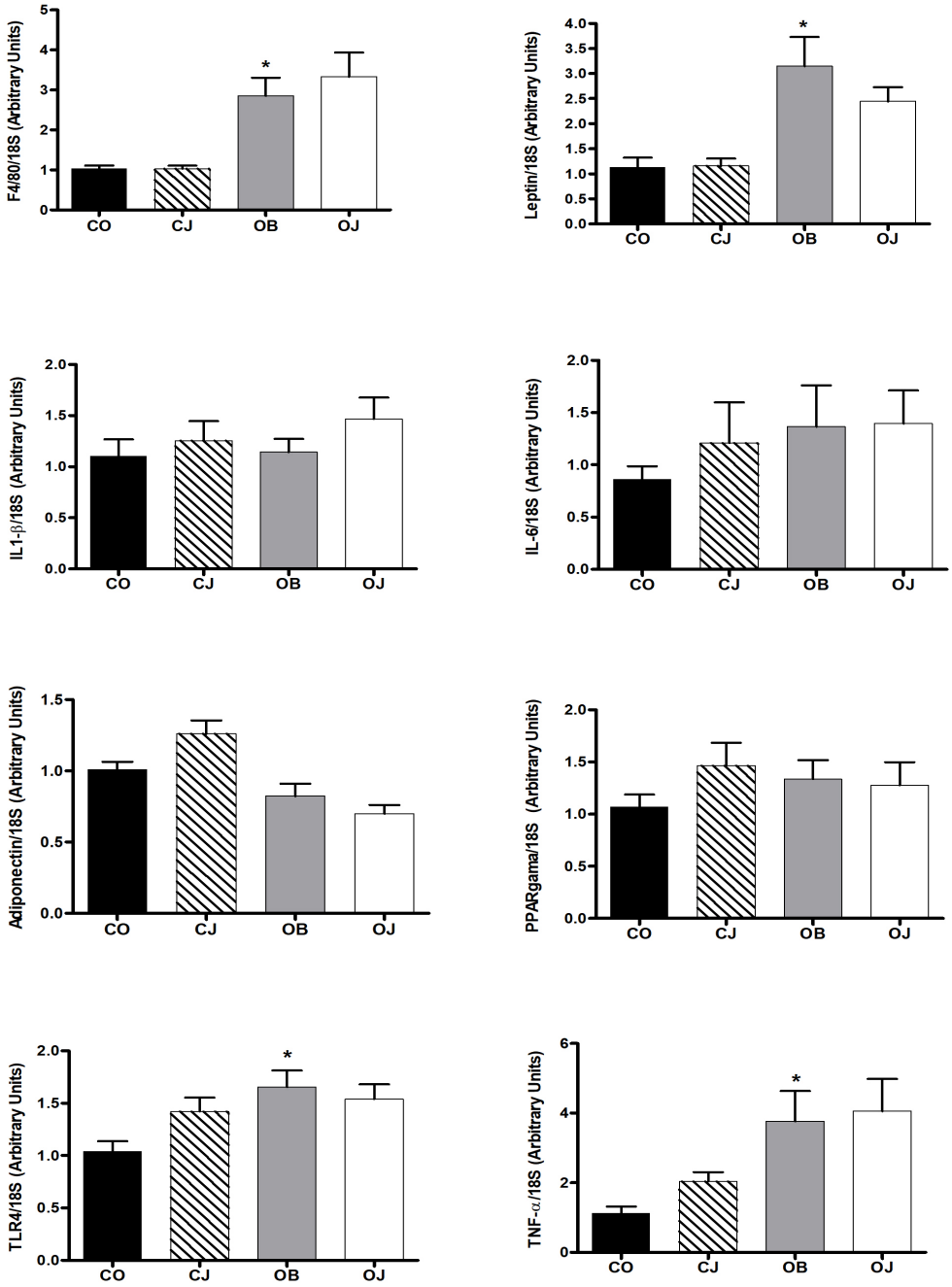


Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=5.

5.3.8 Expression of inflammatory genes in epididymal adipose tissue of male mice (fathers)

Expression of inflammation-related genes (Figure 18) in epididymal adipose tissue of 14-week-old male (fathers) mice are presented below.

Figure 18. Expression of inflammation-related genes evaluated in epididymal adipose tissue of 14-week-old male mice (fathers).



Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=10.

There were no differences (p≥0.05) between the CO and CJ groups regarding the expression of these genes. The expression of F4/80, leptin, TLR4 and TNF-α was higher (p≤0.05) in the OB group when compared to the CO group. No differences (p≥0.05) were observed between the OB and OJ groups regarding these parameters.

5.3.9 Reproductive parameters of male mice (fathers)

Reproductive parameters of 14-week-old male mice (fathers) is presented in Table 13.

Table 13. Paternal reproductive parameters

	CO	**CJ**	**OB**	**OJ**
Testicle (g/100g b.w.)	0,28 ± 0,01	0,28 ± 0,01	0,20 ± 0,00*	0,20 ± 0,01
Epididymis (g/100g b.w.)	0,11 ± 0,00	0,12 ± 0,01	0,08 ± 0,00*	0,08 ±0 ,00
Sperm morphology (% normal)	80,58 ± 1,42	81,50 ± 1,60	60,25 ± 2,01*	64,25 ± 2,65
Daily sperm production (sperm n/ testicle/day)	$3,63x10^6$ ± 0,05	$3,53\ x10^6$ ± 0,07	$2,75x10^6$ ± 0,11*	$3,54\ x10^6$ ± 0,04
Testicular tubular area (μm^2)	81,13 ± 0,01	84,37 ± 0,01*	84,36 ± 0,01*	82,96 ± 0,01
Testicular interstitial area (μm^2)	18,87 ± 0,01	16,72 ± 0,01	15,64 ± 0,01*	17,04 ± 0,01
Testicular tubular diameter (μm^2)	227,00 ± 5,15	221,13 ± 4,47	218,89 ± 6,82	217,10 ± 5,91

Statistically significant difference (p≤0.05) compared to *CO according to ANOVA followed by Tukey or LSD test. Values are expressed as mean ± standard error of the mean. n=6.

Compared to CO group, relative testicle and epididymis weight was lower in OB and OJ groups (p≤0.05; Table 13). The OB group had lower daily sperm production than CO (p≤0.05; $2.75x10^6$ sperm n/testicle/day vs $3.63x10^6$ sperm n/testicle/day, respectively). There were no differences in daily sperm production between OB and OJ groups nor between CJ and CO groups (Table 13). There were fewer normal sperm percentages of animals from OB (60.25 ± 2,01%) and OJ (64.25 ± 2,65%) groups when compared to normal sperm percentage of CO group (80.58 ± 1,42%). There were no differences in sperm morphology of CJ group when

compared to CO. Compared to CO group, OB presented higher tubular area of testicle (p≤0.05) and lower (p≤0.05) interstitial tubular area. In relation to control group CJ presented higher tubular area of the testicle (p≤0.05). No differences were observed in measurements of tubular diameter of testicle between studied groups (Table 13).

