UNIVERSITY OF SÃO PAULO

Faculty of Pharmaceutical Sciences

Graduate Program in Food Sciences

Area of Experimental Nutrition

The effects of maternal obesity during gestation and lactation and orange juice intake on the metabolic profile of male offspring exposed to control and obesogenic diets and breast cancer risk of female offspring

Natália Pinheiro de Castro

São Paulo

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Original Version

Thesis presented to the Faculty of Pharmaceutical Sciences of the University of São Paulo as pre-requisite to obtain the degree of DOCTOR

Supervisor: Prof. Dr. Thomas Prates Ong

São Paulo

2020

Ficha Catalográfica elaborada eletronicamente pelo autor, utilizando o programa desenvolvido pela Seção Técnica de Informática do ICMC/USP e adaptado para a Divisão de Biblioteca e Documentação do Conjunto das Químicas da USP

Bibliotecária responsável pela orientação de catalogação da publicação:
Marlene Aparecida Vieira - CRB - 8/5562

C355t

Castro, Natalia Pinheiro
The effects of maternal obesity during gestation and lactation and orange juice intake on the metabolic profile of male offspring exposed to control and obesogenic diets and breast cancer risk of female offspring / Natalia Pinheiro Castro. - São Paulo, 2020.
88 p.

Tese (doutorado) - Faculdade de Ciências Farmacêuticas da Universidade de São Paulo. Departamento de Alimentos e Nutrição Experimental.
Orientador: Ong, Thomas Prates

1. Obesity. 2. Breast cancer. 3. Early life. 4. Maternal. I. T. II. Ong, Thomas Prates, orientador.

Natália Pinheiro de Castro

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Commission

of

Thesis for the degree of Doctor

Prof. Dr. Thomas Prates Ong

supervisor/president

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3rd examiner

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4th examiner

São Paulo, _____, 2020.

DEDICATION

I dedicate this thesis to my incredible family. Their love and support made me able to dedicate my life to what I love to do most. It is a privilege.

Acknowledgements

To my supervisor, Thomas Prates Ong, for guiding me in what was a great learning experience. Thank you for the light conversations and understanding, even when I deserved to have my ear pulled. Thank you for supervising me even in your vacation. It was and still is much appreciated.

To Prof. Inar de Castro, a role model. Also, to her students, Tamires, Mariana, Matheus and Gabriela Grassman. Special thanks to Marina Nogueira. Weren´t for you, I might have tipped over some of my deadlines. Sharing the lab with you guys is/was such an easy task.

To Prof. Fernando Moreno and to his Lab, a special thanks to Renato and Mayara, for sharing their knowledge and philosophies with me.

To Prof. Neuza Hassimotto and Prof. Franco Lajolo, for the knowledge and expertise with orange juice. Thank you for collaborating with our study.

To Prof. Luis Fernando Barbisan, for the availability, participation and collaboration with ideas regarding histopathological and immunohistochemistry analyzes.

To prof. João Paulo Fabi, for the availability and collaboration with PCR analysis.

To all of the employees of the animal facility and a special thanks to Renata, Flávia, and José, for being so cooperative, for teaching us everything we know about mice and their reproductive life, and for making our lives so much easier.

To my lab, the Lab of Nutrigenomics and Programming, specially to the researchers that preceded me: Camile Fontelles, Luiza Guido and Mariana Rosim.

To the lab friends that came before me and are still very important to me: Vanessa Cardoso Pires and Gabriela Rezende.

To my lab friends and partners, Livia Ribeiro, Gabriela Novaes and Gabriela Laiber. Sharing this experience with you, the pains and gains, made everything lighter and easier. There is too much to learn in coffee and conversation breaks. Thank you for my wake-up call and for understanding my one most difficult thing during this journey and helping me through it. Now I can have that beer.

To Leo Cruz, our lab technician. People that don´t work in a lab are clueless to how much work goes into everything. How many documents are involved in a single purchase, that our tips and water must be sterilized, that we have urgency for everything, that we need liquid nitrogen every other day. Thank you for being so present in our lab.

To Agatha Meei Lin, graduate student of our lab and the person that conducted the protocol with male offspring. Thank you for the companionship and for having followed through with this challenging experiment.

To Monique Sobrinho, graduate student that also helped me with mammary gland-related analysis.

To Walter Turato, for being patient and available. For contributing with the imaging CT of our animals.

To all the understanding, friendly, always available staff from the Food and Experimental Nutrition Department: Edilson, Roberta, Lea and Vanessa. A special thanks to Lourdinha for always being there. Another special thanks to all the lab technicians that contributed to our research, Aline, Lucia, Luciene and Tânia.

I have made great friends and met wonderful people along the way. Luciana Tedesco, you are the most sensitive and awesome person. Every department should have one of you.

To my lifelong friends, Kelly Giudici, Andrea Toledo, Clarissa Fujiwara, Stella Rosati, Priscila Koritar, Fernanda Simões, Andreia Santana and Lígia Kobayashi. May there be more PhDs, blogs/vlogs, weddings, kids and get togethers. Thank you for your support.

To my family. José Luís de Castro Neto, Maria Amélia Arraes de Alencar Pinheiro de Castro, Alexandra Castro, Rosa Stopa, Alice e Cecília. For making me unfocus in the most wonderful way and teaching me that life can be full of different things and that I can still have everything.

To Thiago Campolim, for the support in the past two years, and for handling rather well my ups and downs for the past year.

Finally, to the Conselho Nacional de Desenvolvimento Científico e Tecnológicod (CNPq) for the doctoral scholarship (161523/2015-3) and Food Research Center (FoRC) for financial support.

I will end this section with a saying from Charles Darwin, which is:

"*It is the long history of human kind (and animal kind, too) that those who learned to collaborate and improvise most effectively have prevailed*."

RESUMO

CASTRO, N.P. A obesidade materna durante a gestação e lactação e o consumo do suco de laranja: efeitos nos parâmetros metabólicos da prole masculina exposta a dieta controle e obesogênica e programação do risco do câncer de mama da prole feminina de camundongos. 88 f. Thesis (PhD) – (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo.

A obesidade é um problema de saúde pública e o principal fator de risco para uma série de doenças crônicas. O câncer de mama é outra doença preocupante: é a principal causa de câncer entre as mulheres e tem elevada taxa de mortalidade. Há 30 anos, Barker e Trichopoulos sugeriram que a doença cardiovascular e o câncer de mama, respectivamente, podem ser originados no útero. Nos anos seguintes essas hipóteses foram confirmadas. Compreender como o ambiente intrauterino pode afetar o desenvolvimento da obesidade e o câncer de mama na idade adulta, portanto, é fundamental para prevenir essas doenças. O estado nutricional e a nutrição durante a gestação e lactação são considerados fatores modificáveis e que pode influenciar o ambiente intrauterino. O suco de laranja (SL) é uma excelente fonte de compostos bioativos, incluindo vitamina C e flavonoides, e estudos sugerem que a ingestão de suco de laranja pode minimizar os efeitos deletérios da obesidade. O objetivo dessa tese foi de avaliar os efeitos da obesidade materna durante a gestação e lactação e a ingestão de SL no (a) perfil metabólico da prole masculinas expostos à dietas controle e obesogênica e (b) risco de câncer de mama da prole feminina. Camundongos fêmeas C57BL/6, com quatro semanas de idade, foram distribuídos em três grupos: controle - alimentados com uma dieta de controle e água ad libitum, obesos- alimentados com dieta obesogênica e água ad libitum e obesos+SL- alimentados com dieta obesogênica e SL. Após três semanas na dieta, as fêmeas foram acasaladas com machos controle. A prole masculina de cada grupo foi desmamada e alimentadas com dieta obesogênica ou controle por 21 semanas. A prole feminina foi eutanasiada para a avaliação do desenvolvimento da glândula mamária ou submetida a um protocolo de carcinogênese mamária quimicamente induzida. Parâmetros para avaliar o metabolismo (como a composição corporal e expressão de genes relacionados à obesidade do tecido adiposo), risco de câncer de mama (como desenvolvimento epitelial e número de terminal end buds) e tumorigênese (incidência, latência e multiplicidade dos tumores mamários) foram coletados. Para investigar diferença estatística entre os grupos foi realizada ANOVA, seguida pelo teste de Tukey ou LSD de Fischer e um $p<0,05$ foi considerado significante. A prole masculina de mães obesas alimentadas com dieta de controle apresentou aumento das concentrações de glicose e aumento das expressões de F4/80 e interleucina-6 em relação a prole controle. A prole masculina de mães obesas+SL alimentadas com dieta controle apresentou expressão de F4/80 e interleucina-6 similar à da prole de controle. A prole masculina de mães controle e alimentada com dieta obesogênica apresentou aumento das concentrações de glicose e aumento do tecido adiposo epididimal em comparação à prole de mães obesas. A prole de mães obesas+SL apresentou maior expressão de leptina e TNF-α. A prole feminina de mães obesas apresentou redução do número de *terminal end buds* e aumento da latência para o aparecimento do primeiro tumor. O consumo de SL diminuiu o desenvolvimento epitelial comparado as proles de mães controles e obesas. A obesidade materna teve maior impacto na prole masculina exposta a dieta controle do que na obesogênica. A ingestão materna de SL ajudou com efeitos danosos induzidos pela obesidade materna na prole masculina alimentada com dieta controle. A prole de fêmeas controles e alimentada com dieta obesogênica apresentou perfil metabólico pior que a prole das mães obesas. Neste caso, o SL não foi benéfico para a prole masculina. A obesidade materna induzida por uma dieta rica em banha e açúcares apresentou discreto efeito protetor no risco de câncer de mama, o SL acentuou esta proteção.

Palavras-chave: Câncer de mama, obesidade, suco de laranja, início da vida.

ABSTRACT

CASTRO, N.P. The effects of maternal obesity during gestation and lactation and orange juice intake on the metabolic profile of male offspring exposed to control and obesogenic diets and breast cancer risk of female offspring. ___f. Thesis (PhD) – (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo.

Obesity is a worldwide public health problem and the main risk factor for a number of chronic diseases. Breast cancer is another worrisome disease: it is the leading cause of cancer amongst women and has an elevated mortality rate. Approximately 30 years ago, Barker and Trichopoulos suggested that cardiovascular disease and breast cancer, respectively, may be originated in utero. In subsequent years, studies proved both hypotheses correct. Understanding how in-utero environment can affect development of obesity and breast cancer in adulthood is key for preventing these diseases. Nutrition during gestation and lactation is considered a modifiable factor to impact in-utero environment. Orange juice (OJ) is an excellent source of bioactive compounds, including vitamin C and flavonoids, and reports suggests that intake of orange juice minimizes damaging effects of obesity. The objective of this thesis was to evaluate the effects of maternal obesity during gestation and lactation and OJ intake on (a) metabolic profile of male offspring exposed to control and obesogenic diets and (b) breast cancer risk of female offspring. Four-week-old C57BL/6 female mice were assigned into three groups: control– fed a control diet and water ad libitum, obese– fed obesogenic diet and water ad libitum and obese+OJ– fed obesogenic diet and OJ. After three weeks on the diet, females were mated to control males. Male offspring from each group were weaned into control or obesogenic diets for 21 weeks. Female offspring was either euthanized for evaluation of mammary gland development or submitted to a chemically induced breast carcinogenesis protocol. Parameters to assess metabolism (as body composition and adipose tissue expression of obesity-related genes), breast cancer risk (as epithelial elongation and number of terminal end buds) and tumorigenesis (incidence, latency and multiplicity of mammary tumors) were collected. ANOVA followed by Tukey or Fischer´s LSD test were used to investigate differences between groups and a $p<0.05$ was considered significant. Male offspring of obese mothers fed control diet presented increased glucose concentrations and expression of F4/80 and interleukin-6 compared to control offspring. Male offspring of obese+OJ mothers fed a control diet presented expression of F4/80 and interleukin-6 similar to control offspring. Male offspring to control mothers fed obesogenic diet presented increased glucose concentrations and epididymal fat tissue compared to offspring of obese mothers. Offspring to obese+OJ mothers presented increased expression of leptin and tumor necrosis factor-α. Female offspring of obese mothers had decreased terminal end buds and increased latency of first tumor and OJ intake decreased epithelial elongation compared to offspring of control/obese mothers. Maternal obesity had greater impact in offspring exposed to control than obesogenic environment. OJ intake by mothers helped with harmful effects induced by maternal obesity on male offspring fed control diet. Control offspring exposed to obesogenic diet presented worse metabolic profile than offspring from obese mothers. In this particular case, OJ was not beneficial to male offspring. Whereas obesity induced by a high-fat high-sugar diet presented a somewhat protective effect on breast cancer risk, OJ further protected offspring of obese mothers.

Key words: Breast cancer, obesity, orange juice, early life.

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1. INTRODUCTION

The high prevalence of obesity in adults, adolescents and children has been a priority concern of many international health organizations, which have directed their efforts in creating policies to contain this, still rising, public health problem (BUTTRISS, 2016; MS, 2019; WHO, 2019). The accumulation of excessive adipose tissue is linked to many chronic diseases, such as type 2 diabetes, atherosclerosis, hypertension and cancer, that are responsible for abbreviating and reducing quality of life (BRAY; KIM; WILDING, 2017; QASIM et al., 2018).

Obesity has been defined as a consequence of a chronic positive energy balance, there is, when energy intake exceeds expenditure overtime (ROMIEU et al., 2017). However, the interplay of biological, environmental and social factors involved in this equation is complex. The biological mechanisms of the brain reward system, hormonal production by adipose tissue and the constant exposure to an obesogenic environment, which promotes consumption of energy-dense foods and sedentary behavior, makes this one of the most challenging diseases to treat (MELDRUM; MORRIS; GAMBONE, 2017; QASIM et al., 2018). Therefore, worldwide efforts to prevent obesity is key.

In July of 2019 it was celebrated the 30th anniversary of the seminal studies published by professor David Barker and colleagues (BARKER et al., 1989a, 1989b; LIMESAND; THORNBURG; HARDING, 2019), which proposed that low birthweight, proxy of intrauterine growth restriction, was associated with a higher risk of hypertension and cardiovascular disease in adulthood. Since then, there is a mounting number of evidences indicating that maternal nutrition and nutritional status impacts the health of their offspring later in life (CNATTINGIUS et al., 2012; REYNOLDS et al., 2013; CASTRO et al., 2017). Recent epidemiological evidence has reported a link between maternal overweight and obesity and type 2 diabetes of their offspring in adulthood (LAHTI-PULKKINEN et al., 2019) and an earlier report of the same study group linked maternal obesity to premature death from cardiovascular event (REYNOLDS et al., 2013). It is not understood, however, how the environment the offspring is born to interferes with this causal relationship. What would be the metabolic effects of an obesogenic environment in offspring's born to obese dams? What

if a dietary treatment-approach was implemented and offspring from obese dams were only exposed to a low- energy-dense diet?

As with obesity, breast cancer rates are increasing worldwide. It is the most incident cancer in women, accounting for 25% of all new cancer diagnosis and the number one cause of death from cancer in most Continents (FERLAY et al., 2019). Known risk factors for breast cancer includes inheritance of genetic mutation, younger age of menarche, nulliparity, late menopause and use of oral contraceptives amongst others (ROJAS; STUCKEY, 2016). Professor Dimitri Trichopoulos proposed in the 90s that breast cancer could also be originated *in utero* (TRICHOPOULOS, 1990) from the observation of Herbst et al. (HERBST; ULFELDER; POSKANZER, 1971), whom linked the use of an anti-abortive synthetic estrogen diethylstilbestrol by pregnant women and vaginal cancer of their offspring in adulthood. Trichopoulos hypothesis was later strengthened by a cohort study that followed up offspring of women exposed to diethylstilbestrol in-utero and found a 40% increase risk of breast cancer when compared to non-exposed women (PALMER et al., 2002).

Mammary gland development starts within the fourth week of intrauterine life, when the thickening of the ectoderm, called mammary placodes, evolves to form primitive mammary buds, which will originate the ductal tree at birth (MACIAS; HINCK, 2012). After birth, the mammary gland will remain inert, in its embryonic state, until it fully develops in puberty, with hormonal stimulation, penetrating the surrounding mature tissues (fat, connective tissue and blood vessels) (MACIAS; HINCK, 2012). Given the in-utero development of the mammary gland, this structure is susceptible to the maternal environment, which includes diet and nutritional status.

“Would the womb be a novel target for cancer prevention in the era of the obesity pandemic?” The question was posed by Simmen and Simmen in 2011 (SIMMEN; SIMMEN, 2011), however it remains current, as rates of obesity and breast cancer has only increased since then (GLOBOCAN, 2018). Foods rich in bioactive compounds have been shown to exert anti-obesogenic effect and to have anti-cancer properties (RAMPERSAUD; VALIM, 2017; SILVA et al., 2019). In this respect, orange juice is one of the most micronutrient rich-dense fruit juices, with more micronutrient per gram of juice than any other fruit juice commercially available (FRANKE et al., 2013; RAMPERSAUD; VALIM, 2017). Additionally, it is rich in flavonoids, carotenoids, sugars, and fibers (BRASILI et al., 2019), which is possibly responsible for the

association of this fruit juice with a better diet quality, lower total cholesterol and lower low-density lipoprotein (LDL)-cholesterol (O'NEIL et al., 2012).

Therefore, in this thesis, it was hypothesized that maternal obesity, induced by a high-fat and high sugar diet, can (a) program obesity in male offspring exposed or not to an obesogenic environment, (b) program breast cancer risk in female offspring. It was also hypothesized that orange juice intake by obese mothers could interfere with the potential harmful programming effects of maternal obesity in offspring´s health.

2. REVIEW OF THE LITERATURE

2.1 OBESITY

Overweight and obesity rates have tripled since the 1980s and it is projected that, by 2030, almost 60% of the world population will be overweight or obese (KELLY et al., 2008; CHOOI; DING; MAGKOS, 2019). The World Health Organization defines obesity as an abnormal fat mass accumulation that impairs health (WORLD HEALTH ORGANIZATION, 2019) and this usually happens when the relationship between body weight and the square of height (body mass index-BMI) exceeds 25kg/m^2.

The chronic imbalance between energy intake and expenditure overtime increases fat mass, as this is the body compartment more adaptable to energy variations (YU, 2017). When this happens, there is a break in homeostatic weight control and a new “set point weight” is determined, involving deregulation of hormones and feedback mechanisms (MÜLLER et al., 2018). Weight homeostasis disruption involves the pleasure and reward system, encoded by the corticolimbic structure and implicates complex brain networks. The interplay between homeostatic and non-homeostatic weight control is referred as “tug of war” by Yu et al. (YU, 2017), as the two regulatory systems collide in the era of food abundance.

Classical animal models of dietary-induced obesity employ the use of a high-fat diet (HFD), which may vary in fat content (PINHEIRO-CASTRO et al., 2019). However, animals submitted to a HFD tend to maintain their energy balance, by eating less of the energy-dense diet and maintaining energy intake similar to control animals (DE OLIVEIRA ANDRADE et al., 2014). By combining sweetened condensed milk to a high-fat diet, as proposed by Samuelsson et al. (2007), animals tend to gain more weight and exhibit a metabolic profile similar to that of individuals with obesity (MASI et al., 2017). Additionally, the availability of a highly palatable diet simulates the environment to which most of our population is exposed.

Adiposity gain and disruption of weight homeostasis can be measured by hormones produced by adipose tissue. Leptin is mainly a product of adipocytes and functions as circulating signal for triglycerides store in adipose tissue (ZHANG; CHUA, 2017). In a post-prandial state, leptin, via hormonal and nutritional signals, increases, entering the cerebrospinal fluid and is

subsequently transported through the blood-brain-barrier. Leptin also access neurons of the hypothalamus via tanycytes – ependymal cells found in the third ventricle of the brain. When leptin reaches its receptors, it activates the anorexigenic POMC neurons and silences the oxygenic NPY/AGRP neurons (ZHANG; CHUA, 2017). As obese individuals have increased circulating leptin concentrations, it is hypothesized that transport mechanisms of leptin, through its receptors, is hindered by an obesogenic diet (ZHANG; CHUA, 2017). Therefore, measurements of leptin are valuable to understand the on-set of obesity.

