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

Paternal pre-conceptional nutrition programs breast cancer risk in rat female offspring: opposing effects of animal- and plant- based high fat diets.

Camile Castilho Fontelles

São Paulo
2016

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DEDICATION

To my loving grandmother Rosa Fontelles Burgues de Pau, who crossed an entire ocean without knowing what to expect and with her life story and her endless love taught me to always follow my dreams.

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"The life we want depends on what we do to it."

Christine Baena Castilho Fontelles

FONTELLES, C.C. **Nutrição paterna pré-concepcional programa o risco de câncer de mama na prole feminina de ratos: efeitos opostos de dietas hiperlipídicas de origem animal e vegetal.** 2016. Tese (Doutorado) – Faculdade de Ciências Farmacêuticas, Universidade de São Paulo, 2016.

RESUMO

O câncer de mama é um persistente problema de saúde pública. Hipótese intrigante sugere que a suscetibilidade à doença pode ser modulada em períodos precoces da vida, fenômeno conhecido como programação fetal. Nesse sentido, a maior parte dos estudos de programação fetal refere-se à influência materna, dada a intensa interação existente entre mãe e feto tanto no período fetal, quanto na lactação. Entretanto, estudos recentes mostram que a dieta paterna pré-concepcional também tem um papel de grande importância na suscetibilidade da prole à uma série de doenças crônicas não-transmissíveis de origem metabólica. Portanto, o presente projeto de doutorado direto teve como objetivo avaliar se o consumo paterno de diferentes dietas hiperlipídicas, durante o período de desenvolvimento do sistema reprodutivo de ratos machos, aumentaria a suscetibilidade da prole feminina à carcinogênese mamária. Adicionalmente buscou-se avaliar quais mecanismos poderiam estar envolvidos nesse processo. Utilizaram-se ratos machos da linhagem Sprague-Dawley (n=20/grupo) que consumiram dieta hiperlipídica com 60% de calorias provenientes de lipídeos de banha (grupo LB) ou óleo de milho (grupo CB), ou dieta controle AIN-93G (grupo CO), por nove semanas, durante os períodos de desenvolvimento e maturação sexual. Esses ratos foram acasalados com fêmeas, que consumiram apenas dieta comercial, na proporção 1:1. Sua prole de 50 dias foi submetida ao modelo de carcinogênese mamária com o uso de 7,12-dimetil-benza[*a*]antraceno (50mg/kg). O consumo paterno de dietas hiperlipídicas de origem

animal ou vegetal conferiram efeitos opostos, com o consumo de dieta com alto teor de ácidos graxos saturados (LB) aumentando e o consumo de dieta com alto teor de ácidos graxos poli-insaturados n-6 (CB) diminuindo o risco de desenvolvimento de câncer de mama na prole feminina. Esses efeitos foram associados à alteração da expressão de 89 miRNAS no espermatozoide dos pais e 23 miRNAs na glândula mamária da prole, com sobreposição de 3 miRNAs (miR-1897-5p, miR-219-1-3p e miR-376a#) que estavam alterados em ambos tecidos. Adicionalmente, a prole feminina de machos que consumiram dieta com alto teor de ácidos graxos saturados apresentou menor diferenciação da glândula mamária, maior nível de proliferação celular, menor nível de apoptose e alteração da expressão de proteínas chaves da regulação celular, como na transição epitélio-mesenquimal. Finalmente, essas fêmeas também apresentaram perfil lipídico alterado semelhante à do seu progenitor masculino, bem como modificações epigenéticas que podem estar relacionadas à etiologia do câncer de mama. Assim, concluímos que a dieta paterna hiperlipídica pré-concepcional programou a suscetibilidade da prole feminina à carcinogênese mamária, porém esse efeito é dependente do tipo de ácido graxo consumido e os efeitos observados possivelmente decorrem de alterações no perfil de expressão de miRNAs.

Palavras-chave: câncer de mama, programação fetal, dieta paterna, dieta hiperlipídica.

FONTELLES, C.C. **Paternal pre-conceptional nutrition programs breast cancer risk in rat female offspring: opposing effects of animal- and plant- based high fat diets.** 2016. Thesis (PhD) – Faculty of Pharmaceutical Sciences, University of São Paulo, 2016.

ABSTRACT

Breast cancer is a persistent public health problem. Interesting hypothesis suggests that its risk can be modulated in early life periods, a phenomenon known as fetal programming. In this context, most fetal programming studies focus on maternal influence, due to the greater interaction between mother and fetus in both fetal and lactation periods. However, recent studies show that paternal preconception diet has also a major role in the offspring's susceptibility to metabolic chronic non-communicable diseases. Therefore, this direct doctoral project aimed to assess whether the paternal consumption of different high fat diets during the development period of the reproductive system of male rats increased the susceptibility of female offspring to mammary carcinogenesis. In addition we sought to evaluate which mechanisms could be involved in this process. We used male rats of the Sprague-Dawley strain (n = 20/group) that consumed high fat diet with 60% of calories from lipids from lard (LB group) or corn oil (CB group), or AIN-93G control diet (CO group) for nine weeks, during development and sexual maturation periods. These rats were mated with females who consumed only commercial diet in 1:1 ratio. Their 50 days old offspring were subjected to mammary carcinogenesis model using 7,12-dimethylbenz[*a*]anthracene (50mg/kg). Paternal consumption of high fat diet of animal or plant source had opposite effects, with the paternal consumption of diet with high content of saturated fatty acids (LB) increasing and consumption of diet with high content of n-6 polyunsaturated fatty acids (CB) reducing the risk of breast cancer development in female offspring. These effects were

due to changes in the expression of 89 miRNAs in the father's sperm and 23 miRNAs in the offspring's mammary gland, with overlapping of three miRNAs (miR-1897-5p, miR-219-1-3p and miR-376a #) that were altered in both tissues. Additionally, female offspring of males fed diets with high content of saturated fatty acids showed less differentiation of the mammary gland, higher levels of cell proliferation, lower levels of apoptosis and altered expression of keys proteins that regulate important cellular functions, such as epithelial to mesenchymal transition. Finally, these females had also altered lipid profile of the fat pad similar to their male parent as well as epigenetic changes that may be related to the etiology of breast cancer. Thus, we conclude that the high-fat preconception paternal diet programmed the susceptibility of female offspring to mammary carcinogenesis, but this effect was dependent on the type of fatty acid consumed and the observed effects possibly results from changes in miRNA expression profile.

Keywords: breast cancer, fetal programming, paternal diet, high-fat diet.

ABREVIATIONS

- AA - Arachidonic acid
- AB- Alveolar Bud
- AKT - V-akt murine thymoma viral oncogene
- ANOVA – Analysis of variance
- AOAC - Association of Official Analytical Chemists
- AUC – Area under the curve
- BHT – Butylated hydroxytoluene
- BRCA1 – Breast cancer 1
- c-RAF - V- raf-leukemia viral oncogene
- CASP3 - Caspase 3
- CEBPß - CCAAT/enhancer binding protein beta
- CEUA- Animal use ethical committee
- CFL - Cofilin
- CO – Male rats fed AIN93G diet
- CB - Male rats fed corn-oil-based high fat diet
- DCIS - Ductal carcinoma *in situ*
- DES - Diethylstilbestrol
- DHA - Docosahexaenoic acid
- DMBA - 7,12-dimethylbenz[*a*]anthracene
- DNA - Deoxyribonucleic acid
- DNMT – DNA methyltransferases
- DOHaD - Developmental origins of health and disease
- DTT - Dithiothreitol
- EMT - Epithelial to mesenchymal transition
- EPA - Eicosapentaenoic acid
- ER - Estrogen receptor
- ERK1/2 – Extracelular signal-regulated kinase ½
- FASN – Fatty acid synthase
- FCF – Faculty of pharmaceuticals sciences
- FID - Flame ionization detector
- FOXP3 – Forkhead box P3

- GEO - Gene Expression Omnibus
- GESR - Genomics and Epigenomics Shared Resources
- H3K4 – Histone 3 lysine 4
- H3K9 - Histone 3 lysine 9
- H3K27 - Histone 3 lysine 27
- H4K16 - Histone 4 lysine 16
- H4K20 - Histone 4 lysine 20
- HAT- Histone acetylases
- HCl – Hydrogen chloride
- HDAC – Histone deacetylases
- HER2- Human epidermal growth factor receptor 2
- HMT – Histone methyltransferases
- HPLC-DAD - High-Performance Liquid Chromatography, Diode-Array Detection
- IDC - Infiltrating ductal carcinoma
- IGF1R - Insulin-like growth factor 1 receptor
- IR beta – Insulin receptor beta
- ITT – Insulin tolerance test
- KDM - Histone demethylases
- LA - Linolenic acid
- LB- Male rats fed lard-based high fat diet
- LC3B1 – Microtubule-associated protein 1 light chain 3 beta subunit 1
- LCIS - Lobular carcinoma *in situ*
- LSD – Least Significant Difference
- $MgCl_2$ – Magnesium chloride
- MKK4 - Mitogen activated protein kinase 4
- MNU - 1-methyl-1-nitrosourea
- MTOR - Mechanistic target of rapamycin
- MUFA- Monounsaturated fatty acids
- NAOH – Sodium hydroxide
- NDUFB6 - NADH dehydrogenase (ubiquinone) 1β-subcomplex 6
- NF-kB - Nuclear Factor kappa-B
- NIH – National Institute of Health

- P38 - Mitogen activated protein kinase 14
- p-JNK - Phosphorylated Mitogen-activated protein kinase 8
- PBS – Phosphate-buffered saline
- PCA - Principal Component Analysis
- PKD1- Protein Kinase D1
- PMSF – Phenylmethylsulfonyl fluoride
- PPAR – Peroxisome proliferator-activated receptor
- PR - Progesterone receptor
- PTEN- Phosphatase and tensin homolog
- PUFA - Polyunsaturated fatty acids
- RANKL - Receptor Activator of Nuclear Factor kappa-B Ligand
- RAS - Harvey rat sarcoma virus oncogene
- RASF1a - Ras association domain-containing protein 1a
- RIPA – Radioimmunoprecipitation assay buffer
- RNA – Ribonucleic acid
- SCLB - Somatic cell lysis buffer
- SDS- Sodium dodecyl sulfate
- SEM - Standard error of mean
- SFA - Saturated fatty acids
- SMAD 3 - Smad family member 3
- TDLU- Terminal Ductal Lobular Units
- TEB - Terminal end buds
- TGF- Transforming Growth Factor
- TGFßR1- Transforming growth factor beta receptor I
- TIMP3 - Metalloproteinase inhibitor 3
- TMS1- Target of methylation-induced silencing 1
- TNBC - Triple negative breast cancer
- USA – United States of America
- ZEB - Zinc finger E-box-binding homeobox

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SUMMARY

1. LITERATURE REVIEW

Cancer

Cancer is a wide term used to identify a large number of diseases, which have in common the ability to proliferate uncontrollably (National Institute of Health, 2007). In order to cancer cells evolve into its neoplastic state, they must acquire certain cancer hallmarks, such as sustaining proliferative signals, evading growth suppressors, avoiding immune destruction, enabling replicative immortality, tumor promoting inflammation, activating invasion and metastasis, inducing angiogenesis, genome instability and mutation, resisting cell death and finally deregulating cellular energetics (Hanahan & Weinberg, 2011). By acquiring these hallmarks cancer cells, in an intrinsic relation with tumor associated stroma, can became tumorigenic and ultimately malignant (Hanahan & Weinberg, 2011).

Cancer development is a multi-step process, with three main steps: initiation, that involves one or more stable changes within the cell that occur spontaneously or induced by environmental exposure; promotion, a reversible phase, characterized by a selective growth enhancement provoked in the initiated cells and its progeny by continuous exposure to a promoting agent; and finally the progression, an irreversible phase, when after consecutive alterations in the neoplasia give rise to increasingly malignant sub-populations, resulting in further degrees of independence, invasiveness and metastasis (Devi, 2004).

Though it was present in human beings at ancient times, cancer incidence is currently increasing, with a significant prevalence in modern societies (David & Zimmerman, 2010). Recent studies showed that in 2012 there were 14.1 million new cases and 8.2

million deaths of cancer worldwide, while for 2030 researchers estimate an increase to 21.4 million new cases and 13.2 million deaths due to the disease (Ferlay *et al*., 2015). The most frequently diagnosed cancers are lung and breast cancer in men and women respectively, both worldwide and in less developed countries, while in more developed countries prostate and lung cancers are the most frequently diagnosed cancers in men and women respectively (Torre *et al*., 2015). Further regularly diagnosed cancers worldwide include: liver, stomach, and colorectum among men and stomach, cervix uteri and colorectum among women (Torre *et al*., 2015).

High disease incidence is associated with increase governments budget expenditure and the global costs on oncology treatment were above U$100 billion in 2014, which represents an increase of 10.3% over these costs in the year of 2013 (American Association for Cancer Research, 2015). More specifically in Brazil, the government spent more than R$1.9 billion with oncologic treatment (Tribunal de Contas da União, 2011). Altogether, these data highlight the importance of research directed towards risk reduction strategies.

Mammary gland development

Unlike other organs, during mammary gland development while the adjacent tissues (fat, connective tissue and blood vessels) are completely mature, the gland itself remains in an embryonic state, being stimulated only during puberty when it starts to penetrate into the mature surrounding tissue (Russo, 2015).

The mammary gland development initiates, in humans, at the 4th embryonic week (Hovey *et al*., 2002) and in rodents at embryonic day 10-11 (Hens & Wysolmerski, 2005),

when mammary placodes (thickening of the ectoderm) arises along the milk line that forms the primitive mammary buds and originating the rudimentary ductal tree at birth (Hens & Wysolmerski, 2005). In rodents at approximately 3 weeks of age, the ductal branching towards the fat pad begins, due to hormones stimulation (Russo *et al*., 1990).

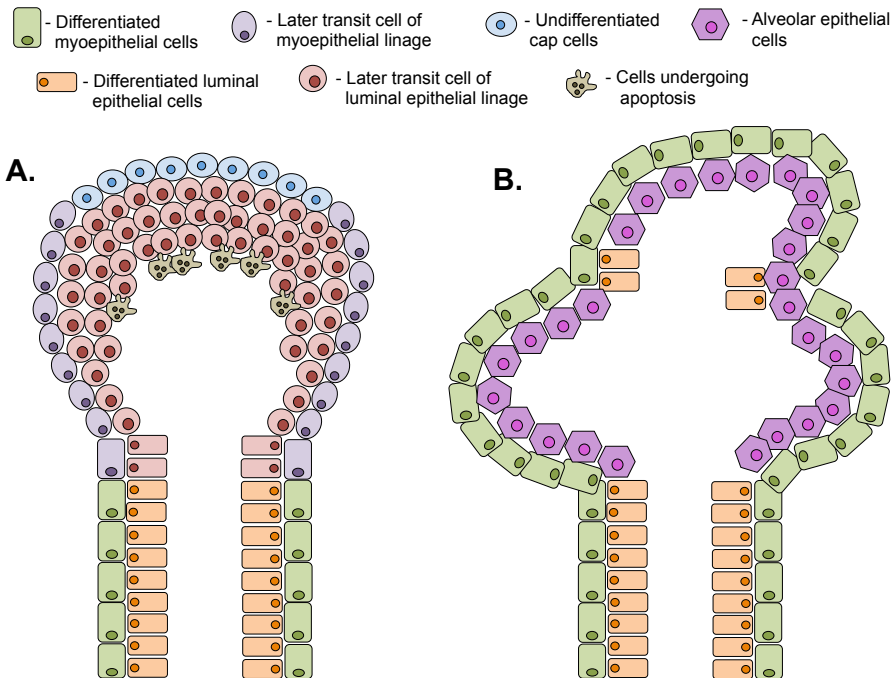

Figure 1: Schematic representation of the structure of mammary gland at different stages of differentiation. A: Prepubertal and pubertal mammary gland contains ducts terminated by highly proliferative terminal end buds. **B:** During pregnancy there is more ductal branching and formation of alveolar structures.

The mammary ducts are capped with terminal end buds (TEBs) that are constituted by an outer layer of undifferentiated cap cells, inner layers of luminal epithelial cells with great proliferative rates and an apoptotic plate behind the luminal cells resulting in ductal canalization (Humphreys *et al*., 1996) (Figure 1A). TEBs are considered a stem cell niche, given its ability to differentiate into both epithelial and myoepithelial cells (Yin *et al*.,

2014). In humans, the corresponding structures of TEBs are the Terminal Ductal Lobular Units (TDLUs) that are considered sites of tumor initiation (Polyak, 2001).

Until puberty, the rudimentary mammary epithelium is subjected to abundant exposure of growth hormones and estrogen, causing an isometric expansion and TEBs can begin to differentiate into alveolar buds (ABs) (Gallego *et al*., 2001). During puberty due to oscillating levels of estrogen and progesterone during the estrous cycle, the mammary ductal branching morphogenesis is potentiated (Masso-Welch *et al.*, 2000). Further continuous rounds of proliferation and incomplete apoptosis, caused by post-puberty ovarian hormones, result in the establishment of a complex network of ducts that infiltrate the fat pad to its boundaries (Atwood *et al*., 2000).