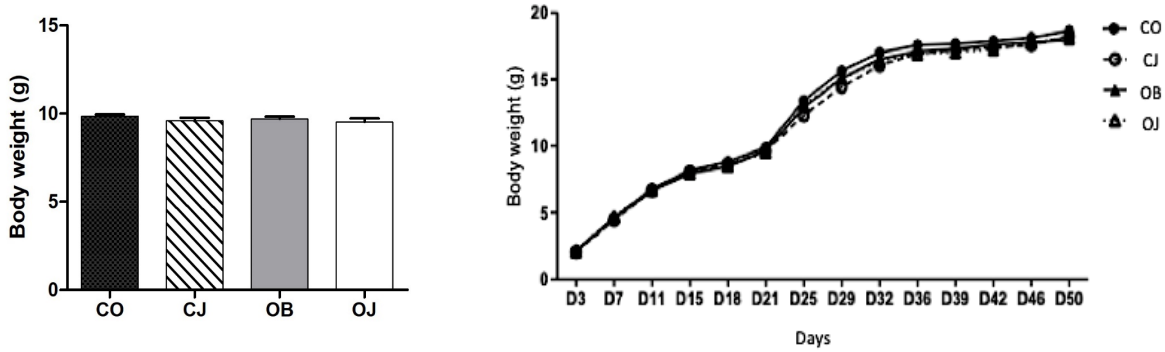
5.4 Female offspring health and metabolic parameters

5.4.1 Weight at weaning and weight gain of 7-week-old female offspring

No differences (p≥0,05) were observed in body weight at weaning (Figure 19A) nor in weight gain (Figure 19B) of 7-week-old female offspring.

Figure 19 A-B. Weight at weaning (A) and weight gain (B) during the first 7-weeks of life of female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

A. B.



Values are expressed as mean ± standard error of the mean. n = 50.

5.4.2 Body composition of 7-week-old female offspring

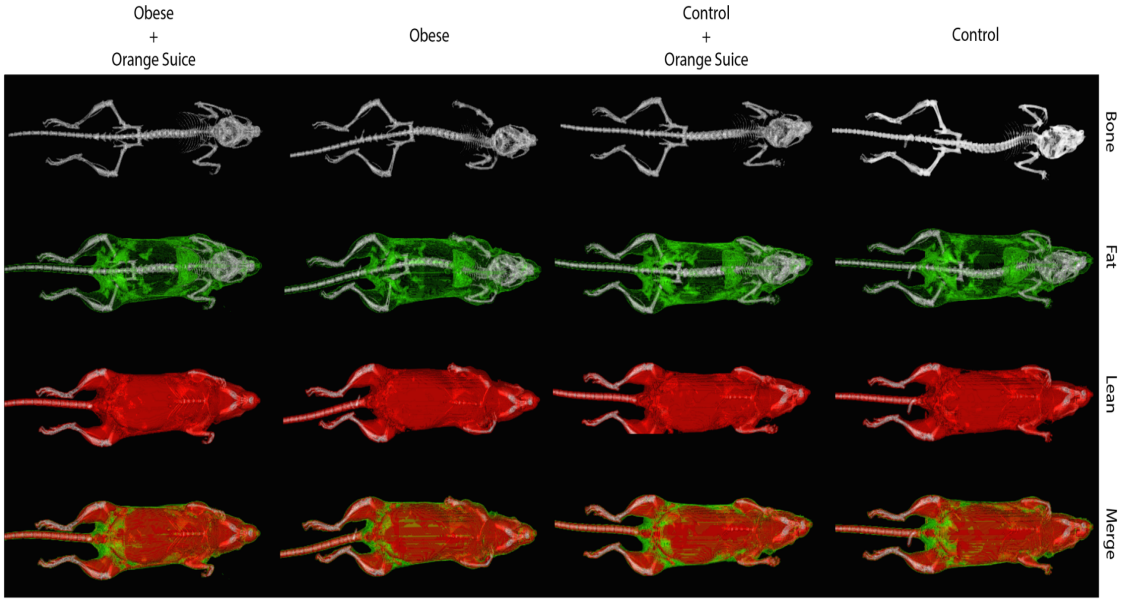
There were no differences between studied groups regarding body composition compartments (fat, lean and bone mass) obtained by computed tomography of 7-week-old female offspring (Table 14 and Figure 20).

Table 14. Fat mass percentage, lean and bone mass of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

	CO	CJ	OB	OJ
Fat (%)	10,45±0,36	10,95±0,47	10,60±1,00	12,71±1,29
Lean mass (%)	79,58±0,24	79,18±0,47	79,80±1,00	77,76±1,21
Bone mass (%)	6,53±0,23	6,60±0,10	6,37±0,08	6,47±0,15

Values are expressed as mean ± standard error of the mean. n = 4.

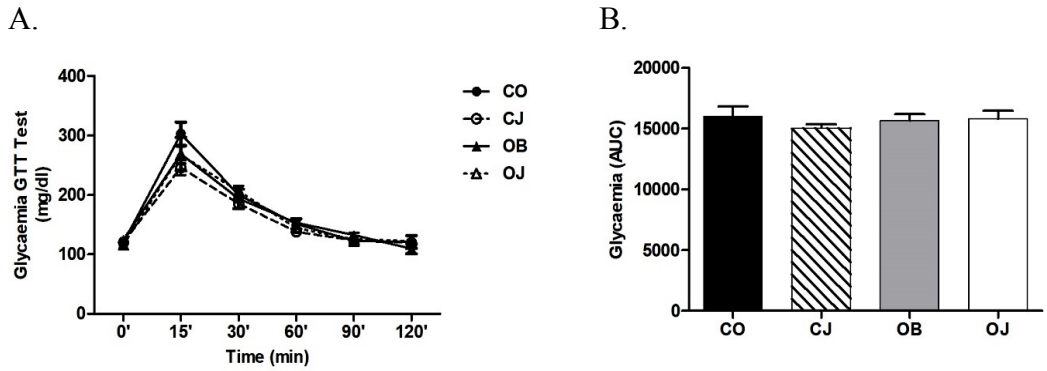
Figure 20. Body composition images of X-ray computed tomography of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).



5.4.3 Intraperitoneal glucose tolerance test in 7-week-old female offspring

No differences ($p \geq 0.05$) were observed between serum glucose values (mg/dL) at the different time points measured nor in the area under the curve of the glucose challenge test (Figure 21 A-B).

Figure 21 A-B. Serum glucose concentration of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) (A) and area under the curve (AUC) of serum glucose (B).



Values are expressed as mean ± standard error of the mean. n = 4.

5.4.4 Relative weight of organs of 7-week-old female offspring

The relative liver weight of offspring from OJ fathers was lower than offspring from OB fathers ($p \leq 0.05$). There were no other organs and tissue weight differences between groups (Table 15).

Table 15. Relative organ weight of 7 weeks old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father), and OJ (obese father and orange juice).

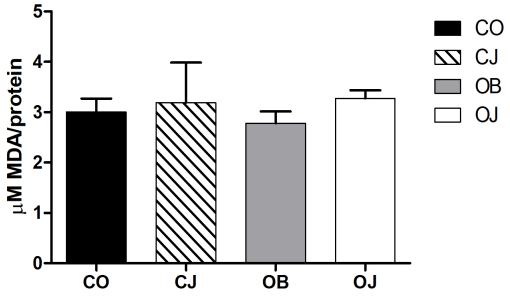
	CO	**CJ**	**OB**	**OJ**
Ovary (g/100g b.w.)	0,23 ± 0,02	0,23 ± 0,01	0,23 ± 0,03	0,25 ± 0,02
Kidney (g/100g b.w.)	0,61 ± 0,02	0,56 ± 0,01	0,59 ± 0,02	0,58 ± 0,01
Liver (g/100g b.w.)	4,43 ± 0,10	4,33 ± 0,09	4,49 ± 0,12	4,09 ± 0,08$^{\#}$
Heart (g/100g b.w.)	0,55 ± 0,01	0,62 ± 0,02	0,58 ± 0,01	0,60 ± 0,02
RAT (g/100g b.w.)	1,68 ± 0,11	1,77 ± 0,14	1,65 ± 0,14	1,83 ± 0,12

RAT (Retroperitoneal Adipose Tissue). Statistically significant difference ($p \leq 0.05$) compared with $^{\#}$OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 20.