Adiponectin is other important adipokine involved in the crosstalk between adipose tissue and many vital organs, as liver, heart, pancreatic β cells and kidney (WANG; SCHERER, 2016). It is known to suppress glucose production in the liver, enhances fatty acid oxidation, has a suggested action in the Central Nervous System and protects cells from apoptosis and inflammation via receptor-dependent mechanisms (WANG; SCHERER, 2016).

Other relevant adipocytokines linked to obesity are markers of low-grade inflammation. In response to a positive energy balance, adipose tissue increases in size (hypertrophy) and number (hyperplasia). It is hypothesized that insufficient blood supply to increased adipose tissue leads to hypoxia and necrosis, which culminates in macrophage infiltration and overproduction of reactive oxygen species, pro-inflammatory cytokines and chemokines (ELLULU et al., 2015).

It is possible to identify oxidative stress related to obesity by measurement of malondialdehyde (MDA), which is an end product of the radical initiated decomposition of poly-unsaturated fatty acids (GIERA; LINGEMAN; NIESSEN, 2012). Furthermore, the endogen antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) act coordinatingly against reactive oxygen species: while SOD catalyzes dismutation of unstable superoxide ($O_2^{\bullet -}$) into molecular oxygen and hydrogen peroxide (H_2O_2), CAT and GPx deactivate the H_2O_2 (MATÉS; PÉREZ-GÓMEZ; DE CASTRO, 1999). When endogenous enzymes are not sufficient to contain stress, MDA concentrations will generally rise.

There are many pro-inflammatory cytokines that can indicate obesity-associated inflammation. F4/80 is a glycoprotein expressed almost exclusively by macrophages (DOS ANJOS CASSADO, 2017) and, therefore, in adipose tissue, is a good marker of macrophage infiltration. Interleukin-6 (IL-6) and Tumor Necrosis Factor (TNF-α) are both products of

macrophage in an inflammation-like state (ELLULU et al., 2017). Toll-like receptors (TLR) are a family of transmembrane proteins of the innate immune response, mainly responsible for recognizing general aspects of molecules associated with pathogens (ROGERO; CALDER, 2018). Toll Like Receptor 4 (TLR-4) is usually activated by lipopolysaccharides present in Gram-negative bacteria, however there are evidences of TLR-4 being activated by saturated fatty acids and fibrinogen concentrations, both increased in obesity (ROGERO; CALDER, 2018). Activation of TLR-4 initiates a cascade of signaling pathways which leads to production of pro-inflammatory cytokines.

2.2 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE – OBESITY

Studies on the developmental programming of health and disease point out that maternal obesity during pregnancy and lactation may be linked to many chronic diseases of the offspring later in life (REYNOLDS et al., 2013; HROLFSDOTTIR et al., 2015). However, because obesity is a multifactorial disease (GONZÁLEZ-MUNIESA et al., 2017), and environment play such an important role for its development (BREHM; D'ALESSIO, 2000), it is difficult to isolate the influence of maternal obesity on offspring obesity risk. It is also a challenge to predict how offspring of obese mothers will handle an obesogenic environment themselves. Are pups/offspring of obese mothers more equipped to deal with an obesogenic environment than offspring born to control mothers? This is what is called a mismatched environment.

The concept of a mismatched environment was also proposed by Hales and Barker (HALES; BARKER, 2001). There are two population-based studies of maternal malnutrition and programming effects of adult disease that are good representations of this theory (CALKINS; DEVASKAR, 2011): the Dutch Famine and the Leningrad Siege. Between years 1944 and 1945, a German blockade interrupted food and fuel transportation to the densely populated cities in the western of Netherlands. As a result, daily nutrition intake of pregnant women varied between 400-1000 calories, much lower than their energy needs (ROSEBOOM; DE ROOIJ; PAINTER, 2006; ROSEBOOM et al., 2011). Once the blockade was lifted, caloric intake was normalized. However, adults born to women exposed to hunger in early, mid and late pregnancy, developed different chronic diseases in adulthood – depending on the timing of

famine *in-utero* (ROSEBOOM; DE ROOIJ; PAINTER, 2006; CALKINS; DEVASKAR, 2011). On the other hand, the siege of Leningrad, now Saint Petersburg, by the German army, interrupted food transportation for 800 days. Therefore, children born between 1941 and 1943 experienced famine not only in-utero but also during infancy. Curiously, contrary to offspring of the Dutch Famine, children born in Leningrad, whom experienced hunger in-utero and in infancy did not develop chronic diseases in adulthood (STANNER et al., 1997; CALKINS; DEVASKAR, 2011). People born in Leningrad and experienced malnutrition in-utero was well adapted to survive in an environment with calorie restriction, there is, similar to what they had in-utero, which protected them from developing chronic diseases in adulthood (CALKINS; DEVASKAR, 2011). This concept was called “thrifty phenotype”, which later evolved into the vast study field of epigenetics. It is now understood that, during in-utero development, epigenetic processes occur to alter gene expression in response to maternal environmental cues, to produce phenotypes best suited for that environment (CALKINS; DEVASKAR, 2011).

Even though there are numerous studies on the metabolic parameters of offspring of obese mothers (SAMUELSSON et al., 2007; DE OLIVEIRA ANDRADE et al., 2014; RIBAROFF et al., 2017; LOCHE et al., 2018; NICHOLAS et al., 2019), not many studies have explored the effect of maternal obesity in offspring weaned into an obesogenic diet (DE ALMEIDA FARIA et al., 2017; HUANG et al., 2020). There is, experimental studies have focused on the mismatch concept – the effects of maternal obesity during gestation and lactation on offspring fed a control diet. Since our population is becoming more obese and is currently exposed to an obesogenic environment, studies on the effect of maternal obesogenic diet and offspring obesogenic diet are necessary.

2.2 BREAST CANCER

It is estimated that most mammary tumors found in our population are adenocarcinomas, which are tumors originated from epithelial cells of the mammary glands (MAKKI, 2015). Additionally, most malignancies initiate in epithelial cells of the milk ducts (where milk is ejected) as opposed to lobules (where milk is produced). According to its histological features, breast adenocarcinomas can be further classified as *in situ* or invasive. The latter is diagnosed

when cancer cells have spread through the basal membrane to surrounding breast tissue (MAKKI, 2015).

Invasive ductal carcinomas are the most frequently diagnosed tumor of the breast. It is graded based on tubule/gland formation, mitotic count and nuclear pleomorphism. Tubule/gland formation is evaluated by how many cancer cells are in tubule formation – clear white space (lumina) surrounded by a continuous string of cell nuclei. A score 1 is when more than 75% of cells are in tubule formation and score 3 is when less than 10% are in tubule formation (worse prognosis). The number of mitotic cells - mitotic count – describes proliferation of cancer cells; score 1 is slowest and score 3 is the most rapid. Pleomorphism is how different in appearance is the nuclei of cancer cells compared to normal cells; grade 1 is closest to normal and grade 3 is the most different (TUTAC et al., 2008; LAKHANI et al., 2012; RUDMANN et al., 2012).

Invasive breast carcinomas can also be classified according to its molecular features. Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2) are proteins that can be expressed in tumor membranes and are targets/indicators of highly effective therapies agaisnt invasive breast cancer in the clinical setting (LAKHANI et al., 2012). ER positive tumors express genes specific to breast luminal cells, whereas ER negative tumors express genes specific to myoepithelial cells. Based on cluster analysis, tumors have been categorized as (a) basal-like tumors, which are ER negative tumors, expressing myoepitlelial/basal genes; (b) HER2-like tumors, which are ER negative tumors that overexpress HER2; (c) luminal A and luminal B, which are both ER positive tumors (LAKHANI et al., 2012).

Although there are known risk factors for breast cancer, all of the mechanisms involved in what will make cells aqcuire the eight hallmark caracteristics of cancerous cells (HANAHAN; WEINBERG, 2011), remains largely unknown. However, it is recognized that there is a strong hormonal component for luminal A and luminal B breast tumors, which represents 2/3 of diagnosed breast cancers. There are two pathways linking estrogen to breast carcinogenesis: the first involves estrogen linking to its receptor in non-cancerous cells, altering gene expression, increasing proliferation and, consequently, the likelihood of mutations (ROJAS; STUCKEY, 2016). The second pathway is linked to the oxidative metabolism of estrogen into quinone metabolites, which will then form DNA adducts (segment of DNA bound to a

carcinogenic molecule) or catechols. Catechols will originate reactive oxygen species and overtime, it will cause DNA damage (ROJAS; STUCKEY, 2016).

In rodent models, breast carcinogenesis can be chemically initiated with the Polyciclic Aromatic Hydrocarbon (PAH) dimethylbenz(a)anthracene (DMBA) (KERDELHUÉ; FOREST; COUMOUL, 2016). Although there are numerous PAH know as carcinogenic or as an endocrine disruptor, DMBA was early established as very effective on inducing mammary adenocarcinomas in Sprague-Dawleys (HUGGINS; GRAND; BRILLANTES, 1961). A single dose of intragastric DMBA is sufficient to promote breast adenocarcinomas in rats. Metabolites of DMBA interact with proliferating cells of the breast, localized in structures called Terminal End Buds (TEB), forming DNA adducts and causing mutations, subsequently initiating malignant cell transformation (KERDELHUÉ; FOREST; COUMOUL, 2016). This chemical model of breast cancer was further developed to include mice, particularly the C57BL/6 strain, which is considered a responsive model in studies of dietary induced obesity (NGUYEN et al., 2017; PINHEIRO-CASTRO et al., 2019). Chemically induced animal model of breast tumorigenesis is suitable because it produces similar characteristics to human breast tumors in terms of latency, histotypes and endocrine responssiveness (RUSSO et al., 1990).

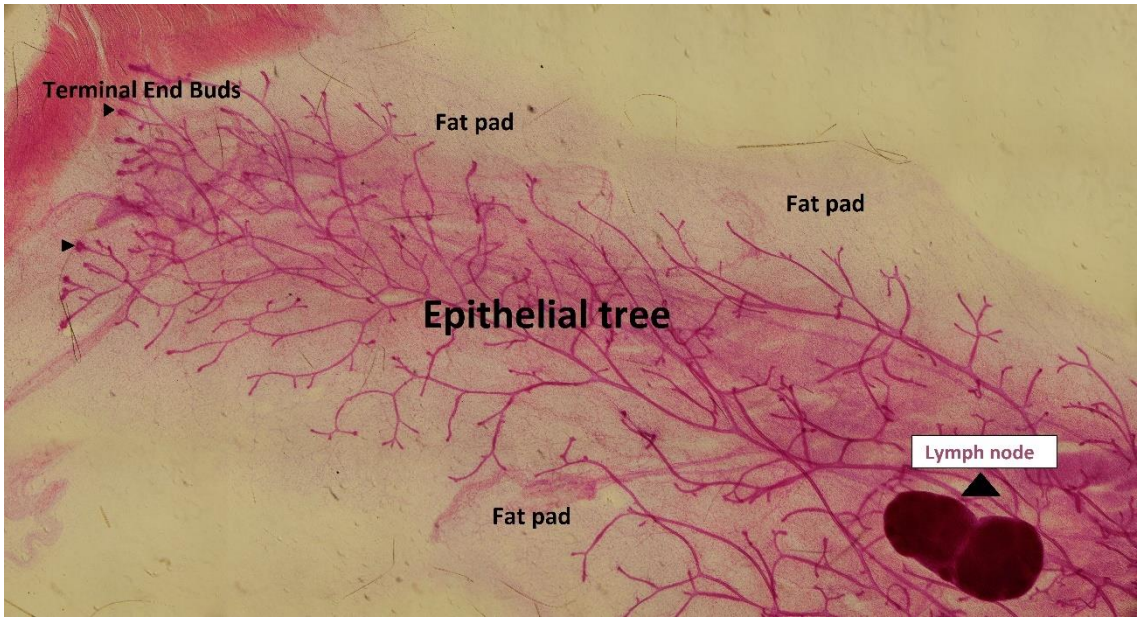
2.4 DEVELOPMENTAL ORIGINS OF HEALTH AND DISEASE – BREAST CANCER

Trichoupoulos first suggested that breast cancer could be originated *in utero* (TRICHOPOULOS, 1990) from observing an association between high birthweight, possibly caused by high hormonal exposure *in utero*, and breast cancer in adulthood (TRICHOPOULOS, 1990; HILAKIVI-CLARKE, 2014). As shown by Barker and colleagues (BARKER; OSMOND, 1986; HALES; P.BARKER, 1992), *in utero* environment is greatly influenced by maternal diet and nutritional status. Therefore, it is reasonable to accept that susceptibility to breast cancer can also be affected by maternal diet and nutritional status. Furthermore, although estrogen is mostly produced in the granulosa cells of the ovary, it is also synthesized by adipose tissue. Obese women, therefore, have another focus of estrogen production (GÉRARD; BROWN, 2018). Therefore, increased circulating concentrations of estrogen may be observed in obese women

when compared to normal weight women, which may also increase risk of breast cancer in their offspring.

There are many studies on the effects of an assortment of maternal obesogenic diets and its relation to breast carcinogenesis of female offspring. Most studies have indicated that offspring breast cancer risk can be programmed by maternal dietary intake. Hilakivi-Clarke et al. (HILAKIVI-CLARKE et al., 1997) observed that a diet rich in n-6 polyunsaturated fatty acids during gestation increased chemically-induced breast cancer risk in female rat offspring. The authors observed that offspring from high-fat fed mothers had increased mammary fat pad, denser epithelial tree and increased number of terminal end buds compared to control offspring. Our study group have observed, unexpectedly, that a lard-based high-fat diet during pregnancy and lactation protected female rat offspring from chemically induced breast carcinogenesis (DE OLIVEIRA ANDRADE et al., 2014). The number of terminal end buds of offspring of mothers fed high lard-based diet were lower than control group, as was the epithelial elongation (distance between lymph node and end of epithelial tree) (Figure 1).

Figure 1: Mammary gland wholemount



Representative image of mammary gland wholemount of a 7-week-old, nulliparous female. The mammary structures generally modified *in-utero* by maternal nutrition are represented here. Microscope picture 40X taken by the author.

It has been established that invasive ductal adenocarcinomas are originated from the hyperplasia of mammary gland epithelium (RUSSO; TAY; RUSSO, 1982b). Therefore, markers of development of this structure are of utmost importance in studies of Developmental Origins of Breast Cancer. Mammary gland of humans and rodents follow similar development and have similar structures (PAINE; LEWIS, 2017). Terminal End Buds, mammary structures found in rodents, are known as Terminal Ductal Lobular Unit in humans, where most diagnosed breast cancers originates (PAINE; LEWIS, 2017). In mammary glands of rodents and humans, increased density is also linked to increased risk of breast cancer (RUSSO; TAY; RUSSO, 1982a; NAZARI; MUKHERJEE, 2018). Measurement of epithelial elongation in rodents is also a marker of risk for breast cancer development, as it indicates increased development of mammary epithelium (RUSSO; TAY; RUSSO, 1982a).

Given the impact of a high-fat diet during gestation and lactation on breast cancer risk of female offspring, it is also important to understand other diet compositions on mammary gland development. A high-fat diet combined with a high sugar diet is more effective on inducing inflammation and insulin resistance compared to a high-fat diet only intervention (MASI et al., 2017; PINHEIRO-CASTRO et al., 2019). Offering sugared condensed milk combined with a high-fat diet has proven successful on programming offspring to chronic diseases in adulthood (SAMUELSSON et al., 2007; LOCHE et al., 2018). Its programming effects on offspring breast cancer risk, however, is unknown. Additionally, this highly palatable diet better represents the obesogenic environment to which our population is exposed to when compared to an exclusive high-fat diet (PINHEIRO-CASTRO et al., 2019).

2.5 ORANGE JUICE

Orange juice is one of the most consumed fruit juices in the world and Brazil is the largest producer of oranges, responsible for the harvest of approximately 17 thousand tons in 2017 (FAO, 2017a). In the same year, China, the second largest producer, was accountable for the harvest of 8 thousand tons of oranges (FAO, 2017a). Although production of oranges is larger in Brazil, Spain is the larger exporter of the fruit (FAO, 2017a). Brazil export its oranges as

orange juice. A preliminary report of the Food and Agriculture Organization attributed approximately 88% of all exported concentrated orange juice to Brazil in 2016 (FAO, 2017b).

Orange juice is an excellent dietary source of bioactive food compound, aside from being recognized as the most micronutrient-dense fruit juice (FRANKE et al., 2013; BRASILI et al., 2019). Hesperidin, narirutin and didymin are the most abundant flavonoids in orange juice and, its associations with other nutrients of orange juice, such as vitamin C, β-carotene, α-carotene and β-cryptoxanthin, are responsible for its antioxidant, anti-obesogenic and cancer preventative properties (FRANKE et al., 2013; RAMPERSAUD; VALIM, 2017; BRASILI et al., 2019). The interest in orange juice remains growing: The Bank of Registry of Clinical Trials have 27 studies presently testing interventions with orange juice (CLINICAL TRIAL REGISTRY, 2019).

Aside from the studies currently underway, there are published studies on the benefits of orange juice consumption on attenuating the harmful effects of obesity. Lima et al. (2019) reported that daily intake of 300mL of orange juice reduced glucose, insulin, triglycerides and LDL-cholesterol compared to basal results in normal weight women. Another study showed that the pro-inflammatory effect induced after intake of a high saturated fatty acid meal was reduced by associating the meal with a 500 mL/day of orange juice in healthy participants (ROCHA et al., 2017).

There are no reports of orange juice intake during pregnancy nor how it may be metabolized by the placenta to reach the fetus. A recent *in vitro* study, with extravillous trophoblasts, which are invasive, highly proliferative and migratory cells, that will be remodeled into the high flow arteries of the placenta and guarantee fetal well-being, showed that upon induced oxidative stress, treatment with a combination of polyphenols, including hesperidin, reduced oxidative stress and promoted adequate trophoblast development (EBEGBONI et al., 2019). The authors further suggests that intake of quercetin or hesperidin during early pregnancy can improve health and function of the placenta (EBEGBONI et al., 2019). A much earlier experimental study (SCHRÖDER-VAN DER ELST et al., 1998), administered via intravenous injection a synthetic, marked flavonoid in 20-day pregnant rats and found that, 24 hours later, 17% of the synthetic flavonoid were in the fetal compartment. The authors concluded that flavonoids can cross the placenta and enter fetal tissues, where they reach higher concentrations than in maternal tissues (SCHRÖDER-VAN DER ELST et al., 1998).

Therefore, using orange juice during gestation and lactation, particularly in the obesogenic context to what women are currently exposed, could be a promising approach to prevent programming effects of maternal obesity on metabolic programming of male offspring exposed to an obesogenic diet and in programming breast cancer of female offspring.

3. OBJECTIVES

3.1 GENERAL OBJECTIVES

To evaluate the effects of maternal obesity during gestation and lactation and orange juice intake on the metabolic profile of male offspring exposed to control and obesogenic diets and breast cancer risk of female offspring.

3.2 ESPECIFIC OBJECTIVES

To evaluate if a maternal obesogenic diet and orange juice intake:

(a) increased weight and fat mass of male mice offspring exposed to an obesogenic and control diets;
(b) produced an internal pro-oxidant metabolic state of male mice offspring exposed to an obesogenic and control diets;
(c) increased production of pro-inflammatory cytokines in adipose tissue of male mice offspring exposed to an obesogenic and control diets;
(d) changed mammary gland development;
(e) changed development of mammary tumors in female offspring submitted to chemical induction of mammary carcinogenesis by 7,12-dimethylbenz[a]anthracene (DMBA).

4. MATERIALS AND METHODS

4.1 EXPERIMENTAL DESIGN AND ANIMAL MANIPULATION

This research project was approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo, Brazil (Protocols CEUA/FCF/USP/505 and 527).