Additional modifications occur at pregnancy, when the mammary epithelium undergoes rapid proliferation resulting in increased branching, with the subsequent development of alveolar epithelium that is capable of milk secretion (Oakes *et al.*, 2008) (Figure 1B). The weaning period is accompanied by the regression of these recently formed alveolar epithelium due to apoptosis, leaving the ductal epithelial nearly as it was before pregnancy (Meier-Abt *et al*., 2013). Figure 2 summarizes the entire mammary gland development process.

The differentiation caused by pregnancy is one of the most significant protective factors against breast cancer (Lee *et al.*, 2014). In women over 25 years old the enduring consequences of pregnancy includes a strong and life-long breast cancer protective effect (Albrektsen *et al.*, 2005). This effect is not exclusive to humans and among rats and mice that were subjected to carcinogen administration, the ones that experienced pregnancy had approximately 75% less mammary tumors' incidence, compared to the virgin control

animals (Medina, 2005). Rodents are often used to study breast cancer given the similarities between theirs and humans' mammary gland development and carcinogenesis, as well as their short life span, easy manipulation, limited genetic heterogeneity and controlled handling environment (Líška *et al*., 2016). In fact, several species, for example dogs, cats and monkeys, have been evaluated for the purpose of breast cancer research, however only rodents presents the necessary criteria to mimic the human disease (Russo, 2015).

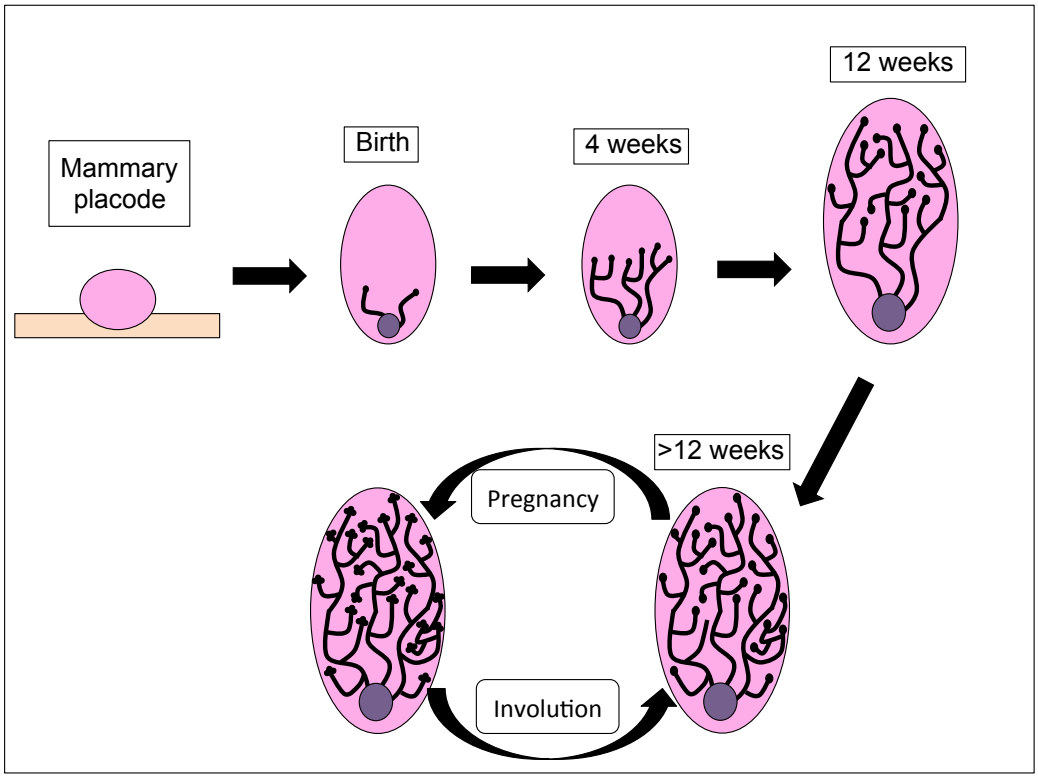

Figure 2: Schematic representation of the mammary gland development.

Since mammary gland development occurs during women's life course, environmental exposures can have a key role in affecting it, emphasizing the importance of focusing on early life exposures for breast cancer development and breast cancer risk (Forman *et al*., 2015).

Breast Cancer

Among women, breast cancer has one of the highest incidence rate, with nearly 1.7 million new cases diagnosed in 2012, representing 25% of all cancers in women worldwide (Ferlay *et al*., 2015). In the United States of America breast cancer is the leading cause of cancer death in women aged 20 to 59 years old (Siegel *et al.*, 2016). Nevertheless, approximately 70% of all breast cancer deaths worldwide occur in the developing countries, where the incidence rate is also increasing, possibly due to increased life expectancy, urbanization and adoption of Western lifestyle (Lee *et al*., 2012). In Brazil, for the years of 2014/2015, 576.000 new cancer cases were estimated, with 57.000 being female mammary tumors (BRASIL, 2014).

Breast cancer is an extremely heterogeneous disease that includes a great number of diverse biological disorders, with specific pathological features and biological behaviors (Dai *et al.*, 2016). Breast cancer can be classified as *in situ* carcinoma and invasive carcinoma, with the *in situ* being further sub-classified as either ductal carcinoma *in situ* (DCIS) or lobular carcinoma *in situ* (LCIS) (To *et al.*, 2014). Additionally from being more common than LCIS, DCIS can be classified, based on histological parameters, in: Comedo, Cribiform, Micropapillary, Papillary and Solid (Ajisaka *et al.*, 2002). Invasive carcinoma can also be divided in: infiltrating ductal, invasive lobular, ductal/lobular, mucinous, tubular, medullary and papillary carcinomas, with the infiltrating ductal carcinoma (IDC) accounting for 70% of all invasive lesions (Li *et al.,* 2005). Finally, IDC can be classified as grade 1 (well differentiated), grade 2 (moderately differentiated) and grade 3 (poorly differentiated), taking into account the levels of nuclear pleomorphism, glandular/tubule formation and mitotic index (Wedemeyer, 2010).

Breast cancer can also be classified based on molecular aspects, such as the expression of estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2), with the additional possibility of absence of ER, PR and HER2, that characterizes a triple negative breast cancer (TNBC) (Anderson *et al.*, 2015). The major breast cancer subtypes based on those molecular aspects are: Luminal A and Luminal B, that are ER+; HER2, that has extra copies of the HER-2 gene; and basal-like, that is a TNBC subtype (Schnitt, 2010). Nevertheless, recent data suggests that in addition to ER, PR and HER2, other markers should be used in order to obtain a more accurate molecular subtype classification (Anderson *et al.*, 2014).

Breast cancer etiological factors includes: age at the first menarche, parity, age at first birth, lifetime duration of breastfeeding, age at menopause, overweight, family history of the disease, alcohol use, use of oral contraceptives, use of menopausal hormone therapy (Barnard *et al.,* 2015) and exposition to radiation (Yalcin, 2013), as well as lifestyle and exposure to environmental factors (Rudolph *et al.,* 2016). Studies have shown that healthier lifestyle changes could prevent 25-30% of breast cancer cases, reinforcing the recommendations given by the World Cancer Research Fund and the American Institute for Cancer Research that provide guidelines for maintaining a healthy weight, undertaking at least 150 minutes of moderate intensity exercise per week, limiting alcohol consumption and eating a plant-based diet (Harvie *et al.,* 2015).

In order to uncover mechanisms of breast cancer prevention, aside from breast cancer development, the use of animal models is highly appropriate and more specifically, the use of rat model has been well described and accepted in the scientific community for over 50 years (Russo, 2015). Mammary carcinoma induction models in rats include:

chemically-induced carcinogenesis, virally-induced carcinogenesis (injection of female rats with adenovirus type 9), oncogene-induced carcinogenesis (incorporation of selected genes *in situ* into mammary epithelial cells) and radiation-induced carcinogenesis. However, only the chemically-induced models have been substantially investigated and the natural history of the disease development is well known (Thompson & Singh, 2000). Both 7,12-dimethylbenz[*a*]anthracene (DMBA) and 1-methyl-1-nitrosourea (MNU) are chemical carcinogens with single dose regime, which allows a distinction between the carcinogenic development steps (initiation, promotion and progression). Additional similarities include the reliability of tumor induction, organ site specificity, tumors of ductal histology mainly carcinomas and tumors of varying hormone responsiveness. On the other hand, important distinctions are that MNU is a direct acting carcinogen, while DMBA requires metabolic activation. Additionally, MNU tumors are more aggressive, have a higher proportion of malignant to benign tumors and are more estrogen-dependent (Russo *et al*., 1990; Thompson & Singh, 2000).

Nutrition and Cancer

The cancer burden will continue to increase in developed countries, however with greater intensity in the developing countries, given that preventive measures are not implemented (Ferlay *et al*., 2015). Cancer prevention is based on the hypothesis that, besides the genetic influence, modifiable external factors, like smoking and diet, can intervene in tumor incidence (Peto, 2001). It is estimated that 35% of all deaths from cancer worldwide are attributable to modifiable risk factors, including unhealthy diet (Danaei *et al.,* 2005). Since 1930 studies have associated diet and cancer outcome, but it

wasn't until the 1960s that studies started to relate dietary interventions and breast cancer (Hill, 1997). Although numerous subsequent cohort studies inspired the World Cancer Research Fund together with the American Institute for Cancer Research to produce, among others, dietary guidelines for prevention of a range of cancers, including breast cancer (World Cancer Research Fund & American Institute for Cancer Research, 2007), few specific food compounds have been conclusively associated with breast cancer incidence.

Weight gain itself is one of the main etiological factors for breast cancers, especially ER+ and ER- subtypes after menopause, and women that gained 20 kg and above during adulthood doubled their breast cancer risk (Vrieling *et al.,* 2010). Regarding dietary intake, it has been shown that every additional 10g of fiber consumed per day was associated with 5% of breast cancer risk reduction, possibly due to the reduction of estrogen and androgen absorption in the bowel and consequently in the circulation, as well as its effects on insulin sensitivity (Aune *et al*., 2012). Intriguingly, soy consumption is beneficial only if it is consumed since childhood, because it has a lifelong effect that leads to a more differentiated breast phenotype in adulthood (Dewi *et al*., 2013), with intake of 5g of soy protein per day being associated with 4% breast cancer risk reduction, although only in Asian postmenopausal women (Dong & Qin, 2011). The consumption of dairy foods, although controversial seems to be related to risk reduction, as shown in a meta-analysis that included more than 1 million participants where the higher consumption of dairy products was associated with 16% lower rates of breast cancer, compared to the lower consumption, and this effect could be related to the content of calcium, conjugated linoleic acids or vitamin D in dairy food (Dong *et al.*, 2011).

Recent meta-analysis indicates slight increase in breast cancer risk associated with women's higher consumption of meat, with each additional 100g of red meat per day linked to 4% increase of risk and each additional 30g of processed meat per day linked to 3% increase of breast cancer risk (Alexander *et al.,* 2010). Animal meat as well as milk, eggs, palm and coconut oil are main sources of saturated fatty acids (SFA), and the most prevalent dietary SFA are palmitic (16:0) and stearic (18:0) acids (Suburu & Chen, 2012). In fact, the European Investigation in Cancer and Nutrition found in a recent study a statistical positive association between SFA intake and breast cancer risk (Sieri *et al*., 2014). However, former meta-analysis showed that although intake of SFA increased the risk of developing breast cancer, this association was true only for postmenopausal breast cancer (Xia *et al.*, 2015).

On the other hand, consumption of polyunsaturated fatty acids (PUFAs), found in many vegetable oils and fatty fish, including arachidonic acid (AA), linolenic acid (LA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), can influence breast cancer proliferation, differentiation, and prognosis (Zhang *et al.*, 2012). Intake of marine omega-3 fatty acids, such as EPA and DHA were associated with reduced breast cancer risk, with each additional 0.7 g of these fatty acids per week linked to 5% decrease of risk (Zheng *et al.,* 2013). However, low intake of n-3 PUFA and high intake of n-6 PUFA increased risk of breast cancer (Kotepui, 2016). Consumption of monounsaturated fatty acids (MUFAs), found in most vegetable oils and some meat, remains especially controversial, although some studies observed positive correlation between MUFA and breast cancers risk, the consumption of oleic acid (C18:1N9c), the most consumed MUFA, was negatively associated with breast cancer risk (Binukumar & Mathew, 2005).

From a molecular perspective, female mice consumption of high fat diet (60% kcal fat), aside from causing higher mammary proliferative rates, increased tumor vascularization and reduced tumor latency, also resulted in elevated mammary gland expression of inflammatory and growth factor genes, such as Receptor Activator of Nuclear Factor kappa-B Ligand (RANKL) that has mitogenic activity in mammary epithelial cells and is a potent inducer of Nuclear Factor kappa-B (NF-kB) activity (Zhao *et al*., 2013). Similarly, mice consumption of high-calorie diet (36% fat from lard, 35% carbohydrates and 18% proteins) induced greater tumor growth, as well as decreased tumor expression of cleaved caspase 3, ER-ß and progesterone receptors and increased expression of Ki67 and other genes that control glycolysis and angiogenesis (Lamas *et al*., 2015). Altogether, these data imply that nutrition can have a deep impact on the genome. The research field dedicated to understand this interface is termed Nutrigenomics, responsible for uncovering how genetic and epigenetic alterations influence the requirements for nutrients aside from the organism responses to food compounds (Zeisel, 2007).

The gene expression profile is closely controlled by epigenetic alterations, which are modifications of the gene expression due to factors other than mutation in the DNA sequence. Epigenetic is a Lamarckian inheritance mechanism, that had it name coined by Conrad Hal Waddington in 1942, based on the Aristotelian term *epigenesis* (González-Recio *et al*., 2015). There are three major epigenetic modifications: DNA methylation, covalent modification that occurs at the 5 position of cytosines present in the CpG dinucleotide; histone modifications, covalent post-translational modifications on N-terminal tails of histones; and RNA-mediated gene silencing, small noncoding RNAs that

can alter gene expression by targeting mRNAs for degradation or by preventing their translation (Karsli-Ceppioglu *et al*., 2014).

Global DNA methylation is a process that involves the addition of a methyl group to a cytosine, mainly at a CpG island at 5-position and it is catalyzed by DNA methyltranferases, resulting in both mitotic and meiotic heritable methylation pattern (Elhamamsy, 2016). CpG islands can be found within the promoter region of numerous genes, and its methylation has been associated with numerous cellular process, for example: transcriptional repression, X chromosome inactivation, embryonic development, genomic imprinting, alteration of chromatin structure and transposon inactivation (Yong *et al*., 2016). DNA methyltranferases (DNMT) in mammals include DNMT1, responsible for DNA methylation maintenance, DNMT3A and DNMT3B, responsible for establishing *de novo* DNA methylation, a key process during embryo implantation and cell development, and DNMT 3-Like (DNMT3L) and DNMT2, that are less studied (Uysal *et al*., 2015). DNA methylation is a critical process in breast cancer development and important genes, such as BRCA1, are silenced by promoter-hypermethylation in sporadic breast cancers (Suijkerbuijk *et al*., 2008). Additionally, the target of methylation-induced silencing 1 (TMS1) gene, involved in the extracellular matrix control of adhesion-dependent survival, is also hypermethylated in the early steps of tumorigenesis (Parsons *et al*., 2009).

Histones modifications occur on histone tails, usually on lysine residues, that can be acetylated, phosphorylated, sumoylated, biotinylated and ubiquinated. Some of these modification are catalyzed by histone methyltransferases (HMTs), acetylases (HATs),

demethylases (HDMs) and deacetylases (HDACs) (Katz *et al.,* 2014). Intriguingly, the presence of a specific type of histone modification at a gene promoter is not sufficient to imply if the gene is silenced or not, with the exception of H3K27me, meaning that is the combination of the histone modifications that will define the gene expression pattern (Justin *et al*., 2010). Anyhow global active marks, such as H3K4me and H3K36me, can open the chromatin conformation and therefore promote gene expression, while repressive marks, for example H3K9me and H4K20, can condense the chromatin and suppress gene expression (Katz *et al*., 2014). Certain histone marks are breast cancer biomarkers, and could therefore be targets of oncological drugs, such as H3K4ac that is a predictor of deregulated cancer related pathway, as well as a strong indicator of progression from initial transformation to aggressive metastatic phenotypes (Messier *et al.,* 2016).