5.4.5 MDA concentration in liver homogenate of 7-week-old female offspring

There was no difference in MDA liver concentration between groups of female offspring (Figure 22).

Figure 22. Liver MDA concentration of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

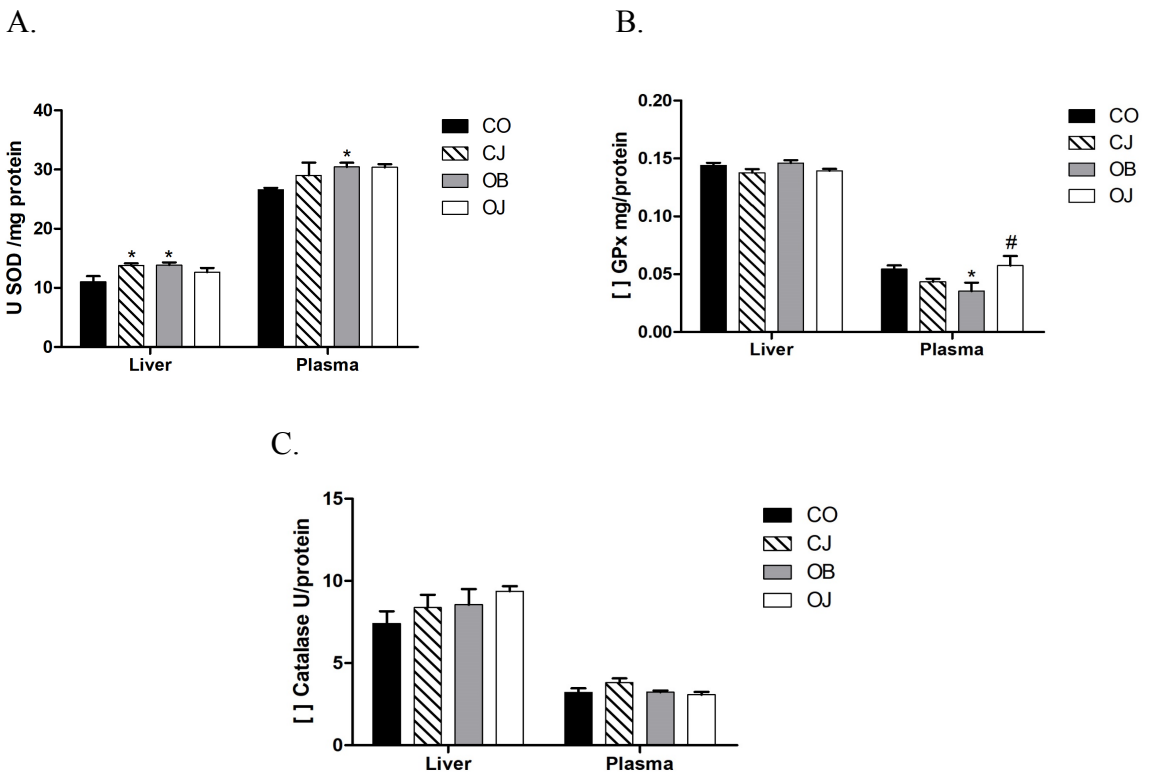


Values are expressed as mean ± standard error of the mean.

5.4.6 Activity of antioxidant enzymes in liver tissue and plasma of 7-week-old female offspring

The offspring from OB fathers presented higher ($p \leq 0.05$) SOD activity in liver and plasma (Figure 23A) and lower ($p \leq 0.05$) GPx activity in plasma (Figure 23B), but not in liver tissue compared to female offspring from CO fathers. The offspring of OJ fathers presented higher ($p \leq 0.05$) GPx plasma activity when compared to the offspring of OB fathers (Figure 25B). There were no differences ($p \geq 0.05$) between the offspring of the CO, CJ, OB and OJ groups regarding GPx activity in liver tissue (Figure 23B) and CAT in liver tissue and plasma (Figure 23C).

Figure 23. Activity of superoxide dismutase (SOD) (A), glutathione peroxidase (GPx) (B) and catalase (CAT) (C), in liver and plasma of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

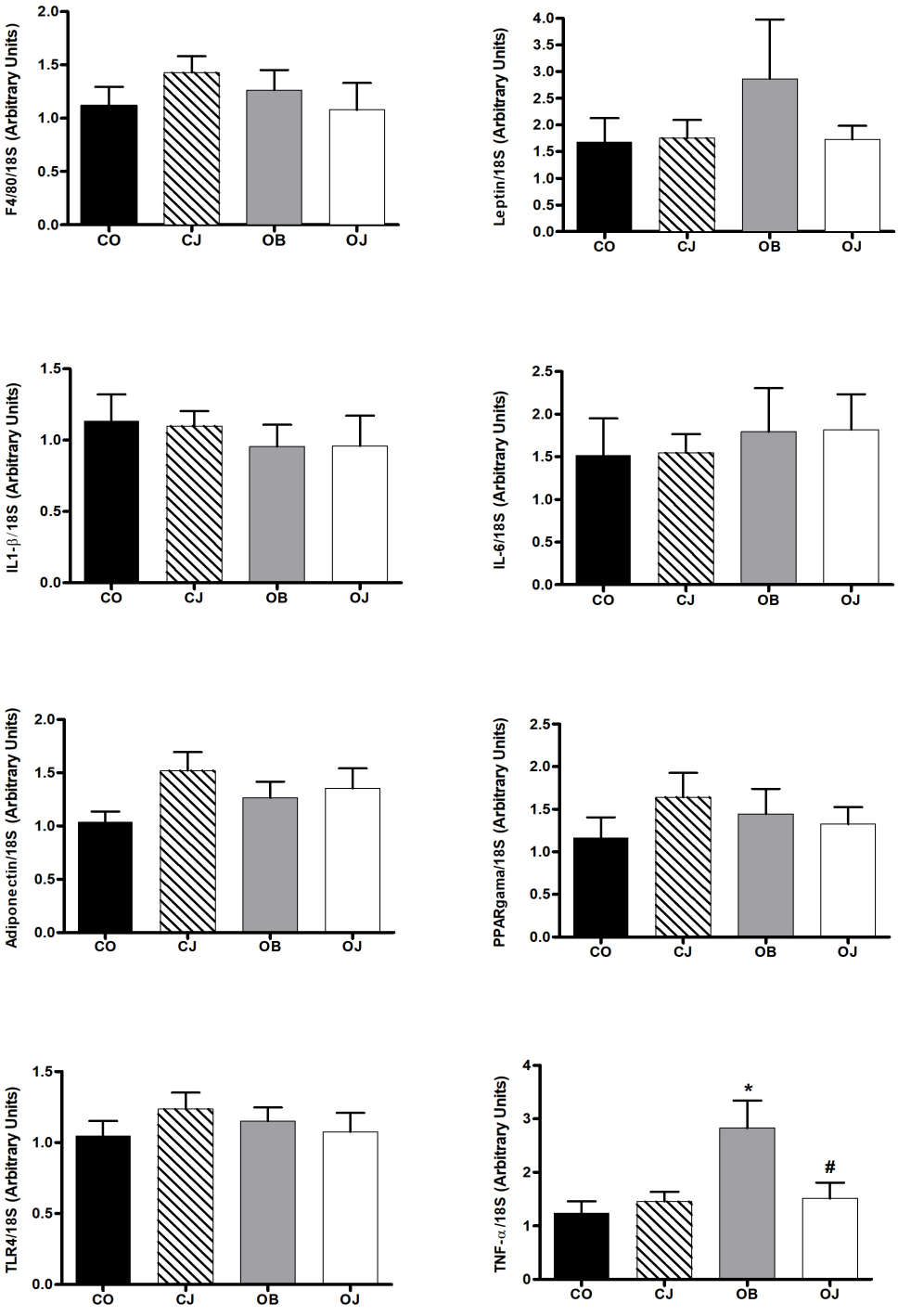


Statistically significant difference ($p \leq 0.05$) compared with *CO and #OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 6.