Four-week old female (n = 90) and male (n = 39) 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 during experimentation. Female mice were randomly divided into 3 groups (n = 30/group), that received control and obesogenic (the latter with water or orange juice *ad libitum*) diets (Table 1) for 11 weeks (3 weeks before mating + 2 weeks of mating +3 weeks of gestation + 3 weeks of lactation), as follows:

(a) control – received AIN-93G diet + water *ad libitum*;
(b) obese – received a high lard-based diet containing 20% of lard, 10% sugars, 28% polysaccharide, 23% protein [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w] + water *ad libitum*;
(c) obese + orange juice – received a high lard-based diet containing 20% of lard, 10% sugars, 28% polysaccharide, 23% protein [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w] + orange juice *ad libitum*.

The dietary intervention/protocol was adapted from Samuelsson et al. (2007).

Mating was performed by housing one male with two females per cage. At day 4 *post-partum*, litter was standardized in order to maintain 6 pups with their respective dams (4 females: 2 males, whenever possible).

At weaning, female offspring was given a control AIN-93M diet until 7 weeks of age. At 7 weeks old, part of female offspring was culled for analysis of mammary gland morphology and the other part was submitted to the chemically induced carcinogenesis protocol.

At weaning, male offspring from each group were randomly assigned into two groups: control and obesogenic diets (Table 1), as follows:

(a) Offspring from control mothers fed a control diet – male offspring were fed a control AIN-93G diet + water *ad libitum*;
(b) offspring from control mothers fed an obesogenic diet – male offspring were fed a high lard-based diet containing 20% of lard, 10% sugars, 28% polysaccharide, 23% protein [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w] + water *ad libitum*;
(c) offspring from obese mothers fed a control diet - male offspring were fed a control AIN-93G diet + water *ad libitum*;
(d) offspring from obese mothers fed an obesogenic diet – male offspring were fed a high lard-based diet containing 20% of lard, 10% sugars, 28% polysaccharide, 23% protein [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w] + water *ad libitum*;
(e) offspring from obese + orange juice mothers fed a control AIN-93G diet – male offspring were fed a control AIN-93G diet + water *ad libitum*;
(f) offspring from obese + orange juice mothers were fed an obesogenic diet – male offspring were fed a high lard-based diet containing 20% of lard, 10% sugars, 28% polysaccharide, 23% protein [w/w], supplemented with sweetened condensed milk fortified with mineral and vitamin mix [w/w] + water *ad libitum*.

During experimentation, body mass and food intake were recorded 3 times a week for all mice. Diets, orange juice and water were changed every other day. Details on the composition of the diets used are summarized in Table 1. All commercial orange juice and sweetened condensed milk used were from the same production batch. Nutritional information of the commercial sweetened condensed milk and orange juice are summarized in tables 2 and 3, respectively. The study protocol is detailed in Figure 2.

When the experiment protocol was concluded, animals were anesthetized by inhalation of the anesthetic isoflurane and exsanguinated by cardiac puncture. Plasma was collected in EDTA microtubes, and, after centrifugation and separation, was stored at −80°C until processed. The tissues were frozen in liquid nitrogen and stored at −80°C until processed.

Table 1 – 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

Table 2 – Nutritional information of the commercial orange juice

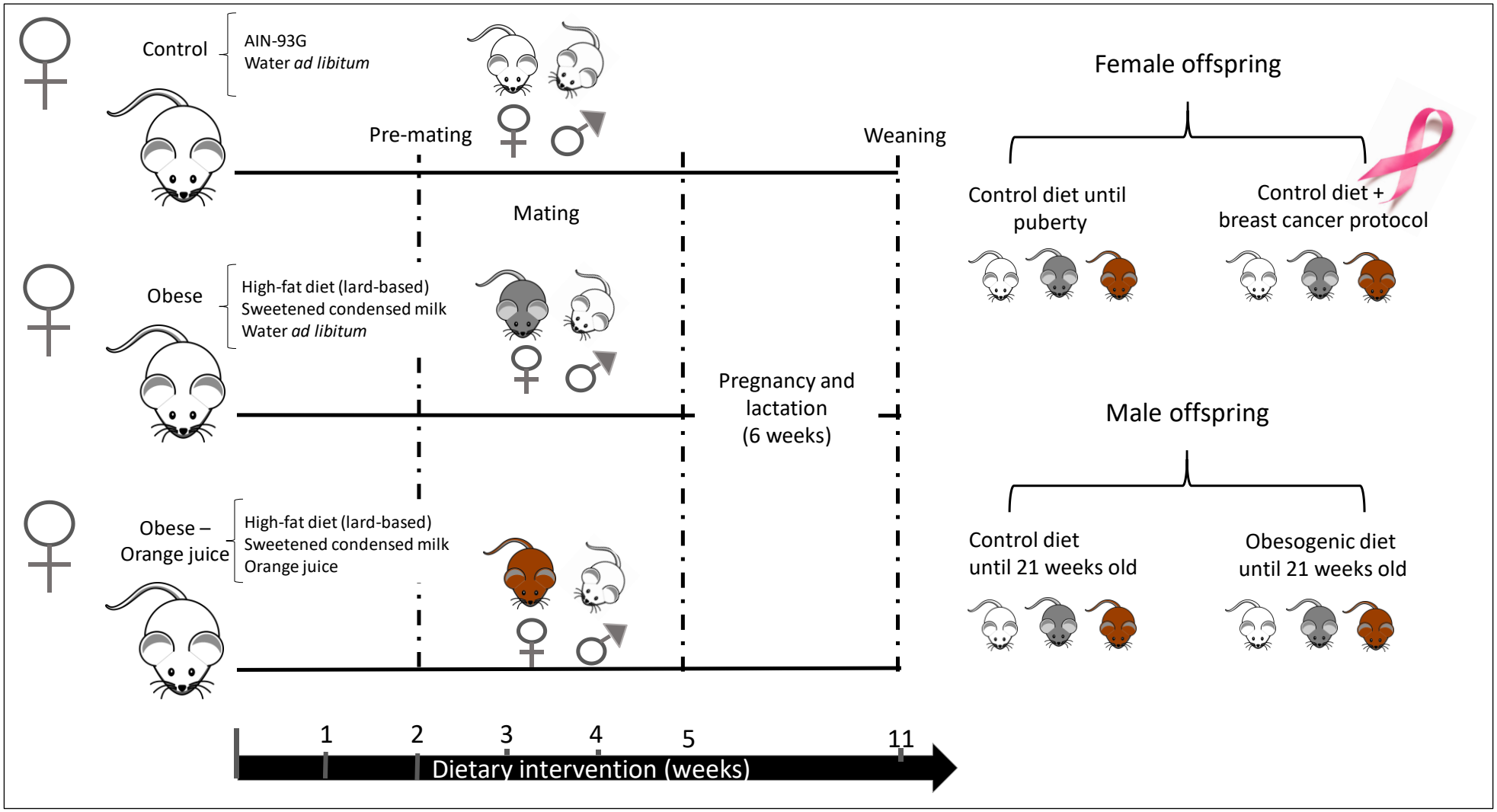
Orange juice	
Carbohydrates	0.10g/mL
Energy	0.80kcal/mL

*Vitamin C is added to the orange juice (0.30mg/mL)

Table 3 – Nutritional information of the sweetened condensed milk

g/g	Sweetened condensed milk
Carbohydrates	0.55
Proteins	0.07
Total fat	0.08
Saturated fatty acids	0.05
Energy	3.25Kcal/g

Figure 2: Study protocol



Protocol designed by the author.

4.2 VITAMIN AND MINERAL MIX SUPPLEMENTATION

The mineral and vitamin mix were weekly supplemented to obesogenic groups according to the average daily consumption of diets by each experimental group in the previous week. Steps for calculation are described below:

(a) Average of mineral and vitamin mix intake by the control and obesogenic groups were calculated (considering quantities of mineral and vitamin mix present in control and high-fat diets).
(b) Calculation of the difference in average mineral and vitamin mix intake between control and obesogenic groups.
(c) Calculation of the average consumption of condensed milk by obesogenic groups.
(d) Dilution of the difference of mineral and vitamin mix in the sweetened condensed milk.

4.3 ANALYSIS OF ORANGE JUICE

4.3.1 Antioxidant activity of orange juice

4.3.1.1 Antioxidant activity by the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH)•

After homogenization and centrifugation (10min, 8000rpm, 4ºC) of orange juice, soluble fraction was filtered and it was added DMSO to the insoluble fraction. Samples were homogenized and remained under agitation for 18 hours. After novel centrifugation (10min, 8000rpm, 4ºC), samples were filtered. Twenty-five microliters of samples, in triplicate, were pipetted and homogenized with DPPH• solution. The mixture was incubated in a microplate reader (Synergy HT, BioTek Instruments, Inc., USA) for 30 min in the dark and absorbance reading was performed at 515nm. Results are presented as µmoL of Trolox equivalent/L, as previously described by Brand Williams et al. and Silva (BRAND-WILLIAMS; CUVELIER; BERSET, 1995; SILVA, 2019).

4.3.1.2 Antioxidant Activity by Oxygen Radical Absorbance Capacity (ORAC)

After homogenization and centrifugation (10min, 8000rpm, 4ºC) of orange juice, soluble fraction was filtered and it was added DMSO to the insoluble fraction. Samples were homogenized and remained under agitation for 18 hours. After novel centrifugation (10min, 8000rpm, 4ºC), samples were filtered and read in a Synergy HT microplate reader (BioTek Instruments, Inc., USA). Following addition of 150 µL of fluorescein to the wells, samples were incubated for 30 minutes. Reading was carried out at 485nm excitation and 528nm emission, after addition of of AAPH 2,2'-azobis (2-amidinopropane) dihydrochloride solution. It was used the Trolox calibration curve. Results are presented as µmoL Trolox equivalent/L, as previously described by Huang et al.(2002) and Silva (2019).

4.3.2 Identification and quantification of flavonoids in orange juice

4.3.2.1 Extraction of flavonoids

The soluble fraction of flavonoids was extracted by homogenizing and centrifuging (10min, 8000rpm, 4ºC) 10 mL of orange juice. The soluble fraction (supernatant) was filtered and estimated by HPLC-DAD (further description in item 4.3.2.2). Twenty milliliters of DMSO was added to the insoluble fraction (precipitate), followed by homogenization and 18 hours of agitation. Samples were centrifuged (10min, 8000rpm, 4ºC), filtered and estimated by HPLC-DAD (further description in item 4.3.2.2). This method has been previously described by Silva (2019).

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

Identity and quantity of phenolic compounds was determined with a 1260 Infinity Quaternary LC System (Agilent Technologies, USA). Detection was performed with a diode array detector, which was coupled to an autosampler and a quaternary pump. It was used a Prodigy 5µ ODS3 column (250x4.60mm; Phenomenex Ltd., United Kingdom) at a flow rate of 1mL/min, 25 °C. Further details of this method is available in the study of Hassimoto et al. (2008). Peaks were identified by similarity of absorption spectra with calibration curve of commercial flavonoids

and phenolic acid standards (Extrasynthese, Genay, France and Sigma-Aldrich), in addition to mass spectra.

4.3.2.3 High performance liquid chromatography coupled to mass/mass spectrometer (LC-ESI-MS/MS)

It was used a Prominence Liquid Chromatograph (Shimadzu, Japan) attached to an ion trap Esquires-LC mass spectrometer (Bruker Daltonics, Billerica, MA) with an electrospray ionization (ESI) interface for identification of flavonoids present in the orange juice (TEIXEIRA et al., 2017). After going through the diode array detector, flow rate was changes to 0.2 ml/min for application to the mass spectrometer. To detect narirutin and hesperidin, the ESI was set in the positive mode. The negative mode was used for detection of other classes of flavonoids. The mass spectrometer settings were as detailed by Teixeira et al. (TEIXEIRA et al., 2017) and analysis was performed with a full scan from *m/z* 100 to 1500. Compounds were identified by comparing results of retention times with commercial standards, absorption spectrum similarity and mass spectral characteristics, also described by Silva (2019).

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4.3.3 Quantification of total phenolics in orange juice

Phenolics were determined by Folin-Ciocalteau (SWAIN; HILLIS, 1959). This is a spectrophotometric method based on phenolics compound reduction of Folin-Ciocalteau reagent into a blue staining product (molybdenum and tungsten) in alkaline medium. To the soluble fraction of orange juice was added 250 µL Folin reagent and 2.0 mL deionized water, followed by the addition of 250 µL of saturated sodium carbonate solution. The mixture was kept in a 37°C water bath for 30 min and absorbance read on a spectrophotometer at 765 nm (Hewlett Packard, 8453). Total phenolic content was presented as gallic acid equivalent (GAE) using a calibration curve – mg GAE equivalent/100mL.

4.3.4 Identification and quantification of organic acids in orange juice

The soluble fraction of orange juice was diluted with metaphosphoric acid and filtered with a 0.45 µm membrane. Samples were inserted into vials and analyzed by high performance liquid chromatography (Hewlett Packard Series 1100) with a SUPELCOGEL C-610H column (30 cm X 7.8 mm). Details on the reagents used and equipment settings are available by Silva (SILVA, 2019). Identification and quantification of organic acids were determined by comparing results with the calibration curves of ascorbic, citric and malic acids. Results are expressed as mg/100mL of orange juice.

4.4 BODY COMPOSITION OF THE STUDIED ANIMALS

Body composition of the studied animals was estimated by computed tomography (Albira PET-SPECT-CT, Bruker Biospin Corp., Woodbridge, CT, USA). For obtention of X-ray CT imaging, mice were individually anesthetized in a closed cage with 3% isoflourane and subsequently moved to an animal bed where inhalatory anesthesia was maintained by a nose cone. Six hundred projections were collected at a 45kV with a 400µA current and reconstructed with the Albira software. Data was analyzed with software PMOD (Zurich, Switzerland) as described by Wathen (WATHEN et al., 2013). Imaging data output was presented as Hounsfield units (HU).

4.5 INTRAPERITONEAL GLUCOSE TOLERANCE TEST (IGTT) OF THE STUDIED ANIMALS

Female mice (mothers), female offspring and male offspring underwent the intraperitoneal glucose tolerance test. Following an 8-hour-fast, animals had their glucose measured with a glucometer (AccuCheck Performa Nano) by caudal puncture (AccuCheck Performa Nano). Animals received an intraperitoneal injection of a dextrose solution (2g/kg body weight) and blood glucose was measured repeatedly at 15, 30, 60, 90 and 120 minutes after injection. From results, data was organized in graphs and area under the curve (AUC) was calculated (TAKADA et al., 2008).

4.6 DETERMINATION OF BLOOD ESTRADIOL OF FEMALE OFFSPRING

Blood estradiol concentrations were determined *via* chemiluminescence with an automated UniCel dxI 800 (Beckman Coulter) in female offspring of control, obese and obese + orange juice mothers.

4.7 DETERMINATION OF ABSOLUTE AND RELATIVE WEIGHTS OF THE ORGANS OF THE STUDIED ANIMALS

Absolute weights of kidney, liver, heart and adipose tissue were measured with an analytical scale immediately after euthanasia. The relative weight of organs was calculated by dividing each organs weight by the last registered measurement of animal´s weight.

4.8 MALONDYHALDEIDE (MDA) CONCENTRATION IN LIVER OF THE STUDIED ANIMALS

Twelve and a half microliters of 0.2% butylated hydroxytoluene and 6.25 µl of 10 N NaOH were added to liver homogenate samples (0.05 ml). Twenty microliters of the mixture, in triplicate, were injected in the HPLC (Agilent Technologies 1200 Series) in a reverse-phase analytical column (250 mm × 4.6 mm; 5 µm; Phenomenex) with an LC8-D8 pre-column (Phenomenex AJ0-1287) (NOGUEIRA et al., 2016). MDA was fluorometrically quantified by reverse-phase HPLC, as described by Hong et al. (2000), at an excitation of 515 nm and emission of 553 nm. Further settings and solutions was described by Nogueira et al. (2016). Results are presented as ηmol MDA/mg protein.

4.9 ANTIOXIDANT ACTIVITY OF ENZYMES IN LIVER OF THE STUDIED ANIMALS

4.9.1 Superoxide Dismutase (SOD) activity

To a microplate already containing 200µl of 0.1mM of ethylenediamine tetraacetic acid (EDTA), 62µM nitrotetrazolium blue chloride (NBT) and 98µM nicotinamide adenine dinucleotide (NADH), was added 25µL of liver homogenate, in triplicate. Twenty-five microliters of a 33µM phenazine methosulphate (PMS) (pH 7.4) containing 0.1mM EDTA initiated the chemical reaction. Absorbance, as index of NBT reduction, was set at 560 nm and analyzed with a BioTek plate reader (Multi-Detection; Synergy) coupled with Gen 5 software. A standard curve was prepared using SOD (Sigma Chemical Co.) (1.5625-25U/ml) as reference. This method has been previously described by Ewing and Janero (EWING; JANERO, 1995).

4.9.2 Glutathione Peroxidase (GPx) activity

Thirty microliters of liver homogenate were incubated at 37°C for 5 min with a 125µl of 0.1 M phosphate buffered saline (PBS) pH 7.4 with 1 mM EDTA, 5 µl of 0.08 M GSH and 5 µl of GR (9.6 U), as described by Nogueira et al. (2016). Into each well were added 30 µl of 1.2 mM NADPH and 5 µl of 0.46% tert-butylhydroquinone (TBHQ). Absorbance was read with a BioTek plate reader (Multi-Detection; Synergy) coupled with Gen 5 software, at 340 nm at 37°C. GPx activity was determined as described by Flohé and Gunzler (1984), with commercial GPx as reference (Sigma Chemical Co.) (0.5-6 U/mg ptn).

4.9.3 Catalase (CAT) activity

To the wells of a UV microplate containing 140 µl of 0.1 M PBS pH 7.4 with 1 mM EDTA, it was added 20 µl of liver homogenate followed by 40 µl of a 30 mM hydrogen peroxide solution. Reading was performed with an absorbance set at 240 nm and 30°C, with a BioTek plate reader (Multi-Detection; Synergy) coupled with Gen 5 software. The calibration curve was prepared from known standards. This methodology was previously described by Bonaventura et al.(1972), Nabavi et al. (2012) and Silva (2019).

4.10 DETERMINATION OF INFLAMMATION'S GENE EXPRESSION IN ADIPOSE TISSUE OF STUDIED ANIMALS

The expression of the following genes: 18S, F4/80, IL-6, TNF-α, TLR-4, leptin and adiponectin were estimated in retroperitoneal adipose tissue of all studied animals. For this analysis, approximately 100 mg of liquid nitrogen-sprayed adipose tissue was homogenized in TRIZOL reagent for total RNA extraction, as described by Chomzynski and Sacchi (CHOMZYNSKI; SACCHI, 1987). One microliter of the solution was placed in a Nano Drop 2000 apparatus (Thermo Scientic, Uniscience, São Paulo, Brazil) for RNA quantification. If samples presented a ratio 260/280nm over 2, cDNA was synthetized with reverse transcriptase from 1µg of RNA. Gene expression profile was determined with QuantStudio 7 Flex™ Real-Time PCR System (Life Technologies, USA) as described by Kubista et al. (2006), using SYBER Green reagent (Invitrogen, Life Technologies) as the fluorescent marker. Primers investigated are detailed in Table 4. Quantification was determined by calculating the standard curve for every gene used, considering 18S expression as control of the adipose tissue.

Table 4 – Sequence of primers used to evaluate gene expression in retroperitoneal adipose tissue

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' – CTTCCATCCAGTTGCCTTCTTG – 3'
		RV 5' – AATTAAGCCTCCGACTTGTGAAG – 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'

Abbreviations: IL - interleukin, TNF-α - tumor necrosis factor alpha, TLR - toll like receptor; 18S – Housekeeping gene. All primers were purchased from Integrated DNA Technologies.

4.11 REPRODUCTION PARAMETERS

To evaluate reproduction among females from each group, the following parameters were observed:

(a) Length of time to impregnate;

(b) number of successful gestations per group, females never impregnated, as well as cannibalism count and other occurrences (such as uterine prolapse and death during birth);

(c) average litter size.