The most prominent small non-coding RNA is the miRNA or microRNA that are molecules of 21-24 nucleotides capable of translation repression through forming partially complementary base pairs within the 3' untranslated regions of protein-encoding mRNAs, which results in mRNA destabilization and consequent translational inhibition (Graves & Zeng, 2012). Remarkably more than half of the protein coding genes are predicted to be modulated by miRNAs, highlighting the key role that miRNAs have on mechanisms that are important for cancer development, such as progression, metastasis, chemotherapeutic multidrug resistance and endocrine resistance in breast cancer (Serpico *et al.,* 2014). In fact, eight differentially expressed miRNAs were found in breast cancer tissues, compared to the normal ones, including: miR-200b, miR-200c, miR-21, miR-378, let-7a, miR-320, miR-23a and miR-22 (Nygaard *et al.,* 2009). The miR-200 family is a significant regulator of the epithelial to mesenchymal transition (EMT), through inhibition of TGF-ß

induced EMT and suppression of ZEB1 and ZEB2 that are responsible for inhibiting EMT (Gregory *et al*., 2008).

The transcriptional response to high-fat diets can be seen in some studies. In Sprague-Dawley female rats the consumption of high extra-virgin olive oil diet increased the levels of global DNA methylation in both mammary gland and tumors, as well as changed histones modifications patterns, while the consumption of high corn-oil diet increased methyltransferase activity in both tissues, resulting in increased promoter methylation of the tumor suppressor genes RASSF1A and TIMP3 (Rodríguez-Miguel *et al*., 2015). These data, together with tumor data showing that corn-oil diet induced higher percentage of tumor-bearing animals, suggested that although both high-fat diets have breast cancer progression effects, high corn-oil diet had a stronger effect than high olive-oil diet (Rodríguez-Miguel *et al*., 2015). Likewise, mice consumption of high-fat diet was also able to increase mammary tumor growth by modulating multiple genes and miRNA expression implicated in cell proliferation, progenitor cell phenotype, epithelial to mesenchymal transition, mammary development and cell communication (De Luca *et al*., 2016). Importantly, the transcriptional response to distinct nutrients can differ from one life period to another (Teegarden *et al*., 2012).

Breast Cancer programming

Both early embryos and parental gametes are especially susceptible to numerous environmental exposures, that can elicit enduring changes in the developmental process that culminate in altered postnatal phenotype that could, in adulthood, be associated with increased disease risk (Fleming *et al.,* 2015). This phenomenon is called fetal

programming/developmental origins of health and disease (DOHaD), hypothesized by Dr. David Barker, and predicts that nutrition among other environmental factors in early life can program the risk of adverse health outcomes later in life (Perrone *et al.,* 2016).

The Dutch famine cohort provided one of the first reliable data on programming. It happened between 1944-1945, when during the end of World War II due to Dutch government support boycott, the German Army banned all food and fuel transport, apart from a very severe winter (Painter *et al.,* 2005). At the height of the famine the average official daily rations were between 400 and 800 kcal, while the adequate caloric intake for pregnant women is on average 2,100 kcal (Hark & Catalano, 2012). The consequences of this restriction in pregnant women was that babies exposed to the famine in early gestation were heavier and had increased coronary heart disease, a more atherogenic lipid profile and higher obesity levels (Painter *et al*., 2005).

Interestingly, when a fetus is exposed to malnutrition during pregnancy the phenotypic consequences include selective protection of certain organs growth, such as the brain, at the expense of other organs adequate growth, for example muscle and pancreas, resulting in poor functional capacity for insulin secretion, among others (Wells, 2011). In addition, if during its life-course this child is exposed to increased food intake and decreased energy expenditure it is particularly prone to develop glucose intolerance and subsequent type two diabetes. This phenomenon is known as the "Thrifty Phenotype" and was hypothesized by Hales and Barker (Hales & Barker, 1992; Hales & Barker, 2001). Though this model elucidates how the fetus adapts to a maternal undernutrition by growth retardation, leaving the adult better prepared to deal with a deprived environment, it doesn't explain the continuous relationship between higher birth size and later disease

risk, nor how can these early events induce long-term changes without any birth weight alteration (Gluckman *et al.,* 2007).

In this context, Gluckman and Hanson (2004) hypothesized the "Predictive Adaptive Response", proposing that the early life conditions induce a developmental plasticity leading to a predicted postnatal phenotype and that a mismatch between the predicted scenario and the later reality leads to worse health outcome in adulthood. Consequently environmental exposures can influence the maternal response that coordinate a range of effects on the body systems of their developing fetus, such as maternal nutrition that can cause, among others, epigenetic changes in the offspring and subsequent childhood adiposity, without difference in birth weight (Bateson *et al.,* 2014).

Dr. Trichopoulos (1990) was the first to hypothesize that, like other non-communicable diseases, breast cancer risk could also be determined in the intra-uterine environment, due to high estrogen exposures. Accordingly, the use of diethylstilbestrol (DES), a synthetic estrogen, by pregnant women around 1940 in order to prevent miscarriage, was associated with a two-fold higher breast cancer incidence in their adult daughters (Palmer, 2006). This effect could be due to modified mammary gland developmental process by the interaction of DES with Wnt/ß-catenin pathway, parathyroid hormone-related protein, bone morphogenic protein 4, insulin growth factor family, among others (Hilakivi-Clarke, 2014).

Among environmental exposures, maternal diet exerts great influence in the female offspring mammary gland development, indicating its major role in determining offspring breast cancer susceptibility (Lof *et al*., 2009). In rodent models, it has been shown that exposure to dietary factors during *in utero* and pubertal periods, developmental phases in

which the mammary gland is undergoing continuous remodeling, alters the daughter's susceptibility to breast cancer, possibly due to changes in the hormonal levels that may induce permanent epigenetic changes (Hilakivi-Clarke, 2007). Maternal consumption of obesity-inducing diet (high levels of soybean and cottonseed oil) was able to stimulate higher leptin levels, higher number of terminal end buds and consequent higher breast cancer incidence in the rat female offspring (De Assis *et al.,* 2006). On the other hand, maternal consumption of a lard-based high-fat diet during pregnancy lowered tumor incidence and multiplicity in the female offspring, possibly through altered protein expression (NFkB p65 and p21) and epigenetic marks (H3K9Tme) (de Oliveira Andrade *et al.*, 2014).

Given the remarkable interaction between mother and fetus, the maternal contribution regarding non-communicable diseases' programming has been more vastly described (Alfaradhi & Ozanne, 2011; Blackmore *et al*., 2014; Hilakivi-Clarke *et al*., 1997; Ong & Ozanne, 2015; Yu *et al.*, 2006) compared to the paternal contribution. Although initial studies underestimated paternal diet, focusing more on smoking, alcohol consumption and higher age (Little, 1987; Lundstrom *et al*., 2010; Pembrey *et al*., 2006), recent data suggest an important role for the paternal nutrition in this context (Ferguson-Smith & Patti, 2011). C57BL/6 male mice fed a low protein diet increased male offspring weight, induced hypotension and vascular dysfunction, elevated heart rate, impaired glucose tolerance, and elevated adiposity and TNF-alpha circulating levels (Watkins & Sinclair, 2014). Similarly, paternal consumption of high-fat diet (with fat derived from butter) induced metabolic and sperm disturbances in male offspring (Fullston *et al*., 2015).

In terms of paternal influences on daughter´s breast cancer risk, few studies are available in literature examining this possibility (Park *et al*., 2008). A case-control study showed a positive association between breast cancer risk in African-descendent women and paternal ageing (Hodgson *et al.,* 2004). Higher paternal age can be accompanied by a decrease in the sperm DNA repair system efficacy, leading to higher breast cancer susceptibility in women (Xue & Michels, 2007). Another case-control study showed association between higher paternal educational level, that is related to his nutritional habits, and breast cancer incidence in the daughters (de Kok *et al*., 2008; Titus-Ernstoff *et al*., 2002).

Therefore, the present study aimed at investigating the influence of different paternal high-fat diets, animal- or plant-based, during development and sexual maturation periods of male Sprague-Dawley rats, on the female offspring susceptibility to chemically-induced mammary carcinogenesis. Additionally, it further aimed at elucidating potential underlying cellular, molecular and epigenetic mechanisms.

1. OBJECTIVES

To evaluate whether paternal consumption of high-fat diets with different compositions, lard- or corn oil-based, during periods of development and sexual maturation, influences the rat female offspring susceptibility to chemically induced mammary carcinogenesis, as well as elucidate potential underlying cellular, molecular and epigenetic mechanisms.

2. DESCRIPTION OF CHAPTERS

This thesis is organized in the format of two scientific articles. The first article, within chapter 1, entitled "**Paternal programming of breast cancer risk in daughters in a rat model: opposing effects of animal- and plant-based high-fat diets**", published in Breast Cancer Research (2016, 18:71, DOI: 10.1186/s13058-016-0729-x), describes that female offspring from male rats fed a lard-based high-fat diet showed lower mammary gland differentiation associated with higher breast cancer susceptibility. On the other hand, female offspring of corn oil-based high-fat diet fed fathers had lower breast cancer susceptibility. Among the plausible mechanisms that underlie this paternal influence, we highlight miRNAs altered expression in both father's sperm and daughter's mammary gland, as well as altered expression of proteins related to key cellular process, for example epithelial to mesenchymal transition.

The second chapter contains the second article entitled "**Paternal animal and plant-based high fat diets can program rat female offspring lipidomic fatty acid profile and epigenetic and protein expression alteration associated with breast cancer risk**" to be submitted to publication. It describes how the consumption of lard- and corn-oil-based high fat diets can modulate adipose tissue fatty acids profile in male rats as well as in their female offspring. Further, the female offspring also showed altered epigenetic marks, more specifically histones modification in addition to changes in protein expression levels. Altogether these findings indicate that paternal diet can have long lasting effects on female offspring's metabolism and gene expression profile that could be associated with breast cancer risk.

3. CHAPTER 1: Paternal programming of breast cancer risk in daughters in a rat model: opposing effects of animal- and plant-based high-fat diets

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Keywords: Paternal diet, breast cancer, high fat diet, female offspring

Abstract

Background: Although males contribute half of the embryo's genome, only recently an interest has started to be directed towards the potential impact of paternal experiences on the offspring health. While there is evidence that paternal malnutrition may increase offspring susceptibility to metabolic diseases, few studies have examined the influence of paternal factors on daughter's breast cancer risk.

Methods: Male Sprague-Dawley rats were fed before and during puberty either a lard-based (high in saturated fats) or a corn oil-based (high in n-6 polyunsaturated fats) high fat diet (60% of fat-derived energy). Control animals were fed AIN-93G control diet (16% of fat-derived energy). At 12 weeks of age male rats were mated. Their 50 days-old female offspring consuming only commercial diet were submitted to the classical model of mammary carcinogenesis based on 7,12-dimethylbenz[*a*]anthracene initiation and mammary tumor development was evaluated. Sperm cells and mammary gland tissue were submitted to cellular and molecular analysis.

Results: Compared to female offspring of control diet fed male rats, offspring of lard fed male rats did not differ in tumor latency, growth and multiplicity. However female offspring of lard fed male rats had increased elongation of mammary epithelial tree, number of terminal end buds and tumor incidence compared to both control diet and corn oil fed male rats. Compared to female offspring of control diet fed male rats, female offspring of corn oil fed male rats showed decreased tumor growth but no difference regarding tumor latency and multiplicity. In addition, female offspring of corn oil fed male rats had longer tumor latency and decreased tumor growth and multiplicity compared to female offspring of lard fed male rats. Paternal consumption of animal or

plant-based high-fat diets elicited opposing effects, with lard that is rich in saturated fatty acids increasing and corn oil that is rich in n-6 polyunsaturated fatty acids decreasing breast cancer risk in their offspring. These effects could be linked to alterations in miRNA expression in fathers' sperm and their daughters' mammary glands as well as to modifications in breast cancer-related proteins expression in this tissue.

Conclusion: Our findings highlight the importance of paternal nutrition in affecting future generation's risk of developing breast cancer.

Introduction

Breast cancer is a global public health problem, with nearly 1.7 million new cases diagnosed in 2012, representing 25% of all cancers in women worldwide (1). Its incidence is projected to significantly rise over the next 20 years despite current efforts to prevent the disease (2). Although the precise reason for this growth is still not clear, it has been suggested that modern women's lifestyle, including postponing first pregnancy and having fewer children can explain the increase (3).

Nutritional habits, such as an adoption of Westernized dietary patterns, are also linked to increased breast cancer risk (4). These patterns are characterized by low consumption of fruits and vegetables, increased energy intake and decreased energy expenditure, leading to obesity, as well as increased intake of saturated, n-6 polyunsaturated and trans fatty acids and decreased intake of n-3 polyunsaturated fats (5, 6). While the majority of epidemiological studies on nutrition and breast cancer risk have focused on women´s diet in adulthood, accumulating epidemiological and experimental evidence highlight early life experiences, including nutrition as relevant factors for later breast cancer risk determination (7). The developmental origins of this cancer has predominantly been considered from a maternal perspective with emphasis on the impact of high fat or energy intake during gestation and lactation on female´s offspring mammary gland development and later breast cancer risk (8, 9).

Although males contribute half of the embryo's genome, only recently an interest has started to be directed towards the potential impact of paternal experiences on the offspring health (10). While experimental studies have shown that paternal malnutrition may increase the offspring susceptibility to metabolic dysregulation, obesity and

cardiovascular diseases (11, 12), few studies have examined the influence of paternal factors on daughter's breast cancer risk. Among them, epidemiological studies show an association between higher paternal educational level, higher age and smoking and increased rate of breast cancer in the daughters (13, 14).

Unlike female production of germ cells that takes place predominantly in early life (15), male production of germ cells starts in utero with mature sperm cells being produced throughout the entire reproductive life (16). Because spermatogenesis can be dramatically influenced by environmental factors, including malnutrition, obesity, and an exposure to toxic compounds, father's health during preconception is now acknowledged as a critical factor in the context of the developmental origins of health and disease (17). In addition to embryogenesis, gametogenesis comprises intense epigenetic (DNA methylation, histone modification, and microRNA expression) remodeling (18, 19). Thus epigenetically inherited increased disease risk could be transmitted not only through the female germ-line but also through the male germ-line (20). Specific windows where male gametes would be especially prone to environmentally elicited epigenetic deregulation comprise prepuberty and the reproductive phase (21).

Given the marked increase in dietary fat intake over the past three decades (22), and the notion that different kinds of dietary fats can lead to different health outcomes (23), we designed this study to investigate in rats whether consumption of high levels of animal or vegetable-based fats by fathers would affect their daughters' risk of breast cancer. We also investigated the underlying cellular and molecular mechanisms. We fed male Sprague-Dawley rats before and during puberty either a lard-based (high in saturated fats) or a corn oil-based (high in n-6 polyunsaturated fats) high fat diet (60% of fat-

derived energy). Control animals were fed AIN-93G control diet containing soybean oil as a fat source (16% of fat- derived energy). Males were mated with female rats consuming a commercial diet. We show that paternal consumption of these high-fat diets elicited opposing effects, with animal fat increasing and vegetable oil decreasing breast cancer risk in their offspring. These effects could be linked to alterations in miRNA expression in fathers' sperm and their daughters' mammary glands as well as to modifications in breast cancer-related proteins expression in this tissue. These novel data show that paternal high-fat diets influence their female offspring's susceptibility to mammary cancer, with consumption of lard increasing and corn oil reducing daughters' risk. Thus, paternal diet before conception sets a stage for his daughter's risk of developing breast cancer.

Material and Methods

Experimental Design: This study was approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo (Protocol Number CEUA/FCF/381). 21 days-old male rats were divided into 3 groups (n=20 rats per group): controls (fed control AIN-93G diet, with 16% of total calories provided by lipids), lard fed males (exposed to high SFA diet, with 60% of total calories provided mainly from lard) and corn oil males (exposed to n-6 PUFA diet, with 60% of total calories provided mainly from corn oil). At 12 weeks of age, male rats were all switched to chow diet and mated by housing 1 male with 1 female per cage. Pregnant female rats and their offspring consumed only commercial laboratory chow (Nuvital, Brazil) (Figure 1). Body weight and food intake was recorded 2-3 times per week.

Figure 1: Experimental Design.

Determination of the diets lipid profile: The lipid profiles of the control, lard and corn oil diets were determined according to the Association of Official Analytical Chemists (AOAC) (24). Fatty acids were esterified to fatty acid methyl esters according to Hartman & Lago (25), and their composition was analyzed with a gas chromatograph (GC 17 A Shimadzu/Class GC 10, Japan) equipped with a flame ionization detector (FID) and a SUPELCOWAX® 10 fused silica capillary column (30 mm × 0.25 mm i.d.). The temperature was set at 170^{o}C, raised to 225^{o}C at a rate of 1^{o}C/min and held for 25 min. The temperatures of the vaporizer and detector were 250^{o}C and 270^{o}C, respectively. Helium was used as the carrier gas (1 mL/min). Identification of the fatty acids was

performed by comparison of the retention times with the standard mixture of fatty acid methyl esters. Each determination was performed in duplicate, using 2 different samples for each diet.