5.4.7 Expression of inflammatory genes in retroperitoneal adipose tissue of 7-week-old female offspring

No differences ($p \geq 0.05$) were observed in the expression of inflammation related-genes, F4/80 leptin, IL-1β, IL-6, adiponectin, PPAR-γ, and TLR-4 in the 7-week-old retroperitoneal adipose tissue between female offspring of all groups CO, CJ, OB and OJ (Figure 24). Compared to CO female offspring, OB female offspring presented higher ($p \leq 0.05$) expression of TNF-α in adipose tissue. OJ female offspring presented lower ($p \leq 0.05$) expression of TNF-α in adipose tissue when compared to OB group.

Figure 24. Expression of inflammation-related genes evaluated in retroperitoneal adipose tissue of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).



Statistically significant difference ($p \leq 0.05$) compared with *CO and #OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n = 10.

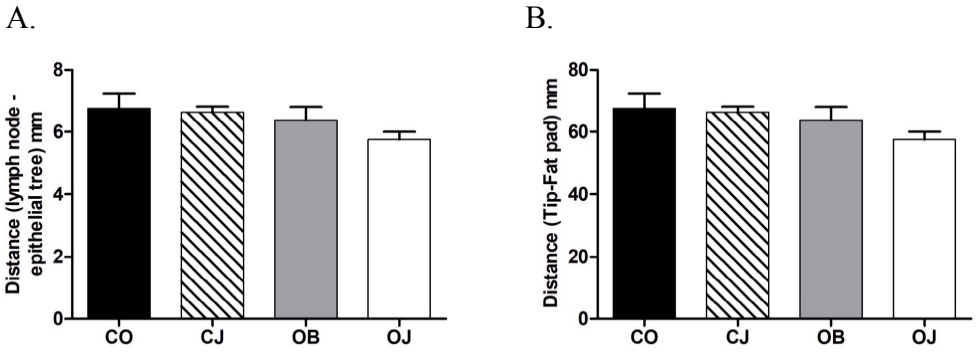
5.5 Female offspring mammary gland development

5.5.1 Mammary gland morphology of 7-week-old female offspring

5.5.1.1 Epithelial growth

Epithelial growth of the mammary gland of 7-week-old female offspring is presented in Figure 25 A-B. No difference ($p \geq 0.05$) was observed between the female offspring of groups in both parameters: in distance between the lymph node and the end of the epithelial tree and in the distance between the nipple and the fat pad.

Figure 25 A-B. Epithelial growth: distance from the lymph node to the end of epithelial tree (A) and distance from the tip of the epithelial tree to the end of fat pad (B), from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).



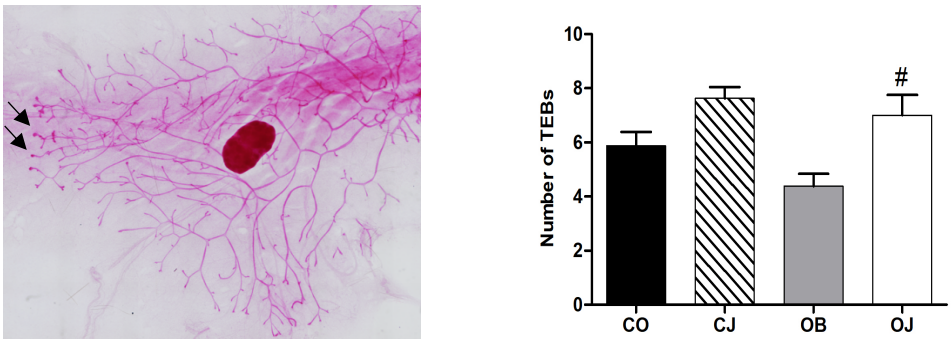
Values are expressed as mean ± standard error of the mean. n=8.

5.5.1.2 Number of TEBs

The potential for malignant transformation in the mammary gland of 7-week-old female offspring was assessed by the number of TEBs (Figure 26 A-B). Female offspring from CJ and OB fathers presented no difference ($p \geq 0.05$) in number of TEBs when compared to female offspring from CO fathers. OJ female offspring presented higher ($p \leq 0.05$) number of TEBs compared to female offspring from OB fathers.

Figure 26 A-B. Terminal end buds (TEBs), indicated by arrows (A); and Number of TEBs (B) of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

A. B.



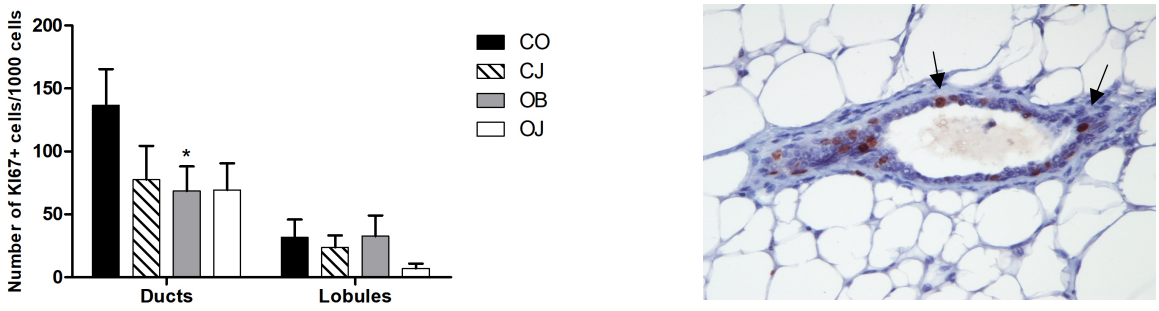
Statistically significant difference ($p \leq 0.05$) compared to #OB according to ANOVA followed by Tukey test. Values are expressed as mean ± standard error of the mean. n=8.

5.5.2 Mammary gland cell proliferation and apoptosis of 7-week-old female offspring

The assessment of cell proliferation in the mammary gland of 7-week-old female offspring is presented in Figure 27A.

Figure 27 A-B. Cell proliferation in the mammary gland ducts and lobules of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice). (A). Photomicrograph (×40 original magnification) of Ki-67 immunostaining (cells indicated by arrows) (B).

A. B.



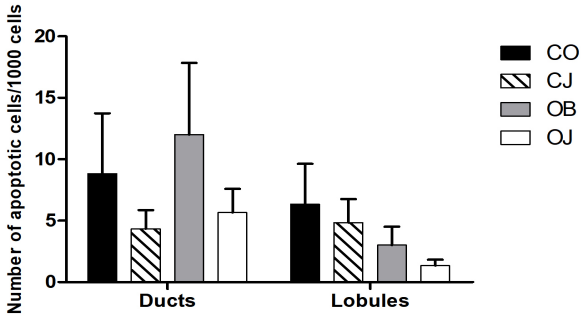
Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by LSD or Tukey test.