4.12 HARVEST OF MAMMARY GLAND OF 7-WEEK-OLD FEMALE OFFSPRING

Abdominal mammary glands of female offspring of experimental groups were collected from 7-week-old female offspring and used for preparing mammary gland wholemounts. Briefly, left abdominal mammary gland was extracted from females and placed in a glass slide. It was then immersed in a solution of Carnoy´s Fixative (75% glacial acetic acid and 25% absolute ethanol) for two days. Mammary gland went through a 70% ethanol solution and distilled water, followed by a Carmine solution for staining. After immersed in several ethanol solutions, mammary gland is immersed in xylene for lipids removal and transparency. The whole process is detailed by de Assis et al. (2010).

4.13 MAMMARY GLAND MORPHOLOGY AND DEVELOPMENT IN 7-WEEK-OLD FEMALE OFFSPRING

Mammary wholemount was used for measurement of epithelial elongation, which is the distance between lymph nodes and the end of the epithelial tree, with a ruler, in millimeters, as described by de Assis et al. (2010). The number of terminal end buds was also counted with the aid of a microscope, as described by de Assis et al., (2010).

4.14 MAMMARY GLAND CELL PROLIFERATION AND APOPTOSIS IN 7-WEEK-OLD FEMALE OFFSPRING – IMMUNOHISTOCHEMISTRY

Immediately after harvest, mammary gland was fixed in 10% buffered formalin. Subsequently, tissue was paraffined and sliced. After deparaffination in xylene and hydration with ethanol, slides were immersed in a 10mM citrate buffer solution and placed in a pressure cooker set at pH 6 for 20 minutes for retrieval of antigen. Blockage with peroxidase was carried out for

10 minutes and nonspecific binding was blocked with 1% skimmed milk in PBS for one hour. Anti-mouse Ki67 primary antibody (Cell Signaling) at a 1:200 dilution was dripped over slices and incubated overnight. Slices were then incubated with the LABS + System – HRP Kit (Dako-Agilent Technologies, USA) and stained with 3,3'-diaminobenzidine (Dako-Agilent Technologies, USA) for 2 min (SILVA, 2019). Slices were immersed in water and hematoxylin for 1.5 minute. Proliferation was quantified by assessing number of Ki67 positive cells amongst 1,000 counted cells. This method has been previously described by Silva (2019). Apoptosis cell count was performed according to Elmore et al. (2016) and results are presented as the average number of apoptotic cells per 1,000 counted cells.

4.15 CHEMICALLY INDUCED MAMMARY CARCINOGENESIS

At post-natal week six, 15mg/100µL of medroxyprogesterone acetate was intraperitoneally injected to female offspring. One week later, four weekly doses of 7,12-dimethylbenz[a]anthracene diluted in corn oil (1mg/100µL; Sigma, USA) were administered orally to mice. This method has been previously described by de Assis et al. (2011), Fontelles et al. (2016) and Silva (2019). Palpation was carried out twice per week, starting at week three after the last DMBA administration and continued for a total of 18 weeks. Animals were euthanized three weeks after the appearance of the first tumor. The end-points for data analysis were (a) latency, which is the number of days until the appearance of the first tumor; (b) incidence, which is the number of animals with tumors; and (c) multiplicity, which is the number of tumors per animal.

4.16 HISTOPATHOLOGICAL ANALYSIS OF MAMMARY TUMORS

Histological slides of tumors stained with hematoxylin and eosin were evaluated under a microscope for histopathological marks that characterizes different types of tumors. Classification was carried out according to Rudmann et al., (2012).

4.17 STATISTICAL ANALYSIS

The statistical analysis was carried out with GraphPad Prism 6.0 (GraphPad software Inc, California, USA). All data were tested for normality, and Tukey or LSD test was used accordingly with two-way ANOVA or one-way ANOVA. Survival statistics were calculated with Kaplan-Meier and long-rank test to determine tumor incidence. A $p \leq 0.05$ was used as threshold for statistical significance and data are presented as mean and standard error of the mean (SEM).

5. RESULTS

5.1 ORANGE JUICE ANALYSES

5.1.1 Antioxidant activity of orange juice

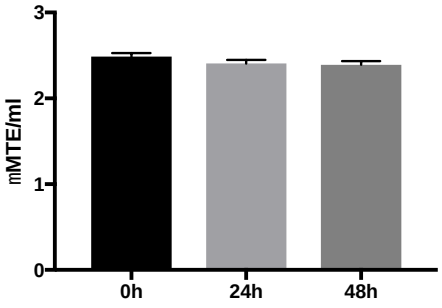
The results of antioxidant activity of orange juice evaluated by both DPPH and ORAC are summarized in Figure 3A. It was also demonstrated that antioxidant activity of orange juice was not altered in two days of exposure, which was the longest period of time orange juice remained at the cages.

Figure 3: Antioxidant activity of orange juice

A

	μmol TE/L
DPPH	2.48 (0.10)
ORAC	7.53 (0.19)

B

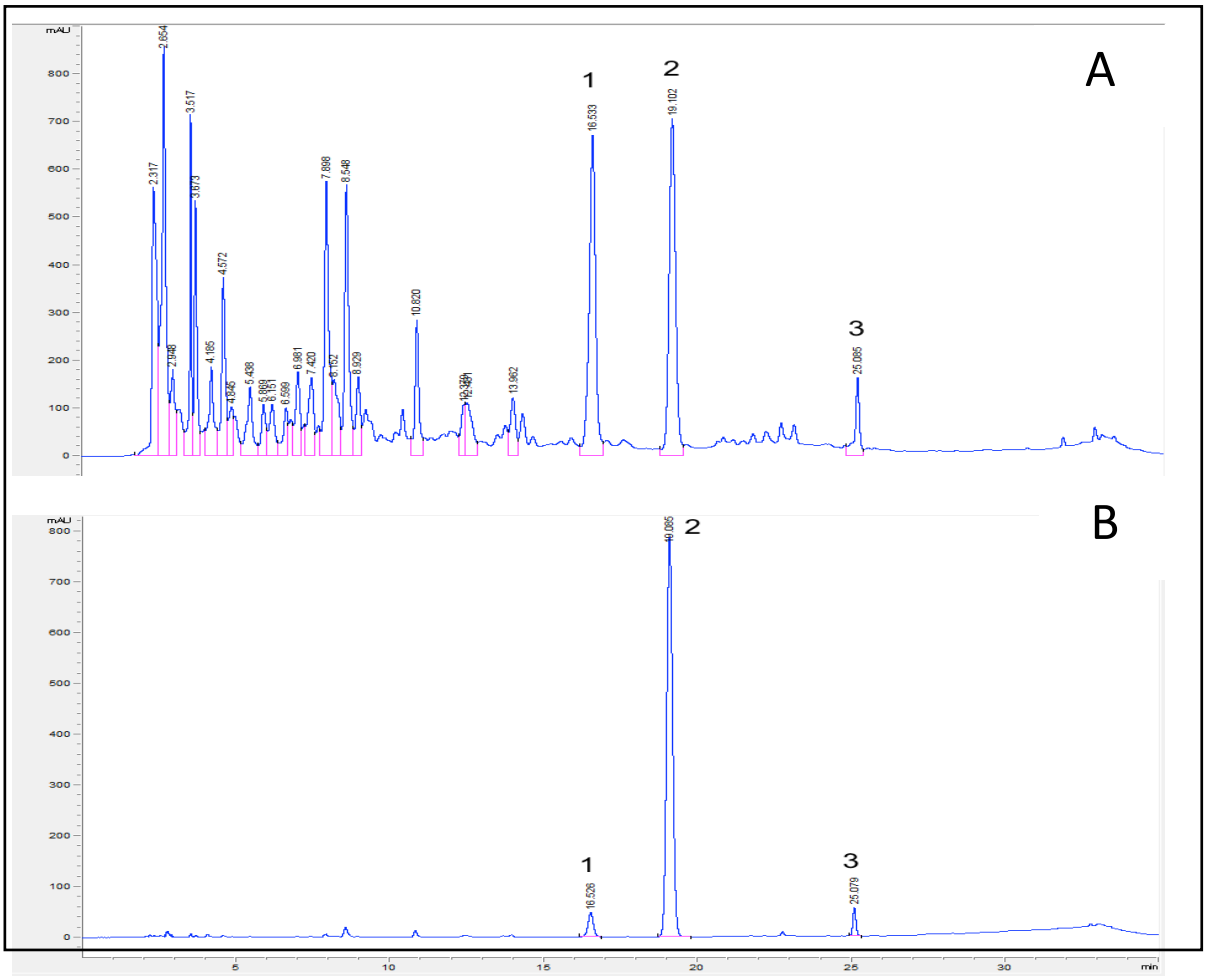


(A) Results of the antioxidant capacity of orange juice as evaluated by the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) and Oxygen Radical Absorbance Capacity (ORAC). Results are presented as mean and standard error of the mean; (B) antioxidant capacity of orange juice, measured by DPPH method, during the time of exposure of orange juice at the animal cages (at 0 hours, 24 hours, 48 hours). Result is expressed as mean and standard error of the mean. Figure reproduced with consent from Silva (2019).

5.1.2. Identification of flavonoids in orange juice

Three major flavonoids (Figure 4A and B) were identified in the supernatant fraction and in the insoluble (precipitated) fraction. The three identified peaks correspond to narirutin, hesperidin and didymin. Identities were confirmed by co-elution with respective standards.

Figure 4:Identification of flavonoids in orange juice



HPLC-DAD chromatogram (280 nm) of the soluble (supernatant) (A) and insoluble (precipitated) fractions (B) of orange juice. The peaks numbered 1, 2 and 3 in both chromatograms are narirutin, hesperidin and didimin, respectively. Figure reproduced with consent from Silva (2019).

5.1.3 Chemical and flavonoid composition of orange juice

The sugars identified in orange juice were glucose, sucrose and fructose, which totaled 70.60ug/mL of sugar in the juice (Table 5). There were two organic acids identified in the juice, citric and ascorbic (Table 5). The three major flavanones identified in orange juice were hesperidin, narirutin and didymin and their quantification are presented in Table 5.

Table 5 – Chemical and flavonoid composition of orange juice

Compounds	**Orange Juice**
Soluble Sugars (µg/ml)	
Glucose	18.60 (0.17)
Sucrose	20.30 (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 (mEq gallic acid/L)	404.70 (2.57)

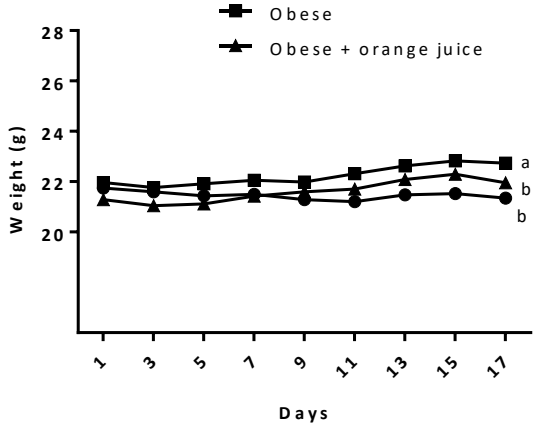
Result presented as mean and standard error of the mean.

5.2 EFFECTS OF THE DIATARY INTERVENTIONS ON MATERNAL CHARACTERISTICS

Immediately before mating, the effects of the 3-week dietary intervention were assessed in female mice. Females offered the obesogenic diet had increased weight in relation to females fed the control diet (Figure 5A). Obese + orange juice females did not gain as much weight as obese females (Figure 5A). It was also observed increased energy intake by the obese group when compared to both control and obese + orange juice groups (Table 5B).

Figure 5: Weight gain and energy intake of females (mothers)

A



B

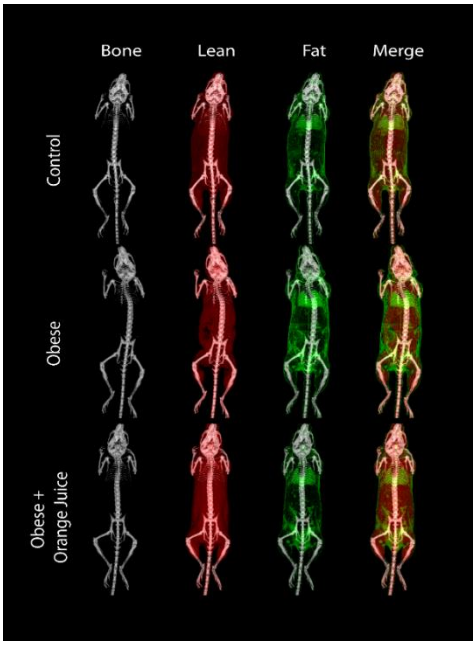
	Mean (SEM)
Control (kcal)	11.88 (0.09)[a]
Obese (kcal)	12.64 (0.13)[b]
Obese + orange juice (kcal)	11.99 (0.09)[a]

(A) Mean weight gain of females from each experimental group, after dietary interventions and until mating. (B) Mean total energy intake after 3 weeks of intervention. Data presented as mean and standard error of the mean (SEM). ANOVA followed by Tukey test (30/group). Figure designed by the author.

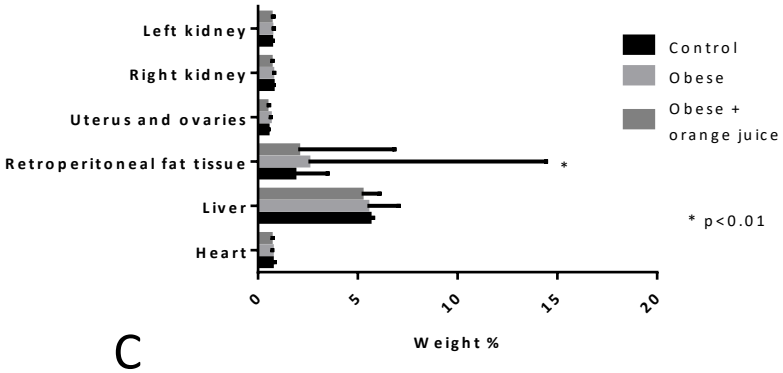
Body composition assessment showed that females from the obese group had increased fat mass percentage when compared to control females (Table 6A-B). There were no differences in bone mass percentage between groups. At culling, females from the obese group presented increased relative retroperitoneal fat mass compared to control and obese + orange juice groups.

Figure 6: Body composition assessment of females and vital organs weight

A



B



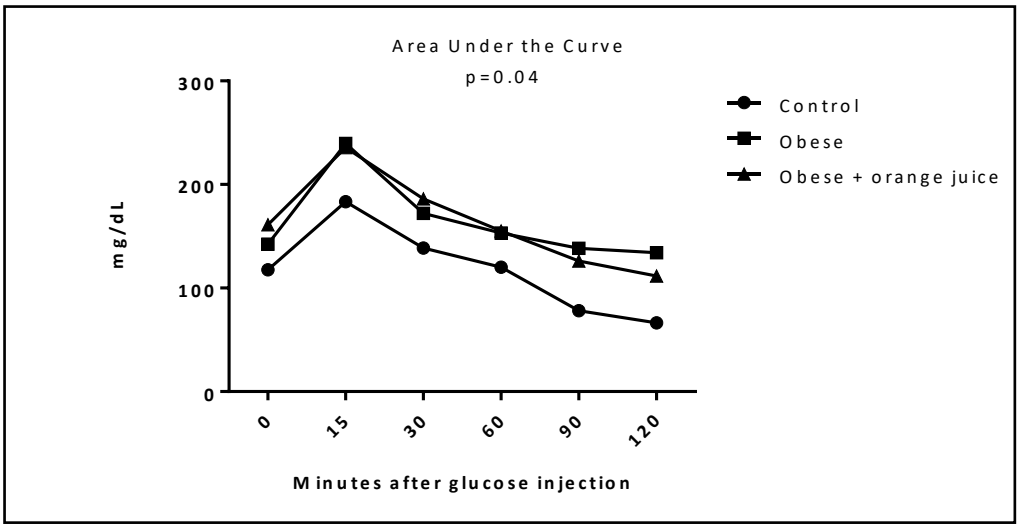
C

	Control	Obese	Obese + orange juice
Fat mass %	8.3 (0.4)[a]	10.9 (0.7)[b]	10.0 (0.6)[a,b]
Lean mass %	81.5 (0.2)[a]	79.3 (0.7)[b]	80.4 (0.6)[a,b]
Bone mass %	7.0 (0.2)	6.6 (0.1)	6.5 (0)

(A) Representative images of females´ body composition assessment by computed tomography (CT) after three weeks on the respective diets. The figure shows bone mass, lean mass (red) and fat mass (green); (B) weight of female´s vital organs in relation to their own body weight (relative weight – g/g); (C) results of body composition assessment by CT. Data are presented as mean and standard error of the mean (SEM). Two-way ANOVA followed by the Tukey test (n=4/group). Figure designed by the author.

Intraperitoneal glucose tolerance test showed that females fed the obesogenic diets presented increased blood glycemia at all time points compared to control females (Figure 7).

Figure 7: Intraperitoneal Glucose Tolerance Test of females (mothers)



Intraperitoneal Glucose Tolerance Test of females before mating. Two-way ANOVA followed by Fischer´s LSD test (n=6/group). Figure made by the author.

There were no indications of oxidative stress on females' hepatic tissues. Malonaldehyde (MDA) and antioxidant enzymes (Table 6) were similar amongst the studied groups.

Table 6 – Estimated oxidative stress in the liver of females (mothers)

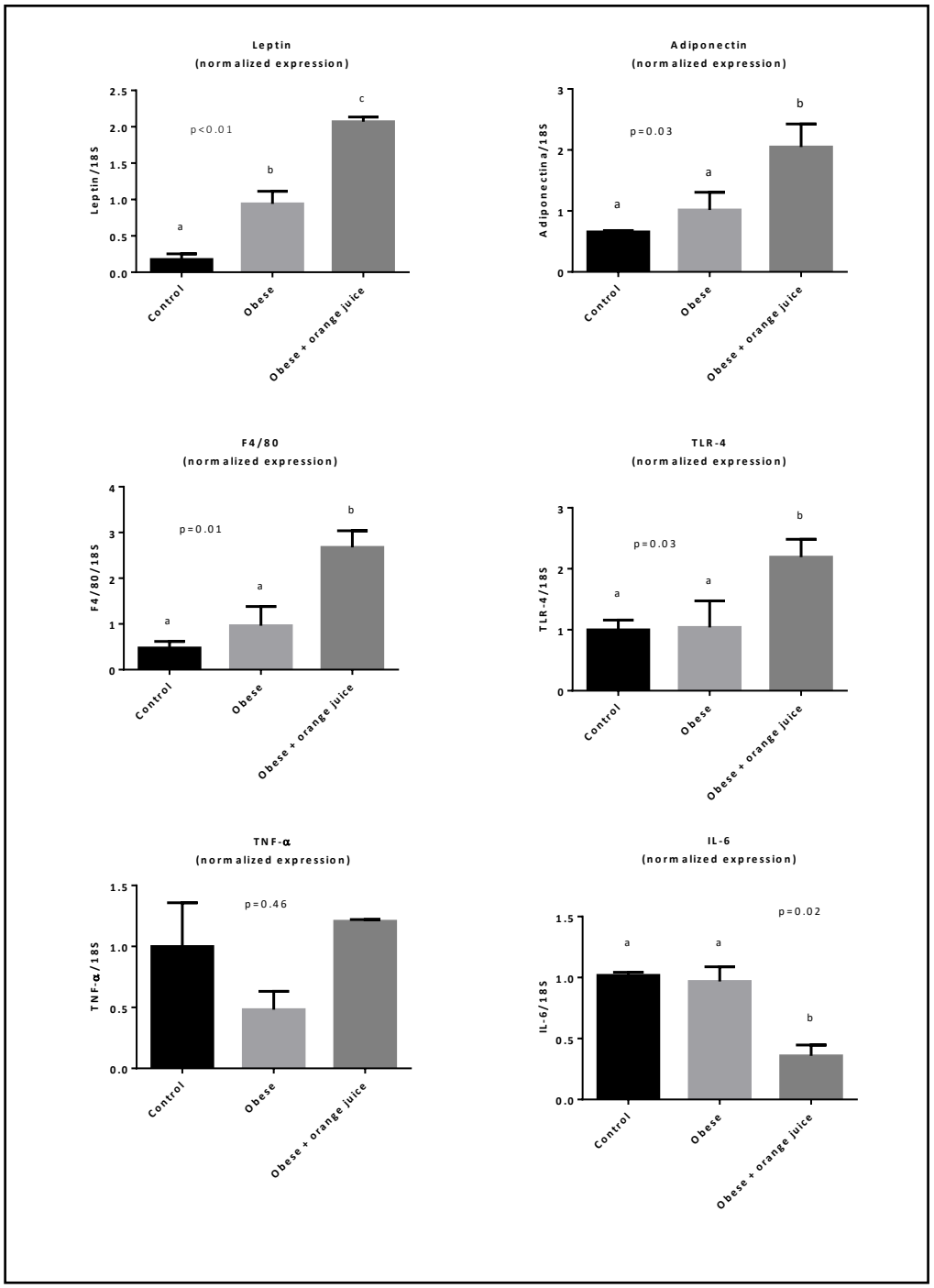
	Control	Obese	Obese + Orange juice	p
MDA nmol/mg ptn	1.120 (0.194)	1.513 (0.078)	1.502 (0.149)	0.18
CAT U/ptn	8.38 (0.29)	8.11 (0.29)	8.33 (0.09)	0.83
SOD U/ptn	8.33 (0.76)	9.69 (2.04)	9.05 (1.31)	0.90
GPx U/ptn	0.12 (<0.01)	0.12 (<0.01)	0.11 (<0.01)	0.13

Data presented as mean and standard error of the mean. MDA: malondialdehyde; ptn: protein; CAT: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase. ANOVA followed by Tukey test (n=4/group).