Insulin Tolerance Test: The tests were performed at 0800 h after 12 h of fasting, according to Takada et al (26). The insulin load (75 mU/100 g b.w.) was injected as a 'bolus' and the blood glucose levels were determined at 0, 3, 6, 9, 12, and 30 min after injection in male rats and their 50 days old female offspring. The area under the curve was calculated according to the trapezoid rule (27).

Mature spermatozoa collection and purification: Control diet, lard and corn oil fed male rats were euthanized once females were pregnant, and caudal epididymis (for sperm collection) dissected. The cauda and vas deferens from male rats were collected, punctured, and transferred to tissue culture dish containing M2 media (M2 Medium-with HEPES, without penicillin and streptomycin, sterile-filtered, suitable for rat embryo, Sigma, U.S.A.) where it was incubated for 1 hour at 37°C. Spermatozoa samples were washed with PBS, and then incubated with SCLB (somatic cell lysis buffer, 0.1% SDS, 0.5% TX-100 in Diethylpyrocarbonate water) for 1 hour, protocol described by Goodrich (28). SCLB was rinsed off with 2 washes of PBS (Phosphate-buffered saline) and the purified spermatozoa sample (at least 95% purity as assessed by microscopy) pelleted and used for miRNA extraction.

Determination of daily sperm production: The right testis was maintained at -20°C until processing to determine the daily sperm production. The technique proposed by Robb (29) is based on the resistance of elongated spermatids present in the phases 17-19 of

spermatogenesis to intense mechanical stress due to the high compaction of chromatin.

Sperm morphology analyses: According to Seed (30), the epididymal was previously frozen at -20°C, underwent incision and subsequently immersed in PBS buffer to promote the dissemination of gametes to the aqueous medium. Then, the obtained solution was placed on slides for examination by light microscope. Two hundred sperm per animal were analyzed microscopically at 400x magnification.

Mammary gland harvesting: Abdominal mammary glands of female offspring of control diet, lard and corn oil male rats (n=6 per group) were collected on postnatal day 50, and used for preparing mammary whole mounts, and miRNA and protein extraction.

Analysis of mammary gland morphology and development: Whole-mount preparations of the 4th abdominal mammary gland from 50 days old female offspring (n = 5/group) were obtained and the epithelium elongation and the number of terminal end buds (TEBs) were determined as described by de Assis et al (31).

Mammary tumor induction: Mammary tumors were induced in 50 days-old female rats offspring (n=24 rats per group) by administration of 7,12-dimethylbenz[*a*]anthracene (DMBA) (50 mg/kg body weight, Sigma, USA). The carcinogen was dissolved in corn oil and administered by oral gavage. Animals were examined for mammary tumors by palpation 2 times per week. Latency of tumor appearance, the number of animals with tumors and the number of tumors per animal (multiplicity) were evaluated. The tumor volume was calculated with tumor measures of length (a), height (b) and width (c) taken with a caliper rule once a week since tumor appearance and throughout the experiment. The formula "(1/6*3,14)*(a*b*c)" was used to calculate the tumor volume, as described

by Rygaard and Spang-Thomsen (32). The tumor growth rate was calculated using the measured volumes of each tumor at a given week (d) and the subsequent week (e) of appearance, with the formula: "[(e-d)/d]*100". Those animals in which tumor burden approximated 10% of total body weight were euthanized. All others animals were sacrificed 19 weeks after carcinogen administration.

Analysis of mammary gland and tumor cell proliferation and apoptosis in female offspring: Cell proliferation was evaluated in mammary gland (ducts and lobules) and tumors from 50 days old female offspring (n = 4/group) by Ki67 immunohistochemistry. After harvesting, mammary tissue was directly fixed in 10% buffered formalin, embedded in paraffin and sectioned. Sections were then deparaffinized in xylene and hydrated through graded ethanol. Antigen retrieval was performed with 10 mM citrate buffer pH 6 for 20 min in pressure cooker. Peroxidase blocking was performed with 10% H_2O_2 for 10 min and nonspecific binding was blocked for 1 h with 1% skimmed milk in PBS. Sections were incubated overnight with anti-rat Ki67 primary antibody (Abcam, UK) at a 1:50 dilution. After washes, sections were incubated with the LABS + System/HRP Kit (Dako-Agilent Technologies, USA) according to the manufacturer's instructions and stained with 3,3'-diaminobenzidine in chromogenic solution (Dako-Agilent Technologies, USA) for 10 min, washed and counterstained for 1.5 min with hematoxylin. Cell proliferation was quantified by assessing the number of Ki67 positive cells among 1000 cells. The slides were evaluated using Image J software (NIH, USA). Apoptosis analysis was conducted in mammary gland (ducts and lobules) and tumors from 50 days old female offspring (n = 4/group), according to Elmore (33) using Image J software (NIH, USA). Results are represented as mean number of apoptotic cells/1000 cells.

microRNA expression profile analysis: Total RNA from paternal sperm and their female offspring total mammary gland was extracted using miRNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. RNA samples was quantified and stored at -80^{O}C until use. miRNAs arrays were performed at the Genomics and Epigenomics Shared Resources (GESR) at Georgetown University using Applied Biosystems TaqMan Rodent MicroRNA Arrays to generate the miRNA expression profile for each experimental group. The TaqMan® Array Rodent MicroRNA A+B Cards Set v3.0 is a two card set containing a total of 384 TaqMan® MicroRNA Assays per card. The set enables accurate quantitation of 641 and 373 unique microRNAs for rat. There are three TaqMan® MicroRNA Assay endogenous controls for each species on each array to aid in data normalization (34). The geNorm algorithm was applied to those endogenous controls to determine the optimal number of stable controls. Geometric mean of these selected controls was used for array normalization. In order to conduct further statistical analysis, the normalized value was log-transformed to meet the t-test requirement. Statistical analysis was conducted by R package limma (35). miRNA that had fdr < 0.1 were considered as significantly altered miRNA and selected for further analysis. Target prediction for microRNAs of interest was conducted using TargetScan (Release 6.2). The predicted targeted mRNA list was then uploaded to Ingenuity pathway analysis (IPA) for gene set enrichment analysis. We selected top canonical pathways for further analysis.

Analysis of protein levels in mammary glands of female offspring: Protein levels of 50 ng/μL were assessed by western blot in total mammary glands obtained from 50 days old female rats (n=5 per group). Total protein was extracted from mammary tissues using RIPA buffer with protease inhibitor (Roche, Switzerland), glycerophosphate (10 mM),

sodium orthovanadate (1 mM), pyrophosphate (5 mM) and PMSF (1 mM). The samples were mixed with the buffer for 5 min, then incubated on ice for 30 min and centrifuged for 10 min on high speed. Protein in the supernatant was quantified with BCA Protein Assay Reagent (Pierce, USA). Protein extracts were resolved on a 4-12% denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred using the iBlot® 7-Minute Blotting System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1 hour at room temperature. Membranes were incubated with the specific primary antibodies at 4°C overnight. After several washes, the membranes were incubated with horseradish peroxidase (HRP)- conjugated secondary antibody (1: 5,000, Santa Cruz Biotechnology, USA) at room temperature for 1 hour. Membranes were developed using the Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., USA), and exposed to Kodak autoradiography films. Optical density of the bands was quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading, expression of the proteins of interest was normalized to the β-actin signal.

Statistical Analysis: Results are expressed as the mean ± standard error of mean (S.E.M.), and all analyses were conducted with R package limma. Multiple-group comparisons were performed using one-way analysis of variance followed by LSD, and two-group comparisons were performed using Student's t-test. Repeated measures ANOVA test was applied for caloric intake data evaluation, and Kaplan Meier Curve and Log-Rank test were applied for determining differences in tumor incidence. For all data analysis, $p \leq 0.05$ was applied as the threshold for statistical significance.

Results

Paternal Dietary and Health Parameters

Compared to control diet fed male rats, the ones that were on the lard or corn oil based high fat diets consumed more ($p \leq 0.05$) saturated fatty acids (SFA) [predominantly palmitic (C16:0) and stearic (C18:0) acids], monounsaturated fatty acids (MUFA) [predominantly oleic acid (C18:1n9c)] and polyunsaturated fatty acids (PUFA) [predominantly linoleic acid (C18:2n6c)] (Figure 2A). Corn oil fed male rats consumed less ($p \leq 0.05$) SFA [predominantly palmitic (C16:0) and stearic (C18:0) acids] and MUFA [predominantly oleic acid (C18:1n9c)] and more ($p \leq 0.05$) PUFA [predominantly linoleic acid (C18:2n6c)] than the lard fed male rats (Figure 2A). Daily caloric intake was approximately 7% higher ($p \leq 0.05$) in both lard and corn oil fed male rats, compared with control diet fed male rats (Data not shown).

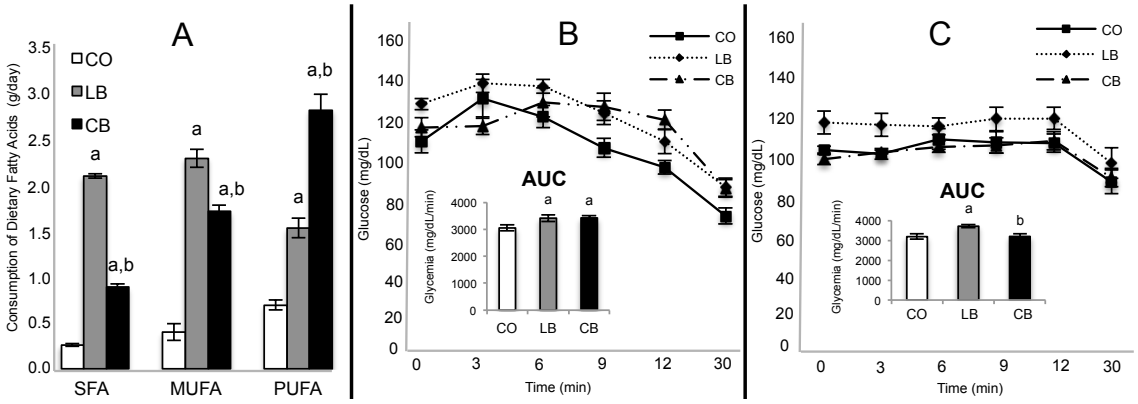

Figure 2: **Paternal fatty acid consumption and insulin tolerance test**. **A:** Control diet (CO) and lard based (LB) and corn oil based (CB) high fat diets fed male rats consumption of dietary fats [Saturated (SFA), Monounsaturated (MUFA) and Polyunsaturated (PUFA)] per day (n=20 per group). **B:** Insulin tolerance test (ITT) of the CO, LB and CB male rats (n=6 per group). Inset: The ITT is shown as the AUC. **C:** and of their 50 days-old female offspring (n=6 per group). Inset: The ITT is shown as the AUC. Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. The data are expressed as mean ± SEM.

Although lard and corn oil fed male rats consumed nearly the same amount of calories per day, lard fed male rats gained more weight than corn oil ($p \leq 0.05$) or control diet fed male rats ($p \leq 0.05$) (Table 1). There was no difference ($p > 0.05$) between control diet and corn oil fed male rats regarding weight gain. Both lard and corn oil fed male rats had higher ($p \leq 0.05$) abdominal, retroperitoneal and epididymal fat pad weights than control diet fed male rats (Table 1). Compared to lard fed male rats, corn oil fed male rats had lower ($p \leq 0.05$) epididymal fat pad weights but no difference ($p > 0.05$) regarding abdominal and retroperitoneal fat pad weights (Table 1). Further, lard fed male rats had lower testicle, epididymis ($p \leq 0.05$) and seminal vesicle ($p \leq 0.08$) weights than the control diet or corn oil fed male rats (Table 1). There was no difference ($p > 0.05$) between control diet and corn oil fed male rats regarding these parameters (Table 1). Lard fed male rats also had ($p \leq 0.05$) less normal sperm cells and lower daily sperm production than control diet or corn oil fed male rats (Table 1). There was no difference ($p > 0.05$) between control diet and corn oil fed male rats regarding these parameters (Table 1). Both lard and corn oil fed male rats had higher ($p \leq 0.05$) fasting glucose levels than control diet fed male rats (Table 1). There was no difference ($p > 0.05$) between lard and corn oil fed male rats regarding this parameter (Table 1). Further, in the insulin tolerance test, lard and corn oil fed male rats had higher ($p \leq 0.05$) area under the curve, compared to control diet fed male rats (Figure 2B), indicating that they were insulin intolerant. There was no difference ($p > 0.05$) between lard and corn oil fed male rats regarding this parameter (Figure 2B).

Female Offspring Health Parameters

Female offspring of both lard and corn oil fed male rats had higher birth weights ($p \leq 0.05$) and weight gain ($p \leq 0.05$; $p \leq 0.08$ for offspring of corn oil fed male rats) than offspring of control diet fed male rats (Table 1). There was no difference ($p > 0.05$) between female offspring of lard and corn oil fed male rats regarding birth weight (Table 1). Female offspring of corn oil fed male rats had lower ($p \leq 0.05$) weight gain than offspring of lard fed male rats (Table 1). Similar to the male rats, both female offspring of lard and corn oil fed male rats had higher ($p \leq 0.05$) retroperitoneal fat weight than offspring of control diet fed male rats (Table 1). There was no difference ($p > 0.05$) between female offspring of lard and corn oil fed male rats regarding this parameter (Table 1). Female offspring of lard fed male rats had higher ($p \leq 0.05$) fasting glucose levels (Table 1) and higher ($p \leq 0.05$) area under the curve compared to female offspring of control diet and corn oil fed male rats (Figure 2C). There was no difference ($p > 0.05$) between female offspring of control diet and corn oil fed male rats regarding these parameters (Table 1 and Figure 2C).

Table 1: Health parameters of male rats and their 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males.

		CO	LB	CB
Fathers	Weight Gain	363.1±6.4g	398.2±12.0g [a]	369.4±7.8g [b]
	Fat weight			
	Abdominal fat	1.4±0.1g/100g b.w.	2.7±0,2g/100g b.w. [a]	2.6±0.2g/100g b.w. [a]
	Retroperitoneal fat	0.8±0.1g/100g b.w.	1.9±0.1g/100g b.w. [a]	1.7±0.2g/100g b.w. [a]
	Epididymal fat	1.2±0.1g/100g b.w.	2.7±0.1g/100g b.w. [a]	2.0±0.2g/100g b.w. [a,b]
	Reproductive Organs			
	Testicle	0.45±0.01g/100g b.w.	0.40 ±0.01g/100g b.w.[a]	0.45±0.01g/100g b.w. [b]
	Epididymis	0.14±0.00g/100g b.w.	0.13±0.00g/100g b.w. [a]	0.14±0.00g/100g b.w. [b]
	Seminal Vesicle	0.33±0.01g/100g b.w.	0.29±0.02g/100g b.w. [c]	0.33±0.02g/100g b.w. [d]
	Sperm morphology (% normal)	66.4 ± 2.8 %	50.1 ± 3.0 %[a]	70.9 ± 2.9 %[b]
	Daily sperm production	31±1.9 sperm n/ testicle/day ($x10^6$)	23±0.9 sperm n/ testicle/day ($x10^6$)[a]	29±0.8 sperm n/ testicle/day ($x10^6$)[b]
	Fasting Glycaemia	100.8±3mg/dL	124.6±4mg/dL [a]	116.1±3mg/dL [a]
50 days-old Female Offspring	Birth Weight	8.0±1.5g	8.8±1.0g [a]	9.1±1.5g [a]
	Weight Gain	138.9±1.5g	147.0±1.2g [a]	142.1±1.1g [c,b]
	Retroperitoneal fat	0.9±0.1g/100g b.w.	1.1±0.0g/100g b.w. [a]	1.2±0.1g/100g b.w. [a]
	Fasting Glycaemia	106.4±2.0mg/dL	112.5±1.9mg/dL[a]	106.3±1.2mg/dL[b]

Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. Marginal difference ($p \leq 0.08$) compared to CO[c] and LB[d], according to T-test. The data are expressed as mean ± SEM (n=20 per group).b.w. = body weight.

Female Offspring Mammary Gland Morphology

Mammary gland morphology was assessed from mammary whole mounts obtained from 50 days old female offspring. Both the elongation of mammary epithelial tree (Figure 3C) and the number of terminal end buds (TEBs) (Figure 3D) were higher in female offspring of lard fed male rats compared to female offspring of control diet and corn oil fed male rats. There was no difference ($p>0.05$) between female offspring of control diet and corn oil fed male rats regarding these parameters (Figure 3C and D).