No differences were observed between cell proliferation in ducts and lobules in mammary gland of female offspring from CJ fathers and CO fathers. Female offspring from OB fathers presented lower ($p \leq 0.05$) cell proliferation in ducts when compare to female offspring from CO fathers, but not in lobules. No differences were observed between female offspring from OJ and CO fathers on this parameter.

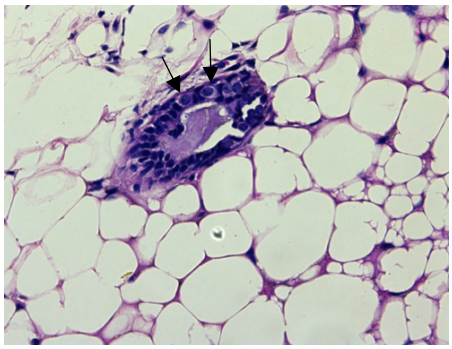
Quantification of apoptotic cells in the mammary gland of 7-week-old female offspring is presented in Figure 28 A. No difference ($p \geq 0.05$) in number of apoptotic cells in ducts and lobules in mammary gland was observed between the groups.

Figure 28 A-B. Quantification of apoptosis in the mammary gland ducts and lobules of 7-week-old female offspring from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice) (A). Photomicrograph (×40 original magnification) of apoptotic cells (indicated by arrows) (B).

A.



B.



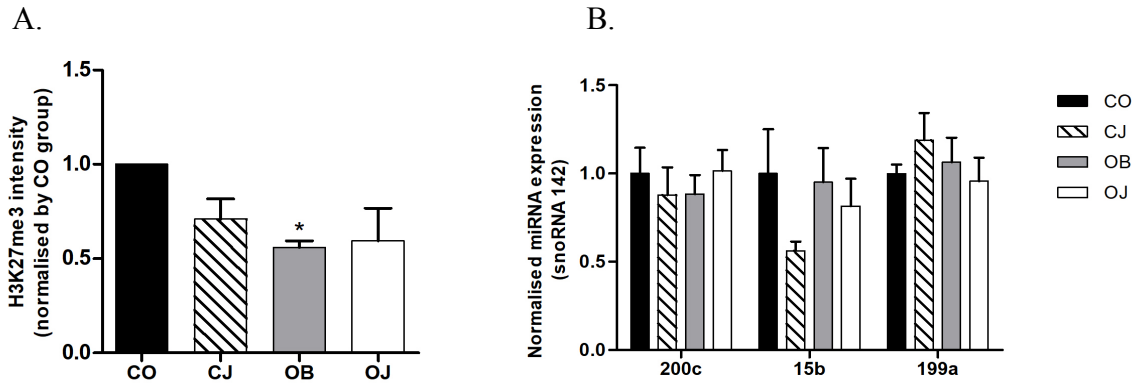
Values are expressed as mean ± standard error. n=6.

5.5.3 Mammary gland histone modification and miRNA expression of 7-week-old female offspring female offspring

Female offspring from CJ fathers presented no difference in trimethylation of lysine 27 on histone H3 (H3K27me3) global levels when compared to female offspring from CO fathers. OB father's female offspring presented lower ($p \leq 0.05$) H3K27me3 global levels when compared to female offspring from CO fathers. Female offspring from OJ father presented no difference ($p \geq 0.05$) in H3K27me3 global levels when compared to female offspring from OB

fathers (Figure 29A). There were no differences in the expression of miRNA 200c, miRNA15b and miRNA199a amongst female offspring from all groups (Figure 29B).

Figure 29 A-B. Mammary gland histone modification (A) and miRNA expression (B) of 7-week-old female offspring, from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).



Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by LSD or Tukey test. Values are expressed as mean ± standard error. (A-B: n=3; n=7, respectively).

5.6 Female offspring mammary carcinogenesis

5.6.1 Incidence, latency and multiplicity of tumors

The female offspring of the CJ group presented higher multiplicity of mammary tumors compared to the CO group ($p \leq 0.05$). Female offspring of the OB group presented lower incidence ($p \leq 0.05$), as well as increased latency of tumors onset ($p \leq 0.05$). Offspring from the OB group presented higher number of tumors per animal when compared to control offspring. Paternal intake of orange juice did not modify tumor incidence, latency or multiplicity in relation to OB offspring (Table 16).

Table 16. Development of mammary tumors in female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

Parameters	**CO**	**CJ**	**OB**	**OJ**
Number of mice	24	24	24	24
Incidence[1]	95,83±0,04	79,16±0,08	66,60±0,09*	54,00±0,10
Latency[2]	68,00±4,87	68,48±5,48	87,12±4,72*	83,17±6,57
Multiplicity[3]	1,17±0,14	1,74±0,18*	1,69±0,21*	1,31±0,13

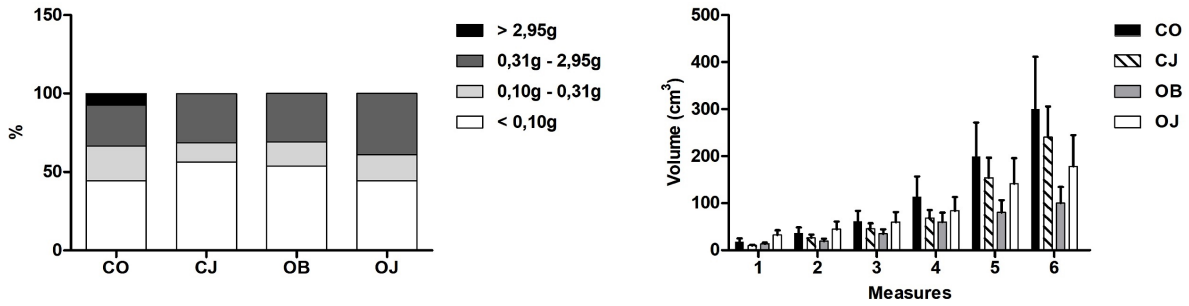
[1] Percentage of animals with tumor; [2] Time of appearance of the first tumor; [3] Mean number of tumors per animal that presented tumor. Statistically significant difference ($p \leq 0.05$) compared to *CO according to ANOVA followed by LSD test and to Kaplan-Meier followed by long-rank test. Values are expressed as mean ± standard error. n = 24.

5.6.2 Tumor weight and volume

There was no difference ($p \geq 0.05$) in tumor weight (Figure 30A) or volume (Figure 30B) amongst offspring submitted to breast carcinogenesis between groups.

Figure 30 A-B. Weight (A) and volume (B) of mammary tumors of female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

A. B.



Values are expressed as mean ± standard error of the mean.

5.6.3 Histopathological classification of tumors

Among the animals that developed tumors, adenocanthoma, adenocarcinoma type A (tubular), adenocarcinoma type B (solid/papillary), adenomyopithelioma and carcinosarcoma were identified (Table 17; Figure 31A-B). No differences ($p \geq 0.05$) were observed in the incidence of each type in female offspring of the different experimental groups.