Except for leptin expression in adipose tissue of female fed obesogenic diet, no other difference in adipokine expression was observed between obese and control groups (Figure

8). Females fed obesogenic diet + orange juice presented increased leptin, F4/80 and TLR-4 when compared to females fed obesogenic diet. Females from obese + orange juice group also presented decreased expression of IL-6 and increased expression of adiponectin.

Figure 8: Relative expression of adipokines in adipose tissue of females (mothers)



Relative expression of adipokines in adipose tissue of females (mothers) before mating. TLR-4: Toll Like Receptor 4; TNF-α: Tumor Necrosis Factor-α; IL-6: Interleukin-6; 18S: housekeeping gene. ANOVA followed by Fischer´s LSD test (n=4/group). Figure made by the author.

The reproductive characteristics of the experimental groups are presented in table 7. The obese + orange juice females took less time to get pregnant compared to control and obesogenic groups. However, there were less successful gestations in this group compared to control. The remaining variables evaluated presented no differences between dietary interventions.

Table 7 – Reproductive characteristics

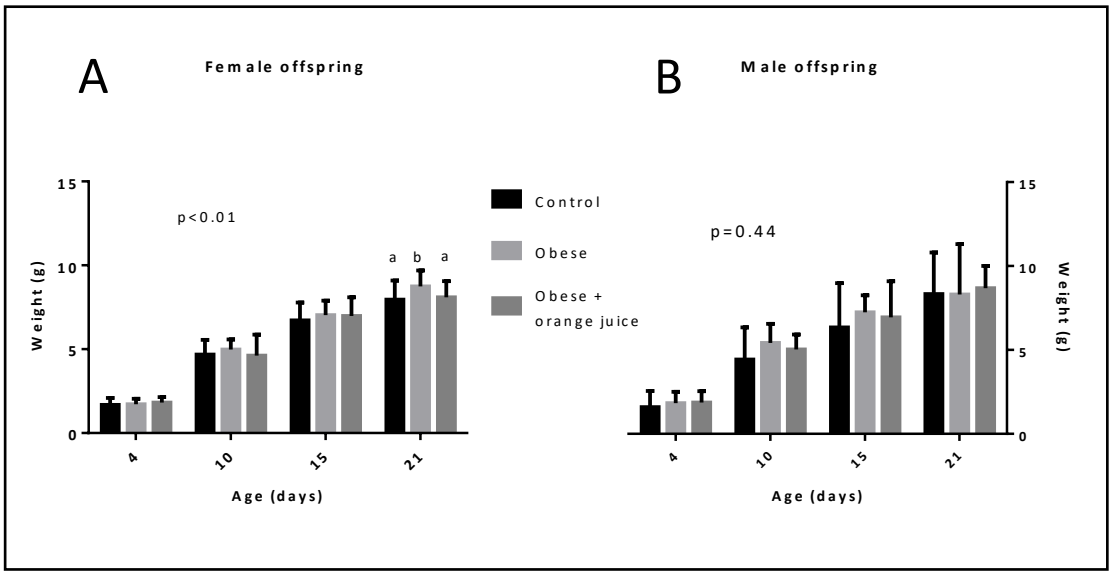
	Control	Obese	Obese + Orange juice	p
Days to pregnancy*	43.5 (4.8)[a]	32.0 (4.7)[a]	27.9 (4.0)[b]	0.05
Number of successful gestations	20.0[a]	21.0[a,b]	14.0[b]	0.04
Number of pups mortality	5.0	5.0	11.0	0.30
Never impregnated	1.0	0	1.0	0.64
Number of pups/litter (size of litter)*	7.4 (0.6)	7.3 (0.5)	6.1 (0.6)	0.24

*Data presented as mean and (standard error of the mean); remaining data are presented in absolute numbers. Two-way ANOVA followed by LSD test (n=26/group).

5.3 EFFECTS OF MATERNAL OBESITY AND ORANGE JUICE INTAKE ON OFFSPRING METABOLIC PROFILE

At weaning female offspring of obese mothers presented greater weight than control and obese + orange juice offspring (Figure 9A). There were no differences in weight of male offspring at any time point after birth until weaning (Figure 9B).

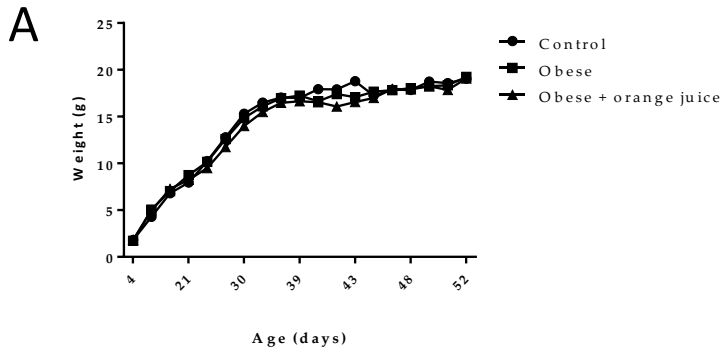
Figure 9: Weight of female and male offspring until weaning.



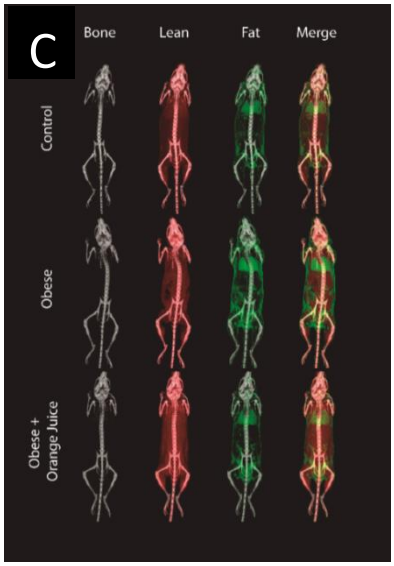
Weight of female (A; n=36/group) and male (B; n=30/group) offspring from day 4 of life until weaning (post-natal day 21). Two-way ANOVA followed by Tukey test. Figure made by the author.

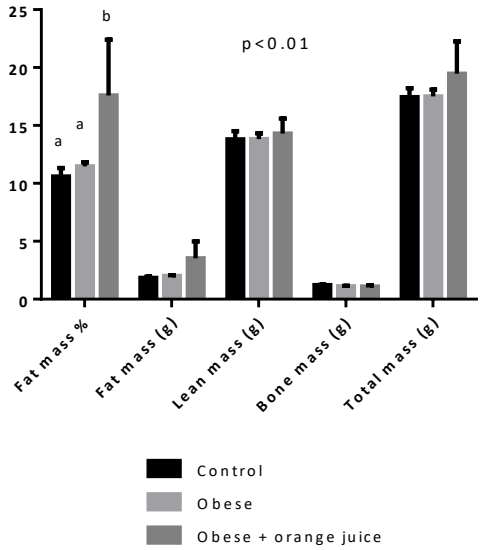
After weaning until post-natal day 50, the difference between weight of female offspring shifted and control and obese groups presented similar weight at the end of the experiment. Obese + orange juice female offspring presented less weight than control and obese offspring (Figure 10A). Curiously, female offspring of obese + orange juice mothers presented increased energy intake compared to obese offspring (Figure 10B). Body composition assessment revealed that female offspring of obese + orange juice mothers had increased fat mass percentage when compared to control and obese groups (Figure 10C-D), which was not observed when vital organs weight were measured (Figure 10F). There were no differences in blood glucose concentrations between groups of female offspring.

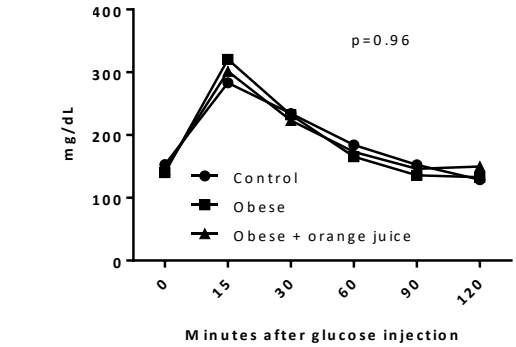
Figure 10: Metabolic parameters of female offspring

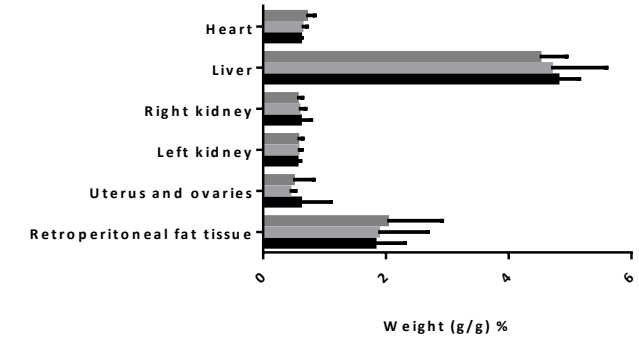


	Control	Obese	Obese + orange juice		Control	Obese	Obese orange juice
Weight (g)	14.40 (1.25)[a]	14.26 (1.25)[a]	13.86 (1.17)[b]	Energy intake (kcal)	9.60 (0.46)[a,b]	9.08(0.53)[a]	9.42(0.58)[b]









(A) Weight gain of female offspring from the 4th day of life until the end of experiment (post-natal day 50; n=30/group); (B) energy intake of female offspring from weaning (post-natal day 21) to the end of experiment (n=36/group)t; (C) representative images of females´ offspring body composition assessment by computed tomography (CT) at the end of experiment (day 50; n=1/group). The figure shows bone mass, lean mass (red) and fat mass (green); (D) results of body composition assessment by CT (n=4/group); (E) : Intraperitoneal glucose tolerance test of female offspring at post-natal day 50; mg of glucose per dL of blood (n=6/group); (F) differences between weight of female offspring´s vital organs / vital organs weight in relation to their own body weight (relative weight – g/g; n=16/group). The following statistical analysis were done: two-way ANOVA followed by Tukey test, one-way ANOVA followed by Tukey or Fischers´ LSD test. Table results are presented as mean and standard error of the mean (SEM). Figure made by the author.

There was no difference in the oxidative stress parameters evaluated between groups of female offspring, as presented in table 8.

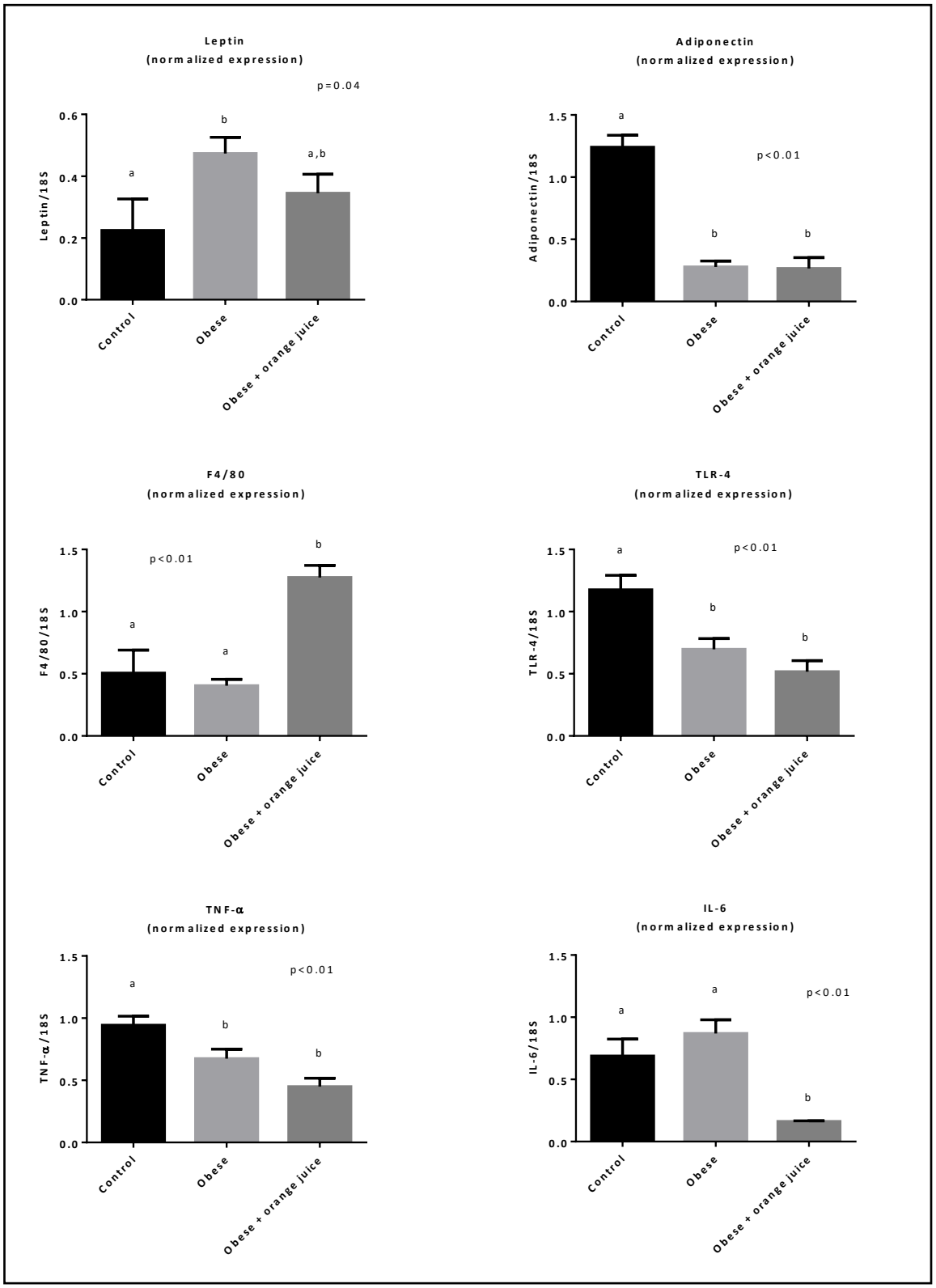
Table 8 – Estimated oxidative stress in the liver of female offspring

	Control	Obese	Obese+ Orange juice	p
MDA / mg ptn	1.79 (0.19)	1.65 (0.07)	1.22 (0.01)	0.48
CAT U/ptn	8.84 (0.32)	8.46 (0.30)	8.07 (0.33)	0.45
SOD U/ptn	8.46 (1.05)	9.91 (0.32)	6.57 (0.83)	0.06
GPx U/ptn	0.12 (0.01)	0.13 (0.01)	0.10 (0.01)	0.08

Data presented as mean and (standard error of the mean). MDA: malondialdehyde; ptn: protein; CAT: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase. ANOVA followed by Fischer´s LSD test (n=7/group).

Whereas female offspring of obese mothers presented increased expression of leptin and decreased in adiponectin compared to control, a few markers of inflammation in adipose tissue presented lower expression than control offspring, such as TLR-4 and TNF-α. Female offspring of obese + orange juice mothers presented increased expression of the macrophage marker F4/80 and decreased expression of IL-6 in adipose tissue compared to offspring of obese mothers (Figure 11).

Figure 11: Relative expression of adipokines in adipose tissue of female offspring

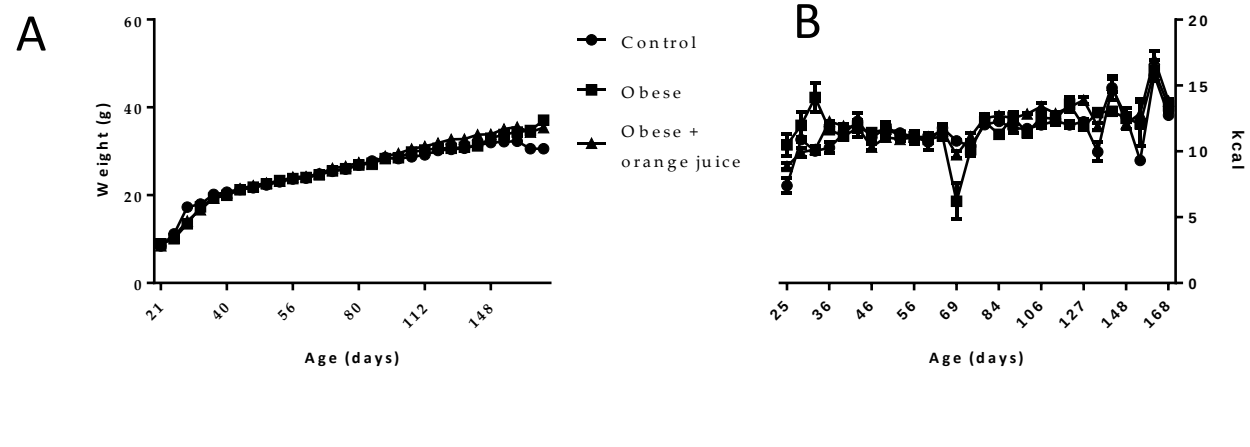


TLR-4: Toll Like Receptor 4; TNF-α: Tumor Necrosis Factor-α; IL-6: Interleukin-6; 18S: housekeeping gene. ANOVA followed by Fischer´s LSD test (n=8/group). Figure made by the author.

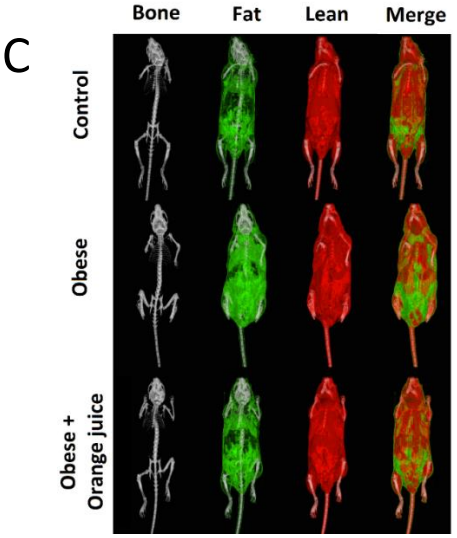
There were no differences in weight (Figure 12A), body composition (12C-D) and vital organs/tissues weight (Figure 12F) between control-fed offspring of obese and control mothers (Figure 12A). There was increased calorie intake by offspring of obese mothers when compared to control (Figure 12B). Glucose was increased amongst male offspring of obese mothers when compared to control offspring (Figure 12E).