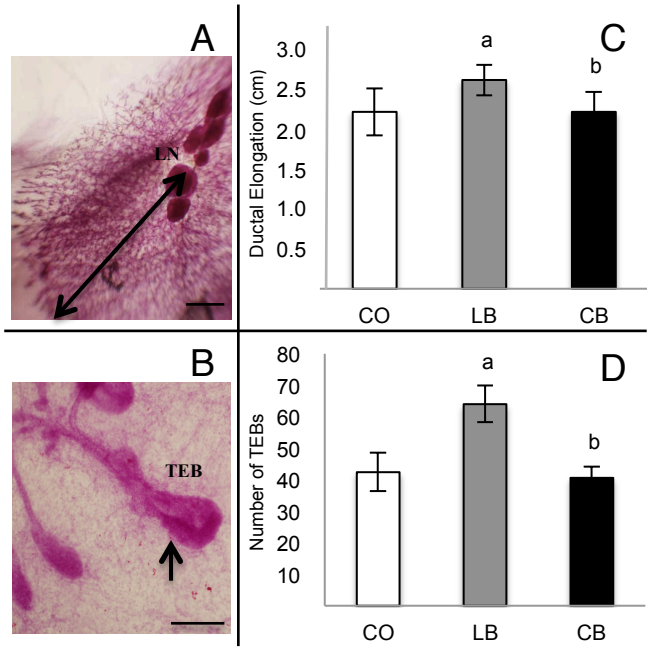

Figure 3: Mammary gland development of 50 days-old female offspring of control diet (CO), lard (LB) and corn oil (CB) fed males: A: histological depiction of the 4th abdominal mammary gland showing ductal elongation, indicated by arrow. **B:** terminal end buds (TEBs), indicated by arrows. **C:** Ductal elongation. **D:** Number of TEBs. All values are expressed as the mean ± SEM. Statistically significant difference ($p\leq0.05$) compared to CO^{a} and LB^{b}, according to ANOVA followed by LSD test. The data are expressed as mean ± SEM (n=5 per group).

Female Offspring Mammary Gland Tumors data

Mammary tumors in the female offspring were induced by the carcinogen 7, 12-dimethyl-benzo[a]anthracene (DMBA). Female offspring of lard fed male rats had increased mammary tumor incidence ($p \leq 0.05$) compared to both offspring of control diet and corn oil fed male rats (Figure 4A). There was no statistical difference ($p > 0.05$) between offspring of control diet and corn oil fed male rats regarding tumor incidence. Female offspring of corn oil fed male rats exhibited longer ($p \leq 0.05$) tumor latency and lower tumor multiplicity ($p \leq 0.05$) compared to female offspring of lard fed male rats (Figure 4B and 4D). Compared to female offspring of control diet fed male rats, female offspring of lard or corn oil fed male rats did not show differences ($p > 0.05$) regarding tumor latency and multiplicity. Further, female offspring of corn oil fed male rats showed lower ($p \leq 0.05$) tumor growth, on the first week of tumor appearance, compared to both offspring of control diet and lard fed male rats (Figure 4C). There was no statistical difference ($p > 0.05$) between offspring of control diet and lard fed male rats regarding tumor growth. Additionally, there was no statistical difference among groups on the tumor growth rate for the remaining experimental weeks.

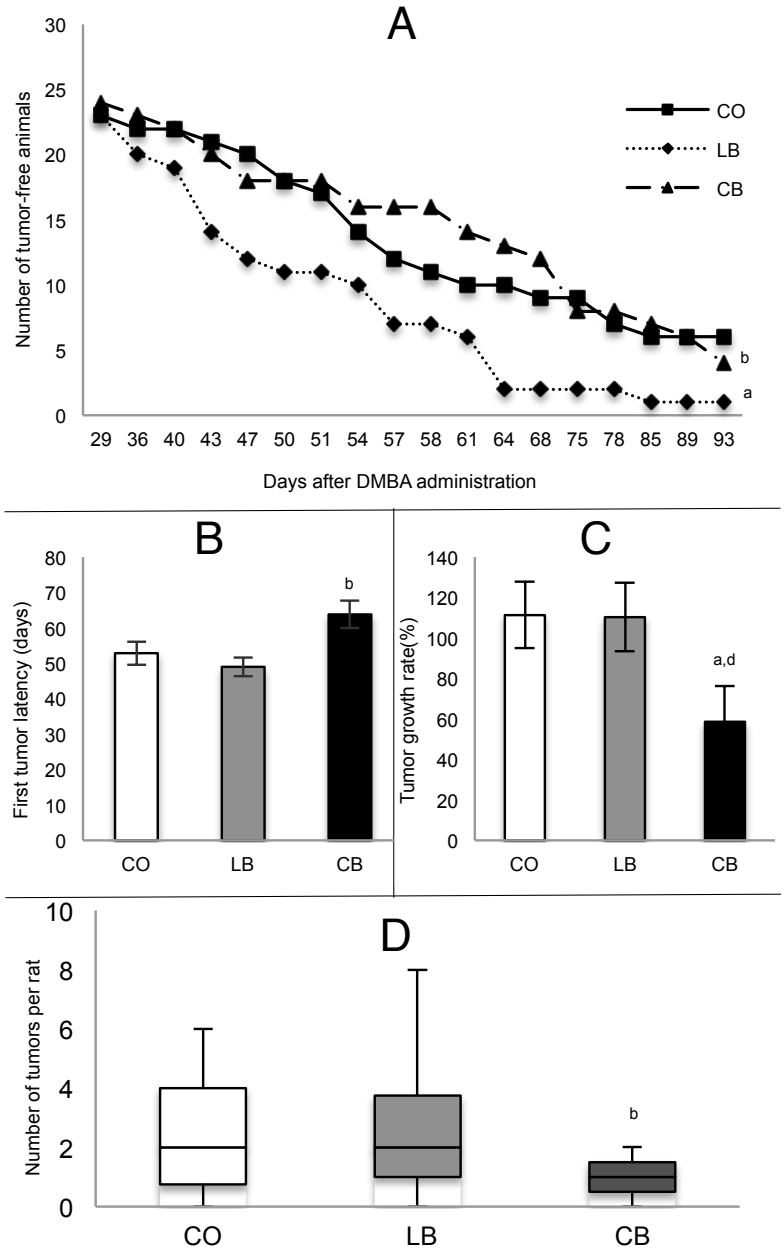

Figure 4: **Mammary tumorigenesis in female offspring of control diet (CO), lard (LB) and corn oil (CB) fed males**. **A:** number of tumor-free rats. **B:** number of days before the appearance of the first tumor. **C:** tumor growth rate on the first week of appearance. **D:** total number of tumors per rat (multiplicity). Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. Marginal difference ($p \leq 0.08$) compared to LB[d], according to T-test. The data are expressed as mean ± SEM (n=24 per group).

Female Offspring Mammary Gland and Tumor Cell Proliferation and Apoptosis

Female offspring of lard fed male rats exhibited increased ($p \leq 0.06$) number of proliferative cells (Figure 5B) and decreased ($p \leq 0.05$) number of apoptotic cells (Figure 5E) in mammary gland lobules compared to female offspring from control diet and corn oil fed male rats. There was no difference ($p > 0.05$) between female offspring of control diet and corn oil fed male rats regarding these parameters (Figure 5B and 5E). Further there was no difference ($p > 0.05$) regarding cell proliferation and apoptosis in mammary gland ducts among female offspring of all groups. Female offspring from both lard and corn oil fed male rats exhibited decreased ($p \leq 0.05$) number of apoptotic cells (Figure 5F) in mammary tumors compared to female offspring from control diet fed male rats. There was no difference ($p > 0.05$) between female offspring of lard and corn oil fed male rats regarding this parameter (Figure 5F). In addition there was no difference ($p > 0.05$) among groups regarding cell proliferation in the mammary tumors (Figure 5C).

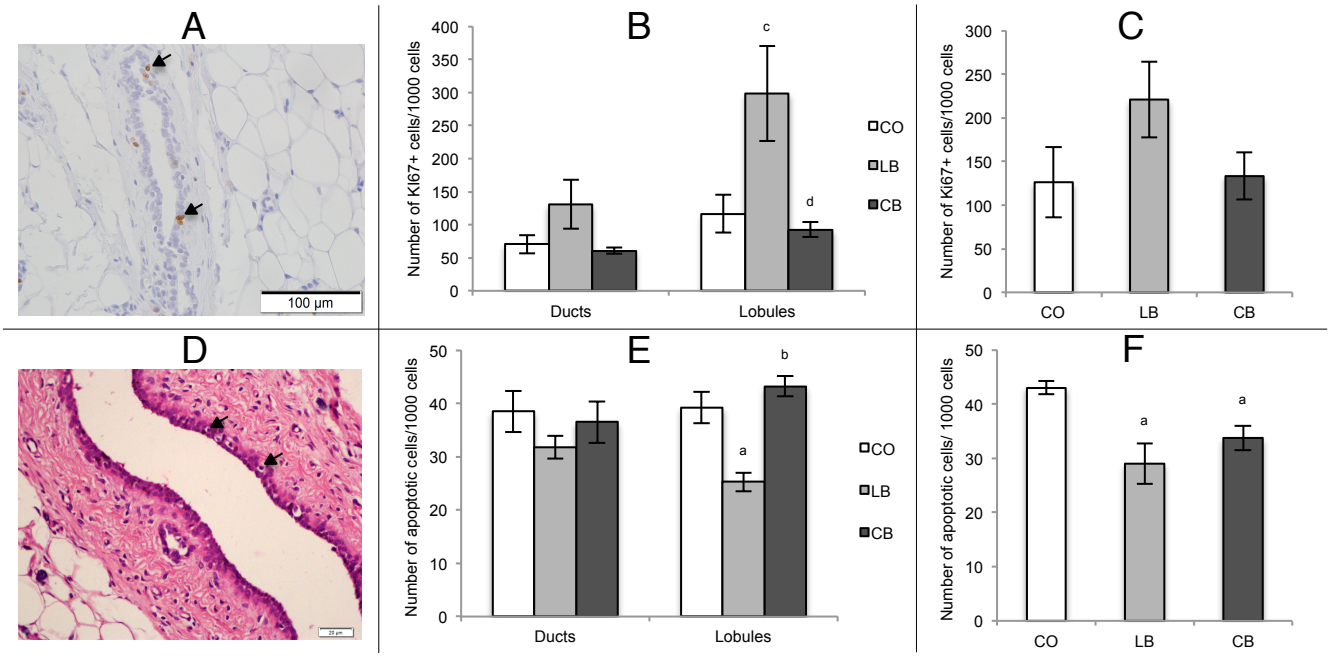

Figure 5: Cell proliferation and apoptosis quantification in mammary gland and tumors. **A:** Photomicrography (20X) of Ki67 immunostaining (i.e. cells indicated by arrows). **B:** Cell proliferation quantification in the mammary gland ducts and lobules of 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. **C:** Cell proliferation quantification in mammary tumors of female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. **D:** Photomicrography (40X) showing apoptotic cells (i.e. cells indicated by arrows). **E:** Apoptosis quantification in the mammary gland ducts and lobules of 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. **F:** Apoptosis quantification in mammary tumors of female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. Marginal difference ($p \leq 0.06$) compared to CO[c] and LB[d], according to T-test. The data are expressed as mean ± SEM (n=4 per group).

miRNA expression profile in fathers' sperm cells and in their daughters' mammary gland

In order to compare the outcomes of the distinct paternal high fat diets on miRNAs expression, Applied Biosystems TaqMan Rodent MicroRNA Arrays were used to generate the miRNA profile for lard and corn oil fed fathers' sperm cells, as well as for their respective daughters' mammary glands. The microarray data is deposited in the public repository Gene Expression Omnibus (GEO) under the access number GSE77012. Corn oil fed male rats showed eighty-nine downregulated ($p \leq 0.05$) miRNAs in the sperm compared to lard fed male rats (Figure 6A). Furthermore, female offspring of corn oil fed male rats showed twenty-one downregulated ($p \leq 0.05$) and two upregulated ($p \leq 0.05$) miRNAs in their mammary gland compared to female offspring of lard fed male rats (Figure 6B). There were three miRNAs that were downregulated in both the sperm and mammary glands in the corn oil fathers and daughters: miR-1897-5p, miR-219-1-3p and miR-376a#. Ingenuity pathway analysis (Supplementary Table 1) indicated that these miRNAs could regulate signaling pathways associated with key physiological processes like growth hormone, PTEN and prolactin signaling, as well as diseases such as Huntington's disease, Cardiac Hypertrophy, Type II Diabetes Mellitus and Breast Cancer.

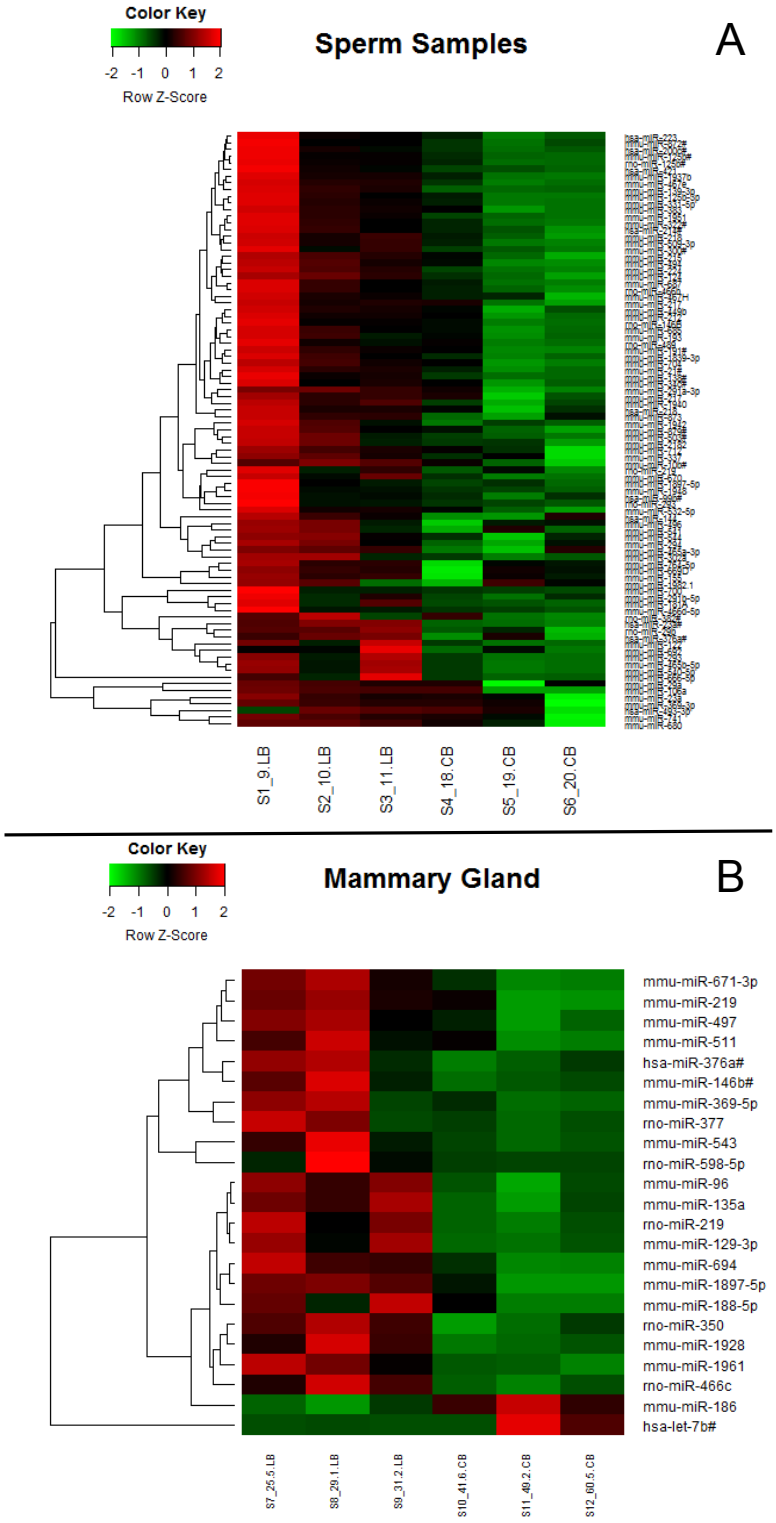

Figure 6: Heatmap of miRNA expression profiles. A: Heatmap of miRNA expression profile in sperm of the male rats from control diet (CO), lard (LB) and corn oil (CB) fed males. **B:** Heatmap of miRNA expression profile in normal mammary tissue of 50 days-old female offspring (n=3 per group).

Supplementary Table 1: Ingenuity Canonical Pathway analyses of the target pathways and molecules modulated by altered miRNA from father's sperm and 50 days old female offspring mammary gland from lard (LB) and corn oil (CB) fed males.