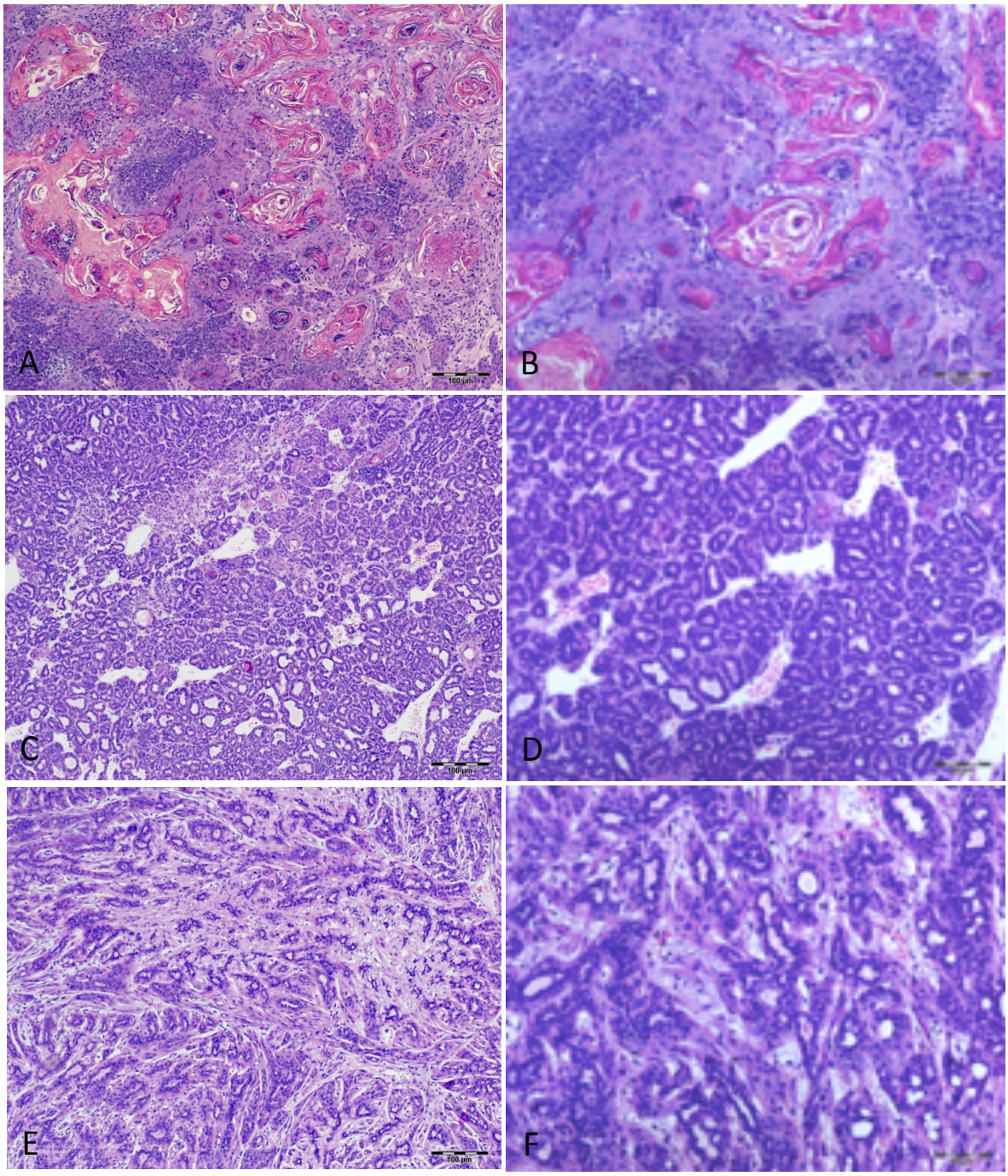
Table 17. Histopathological classification of mammary tumors of female offspring submitted to mammary carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

Histological types	**CO**	**CJ**	**OB**	**OJ**
Adenocanthoma	12 (44%)	19 (58%)	13 (48%)	11 (65%)
Tubular adenocarcinoma	1 (4%)	2 (6%)	1 (4%)	0 (0%)
Solid/Papillary adenocarcinoma	9 (33%)	7 (21%)	8 (30%)	5 (29%)
Adenomyoepithelioma	3 (15%)	1 (3%)	3 (11%)	1 (6%)
Carcinosarcoma	1 (4%)	4 (12%)	2 (7%)	0 (0%)
Total number	27	33	27	17

Values are expressed as absolute number and percentage of tumors.

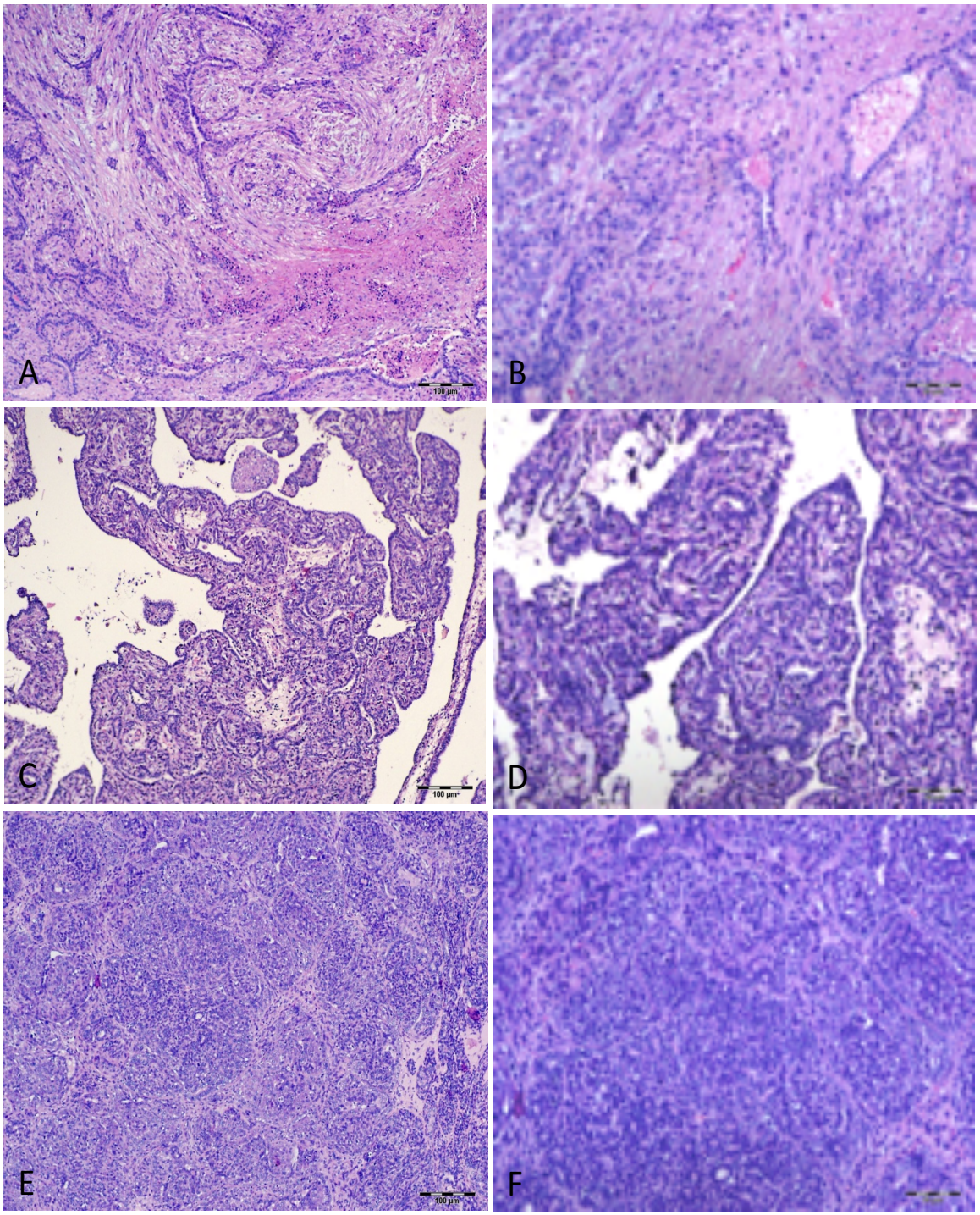
Figure 31 A-B. Representative images of mammary tumors of female offspring, submitted to breast carcinogenesis from groups CO (control father), CJ (control father and orange juice), OB (obese father) and OJ (obese father and orange juice).