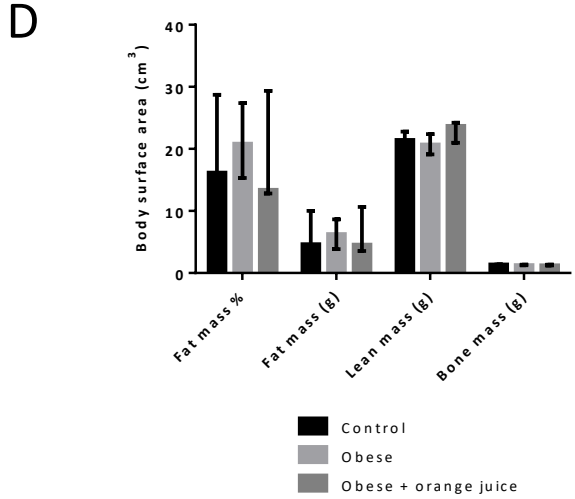
Offspring to obese + orange juice mothers presented similar weight to obese mice (Figure 12A) and increased calorie intake compared to both offspring of control and obese mothers (Figure 12B). Body composition assessment as well as vital organs/tissues weight weren't different between groups. Glucose tolerance test showed that offspring of control and obese + orange juice mothers presented similar blood concentrations of glucose (as estimated by area under the curve), as opposed to offspring of obese mothers, which presented increased glucose concentrations (Figure 12E).

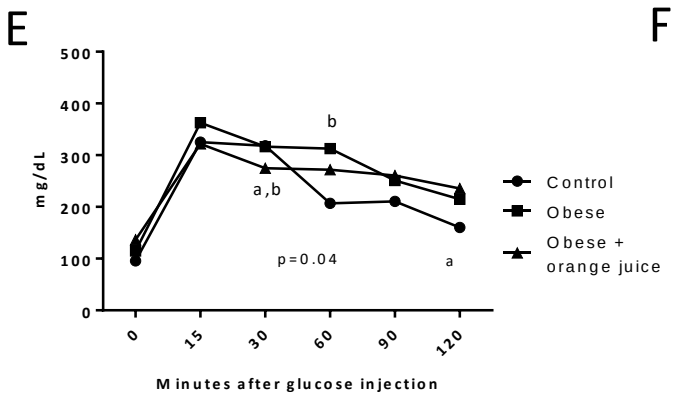
Figure 12: Metabolic parameters of male offspring fed control diet

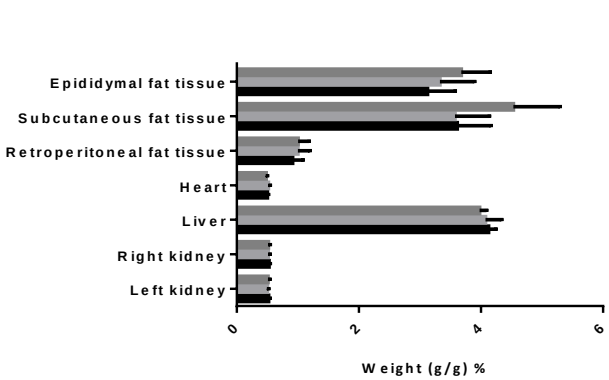


	Control	Obese	Obese + orange juice		Control	Obese	Obese + orange juice
Weight (g)	25.24 (1.11)[a]	25.65 (1.29)[a,b]	26.17 (1.34)[b]	Energy intake (kcal)	11.52 (0.30)[a]	11.90 (0.53)[b]	12.10 (0.32)[c]









(A) Weight gain of male offspring fed control diet from weaning until the end of experiment (week 21 – post-natal day 148) and differences between groups in mean body weight at the end of experiment (n=10/group); (B) energy consumption of male offspring fed control diet from weaning (post-natal day 21) to the end of experiment and differences between energy intake between experimental groups (n=10/group); (C) representative image of body composition assessment of male offspring fed control diet by computed tomography (CT) at the end of experiment (week 21). The figure shows bone mass, lean mass (red) and fat mass (green) (n=1/group); (D) results of body composition assessment by CT (n=4/group); (E) intraperitoneal glucose tolerance test of male offspring fed control diet (n=6/group); (F) differences between male offspring´s fed control diet vital organs weight in relation to their own body weight (relative weight – g/g) (n=10/group). The following statistical analysis were done: two-way ANOVA followed by Tukey test, one-way ANOVA followed by Tukey or Fischers´ LSD test. Table results are presented as mean and standard error of the mean (SEM). Figure made by the author.

Offspring from obese + orange juice mothers and fed control diet, presented lower liver MDA concentrations than offspring from obese mothers (Table 9). This group also presented less glutathione peroxidase activity compared to control offspring.

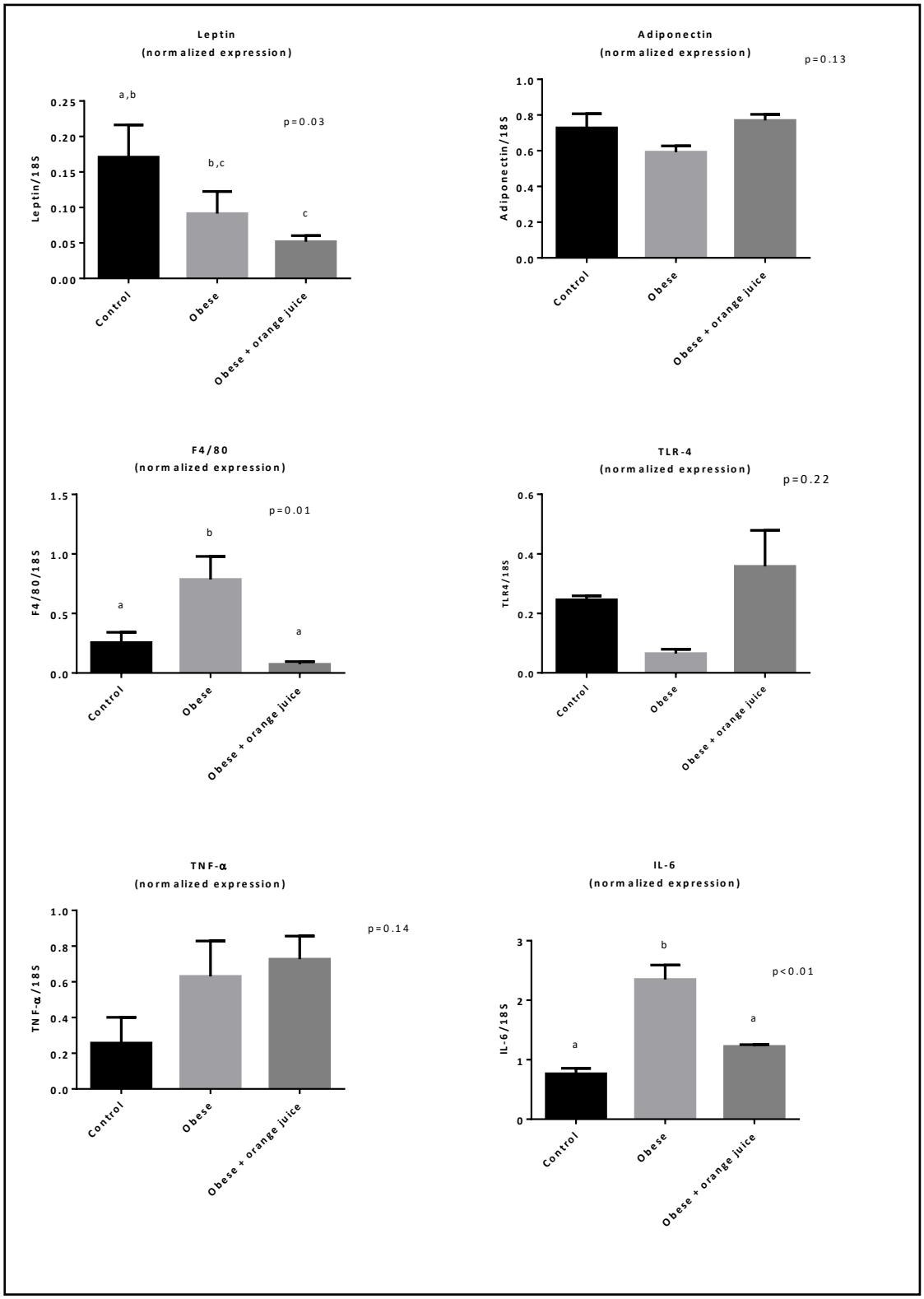
Table 9 – Estimated oxidative stress in the liver male offspring fed control diet

	Control	Obese	Obese+ Orange juice	p
MDA / mg ptn	2.09 (0.09)[a]	2.43 (0.11)[a]	1.49 (0.19)[b]	<0.01
CAT U/ptn	8.21 (0.46)	8.80 (0.50)	8.03 (0.67)	0.71
SOD U/ptn	9.04 (0.62)	10.28 (0.46)	8.31 (0.45)	0.09
GPx U/ptn	0.118 (0.003)[a]	0.095 (0.004)[b]	0.090 (0.001)[b]	<0.01

Data presented as mean and (standard error of the mean).. MDA: malondialdehyde; ptn: protein; CAT: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase. ANOVA followed by Fischer´s LSD test (n=6/group).

Obese male offspring fed a control diet presented increased expression of F4/80 and increased expression of IL-6 (Figure 13). Interestingly, obese + orange juice male offspring fed a control diet had expression of F4/80 and IL-6 similar to control offspring.

Figure 13: Relative expression of adipokines in adipose tissue of male offspring fed a control diet

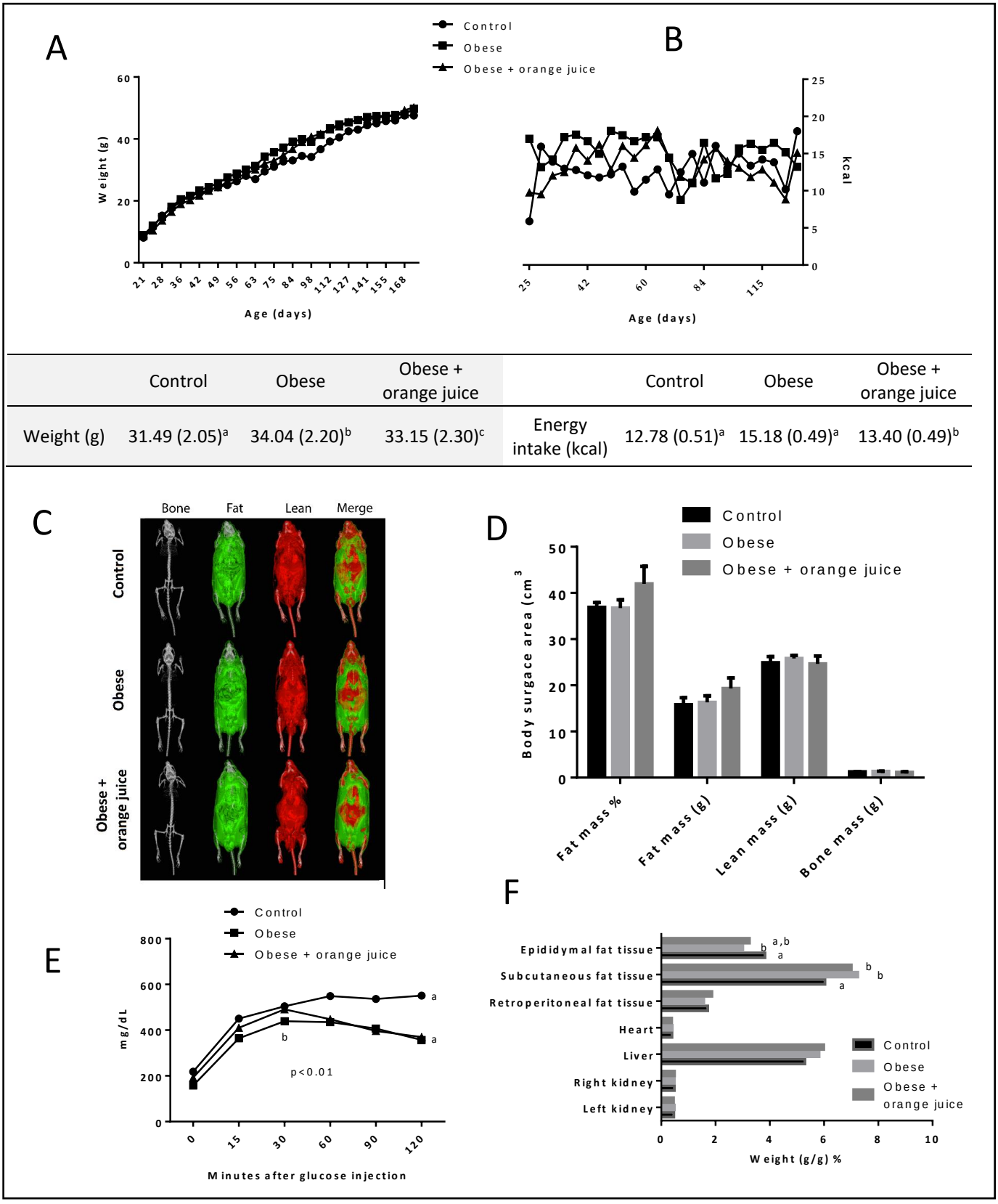


Males tissues were harvested at the end of the experiment (post-natal week 21). TLR-4: Toll Like Receptor 4; TNF-α: Tumor Necrosis Factor-α; IL-6: Interleukin-6; 18S: housekeeping gene. ANOVA followed by Fischer´s LSD test (n=6/group). Figure made by the author.

Offspring of obese mothers fed a high-fat high-sugar diet presented greater weight than control and obese + orange juice offspring fed the same diet (Figure 14A). This weight gain pattern is justified by energy intake: offspring from obese mothers fed obesogenic diet presented greater energy intake (Figure 14B). Body composition assessment did not show differences in body compartments of mice offspring fed obesogenic diet (Figure 14C-D). However, at euthanasia, it was observed that male offspring from obese mothers and fed an obesogenic diet presented decreased epididymal fat tissue and increased subcutaneous fat compared to control mice (Figure 14F). Curiously, glucose was decreased amongst obese offspring fed obesogenic diets when compared to control offspring fed obesogenic diet.

Despite having increased weight compared to control offspring, offspring of obese + orange juice mothers presented less weight than offspring from obese mothers (Figure 14A). However, body composition assessment showed similar fat mass with offspring of obese mothers, which was confirmed by weight of fat tissues at euthanasia. Offspring of obese + orange juice mothers and fed an obesogenic diet also presented similar glucose concentrations to offspring from control diet, which is increased compared to offspring from obese mothers.

Figure 14: Metabolic parameters of male offspring fed a high-fat and high-sugar diet



	Control	Obese	Obese + orange juice		Control	Obese	Obese + orange juice
Weight (g)	31.49 (2.05)[a]	34.04 (2.20)[b]	33.15 (2.30)[c]	Energy intake (kcal)	12.78 (0.51)[a]	15.18 (0.49)[a]	13.40 (0.49)[b]

(A) Weight gain of male offspring fed high-fat high sugar diet from weaning until the end of experiment (week 21 – day 148) and differences between groups in mean body weight of male offspring fed obesogenic diet at the end of experiment (n=10/group); (B) energy consumption of male offspring fed obesogenic diet from weaning (post-natal day 21) to the end of experiment and differences between energy consumption of experimental groups (n=10/group); (C) representative image of body composition of male offspring fed obesogenic diet by computed tomography (CT) at the end of experiment (week 21). The figure shows bone mass, lean mass (red) and fat mass (green) (n=1/group); (D) results of body composition assessment by CT (n=4/group); (E) intraperitoneal glucose tolerance test of male offspring fed obesogenic diet (n=6/group); (F) differences between male offspring´s fed obesogenic diet vital organs weight in relation to their own body weight (relative weight g/g) (n=10/group). The following statistical analysis were done: two-way ANOVA followed by Tukey test, one-way ANOVA followed by Tukey or Fischers´ LSD test. Table results are presented as mean and standard error of the mean (SEM). Figure made by the author.

Male offspring of obese and obese + orange juice mothers and fed obesogenic diet, presented decreased liver concentrations of MDA and decreased activity of GPx compared to control offspring fed obesogenic diet (Table 10).

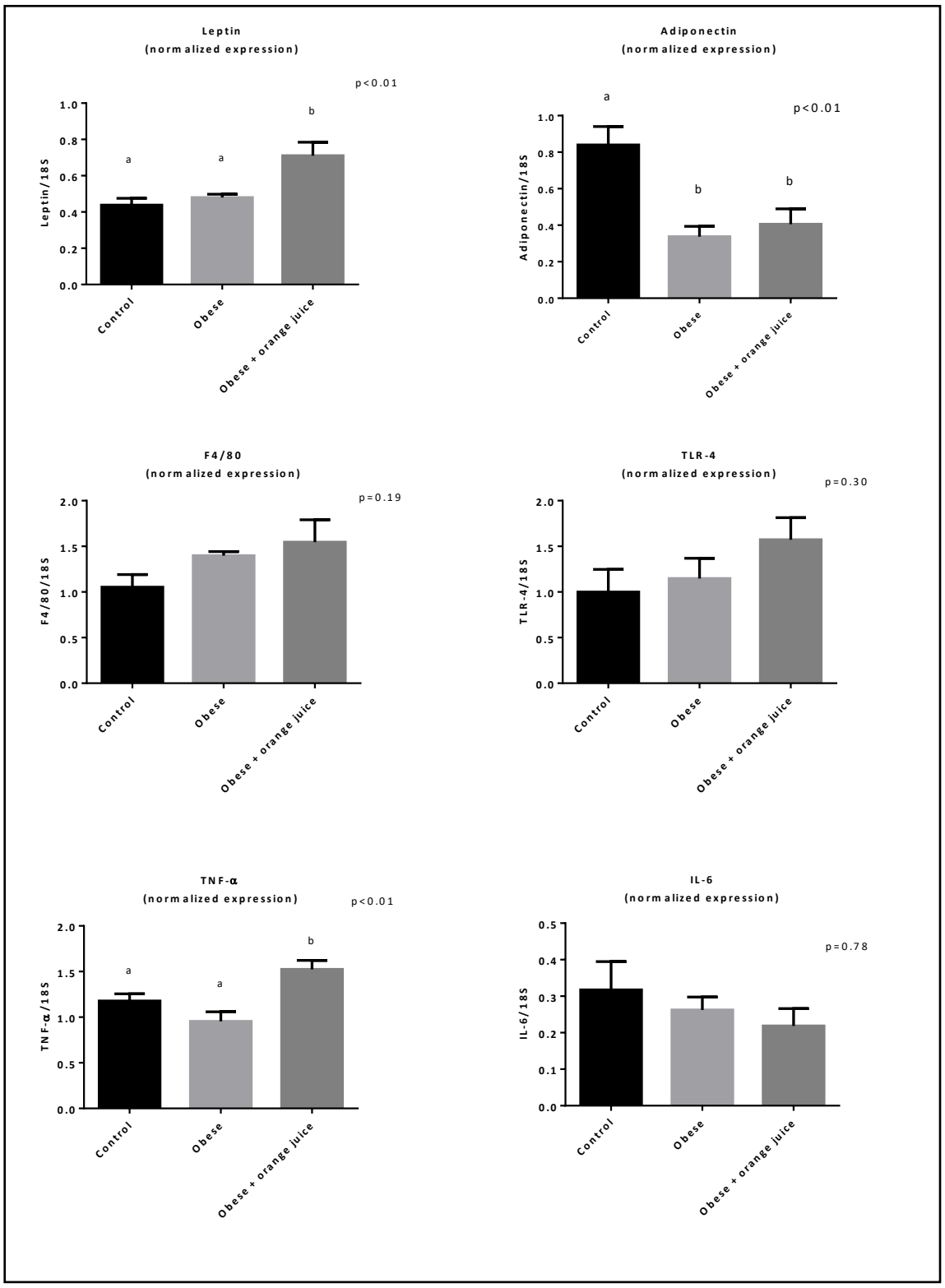
Table 10 - Estimated oxidative stress in the liver of male offspring fed obesogenic diet

	Control	Obese	Obese+ Orange juice	p
MDA / mg ptn	8.10 (0.13)[a]	7.17 (0.24)[b]	7.19 (0.27)[b]	<0.01
CAT U/ptn	9.47 (0.58)	9.75 (0.35)	8.02 (0.73)	0.17
SOD U/ptn	0.084 (0.002)	0.086 (0.004)	0.088 (0.006)	0.67
GPx U/ptn	8.10 (0.13)[a]	7.17 (0.24)[b]	7.19 (0.27)[b]	<0.01

Data presented as mean and standard error of the mean. MDA: malondialdehyde; ptn: protein; CAT: catalase; SOD: superoxide dismutase; GPX: glutathione peroxidase. ANOVA followed by Fischer´s LSD test (n=7/group).