Ingenuity Canonical Pathways	-log (p-value)	Ratio	Molecules
Prolactin Signaling	4.62E00	9.59E-02	PRKCI,SP1,PIK3R1,PRKCE,SOCS4,**CEBPB**,**PRKD1**
PTEN Signaling	4.11E00	6.78E-02	**TGFBR1**,**CASP3**,PIK3R1,FGFR1,**IGF1R**,BMPR2, FOXG1,BCL2L11
Huntington's Disease Signaling	4.03E00	4.78E-02	GRM5,HDAC9,PRKCI,**CASP3**,SP1,PIK3R1,**IGF1R**, PRKCE,CDK5R1,SIN3A,**PRKD1**
Growth Hormone Signaling	3.79E00	8.7E-02	PRKCI,PIK3R1,**IGF1R**,PRKCE,SOCS4,**PRKD1**
HER-2 Signaling in Breast Cancer	3.56E00	7.89E-02	PRKCI,PIK3R1,PRKCE,PARD6B,ITGB8,**PRKD1**
Role of NFAT in Cardiac Hypertrophy	3.56E00	5.03E-02	HDAC9,PRKCI,**TGFBR1**,PIK3R1,IGF1R,MEF2A, PRKCE,**PRKD1**,CABIN1
Type II Diabetes Mellitus Signaling	3.33E00	5.98E-02	PRKCI,PIK3R1,PRKCE,SOCS4,**CEBPB**,ACSL1,**PRKD1**

Protein expression in female offspring mammary gland

Since miR-1897-5p, miR-219-1-3p and miR-376a# can directly or indirectly modulate several targets (shown on the Supplementary Table 1), we decided to analyze through Western Blot proteins linked to breast cancer: CCAAT/enhancer binding protein beta (Cebpß), Caspase 3 (Casp3), Insulin-like growth factor 1 receptor (Igf1r), Protein Kinase D1 (Pkd1) and Transforming growth factor beta receptor I (Tgfßr1). There was no difference ($p>0.05$) among female offspring of control diet, lard and corn oil fed male rats regarding Cebpß, Casp3 and Igf1r levels (data not shown). On the other hand, female offspring of corn oil fed male rats had higher ($p\leq0.05$) Pkd1 levels in the mammary glands compared to female offspring of lard but not control diet fed male rats (Figure 7).

There was no difference ($p>0.05$) between female offspring of lard and control diet fed male rats regarding this protein (Figure 7). In addition, Tgfßr1 levels were significantly increased in the offspring of lard fed male rats (Figure 7) compared to both offspring of control diet ($p\leq0.05$) and corn oil ($p\leq0.06$) fed male rats. There was no difference ($p>0.05$) between female offspring of corn oil and control diet fed male rats regarding this protein (Figure 7). Interestingly both proteins are involved in regulating epithelial to mesenchymal transition (EMT): Pkd1 inhibits it (36) and Tgfßr1 promotes this process (37).

We further explored if Tgfß and key regulators of its activity were altered by measuring protein levels of V-akt murine thymoma viral oncogene (Akt), Cofilin (Cfl), V- raf-leukemia viral oncogene (c-Raf), Mitogen-activated protein kinase (Erk1/2), phosphorylated Mitogen-activated protein kinase 8 (p-Jnk), Mitogen activated protein kinase 4 (Mkk4), Mechanistic target of rapamycin (Mtor), Mitogen activated protein kinase 14 (p38), phosphorylated Smad family member 3/Smad family member 3 ratio (p-Smad3/Smad3) and Harvey rat sarcoma virus oncogene (Ras). Tgfß protein expression was higher ($p\leq0.05$) in the mammary glands of the female offspring of lard fed male rats than in the offspring of control diet or corn oil fed male rats. There was no difference ($p>0.05$) between female offspring of corn oil and control diet fed male rats regarding this protein (Figure 7). Further, the levels of Akt and p-jnk were higher ($p\leq0.05$) in the female offspring of lard fed male rats compared to female offspring of control diet fed male rats (Figure 7). There was no difference ($p>0.05$) between female offspring of corn oil and control diet or lard fed male rats regarding these proteins (Figure 7). Female offspring of corn oil fed male rats had lower levels of Mtor, Mkk4 ($p\leq0.06$) and p-Smad3/Smad3

(p≤0.05) compared to female offspring of lard but not control diet fed male rats (Figure 7). There was no difference (p>0.05) between female offspring of lard and control diet fed male rats regarding these proteins (Figure 7). In addition to promoting EMT, all these altered proteins also are involved in increasing cell survival, growth, migration and invasion.

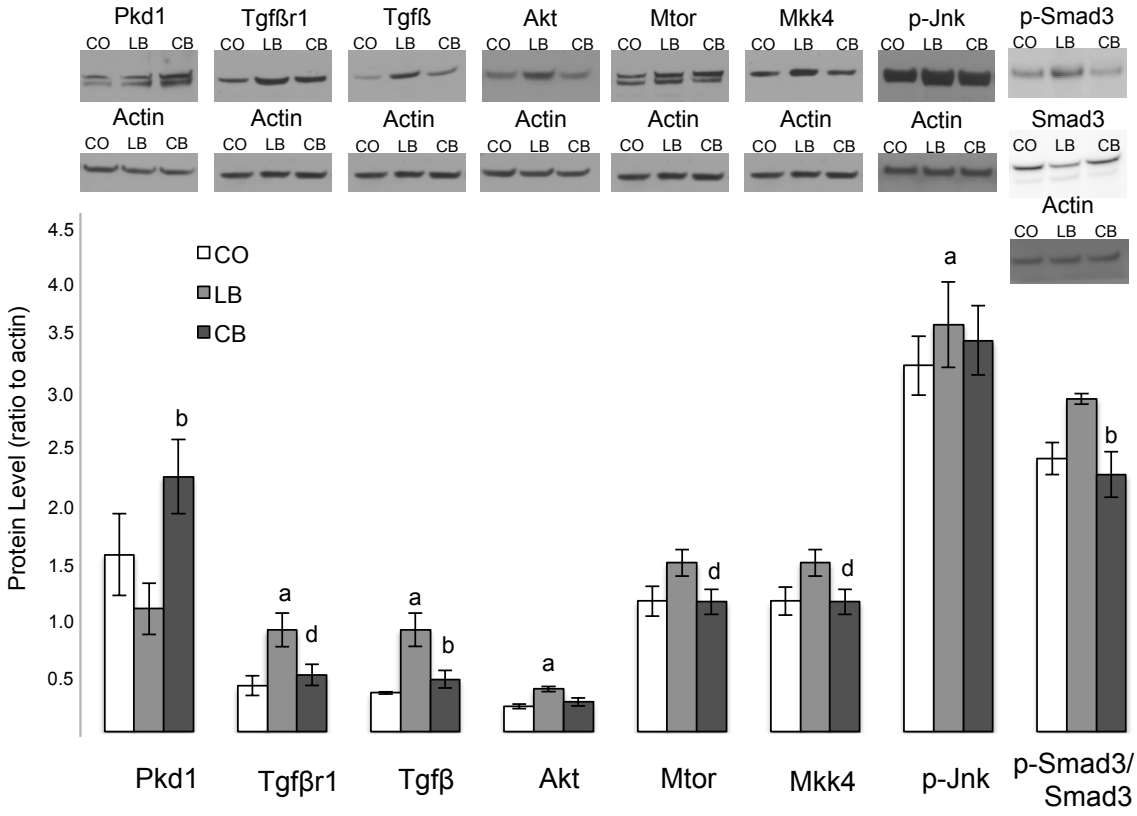

Figure 7: Protein alterations associated with miRNA expression. Western blot analysis of Pkd1, Tgfßr1, Tgfß, Akt, Mtor, Mkk4, p-Jnk and p-Smad3/Smad3 ratio protein expression in mammary gland of 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. Statistically significant difference (p≤0.05) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. Marginal difference (p≤0.06) compared to LB[d], according to T-test. The data are expressed as mean ± SEM (n=5 per group).

Discussion

Breast cancer is a complex disease with a multi-factorial etiology (38). It is increasingly evident that in utero environment can program later susceptibility to breast cancer (39). Findings obtained in our study suggest that breast cancer risk can be determined even earlier through diet-induced changes in paternal germ cells before conception. Our study shows that compared to female offspring of control diet fed fathers, offspring of lard fed fathers did not differ in tumor latency, growth and multiplicity. However female offspring of lard fed fathers had increased elongation of mammary epithelial tree, number of TEBs and tumor incidence compared to both control diet and corn oil fed fathers, showing that paternal exposure to lard based high fat diet containing SFA increased daughter's mammary cancer risk. TEBs are considered sites of tumor initiation (40) and increased epithelial elongation reflects rapid epithelial growth (41). Additionally, female offspring from lard fed fathers showed increased cell proliferation and decreased apoptosis in the mammary glands lobules compared to female offspring from both control diet and corn oil fed fathers. Altogether these findings support the view that altered mammary gland development represents a potential underlying mechanism of increased breast cancer risk (42).

Compared to female offspring of control diet fed fathers, female offspring of corn oil fed fathers showed decreased tumor growth. There was no difference regarding tumor latency and multiplicity between female offspring of control diet and corn oil fed fathers. In addition female offspring of corn oil fed fathers had longer tumor latency and decreased tumor growth and multiplicity compared to female offspring of lard fed fathers. These data show that paternal exposure to corn oil based high fat diet containing n-6

PUFA had an opposite effect, compared to paternal lard based high fat diet, and reduced daughter´s mammary cancer risk.

Although male rats on the lard and corn oil high fat diets consumed the same amount of calories, lard increased body weight and size of epididymal fat pad more than corn oil did. Thus, different fatty acids can have distinct effects on adipose accumulation, as already shown by others (43). Our results further showed that lard, but not corn oil, elicited detrimental effects on male reproductive parameters (few normal sperm cells and lower daily sperm production). This is in line with earlier human and animal data showing that SFA disrupt testicular metabolism and sperm quality, while PUFA are essential for sperm cell membrane fluidity and flexibility, and fertilization (44). Excessive epididymal fat in lard fed males may have been detrimental for spermatogenesis, as this tissue is an essential depot for spermatogenesis (45). The adverse effects of lard may not be mediated through increased insulin resistance. Although a correlation between insulin resistance and impaired sperm production has been reported in rats fed high SFA diet (46), as also found here, corn oil based high fat diet also impaired insulin tolerance, but did not impair male reproductive parameters. We propose that impaired sperm quality and function in lard fed fathers could be associated with disruption in metabolic programming and increased breast cancer risk among their daughters.

The impact of obesity in fathers leading to metabolic dysfunction in their female offspring has been previously observed in rodent's studies (10), and it was also seen in our study. Female offspring of both lard and corn oil fed fathers exhibited increased body weight and adiposity. However, only female offspring of lard fed fathers displayed impaired insulin response indicating that the type of dietary fatty acids represents a key

factor in the metabolic programming through the male germline.

Epigenetic modifications that are necessary for achieving reproductive capacity of male gametes include DNA methylation, histone retention and expression of non-coding RNAs such as miRNAs (47). Because miRNAs can modulate the expression of hundreds of mRNAs that affect embryonic development as well as the establishment of the offspring's epigenome (48), they have been proposed to mediate paternal programming effects on the offspring (49). The epididymis has been implicated as the site of the alterations of microRNA signatures occurring during the maturation of sperm cells, and therefore an increase in epididymal fat pad size could potentially impact inheritance of miRNA signature and/or the developmental trajectory of the offspring (50). The impact of high-fat diet-induced male obesity on the miRNA profile in mature spermatozoa has been examined in rodent studies (51). In a study by Fullston et al (52) males fed a high saturated fat diet exhibited changes in miRNAs in the testes and mature spermatozoa that target mRNA associated with spermatogenesis, embryonic development and metabolic diseases in the offspring. We provide here further evidence that paternal nutrition can impact sperm miRNA profile and possibly subsequent mammary gland miRNA profile that in turn target genes implicated in breast cancer and other diseases.

Some of the miRNAs that were differentially expressed in the lard or corn oil consuming fathers' germ cells also were differentially expressed in their daughters' mammary glands, although the daughters were never directly exposed to the high fat diets. When compared to lard fed fathers and their daughters three miRNA were significantly altered in both the sperm and mammary gland of corn oil fed fathers and their daughters: miR-1897-5p, miR-219-1-3p and miR-376a#. Since miRNAs can

modulate gene expression by inhibiting the translation of messenger RNA (mRNA) or by directing their degradation (53), we focused on determining if the expression of the top potentially targeted proteins (Supplementary Table 1) was altered in the daughters of lard or corn oil fed fathers. Among them we highlight Pkd1 and Tgfßr1. PKD1 is a serine/threonine kinase that is expressed in ductal epithelial cells of the mammary gland and maintains the epithelial phenotype and prevents EMT (54). Inhibition of PKD1 can lead to pathological conditions, such as cancer (55). Thus, increased Pkd1 levels in the mammary glands of corn oil consuming fathers' offspring, compared to female offspring of lard fed fathers, are in line with their lowest susceptibility to breast cancer. In addition, compared to female offspring of control diet and corn oil fed fathers, another miRNAs target, Tgfβr1, was increased in the daughters of lard fed fathers that displayed the highest susceptibility to mammary cancer. TGFßR1 expression is related to promoting breast carcinogenesis through multiple mechanisms, including enhancing EMT (56). We assessed the protein levels of its up- and downstream signaling partners. Female offspring of lard fed fathers showed higher protein levels of Tgfβ, compared to both female offspring of control diet and corn oil fed fathers, and of Akt and p-jnk compared to control diet fed fathers. In addition, female offspring of corn oil fed rats had lower levels of Mtor, Mkk4 and p-Smad3/Smad3 compared to female offspring of lard fed fathers. These proteins collectively play important roles in cell survival, growth, migration and invasion (57, 58). These findings indicate that other mechanisms besides miRNAs contribute to causing changes in gene expression in the daughter´s mammary tissue following paternal exposure to lard based high fat diet.

Because fathers, mothers and their daughters tend to share the same nutritional

habits (59), it would be important to further investigate if the paternally programmed breast cancer risk is affected by maternal and female offspring's fat intake. Maternal intake of high corn oil diet during pregnancy increases female offspring's mammary cancer risk (8), whilst intake of lard has opposite effects (9). In addition, because obesity-induced altered sperm miRNA expression in the fathers can be normalized through exercise or dietary intervention (consumption of balanced diet), and that then improves the metabolic health of female offspring (60), the efficacy of similar intervention on daughter's breast cancer risk should be investigated.

Conclusion

In conclusion, we show here that paternal intake of lard based high fat diet rich in SFA increases female offspring's mammary cancer risk as indicated by the increased elongation of mammary epithelial tree, number of TEBs and tumor incidence in female offspring of lard fed fathers compared to both female offspring of control diet and corn oil fed rats. However, if the paternal fat source is corn oil that is high in n-6 PUFAs, offspring's mammary cancer risk is reduced as indicated by decreased tumor growth in female offspring of corn oil fed fathers as compared to both female offspring of control diet and lard fed fathers, and longer tumor latency and decreased tumor multiplicity compared to female offspring of lard fed fathers. Altered miRNAs expression in fathers'´ sperm and daughters´ mammary glands may at least underlie these effects, but other epigenetic changes are likely to be involved. Our findings highlight the importance of paternal nutrition in affecting future generation's risk of developing breast cancer.

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4. CHAPTER 2: Paternal animal and plant-based high fat diets can program rat female offspring lipidomic fatty acid profile and epigenetic and protein expression alteration associated with breast cancer risk

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Keywords: Paternal diet, pre-conceptional programming, breast cancer, lipidomic profile, epigenetic

Abstract

Background: The impact of paternal nutrition on female offspring breast cancer susceptibility has been overlooked. We recently showed that female offspring of male Sprague-Dawley rats fed, before and during puberty, a lard based (high in saturated fat) had higher breast cancer susceptibility than the female offspring of male Sprague-Dawley rats fed corn oil based diet (high in n-6 polyunsaturated) or an AIN-93G control diet. Additionally, the female offspring of rats fed corn oil based diet showed decreased breast cancer susceptibility compared to the control group. Although, initial data indicated that these effects could be mediated through miRNA alterations, we additionally hypothesized that other mechanisms such as DNA methylation and histones post-translational modifications could also mediate the paternal high-fat diet on offspring's breast cancer risk.

Methods: Fatty acid profile, global DNA methylation, global histones modifications and protein levels were assessed in the tissues (fat pads, liver, epididymis and testicle) of male rats that were fed either a lard based, corn oil based or control diet, as well as in the tissues (fat pad, liver and mammary gland) of their 50-days-old female offspring.

Results: Lard-based diet fed male rats had more saturated fatty acids (SFA) and monounsaturated fatty acids (MUFA) while corn oil fed male rats had more polyunsaturated fatty acids (PUFA) in the abdominal, retroperitoneal and retroepididymal fat pad compared to control. Similarly, female offspring of lard-based fed male rats had more SFA, while female offspring of corn oil fed male rats had more PUFA in its retroperitoneal fat pad. Corn oil fed male rats had lower global DNA methylation levels in their testicles and lard fed male rats had higher global DNA methylation levels in their

epididymis. Female offspring of lard fed fathers had lower levels of global H3K4me, H3K9ac and H3K27Tme and higher DNMT3a, PPARgamma and ERalpha protein levels. On the other hand, female offspring of corn oil fed male rats had lower FOXP3, IRbeta and LC3B1 protein levels.