A)



A-B) Adenocanthoma; C-D) Tubular adenocarcinoma E-F) Adenomyoepithelioma with myxoid stroma, 10X e 20X original magnification, respectively.

B)



A-B) Carcinosarcoma; C-D) Papillary Adenocarcinoma; E-F) Solid Adenocarcinoma, 10X e 20X original magnification, respectively.

6. Discussion

6.1 Dietary intervention

Paternal obesity was induced with a high-fat/high-sugar diet, as proven successful to promote obesity in male mice (PINHEIRO-CASTRO et al., 2019). Furthermore, the disturbances caused by the high-fat/high-sugar diet is similar to what is currently observed in dietary pattern of most western societies (PINHEIRO-CASTRO et al., 2019), the main public health problem of actuality (GBD Obesity Collaborators, 2017). Animals fed with high-fat/high-sugar diet presented approximately 10g more in weight than control groups at the end of experiment. Obesity was also confirmed by amount of fat mass % of animals fed with a high-fat/high-sugar diet when compared to control diets. Orange juice consumption had no effect on weight gain of animals at the end of experiment. OJ and OB groups had similar weight as did CJ and CO, indicating that orange juice did not prevent weight gain with the high-fat/high-sugar diet, nor promoted weight gain when consumed with control diet, despite its macronutrient content.

Concerning paternal nutrition, some differences were observed between experimental groups. Compared to CO, CJ father's diet was higher in carbohydrates, lower in total lipids (mainly PUFA), flavanones and vitamin C. OB fathers consumed greater amounts of MUFA (n-9) and saturated fatty acids, lower PUFA fatty acids, increased amounts of minerals (calcium, magnesium, zinc, phosphorus, potassium) and higher amounts of vitamins (pyridoxin, riboflavin, vitamin C). OJ fathers presented higher intake of flavanones and vitamin C than OB fathers. Although, there was a slight difference in vitamin and mineral intake between high-fat/high-sugar diet fed groups and control, the supplementation in the sweetened condensed milk was essential to meet the requirements of a nutritionally balanced diet for animals (REEVES, et al 1993), as male mice decreased in approximately 69% the consumption of the pellet diet.

In addition, fatty acid profile in liver, plasma and epididymal adipose tissue of OB fathers were higher in MUFA content, suggesting that fat deposition and lipid metabolism in obese fathers was influenced by the consumption of high-fat/high sugar diet. The type of fat in the diet is directly related to the severity of lipid accumulation in liver and also to the type of lipid stored (CASTRO et al., 2012).

As with obesity in humans, animals fed with high-fat/high-sugar diet presented increased circulating glucose compared to control animals. This suggests that obese animals

are glucose intolerant and less sensitive to insulin. In addition, obese animals also presented higher expression of the pro-inflammatory genes, F4/80, leptin, TLR4 and TNF-α when compared to control animals. Orange juice apparently had no effect on these pro-inflammatory gene expression profile. An increased inflammatory response has been pointed as a significant factor in the etiology of insulin-action resistance (ROGERO; CALDER, 2018). Through the activation of protein kinases related to Toll signaling pathways and TNF-α receptors, such as the inhibitor of kappa B kinase (IKK) and c-jun N-terminal kinase (JNK)-1, which are capable of phosphorylating insulin receptor substrate 1 (IRS-1). This reduces IRS-1 interaction with the insulin receptor beta subunit decreasing insulin signal transduction (ROGERO; CALDER, 2018).

Despite not presenting alterations in pro-oxidant marker of liver MDA, obese animals had increased liver and plasma SOD activity and increased catalase activity. Increased antioxidants enzymes may be a consequence of a general pro-oxidant state. Under metabolic stress, with great production of reactive oxygen species, antioxidant enzymes increase in order to minimize oxidative stress (RINDLER et al., 2013). Rindler et al. (2013), observed a rapid induction of catalase in heart of mice in response to a high-fat diet, suggesting that catalase is an important component of the immediate antioxidant response. Although, there is association between decreased SOD activity in obese adults (ROH; CHO; SO, 2017) there is also reported that SOD activity increases in response to obesity (STEFANOVIĆ et al., 2008). Interestingly, control-orange juice group also presented increased concentration of SOD activity. It is possible that increased SOD activity in this group was due to orange juice antioxidant effect (RANGEL-HUERTA et al., 2015). In a rat model, hesperidin the main flavanone present in orange juice, showed antioxidant activity by decreasing liver reactive oxygen species production and increasing SOD activity in liver (ADEFEGHA et al., 2017). The obese-orange juice group presented similar SOD activity to control group. We hypothesize that antioxidant effects of orange juice may have compensated obesity´s response in the liver.

Therefore, obesity was well established with the dietary intervention used.

6.1 Paternal dietary intervention and reproductive parameters

In the context of developmental programming, changes in male germ cells structure, function and in epigenetic information is crucial for uncovering the potential mechanism underlying the susceptibility of diseases in offspring (MCPHERSON et al., 2014; DONKIN; BARRÈS, 2018).

Obese fathers failed at almost all parameters evaluated when compared to control animals. Obese fathers had lower relative weight of testicle and epididymis, lower daily production of spermatozoids, lower percentage of normal spermatozoids and reduced interstitial tubular area of testicle compared to CO fathers. Other studies have reported that obesity may impair sperm quality, such as quantification and morphology and, therefore, can impact male fertility (HOUFFLYN; MATTHYS; SOUBRY, 2017). Decreased testicle weight may reflect reduced interstitial tissue, which is composed of Leydig cells and is responsible for male sex hormones production (OLUWAKEMI; OLUFEYISIPE, 2016). OB fathers also presented higher tubular area, site of all the processes involved in male sperm cells production (RAAD et al., 2017).

It is known that obesity impair sperm at the molecular level (epigenetic changes) and at clinical characteristics (morphology and daily sperm production), the last shown to affect male reproductive health (MCPHERSON et al., 2014). However, the interaction between these molecular and clinical characteristics of sperm are largely unknown. It is unclear whether sperm characteristics are affected by epigenetic changes (HOUFFLYN; MATTHYS; SOUBRY, 2017). However, it is recognized that epigenetic regulation is the mechanism underlying paternal programming (FREEMAN; WANG, 2019).

6.2 Programming effects of paternal obesity in female offspring metabolic health

There were no differences in 7-week-old female offspring´s body weight, body composition and glucose tolerance between the studied groups. Despite the absence of differences in these parameters, it was observed that offspring from obese fathers presented higher liver and plasma SOD activity and lower plasma GPx activity when compared to control offspring. This suggests an imbalance in the antioxidant defense system in offspring of obese fathers. Inflammation of adipose tissue, a common feature of obesity, has been associated with insulin resistance and an imbalance of the antioxidant defense system (MASI et al., 2017; SURIYAPROM et al., 2019). Both have been implicated in several diseases, such as atherosclerosis, diabetes and obesity (SALTIEL; OLEFSKY, 2017; SURIYAPROM et al., 2019).