Adiponectin expression in adipose tissue of offspring of control mothers and fed obesogenic diet was increased when compared to offspring from obese mothers. Offspring from obese + orange juice mothers exposed to an obesogenic diet presented increased leptin and TNF-α when compared to control and obese offspring.

Figure 15: Relative expression of adipokines in adipose tissue of male offspring fed a high-fat high-sugar diet

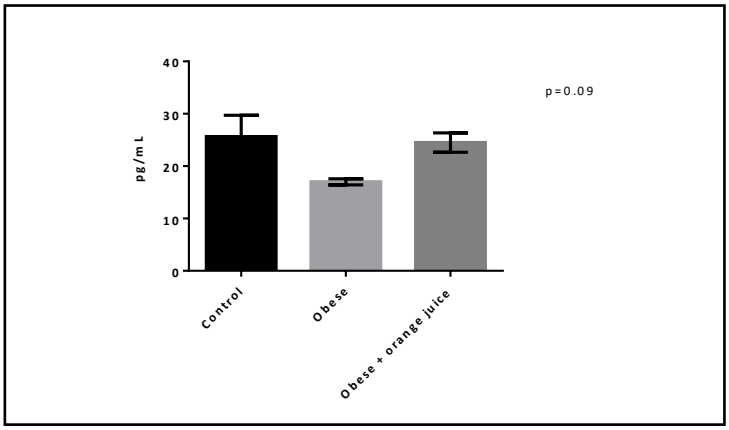


Males tissues were harvested at the end of experiment (post-natal week 21). TLR-4: Toll Like Receptor 4; TNF-α: Tumor Necrosis Factor-α; IL-6: Interleukin-6; 18S: housekeeping gene. ANOVA followed by Fischer´s LSD test (n=6/group).

5.4 EFFECTS OF MATERNAL OBESITY AND ORANGE JUICE INTAKE ON OFFSPRING FEMALE BREAST CANCER RISK

There was no difference in blood estradiol concentration between groups of 50-day old female offspring (Figure 16).

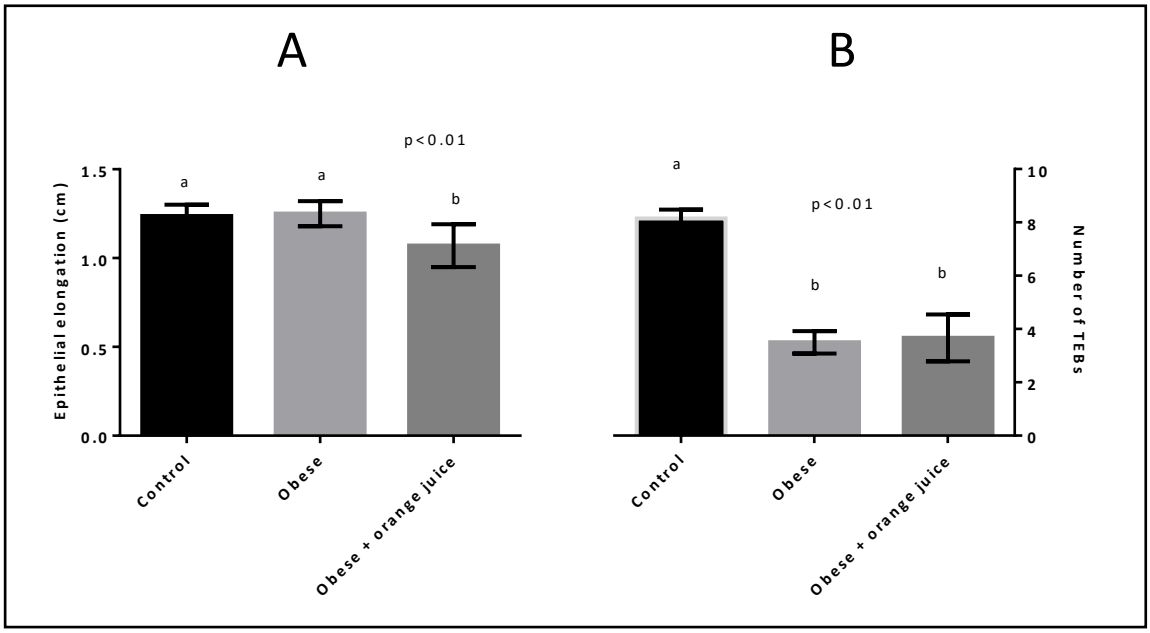
Figure 16: Estimated estradiol concentrations in blood of female offspring



ANOVA followed by LSD Fischer´s test (n=5/group). Figure made by the author.

Female offspring from obese mothers presented decreased number of Terminal End Buds (TEBs) when compared to control offspring (Figure 17B). Whereas female offspring from obese + orange juice mothers presented decreased epithelial elongation (Figure 17A) and decreased number of TEBs (Figure 17B) compared to control offspring.

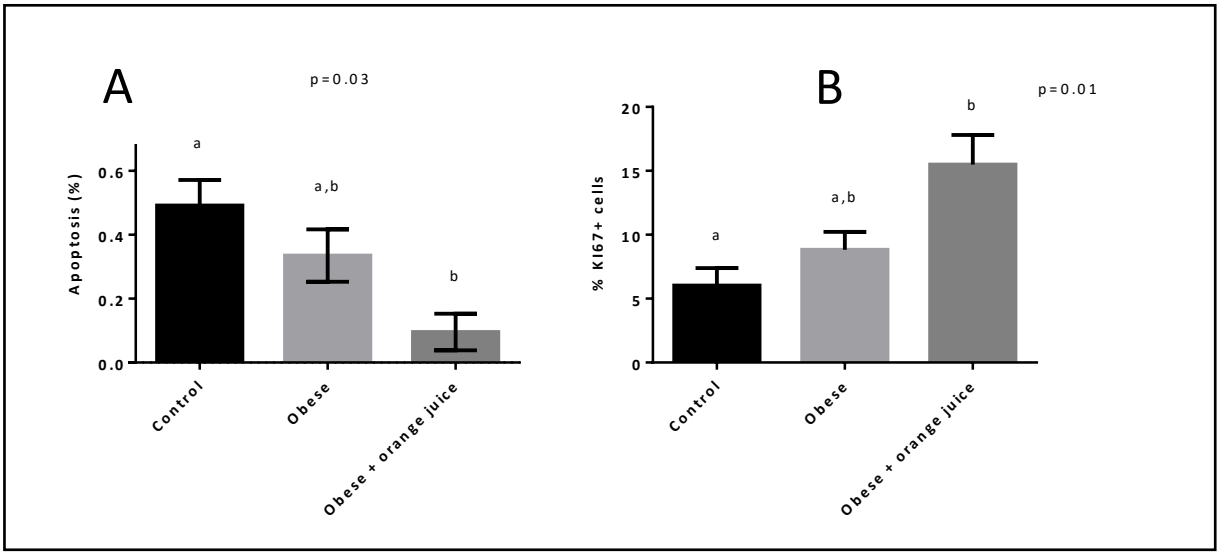
Figure 17: Evaluation of potential mammary gland tumorigenesis



Risk of mammary gland tumorigenesis in 50-day old mammary gland of female offspring. (A) Epithelial elongation - distance between lymph node and the end of epithelial tree; (B) Number of Terminal End Buds (n=6/group). ANOVA followed by Fischer´s LSD test. Figure made by the author

Whereas offspring of obese + orange juice mothers presented similar rates of apoptosis (Figure 18A) and proliferation (Figure 18B) to obese offspring, offspring of obese + orange juice mothers presented decreased and increased apoptosis and proliferation, respectively, in relation to offspring of control females.

Figure 18: Evaluation of potential to mammary carcinogenesis

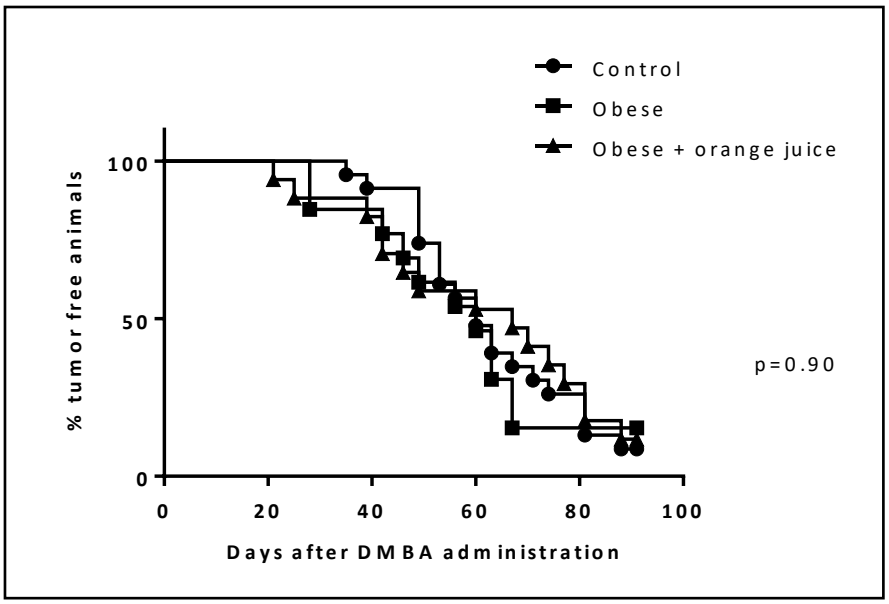


(A) Number of apoptotic cells (per 1000 cells) in mammary gland of 50-day old female offspring; (B) number of immunostained KI67+ cells on mammary gland of 50-day old female offspring (marker of cell proliferation). ANOVA followed by Fischer´s LSD test (n=6/group). Figure made by the author.

5.5 EFFECTS OF MATERNAL OBESITY AND ORANGE JUICE INTAKE ON OFFSPRING FEMALE BREAST CANCER RISK – CARCINOGENESIS

There was no difference in the incidence of mammary tumorigenesis between the studied groups, as represented with the survival statistics below (Figure 19).

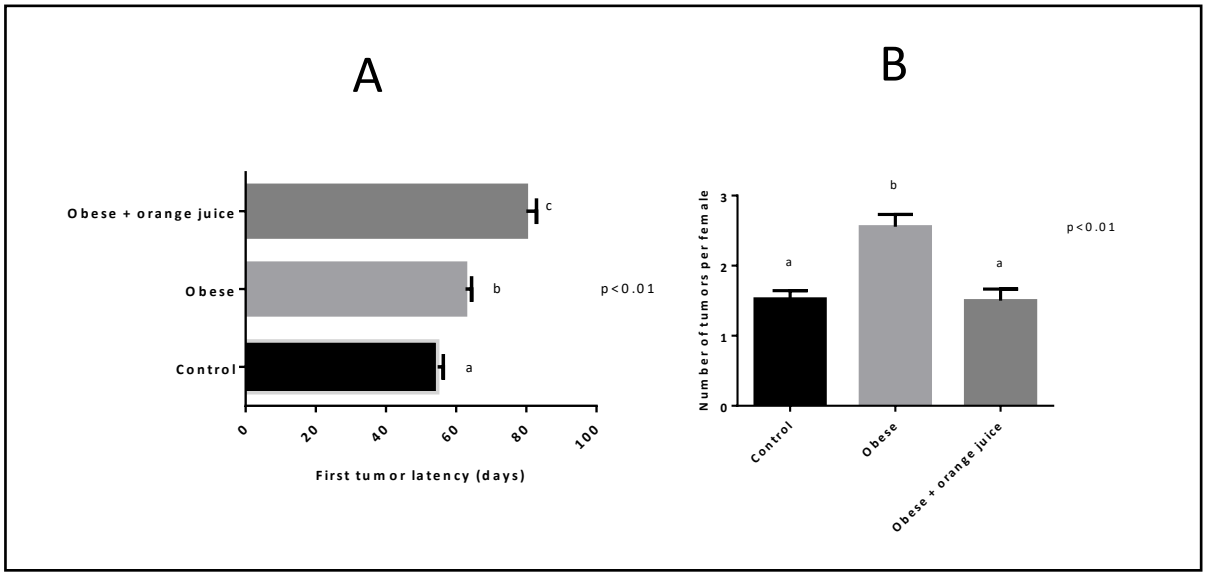
Figure 19: Number of tumor-free mice



Number of tumor free female offspring exposed to control, obesogenic and obesogenic + orange juice during early life development. Kaplan-Meier survival statistics (n=15/group). Figure made by the author.

Tumors in chemically induced female offspring of obese + orange juice developed later than obese and control groups (Figure 20A). Offspring of obese mothers developed tumors later than control offspring (Figure 20A). Offspring of obese mothers presented greater average of tumors per female, whereas maternal orange juice intake appeared to reduce number of tumors in female offspring (Figure 20B).

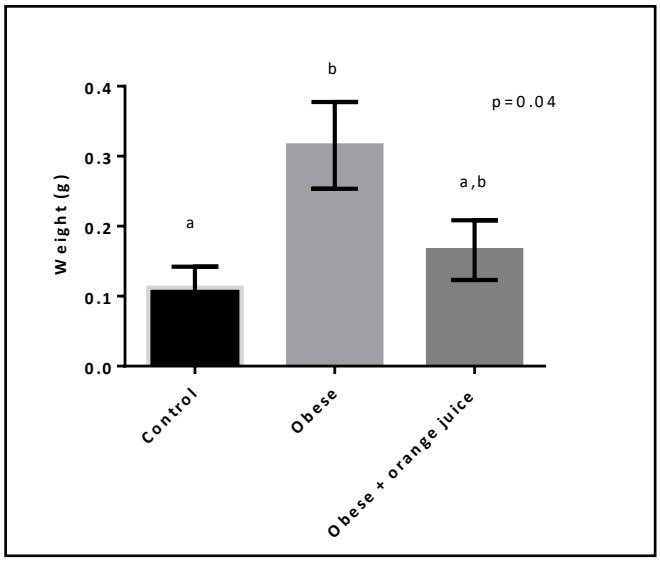
Figure 20: Mammary tumorigenesis in female offspring



Mammary tumorigenesis in offspring of control, obese and obese + orange juice mothers. (A) Tumor latency - number of days for the appearance of the first tumor; (B) Multiplicity – number of tumors per mouse. ANOVA followed by Fischer´s LSD test (n=16/group). Figure made by the author.

The first tumor of offspring of obese mothers were heavier than tumors from offspring control and obese + orange juice mothers (Figure 21).

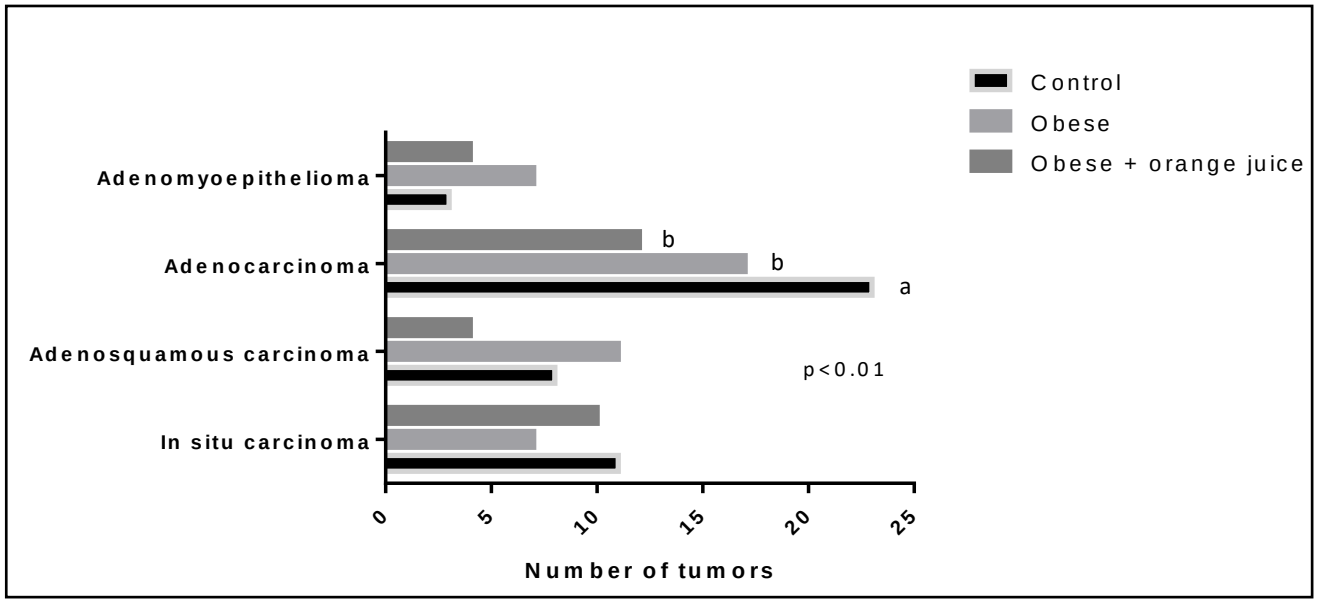
Figure 21: Weight of the first tumor to appear in female offspring



ANOVA followed by Fischer´s LSD test (n=16/group). Figure made by the author

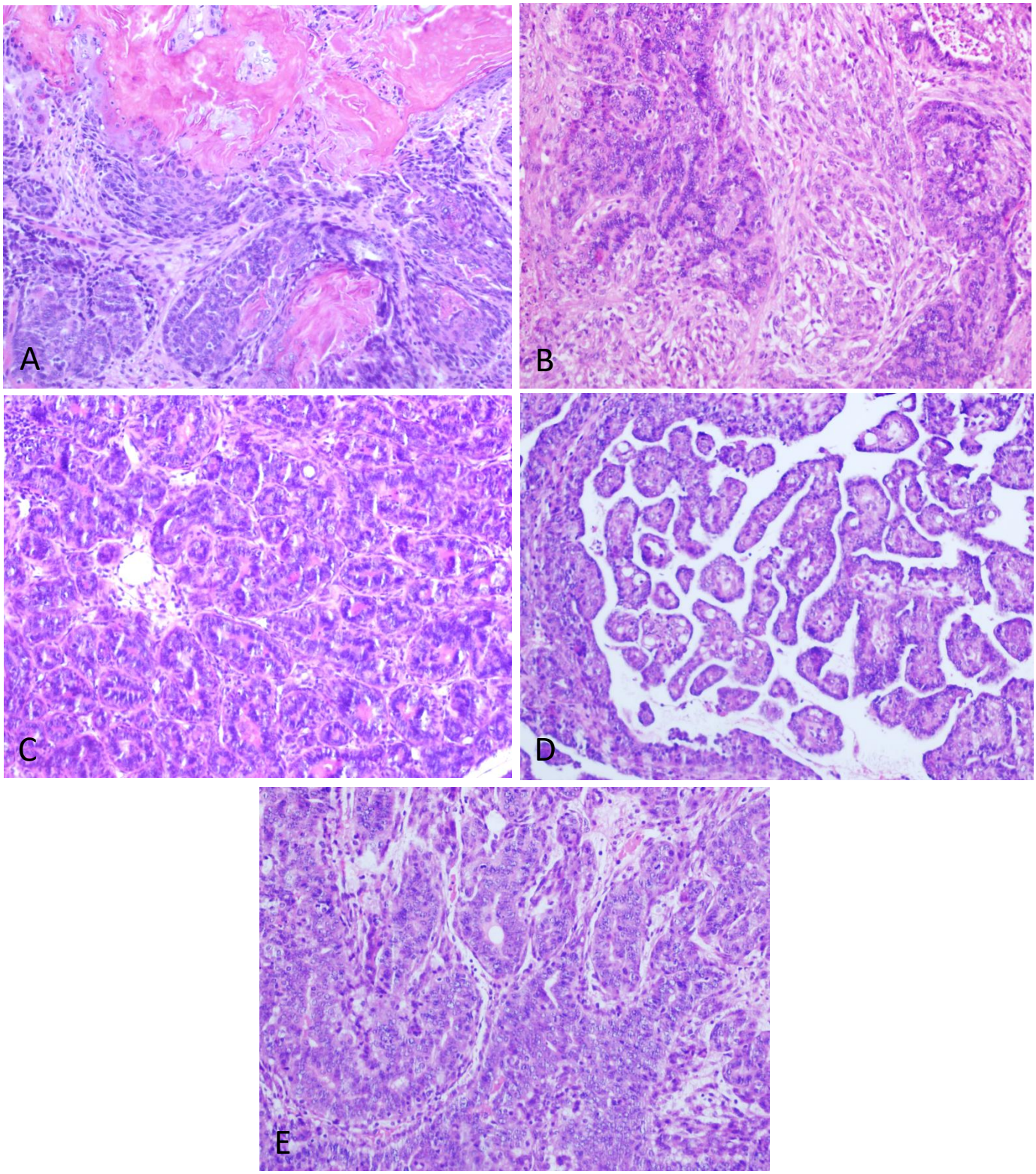
The number and types of tumor found with the tumorigenesis protocol are represented in the figure below. Female offspring of control mothers presented increased adenocarcinoma compared to obese and obese + orange juice offspring (Figures 28 and 29).