Conclusions: These findings highlight that paternal animal or plant based high fat diets can cause modifications in the fatty acid profile, as well as in epigenetic and protein expression changes, that could modulate the susceptibility to breast cancer in the female offspring.

Introduction

In the last decades of the 20th century, scientists observed worldwide changes in the population diet and health outcome, with a shift from diseases associated with malnutrition and periodic famine to diseases associated with urban–industrial lifestyles [1,2]. In fact, the consumption of a Western diet, characterized by a diet high in saturated fats, sugar, and refined foods but low in fiber is globally increasing, reflecting in people health outcome [3]. Regarding breast cancer, there is evidence for the association between consumption of a Western dietary pattern and breast cancer risk and although the results from studies remain inconsistent, many researchers believe that the type of dietary fatty acid consumed can have a key role in it [4]. It has been reported that breast cancer patients presents different fatty acid profile in the blood serum and erythrocyte membrane from cancer free-women, specifically three saturated fatty acids: Myristic (C14:0), Palmitic (C16:0) and Stearic (C18:0) acids and three unsaturated fatty acids: Linoleic (C18:2), alpha-Linoleic (C18:3) and Eicosapentanoic (C20:5) acids were significantly altered [5].

The adipose tissue is of particular importance to breast cancer risk since the mammary gland is confined to a fat pad, and there is cumulative evidence supporting that adipocytes surrounding the mammary gland are key components of breast cancer progression and carcinogenesis [6]. Moreover, adipocytes can increase the systemic levels of estrogen, due to its aromatase activity, which could increase the risk of estrogen receptor-positive (ER+) breast cancer in post-menopausal women [7]. Accumulation of adipose tissue can induce inflammation, that in return can stimulate low or intermediate levels of oxidative stress causing DNA damage, which results in mutations, inflammatory reactions and increase cell proliferation, eventually inducing breast cancer initiation and

promotion [8].

Dietary fats could influence the carcinogenic process through modification of structural components of cells (i.e. membrane lipids), metabolic effects, signal transduction, and gene expression modulation [9]. For example, consumption of polyunsaturated fatty acids (PUFAs) triggers the peroxisome proliferator-activated receptor-alpha (PPARalpha), which is responsible for regulating fatty acid oxidation and fatty acid and triacylglycerol synthesis [10]. Furthermore, Wistar rats fed a high-fat and treated with PPARalpha agonist or peroxisome proliferator-activated receptor-alpha gamma (PPARgamma) agonists, showed reduced lipid levels in the muscle and adipose tissue, as well as improved insulin sensivity [11]. Interestingly, PPARgamma plays an important role in improving the metabolic syndrome effects caused by the consumption of a high fat diet, as well as in regulating breast tumors' growth, apoptosis and differentiation [12].

Since epigenetic modifications are important to the adipogenesis process, i.e. hipomethylation of gene's promoter and global histone methylation and acetylation [13], researchers began to focus in epigenetic mechanisms that could be altered in the adipose tissue in response to high-fat diets. In Wistar rats, for example, it has been shown that consumption of obesogenic diet could alter DNA methylation pattern of leptin in retroperitoneal adipocytes and Fasn and NADH dehydrogenase (ubiquinone) 1β-subcomplex 6 (NDUFB6) in epididymal adipose tissue, which might change gene expression impairing metabolic functions [14].

Although there is increasing number of studies that focus on dietary interventions that modify the mammary gland surrounding, few began to be directed towards the influence

of parental diet. It has been shown that maternal consumption of trans fatty acids, palm oil or interesterified fat during pregnancy and lactation periods could elicit alterations in the brain fatty acids profile in adulthood of male offspring [15]. Regarding breast cancer, study showed that maternal consumption of conjugated linoleic acids also during pregnancy and lactation could trigger its incorporation into the offspring tissues, which modify the efficiency of fatty acids metabolism, reducing breast cancer risk [16]. Interestingly, these changes in the fatty acid profile could be accompanied by changes in gene expression, as described by our research group, showing that maternal consumption of lard based high fat diet during pregnancy and lactation changed the transcriptional network and the fatty acid profile in the mammary gland of the female offspring, and this modification seams to be related to a reduced breast cancer risk [17].

Given the fact that by 2020 poor diet is probably going to contribute to nearly 75% of all deaths from non-communicable diseases such as heart disease, type 2 diabetes, obesity and cancer, and that the global consumption of saturated fats averaged 9,4%, with country specific intakes varying between 2.3 to 27.5% [18], attention should be focused on both maternal and paternal diets in order to prevent those diseases. Paternal diet has been overlooked for the past years, just recently its influence has been investigated in the context of breast cancer [19,20]. However, little is known about the influence of paternal diet in the lipidomic profile of the female offspring and its associated epigenetic and proteomic changes.

We previously shown that male Sprague-Dawley rats consumption of animal- or plant-based high fat diets elicited opposing effects on female offspring breast cancer risk, with lard based high fat diet increasing and corn oil based high fat diet decreasing it [21].

In order to understand these opposing effects, we used lipidomics and epigenetics approaches to explore the fatty acid profile of fathers and daughters' fats, as well as epigenetic marks and mammary gland global protein expression.

Material and Methods

Experimental protocol: The fad pad tissues used in the present study were obtained from our previously study [21]. In summary, Sprague-Dawley male rats were fed before and during puberty either a lard-based (high in saturated fats) or a corn oil-based (high in n-6 polyunsaturated fats) high fat diet (60% of fat-derived energy). Control animals were fed AIN-93G control diet (16% of fat-derived energy). At 12 weeks of age male rats were mated, with females that consumed only commercial diet, and their 50 days-old female offspring, that also consumed only commercial diet, were submitted to the classical model of mammary carcinogenesis based on 7,12-dimethylbenz[*a*]anthracene initiation. It is noteworthy that the fad pads from female offspring used in this experiment were obtained previously to the breast cancer induction. The experiments were approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo (Protocol Number CEUA/FCF/381).

Fatty acid lipidomic analysis: Fad pad from control diet, lard and corn oil fed male rats and their female offspring tissues (10mg) were transferred to tubes containing 1 mg of IS (tricosanoic acid methyl ester (C23:0), 50 μl 0·5 % BHT and 1 ml 0·5 M-methanolic NaOH. Fatty acids quantification was carried out using a GC equipped with a G3243A MS detector (Agilent 7890A GC System; Agilent Technologies Inc.). A fused silica capillary column (J&W DB-23 Agilent 122–236; 60 m × 250 mm inner diameter) was used to inject 1 μl of the sample. High-purity He was used as the carrier gas at a flow rate

of 1·3 ml/min with a split injection of 50:1. The oven temperature was programmed from 80 to 175°C at a rate of 5°C/min, followed by another gradient of 3°C/min to 230°C, which was maintained for 5 min. The GC inlet and transfer line temperatures were 250 and 280°C, respectively. GC-MS was performed using 70 eV EI in scan acquisition and quantified by TIC. The fatty acids were identified by NIST and by comparing the retention time with those of four purified standard mixtures o fatty acid methyl esters (4-7801; 47085-U; 49453-U and 47885-U; Sigma Chemical Co.). All mass spectra were acquired over an m/z range of 40–500. Results are expressed as mg/100 mg of fad pad tissue.

Global DNA methylation: Global DNA methylation was assessed by High-Performance Liquid Chromatography with Diode-Array Detection (HPLC-DAD) in epididymis, testicle and liver from control diet, lard and corn oil fed male rats and mammary and liver tissues from their female offspring. For DNA extraction, 100 mg of tissue was homogenized with lysis buffer (Gentra Puregene Kit, QIAGEN Mayland Sciences, USA) containing 0.5 mM deferoxamine (Sigma Aldrich, USA). The homogenized samples were incubated with proteinase K (20 mg/mL, Sigma Aldrich, USA) overnight at room temperature. Samples were treated with RNase A (15 mg/mL, Sigma Aldrich, USA) for 2 h, and protein was precipitated with protein precipitating solution (Gentra Puregene Kit, QIAGEN, Maryland, USA). The homogenate was centrifuged at 3,000 rpm for 15 min at 4°C, and DNA was precipitated with cold isopropanol. After centrifugation, Tris buffer (10 mM Tris with 1 mM deferoxamine, pH = 7.0) and chloroform (96%) and isoamyl alcohol (4%) were added to the pellet. After centrifugation, 5 M NaCl and 70% ethanol were added to the supernatant and incubated overnight. Samples were centrifuged for 10

min at 3,000 rpm, and the DNA was resuspended in water with 0.1 mM deferoxamine. The samples were quantified by spectrophotometry (Nanodrop1000, ThermoScientific). DNA (5 μg) was hydrolyzed with Tris buffer including 200 mM HCl/$MgCl_2$ (pH 7.4) and DNAse 1 (Sigma Aldrich, EUA) for 1 h at 37°C. DNA was incubated with phosphodiesterase (Sigma Aldrich, USA) and alkaline phosphatase for 1 h at 37°C. Aliquots of hydrolyzed DNA were injected into the HPLC-DAD analytical system (Shimadzu Corporation, Japan) using Luna column C18(2), 250 mm x 4.6 mm ID, 5 microns (Phenomenex, Torrance, CA), with a C18 pre-column of 4.0 x 3.0 mm (Phenomenex, Torrance, CA) eluted with a gradient of formic acid (0.1% in water) and CH_3OH (0 - 25 min, 0 - 18% CH_3OH; 25th - 27th min 18 - 0% CH3 OH, 27 - 37 min, 0% CH3 OH) with a flow rate of 1 mL/ min, at 30°C. The DAD detector was set at 260 nm for quantification of dC and at 286 nm for quantification of the 5-methyl-dC. Calibration curves were performed in the ranges of 0.05 to 6 nmol for dC and 0.005 to 0.4 nmol for 5-methyl-dC.

Global histones modifications: Global acetylation levels of histone H3 lysine 9 (H3K9) and H4 lysine 16 (H4K16) and methylation levels of H3 lysine 4 (H3K4), H3K9, H3 lysine 27 (H3K27) and H4 lysine 20 (H4K20) were assessed by western blot in testicle of control diet, lard and corn oil fed male rats and mammary tissue of their female offspring. Histones were extracted with Abcam Histone Extraction kit (cat nº ab113476). Approximately 100 mg of tissue was disaggregate in Pre-Lysis buffer and centrifuged at 10000 rpm for 1 minute, at 4°C. The supernatant was removed and the pellet was resuspended in 3 volumes of Lysis Buffer and incubated on ice for 30 minutes. Then it was centrifuged at 12000 rpm for 5 minutes at 4°C and the supernatant fraction containing

the acid-soluble proteins was transferred into a new vial. Upon addition of 0.3 volumes of Balance-DTT Buffer to the supernatant, the samples were quantified with a Biotek ELx 808 Absorbance Reader. Histone extracts were resolved on a NuPAGE 4-12% Bis-Tris gel. Histones were transferred using the iBlot® 7-Minute Blotting System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1 hour at room temperature. Membranes were incubated with the specific primary antibodies at 4°C overnight. After several washes, the membranes were incubated with specific secondary antibody at room temperature for 1 hour. Membranes were developed using the Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., USA), and exposed to Kodak autoradiography films. Optical density of the bands was quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading, expression of the proteins of interest was normalized to the β-actin signal.

Mammary gland protein expression: Protein levels of DNMT3a, PPARg1, ERalpha, FOXP3, IRbeta and LC3B1 were assessed by western blot in mammary tissue of control diet, lard and corn oil female offspring. Total protein was extracted from mammary tissues using RIPA buffer with protease inhibitor (Roche, Switzerland), glycerophosphate (10 mM), sodium orthovanadate (1 mM), pyrophosphate (5 mM) and PMSF (1 mM). The samples were mixed with the buffer for 5 min, then incubated on ice for 30 min and centrifuged for 10 min on high speed. Protein in the supernatant was quantified with BCA Protein Assay Reagent (Pierce, USA). Protein extracts were resolved on a 4-12% denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred using the iBlot® 7-Minute Blotting System (Invitrogen, USA) and blocked with 5% non-fat dry milk for 1 hour at room temperature. Membranes were incubated with the specific primary

antibodies at 4°C overnight. After several washes, the membranes were incubated with specific secondary antibody at room temperature for 1 hour. Membranes were developed using the Chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., USA), and exposed to Kodak autoradiography films. Optical density of the bands was quantified using Quantity-one software (BIO-RAD, USA). To control for equal protein loading, expression of the proteins of interest was normalized to the β-actin signal.

Statistical Analysis: Results are expressed as the mean ± standard error of mean (S.E.M.), and all analyses were conducted with R package limma. Multiple-group comparisons were performed using one-way analysis of variance followed by LSD, and two-group comparisons were performed using Student's t-test. For all data analysis, $p \leq 0.05$ was applied as the threshold for statistical significance.

Multivariate analysis: Principal Component Analysis (PCA) is a bilinear decomposition method used for overviewing clusters within multivariate data. The data were represented in K-dimensional space and reduced to a few principal components, which described the maximum variation within the data. The data were analyzed by PCA to establish any 'groupings' with respect to the three groups. A score plot of Factor1 versus Factor2 was used to examine separation for the three groups.

Results

Lipidomic Profile

- *Abdominal, retroperitoneal and retroepididymal fat pad lipid profile of control diet, lard and corn oil fed male rats*

Compared to both control diet and lard fed male rats, corn oil fed male rats had less ($p \leq 0.05$) SFA in its abdominal fat pad (Figure 1A). There was no statistical difference ($p > 0.05$) between lard fed male rats and control diet fed male rats regarding this parameter. Compared to both control diet and corn oil fed male rats, lard fed male rats had more ($p \leq 0.05$) MUFA in its abdominal fat pad (Figure 1A). There was no statistical difference ($p > 0.05$) between corn oil fed male rats and control diet fed male rats regarding this parameter. Compared to both control diet and lard fed male rats, corn oil fed male rats had more ($p \leq 0.05$) PUFA in its abdominal fat pad (Figure 1A). Additionally lard fed male rats had less ($p \leq 0.05$) PUFA than control diet fed male rats, in its abdominal fat pad (Figure 1A). According to the principal component analysis, the lipid profile of the paternal abdominal fad pad clearly differentiated between control diet, lard and corn oil fed male rats (Figure 3A), with the specific fatty acids, predominantly palmitic (C16:0), stearic (C18:0), oleic (C18:1N9c) and linoleic (C18:2N6c) acids accounting for 61,82% variability among groups (Figure 3B).

Regarding to the lipid profile in the retroperitoneal fat pad, lard fed male rats had more ($p \leq 0.05$) SFA compared to both control diet and corn oil fed male rats (Figure 1B). Additionally corn oil fed males had less ($p \leq 0.05$) SFA than control diet fed male rats (Figure 1B). Compared to both control diet and corn oil fed male rats, lard fed male rats had more ($p \leq 0.05$) MUFA (Figure 1B). Additionally corn oil fed males had less ($p \leq 0.05$) MUFA than control diet fed male rats (Figure 1B). Compared to both control diet and lard fed male rats, corn oil fed male rats had more ($p \leq 0.05$) PUFA in its retroperitoneal fat pad (Figure 1B). Additionally lard fed males had less ($p \leq 0.05$) PUFA than control diet fed males (Figure 1B). According to the principal component analysis, the lipid profile of the

paternal retroperitoneal fad pad clearly differentiated between control diet, lard and corn oil fed male rats (Figure 3C), with the specific fatty acids, predominantly heptadecanoic (C17:0), stearic (C18:0), oleic (C18:1N9c) and linoleic (C18:2N6c) acids accounting for 78,06% variability among groups (Figure 3D).

Regarding to the lipid profile in epididymal fat pad, lard fed male rats had more ($p \leq 0.05$) SFA compared to both control diet and corn oil fed male rats (Figure 1C). There was no statistical difference ($p > 0.05$) between corn oil fed male rats and control diet fed male rats regarding this parameter. Compared to both control diet and corn oil fed male rats, lard fed male rats had more ($p \leq 0.05$) MUFA (Figure 1C). There was no statistical difference ($p > 0.05$) between corn oil fed male rats and control diet fed male rats regarding this parameter. Compared to both control diet and corn oil fed male rats, lard fed male rats had less ($p \leq 0.05$) PUFA (Figure 1C). There was no statistical difference ($p > 0.05$) between corn oil fed male rats and control diet fed male rats regarding this parameter. According to the principal component analysis, the lipid profile of the paternal abdominal fad pad didn't clearly differentiated, although a trend can be seen, between control diet, lard and corn oil fed male rats (Figure 3E), with the specific fatty acids, predominantly myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1N9c) and linoleic (C18:2N6c) acids accounting for 48,95% variability among groups (Figure 3F).