Offspring from obese fathers presented higher expression adipose tissue of TNF-α. Alfaradhi et al. (2016) observed that lean male offspring of obese dams presented increased adipose tissue TNF-α expression, which the authors attributed to miRNAs regulating expression of inflammatory signaling molecules. Consumption of orange juice by obese fathers

reduced adipose tissue TNF-α expression and increased plasma GPx activity of female offspring when compared to OB offspring. This is a promising perspective, as it suggests that orange juice may have a therapeutic function against programming effects of adipose tissue inflammation, caused by paternal obesity, added by an improvement of plasma antioxidant profile. Although few studies have investigated paternal consumption of bioactive compounds during preconception in offspring metabolic health (SILVA, et al. 2019). McPherson et al. (2016) showed that supplementing malnourished male mice with a blend of green tea extract, lycopene and micronutrients (vitamin C, vitamin E, folate, zinc and selenium) can reduce oxidative damage to sperm DNA and prevent fat accumulation and dyslipidemia in offspring. Therefore, results of the present study indicate that female offspring from obese parents may be susceptible to a phenotype of adipose tissue inflammation and alterations in the antioxidant defense system, which may favor development of metabolic disorders, such as obesity and insulin resistance. Consumption of orange juice by obese parents, on the other hand, protected offspring from these metabolical alterations.

It is possible that we were not able to observe all of the metabolic alterations programmed by paternal obesity and orange juice consumption, because most metabolical complications due to programming effects occurs at adulthood (NG et al., 2010; FULLSTON et al., 2015; MCPHERSON et al., 2016). As the main objective of this study was to evaluate mammary gland morphology in puberty (7-week-age), offspring did not reach adulthood in the present study.

6.3 Mammary gland development and susceptibility to carcinogenesis in programmed female offspring

Considering mammary gland morphology and breast cancer risk, compared to CO female offspring, no differences were observed between CJ nor OB female offspring. However, female offspring from OJ group showed an increase in number of TEBs compared to OB offspring. Consumption of orange juice by obese fathers seems to not have protect offspring from breast cancer risk regarding this parameter. Although overall there is a positive association between number of TEBs and breast cancer risk (HILAKIVI-CLARKE, 2007), one study has reported an early-life dietary modification that reduced number of TEBs and still resulted in increased susceptibility to breast tumorigenesis (OLIVO; HILAKIVI-CLARKE, 2005).

Regarding breast cancer susceptibility, CJ female offspring presented higher multiplicity of mammary tumors when compared to CO offspring. This was not observed in

obese fathers that consumed orange juice. Therefore, consumption of orange juice on breast cancer may be dependent on paternal metabolic state. This was also shown in a maternal model of programming: consumption of some classes of polyphenols had opposing effects, depending on maternal nutritional status (DEL BAS et al., 2015).

Offspring from OB group showed higher tumor latency, lower tumor incidence, higher multiplicity of tumors and lower cell proliferation (Ki67) in mammary ducts when compared to CO offspring. During peri-puberty (post-natal day 42), the proliferative edge of the TEB is usually not surrounded by connective tissue, therefore proliferation and cell migration results in invasion into the fat pad and elongation of the duct, a process associated with increased risk of cancer development in a chemically-induced mammary cancer (GOPALAKRISHNAN et al., 2018; WANG; JENKINS; LAMARTINIERE, 2014). Female offspring of OB fathers had lower cell proliferation in mammary gland ducts in puberty, suggesting decreased breast cancer risk (WANG; JENKINS; LAMARTINIERE, 2014). Therefore, paternal obesity appears to have protected female offspring from breast cancer risk.

Another parameter that was observed in mammary gland of OB offspring was lower global levels of H3K27me3. Catalyzing trimethylation of lysine 27 on histone H3 (H3K27) is the polycomb repressive complex 2 (PRC2) function, a chromatin associated methyltransferase (LAUGESEN; HØJFELDT; HELIN, 2019). Enhancer of zeste homolog 2 (EZH2) is a domain of PCR2, containing methyltransferase that catalyzes the formation H3K27me3 (LAUGESEN; HØJFELDT; HELIN, 2016). It was demonstrated that knocking down EZH2 in mice results in delayed outgrowth of the mammary epithelium during puberty, caused by impaired terminal end bud formation and ductal elongation (MALGORZATA MICHALAK et al., 2013). A delay in mammary gland development was previously associated to increased breast cancer risk (FONTELLES et al., 2016a), as well as abnormal amounts of EZH2 (GAN et al., 2018). Therefore, further investigation would be important to determine whether the protective effects of breast cancer risk in OB offspring could be due to delayed mammary gland development or if there is another underlaying mechanism. Although number of TEBs were higher in OJ offspring when compared to OB, no differences were observed between OB and OJ female offspring regarding incidence, latency and multiplicity of mammary tumors. MiRNAs previously linked to breast cancer risk were also evaluated in mammary gland, however no differences were found between miRNAs expression in the mammary gland of female offspring between the studied groups.

Although this study provided interesting results, there are important questions that still need to be answered, as the mechanisms by which paternal obesity/nutrition led to reduction of

breast cancer susceptibility. Also, what mechanisms explain why orange juice presented protective metabolic effects in female offspring, but did not prevent breast cancer. Furthermore, the potential benefits of paternal consumption of polyphenols should be further investigated, as it has been shown that some classes of these bioactive compounds have positive effects on male reproductive parameters (JIANG et al., 2018; RAHMAN et al., 2018). Understanding orange juice and polyphenols consumption by fathers in their specific metabolic context (i.e. lean vs obese man) looking at offspring for the outcomes can produce different results (protection, absence of effects or even harm) depending on paternal health status. This is essential to adequately translate experimental evidence into potential dietary recommendations (SILVA, et al. 2019).

One limitation of this study, is that epigenetic changes in sperm cells were not evaluated. However, it has been demonstrated that obese man presents distinct sperm miRNA expression and DNA methylation profiles than lean men (DONKIN et al., 2016), as well as in animal models (FONTELLES et al., 2016b; MCPHERSON et al., 2016). These markers when altered, are known to be implicated in abnormal phenotypes in offspring and perturbations in embryogenesis (CRAIG et al., 2017).

This study confirms that breast cancer risk in female offspring can be programmed by paternal diet. However, contrary to expected, paternal high-fat/high-sugar diet decreased susceptibility to breast cancer. Orange juice intake, on the other hand, increased breast cancer susceptibility.

7. Conclusions

In conclusion, consumption of orange juice by non-obese fathers during preconception increased susceptibility of female offspring to mammary carcinogenesis. Although paternal consumption of a high-fat/high-sugar diet during preconception decreased incidence and increased latency of tumors, the multiplicity of lesions increased. There is indication that offspring response to paternal orange juice consumption depends of paternal metabolic context.

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Attachment 1

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Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF **84.2015-P504**

CERTIFICADO

A Comissão de Ética no Uso de Animais, da Faculdade de Ciências Farmacêuticas, da Universidade de São Paulo, **CERTIFICA** que o Projeto de Pesquisa "**Obesidade paterna e programação do risco de câncer de mama: efeitos do consumo de suco de laranja**" (Protocolo CEUA/FCF/504), de responsabilidade do(a) pesquisador(a) **Prof. Dr. Thomas Prates Ong**, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal (CONCEA) e foi APROVADO em reunião de **21 de agosto de 2015**. Conforme a legislação vigente, deverá ser apresentado, no encerramento deste Projeto de Pesquisa, o respectivo **relatório final**.

São Paulo, 17 de novembro de 2015.

Prof. Dr. Joilson de Oliveira Martins
Coordenador da CEUA/FCF/USP

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