Figure 22: Subtypes of tumors found in DMBA-initiated female offspring



ANOVA followed by Fischer´s LSD test (n=16/group). Figure made by the author.

Figure 23: Representative images of invasive mammary tumors identified histological sections HE-stained (Objective 40x).



(A) Adenosquamous carcinoma; (B) Adenomyoepithelioma; (C-E) tubular, papillary and solid adenocarcinoma, respectively. Images made by Prof. Luis Fernando Barbisan.

6. DISCUSSION

6.1 MATERNAL DIETARY INTERVENTION

Three-weeks of dietary intervention promoted increased body weight and fat mass % in females fed obesogenic diet, which was justified by increased energy intake. Other studies that used a similar protocol of a high-fat and high-sugar diet also described rapid weight gain compared to control animals (PINHEIRO-CASTRO et al., 2019). Loche et. al (2018) reported that female mice fed 8 weeks with a similar, palatable diet, presented a 21.2% increase in weight compared to control mice. In our study, female mice fed the obesogenic diet had a 2% increase in weight compared to control females before mating, whereas obese + orange juice females presented similar weight to control females. Induction of obesity by diets differs in studies: mostly, variations occur in the types of diets used and length of experiment. Our study group has reviewed the differences between diets used to induce obesity in animal models (PINHEIRO-CASTRO et al., 2019). The high-fat and high-sugar diet was chosen because it was proven successful in other studies of programming chronic diseases in offspring later in life (SAMUELSSON et al., 2007; BLACKMORE et al., 2014; LOCHE et al., 2018). Additionally, this diet better represents the diets to which we are presently exposed to: the Western diet (LUMLEY et al., 2016; STEELE; PIRKLE; KIRKPATRICK, 2017), responsible for the increasing rates of obesity worldwide.

Previous experience with this specific dietary model in female mice was responsible for the adaptations we have made to the method originally described by Samuelsson et al. (2007). Modifications were made to the calculation of mineral mix, less length of dietary intervention and nulliparous females were used for mating. Even after reducing time of intervention to three weeks (plus gestation and lactation), female mice presented increased weight, increased fat mass % and deregulation of adipokine production by adipose tissue. Proving that high-fat diet associated with condensed milk is an effective model to promote obesity in mice (SAMUELSSON et al., 2007).

Leptin expression was increased in the obese and obese + orange juice groups, indicating greater fat mass of these groups when compared to control, which also reinforces results in the differences observed in weight, body composition and weight of adipose tissue amongst obese females. Increased leptin in adipose tissue is also associated with insulin resistance, which was established in the intraperitoneal glucose tolerance test. Leptin is also linked to

other disturbances known to compromise health, such as platelet aggregation, arterial thrombosis and angiogenesis (VAN GAAL; MERTENS; DE BLOCK, 2006). Aside from leptin, no other changes were observed in adipokine production of obese females when compared to control females.

Obese + orange juice fed females presented same weight as well as fat mass of control fed females, revealing a potential protective effect of orange juice in weight and body composition of females fed an obesogenic diet. Energy consumption was also similar to control animals. Therefore, orange juice might have acted as an appetite regulator. A recent study reported that orange juice extract supplementation for 4 weeks to overfed zebrafish, reduced their weight, body mass index and visceral adipose tissue when compared to overfed zebrafish (MONTALBANO et al., 2019). The authors observed that the overfed + orange extract group presented altered obesity-related genes, such as leptin, ghrelin, orexin, pro-opiomelanocortin, and neuropeptide Y, in both gut and brain, which are appetite regulators (MONTALBANO et al., 2019). This contributes to the theory that orange juice may be anti-obesogenic by reducing appetite and, consequently, energy intake. The orange juice extract the researchers used on zebrafish had 62.8mg/L of narirutin, 56.4mg/L of hesperidin, whereas the orange juice used in our research had 81.42mg/L and 123.87mg/L, respectively. It is noteworthy that the concentrated orange juice we used had 22.6% and 54.5% more narirutin and hesperidin, respectively, than the orange juice extract used by Montalbano et al. (MONTALBANO et al., 2019).

Despite improvements in weight gain, energy intake and fat mass of the orange juice supplemented group compared to the obese group, there was no change in insulin resistance, as measured by Intraperitoneal Glucose Tolerance Test. Additionally, expression profile of adipokine of adipose tissue showed greater expression of the pro-inflammatory markers leptin, F4/80 and TLR-4, whereas obese group presented similar expression levels to control. Therefore, apparently, orange juice affected expression profiles of these cytokines. Adiponectin and IL-6, on the other hand, presented increased and decreased expression, respectively, compared to adipose tissue of control and obese females. In an *in vivo* and *in vitro* study (DOURADO et al., 2014), mice received oral doses of either saline or hesperidin or orange juice concentrate. The authors stated that when macrophage of mice fed hesperidin were activated by LPS there was a reduction in nitric oxide production (NO), decreasing oxidative stress (DOURADO et al., 2014). However, when macrophage of animals fed orange juice was activated by LPS, there was an increase in NO production and

exacerbation of oxidative stress (DOURADO et al., 2014). If orange juice could increase production of oxidative stress, it would explain increased expression of TLR-4, which is a LPS-activated receptor that can also be triggered by saturated fatty acids (ROGERO; CALDER, 2018) and F4/80, which is a marker of macrophage infiltration.

There are reports of citrus fruits being able to increase adiponectin and decrease pro-inflammatory cytokine IL-6. *Citrus unshiu* peel ethanol extract, a common Korean fruit, with peel rich in hesperidin and narirutin, have shown that supplementation of *Citrus unshiu* peel ethanol powder extract with a high-fat diet to mice, increased adiponectin and decreased IL-6 in relation to high-fat diet fed mice (PARK et al., 2013).

Regarding reproductive parameters, females fed obese + orange juice diets had less successful gestations, with more newborn mortality and female to never impregnate than control and obese groups. Dietary intervention did not alter reproductive characteristics of obese females when compared to control. It was reported that dietary quercetin (a flavonoid) can negatively affect reproductive potential in female mice by inhibiting enzyme transglutaminase 2, which regulates ovarian ageing (BEAZLEY; NURMINSKAYA, 2016). However, no reports were found of how hesperidin or narirutin, the most studied flavones of orange juice, or even orange juice itself, can affect female reproductive parameters.

6.2 EFFECTS OF MATERNAL OBESITY AND ORANGE JUICE INTAKE ON OFFSPRINGS METABOLIC PROFILE

6.2.1 Female offspring

At weaning, after 11 weeks of maternal dietary intervention, female offspring of obese mothers presented increased weight compared to control and obese + orange juice offspring. Were differences in weight observed in 4-day old offspring, weight at weaning could be attributed to *in-utero* development. However, because it is evidenced only at post-natal day 21, it can be explained by lactation. In a cross-fostering study (OBEN et al., 2010), it was observed that offspring born to lean mothers and suckled by obese mothers presented greater calorie intake, body weight and increased plasma leptin and insulin than control offspring suckled by lean mothers. In our study, when the environment changed, post weaning, with only control diet available, body weight differences between control and obese offspring were no longer observed. There was also no indication of increased fat mass,

insulin resistance or oxidative stress between control and obese offspring. Vital organs weight of control and obese 50-day old offspring were also similar, there is, with no indication of a possible disfunction in the physiology of organs and tissues. However, pro-inflammatory adipokine expression showed that female offspring of obese mother presented increased leptin and decreased adiponectin compared to control offspring, which is usually observed in obesity. Contrary to what was expected, obese offspring presented decreased TLR-4 and TNF-α, which are pro-inflammatory cytokines, when compared to control offspring. Previously, our study group has reported that a maternal lard-based diet protected female offspring from mammary carcinogenesis in Wistar rats (de Oliveira Andrade et al., 2014). Since mammary gland is composed mostly of adipose tissue, it is possible that a lard-based diet could have had the same protective effect on female offspring of obese mothers. However, the diet used by de Oliveira Andrade et al. (2014) was not supplemented by sweetened condensed milk. Alfaradhi et al. (2014) used exactly the same diet we used in our study, but with a greater intervention period – female mice were fed obesogenic diet for 11 weeks before mating *vs* the 3 weeks we used. The authors described increased TNF-α protein concentrations in liver of female offspring of obese mothers when compared to control. This conflicts in results regarding TNF-α expression can be attributed to differences in the studied tissues/organ. Additionally, our results are strengthened by decreased expression of TLR-4 in offspring of obese mothers compared to control mice. Two reduced markers of inflammation in adipose tissue is suggestive of a protective effect of maternal obesity induced by a high-fat lard-based diet.

Female offspring of obese-orange juice mothers, in post-natal day 50 presented lower weight compared to control and obese offspring. This difference in weight cannot be explained by energy intake, as calorie intake of obese + orange juice offspring was similar to control. Despite body weight, body composition analysis showed greater fat mass in offspring of obese + orange juice mothers compared to offspring of control and obese mothers. However, since this difference was not observed when tissues were weighted at euthanasia, findings of body composition assessment of female offspring from obese + orange juice mothers might be a chance finding.

Unexpectedly, female offspring from obese + orange juice mothers presented increased expression of the macrophage marker F4/80 when compared to control and obese offspring, which contrasts with reduced expression of IL-6 compared to control and obese offspring. Interestingly, adipose tissue of females (mothers) also presented a similar pattern of

expression: increased F4/80 and reduced IL-6. Whereas we can only speculate that orange juice may have increased NO production in adipose tissue, as suggested by Dourado et al. (DOURADO et al., 2014), thus causing macrophage activation (CASTANEDA et al., 2017) and possibly increasing F4/80 expression in adipose tissue, further *in vitro* studies should be carried out to prove this hypothesis. As for the reduced IL-6 expression it can be an effect of orange juice intake by mothers, as this fruit juice is known to decrease IL-6 (BUSCEMI et al., 2012).

As previous studies have described, there are sex-differences in programming effects of maternal obesity (BERENDS et al., 2018; EUCLYDES et al., 2018). Additionally, as female offspring was euthanized at post-natal day 50 and males at post-natal day 150, results of metabolic programming of maternal obesity between female and male offspring, in this study, are not comparable.

6.2.2 Male offspring fed control diet

There were no differences in weight, fat mass percentage as evaluated by computed tomography nor weight of fat mass at euthanasia between control and obese male offspring fed control diet. Male offspring to obese + orange juice mothers, however, presented increased weight when compared do control offspring. This group also presented increased calorie consumption, which justifies increased weight findings. However, increased weight was neither observed in the body composition assessment nor in adipose tissues weight at euthanasia.

There were no differences in liver MDA concentrations, CAT and SOD activities between control and obese offspring. However, it was observed a decrease in GPx activity in male offspring of obese mothers when compared to control. As a response to increased oxidative stress, Afaradhi et al. (2014) also reported decreased GPx expression in liver of female offspring to obese mothers. Therefore, liver decreased activity of GPx could have contained oxidative stress and normalized MDA concentrations. This is in line with the glucose tolerance test, as male offspring of obese mothers presented increased glucose intolerance when compared to control offspring. Male offspring from obese mothers fed control diet also presented increased adipose tissue expressions of F4/80 and IL-6. Therefore, offspring from obese mothers presented increased risk for developing metabolic/chronic diseases compared to offspring of control mothers.

Orange juice intake by mothers, however, appeared able to contain damages induced by maternal obesity on male offspring fed control diet. Orange juice intake by mothers did not reduce weight gained by offspring of obese mothers, neither it was able to contain increased energy intake by offspring of obese mothers. However, liver MDA concentrations were decreased when compared to offspring from control and obese mothers, demonstrating a reduction in liver oxidative stress. It was also observed decreased activity of GPx, possibly promoted in response to maternal obesogenic diet. The protective effect of maternal orange juice intake is further observed in the glucose tolerance test, as offspring from obese + orange juice mothers presented similar results to control male offspring. Furthermore, orange juice intake by mothers normalized expression of the pro-inflammatory cytokines F4/80 and IL-6.

Together, these results suggest that use of orange juice during gestation and lactation may contain damages induced by maternal obesity in male offspring exposed to a control diet.

6.2.2 Male offspring fed obesogenic diet

Male offspring of obese mothers and exposed to an obesogenic environment presented greater weight gain than offspring of control mothers, despite having similar calorie intake. Although increased weight was not observed in body composition assessment, offspring of obese mothers presented decreased weight of epididymal fat tissue and increased subcutaneous fat tissue, showing a clear difference in response to an obesogenic environment. Epididymal and retroperitoneal fat tissues in mice are comparable to human visceral adipose tissue, there is, the body compartment most linked to inflammation and chronic diseases in adulthood (CHUSYD et al., 2016). In fact, removal of this body compartment from mice ameliorates insulin action, reduces tumorigenesis and improves longevity (GABRIELY et al., 2002; CHUSYD et al., 2016). Regardless of being lighter than offspring of obese mothers when exposed to an obesogenic environment, fat storage by offspring of control mothers in the epididymal region is a disadvantage when compared to the subcutaneous storage of offspring of obese mothers. This is further observed in oxidative stress parameters, as offspring from obese mothers presented less liver MDA than control offspring, which indicates oxidative stress if coupled with reduced GPx activity. The glucose tolerance test supports this theory, as male offspring from control mothers presented greater glucose concentrations at all time points after intraperitoneal glucose injection when

compared to offspring from obese mothers. There were no differences identified in the expression profile of adipose tissues pro-inflammatory cytokines between offspring from control and obese mothers. Despite results suggesting that male offspring from control mothers are less equipped to deal with an obesogenic environment than offspring of obese mothers, other variables need to be investigated to confirm this hypothesis.

Orange juice intake by mothers did not protect offspring of obese mothers from obesogenic environment. Obesogenic fed mice from obese + orange juice mothers presented similar blood glucose concentrations to obese mice and similar oxidative stress profile. However, expression of leptin and TNF-α, both cytokines positively linked to a pro-inflammatory status, were increased in offspring from obese + orange juice mothers, but not from obese mothers.

6.3 MATERNAL OBESITY AND ORANGE JUICE INTAKE ON OFFSPRING BREAST CANCER RISK

Despite female offspring to obese mothers having decreased number of terminal end buds, other parameters evaluated in 50-day-old female offspring's´ mammary gland indicated that there was no difference in mammary gland development between offspring from control and obese mothers. A decreased number of terminal end buds by offspring from obese mothers was also observed in the study of de Oliveira Andrade et al. (2014), which attributed a protective effect to a maternal high-fat lard-based diet on breast cancer risk when compared to control female offspring. As with our study, the authors did not observe differences in blood estradiol concentrations between groups of offspring (DE OLIVEIRA ANDRADE et al., 2014). However, we did not observe difference in epithelial development, neither in apoptotic and proliferative cell count of mammary ducts and lobules in offspring of control and obese mothers.

There was no difference in tumor incidence between experimental groups of offspring, as indicated by tumorigenesis protocol. However, female offspring of obese mothers presented increased latency when compared to control offspring, suggesting a protective effect of maternal obesogenic high-fat lard-based diet. It was observed an increase in multiplicity and weight of the first tumor to appear in females amongst offspring of obese mothers when compared to control offspring.

Orange juice intake by obese mothers, on the other hand, revealed reduced development of epithelium, marked by decreased epithelial elongation and decreased number of terminal end buds (TEB), both signs of reduced breast cancer risk. The tumorigenesis protocol revealed that offspring from obese + orange juice mothers presented increased latency, reduced number of tumors per mice and decreased weight of the first tumor when compared to offspring from obese mothers. This indicates that orange juice intake by obese mothers may potentialize the protective effect of a lard-based high-fat diet to offspring breast cancer risk.

Histopathological tumor analysis also showed that offspring from control mothers had increased adenocarcinomas compared to offspring from obese and obese + orange juice mothers. Since these are the most frequent diagnosed tumors in our population, the protective effects of the maternal diet used to induce obesity should be explored in future studies.

Despite female offspring from obese + orange juice groups having presented similar apoptotic and proliferative cell counts in mammary ducts and lobules to offspring from obese mothers, compared to control offspring, offspring of obese + orange juice mothers present decreased apoptosis and increased proliferation. There are evidences to support that the imbalance between apoptosis and increased proliferation is a strong predictor of epithelial malignant transformation (KUTANZI et al., 2010; YE et al., 2016). Since our study does not include a maternal control diet and orange juice intake, information regarding orange juice intake by this specific metabolic group remains unknown.

If we consider metabolic parameters of female offspring measured in adipose tissue, there is a reduction in expression of pro-inflammatory markers, such as TLR-4 (for both offspring of obese and obese + orange juice), TNF-α (for both obese and obese + orange juice groups) and in IL-6 (only for obese +orange juice group). Since one of the most abundant tissue in mammary gland is adipose tissue, it is hypothesized that there is less inflammation in mammary gland of female offspring from obese and obese + orange juice group. However, further investigation needs to be carried out to confirm this hypothesis.

7. CONCLUSIONS

The first objective of this thesis was to investigate the effects of maternal obesity and orange juice intake on male offspring exposed or not to an obesogenic environment. A maternal obesogenic diet programmed increased oxidative stress and pro-inflammatory profile in their offspring even when exposed to a control diet (environment), when compared to offspring from control mothers. Orange juice intake by obese mother was able to reverse the metabolic effects brought on by maternal obesity.

When offspring from control mothers were exposed to an obesogenic diet, mice were apparently less equipped to deal with an environment with excessive calories available, as animals presented increased glucose concentrations and liver oxidative stress when compared to offspring from obese mothers. Orange juice intake by obese mothers further deteriorated offspring´s general health, by increasing expression of pro-inflammatory cytokines of adipose tissue.

A study of mammary gland development, revealed that offspring from obese mothers had decreased number of terminal end buds, suggesting a protective effect of maternal obesity on breast cancer risk. Tumorigenesis confirmed protective effects of maternal obesity, as tumors took longer to develop (latency) in offspring of obese mothers when compared to control, despite presenting increased number of tumors per mice (multiplicity) and increased weight of the first tumor when compared to control. Orange juice intake by obese mothers improved protective markers of breast cancer risk by decreasing epithelial development as compared to offspring from obese mothers, increasing latency and reducing multiplicity and weight of the first tumor when compared to offspring from obese mothers.

Programming effects of maternal obesity and orange juice intake on offspring health are dependent on the environment the offspring is exposed to after weaning. A mismatched environment, in our study, was linked to a worse projection. Orange juice intake by obese mothers also presented conflicting results, as it was beneficial for male offspring exposed to a control diet, but not exposed to an obesogenic environment. As for female offspring, maternal obesity reduced offspring breast cancer risk and orange juice intake by obese mothers strengthened protective effects of a high-fat lard-based diet. We suggest further studies to explore the protective effects of maternal high-fat lard-based diet and orange juice

on female offspring breast cancer risk. A further understanding of the epigenetics mechanisms responsible for the differences observed between intervention groups may elucidate how orange juice was able to revert effects of maternal obesity in male offspring exposed to control diet and how maternal high-fat lard-based diet and orange juice was able to protect female offspring of obese mothers from increased breast cancer risk.

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