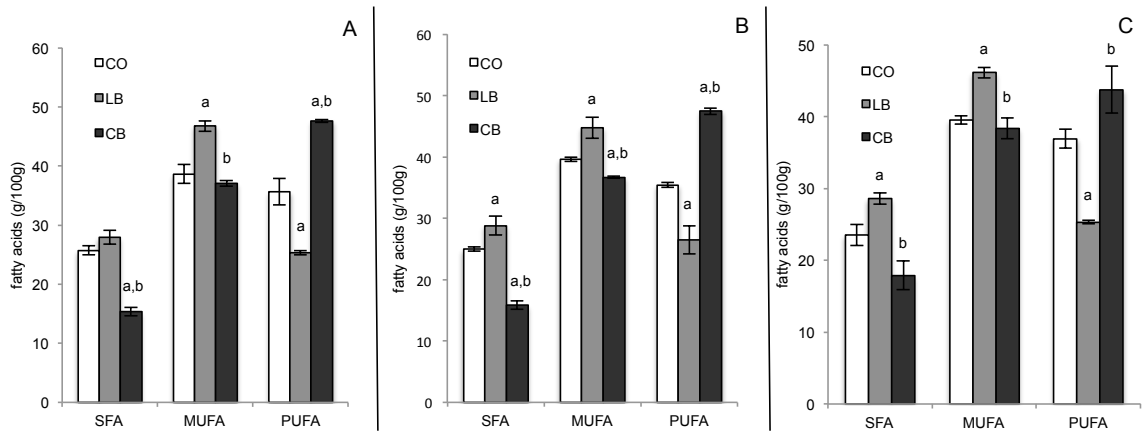

Figure 1: Adipose tissue lipidomic profile of control diet (CO), lard (LB) and corn oil (CB) fed male rats. A: Abdominal fat pad. **B:** Retroperitonial fat pad. **C:** Epididymal fat pad. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Statistically significant difference ($p \leq 0.05$) compared to CO^a and LB^b, according to ANOVA followed by LSD test. The data are expressed as mean ± SEM (n=5 per group).

- *Retroperitoneal fat pad lipid profile of female offspring of control diet, lard and corn oil fed male rats*

Female offspring of lard fed male rats had more ($p \leq 0.05$) SFA in its retroperitoneal fat pad (Figure 2) than female offspring of control diet fed male rats. There was no statistical difference ($p > 0.05$) between female offspring of corn oil fed male rats and female offspring of control diet or lard fed male rats regarding this parameter. There was no statistical difference ($p > 0.05$) among groups regarding MUFA levels in the female offspring retroperitoneal fat pad. Compared to both female offspring of control diet ($p \leq 0.05$) and corn oil ($p = 0.08$) fed male rats, female offspring of lard fed male rats had less PUFA in its retroperitoneal fat pad (Figure 2). There was no statistical difference ($p > 0.05$) between female offspring of corn oil fed male rats and female offspring of control diet fed male rats regarding this parameter. According to the principal component

analysis, the lipid profile of the paternal abdominal fad pad didn't clearly differentiated, although a trend can be seen, between control diet, lard and corn oil fed male rats (Figure 3G), with the specific fatty acids, predominantly palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1N9c), oleic (C18:1N9c) and linoleic (C18:2N6c) acids accounting for 42,25% variability among groups (Figure 3H).

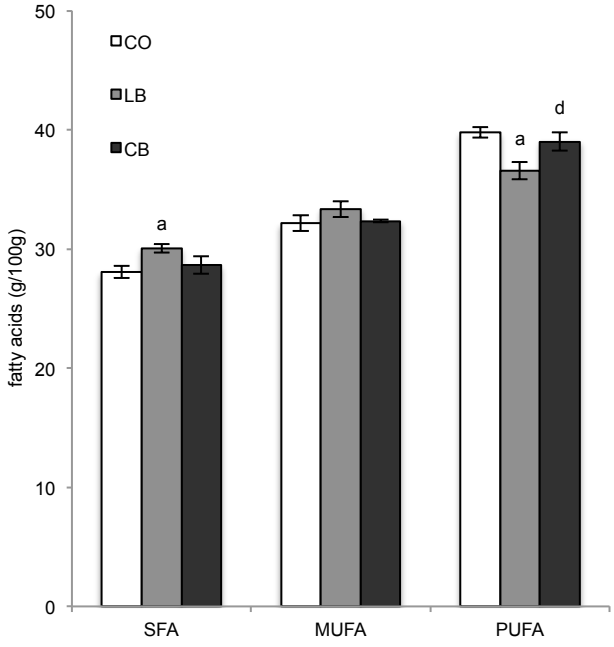

Figure 2: Adipose tissue lipidomic profile of female offspring of control diet (CO), lard (LB) and corn oil (CB) fed male rats. Saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA). Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. The data are expressed as mean ± SEM (n=5 per group).

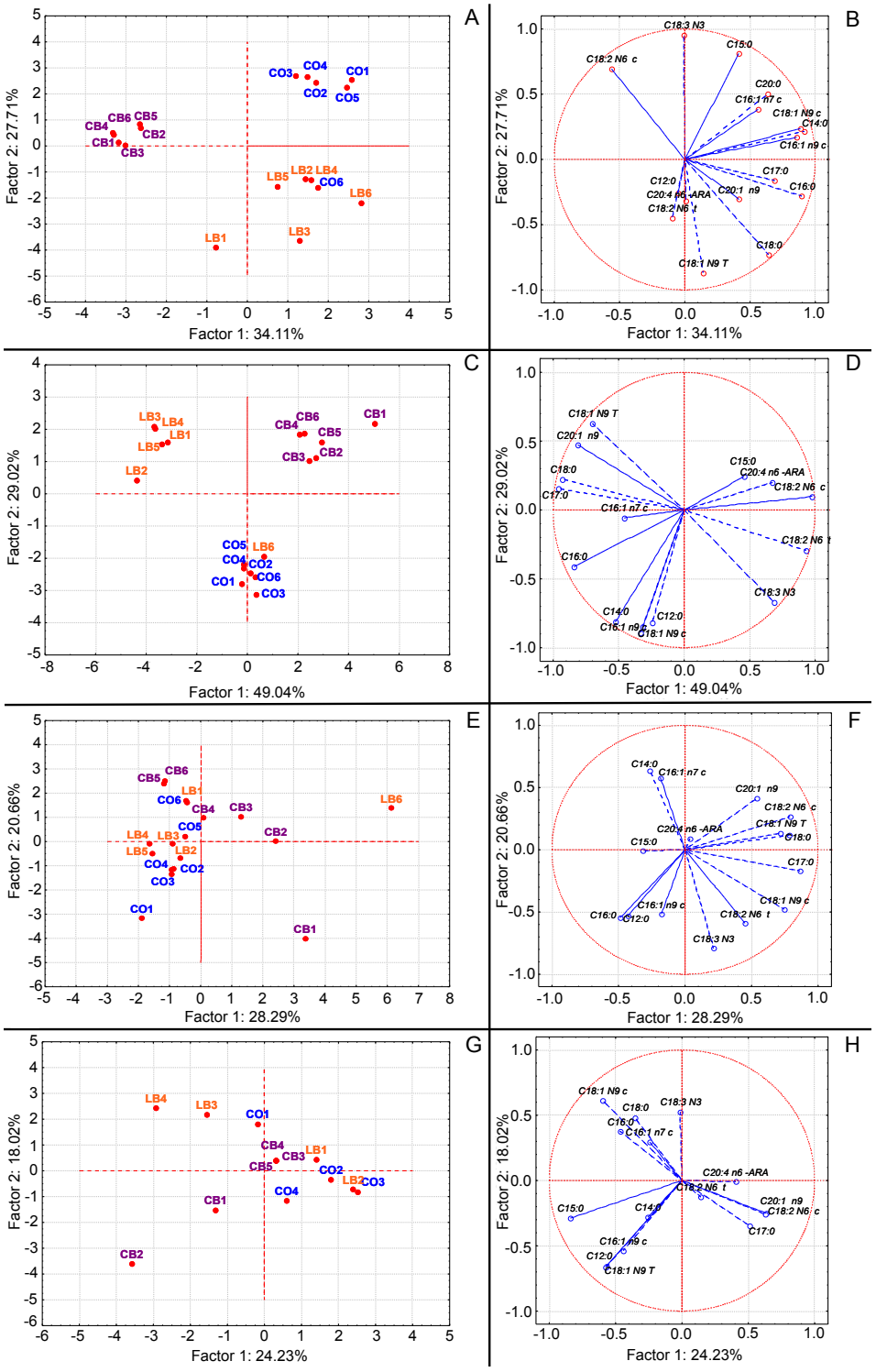

Figure 3: Principal component analysis (PCA) of lipid profile. PCA of control diet (CO), lard (LB) and corn oil (CB) fed male rats by group of: **A**: abdominal fat pad. **C:** Retroperitonial fat pad.**E:** Epididymal fat pad. By fatty acids **B**: abdominal fat pad. **D:** Retroperitonial fat pad.**F:** Epididymal fat pad. PCA of female offspring of control diet (CO), lard (LB) and corn oil (CB) fed male rats **G:** by group, and **H:** by fatty acid.

Global DNA methylation

-Epididymis, testicle and liver tissues global DNA methylation of control diet, lard and corn oil fed male rats

Corn oil fed male rats had lower ($p \leq 0.05$) global DNA methylation levels in their testicles than control diet fed male rats (Figure 4A). There was no statistical difference ($p > 0.05$) between lard fed male rats and control diet or corn oil diet fed male rats regarding this parameter. Lard fed male rats had higher ($p \leq 0.05$) global DNA methylation levels in their epididymis than control diet fed male rats (Figure 4A). There was no statistical difference ($p > 0.05$) between corn oil fed male rats and control diet or lard diet fed male rats regarding this parameter. There was no statistical difference ($p > 0.05$) among groups regarding global DNA methylation in liver tissue of control diet, lard and corn oil fed male rats.

- *Mammary gland and liver tissue global DNA methylation of female offspring of control diet, lard and corn oil fed male rats*

There was no statistical difference ($p > 0.05$) among groups regarding global DNA methylation in mammary and liver tissue from female offspring of control diet, lard and corn oil fed male rats (Figure 4B).

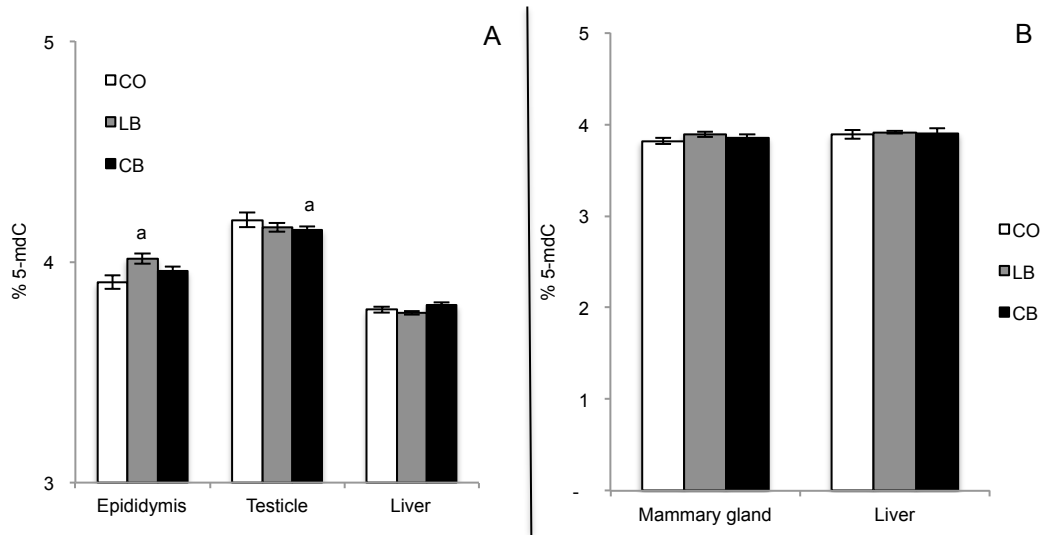

Figure 4: Global DNA methylation. A: Global DNA methylation of epididymis, testicle and liver from control diet (CO), lard (LB) and corn oil (CB) fed male rats. **B:** Global DNA methylation of mammary gland and liver from female offspring of control diet (CO), lard (LB) and corn oil (CB) fed male rats. Statistically significant difference ($p \leq 0.05$) compared to CO^a and LB^b, according to ANOVA followed by LSD test. The data are expressed as mean ± SEM (n=5 per group).

<u>*Global histones modifications*</u>

- *Testicle tissue global histones modifications of control diet, lard and corn oil fed male rats*

There was no statistical difference ($p>0.05$) among groups regarding global histones modifications in testicle tissue from control diet, lard and corn oil fed male rats (data not shown).

- *Mammary Gland global histones modifications of female offspring of control diet, lard and corn oil fed male rats*

Female offspring of lard fed male rats had lower ($p \leq 0.05$) levels of methylated H3K4,

acetylated H3K9 and tri-methylated H3K27 than female offspring of control diet fed male rats (Figure 5). Additionally, female offspring of corn oil fed male rats had lower ($p \leq 0.05$) levels methylated H3K4 and tri-methylated H3K27 than female offspring of control diet fed male rats (Figure 5). There was no statistical difference ($p > 0.05$) between female offspring of lard fed male rats and female offspring of control diet or corn oil diet fed male rats regarding these parameters. In addition, there was no statistical difference ($p > 0.05$) among groups regarding di/tri-methylated H3K9, acetylated H4K16 and tri-methylated H4K20 in the mammary tissue from female offspring of control diet, lard and corn oil male rats (Figure 5).

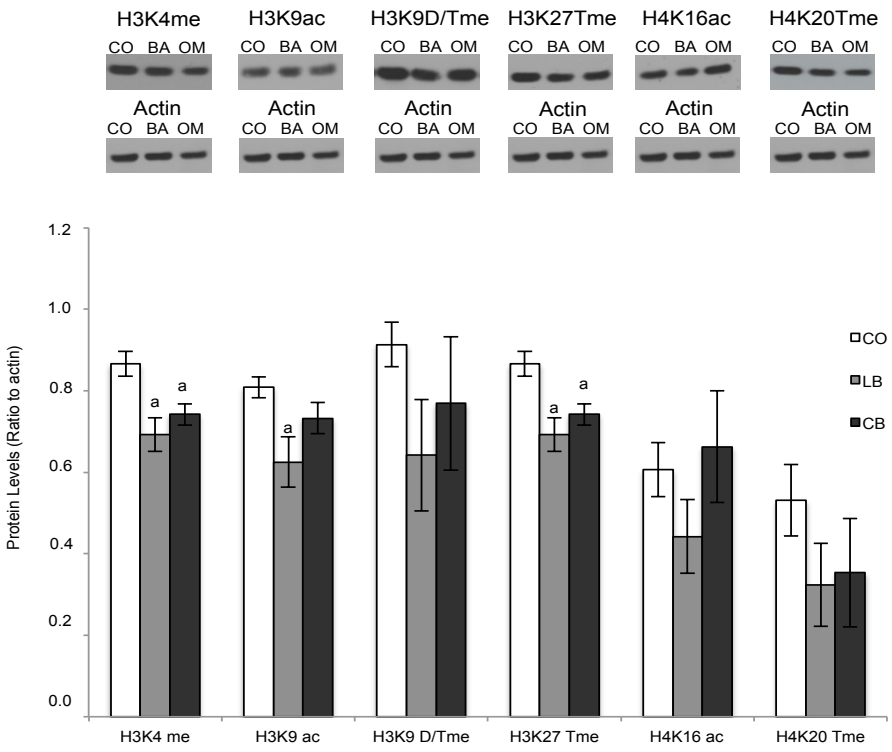

Figure 5: Global histone expression. Western blot analysis of H3K4me, H3K9ac, H3K9D/Tme, H3K27Tme, H4K16ac and H4K20Tme expression in mammary gland of 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. Statistically significant difference ($p \leq 0.05$) compared to CO^a according to ANOVA followed by LSD test. The data are expressed as mean ± SEM (n=5 per group).

Mammary gland protein expression of female offspring of control diet, lard and corn oil fed male rats

Female offspring of lard fed male rats had higher ($p \leq 0.05$) DNMT3a and PPARgamma1 protein levels than female offspring of control diet fed male rats (Figure 6). There was no statistical difference ($p > 0.05$) between female offspring of corn oil fed male rats and female offspring of control diet or lard diet fed male rats regarding these parameters. Female offspring of corn oil fed male rats had lower ($p \leq 0.05$) ERalpha protein levels than female offspring of control diet and lard fed male rats (Figure 6). There was no statistical difference ($p > 0.05$) between female offspring of lard fed male rats and female offspring of control diet fed male rats regarding this parameter. Female offspring of corn oil fed male rats had lower FOXP3 ($p \leq 0.05$), IRbeta ($p = 0.07$) and LC3B1 ($p = 0.07$) protein levels than female offspring of lard fed male rats (Figure 6). Compared to female offspring of control diet fed male rats, both female offspring of lard and corn oil male rats showed no statistical difference ($p > 0.05$) regarding these parameters.

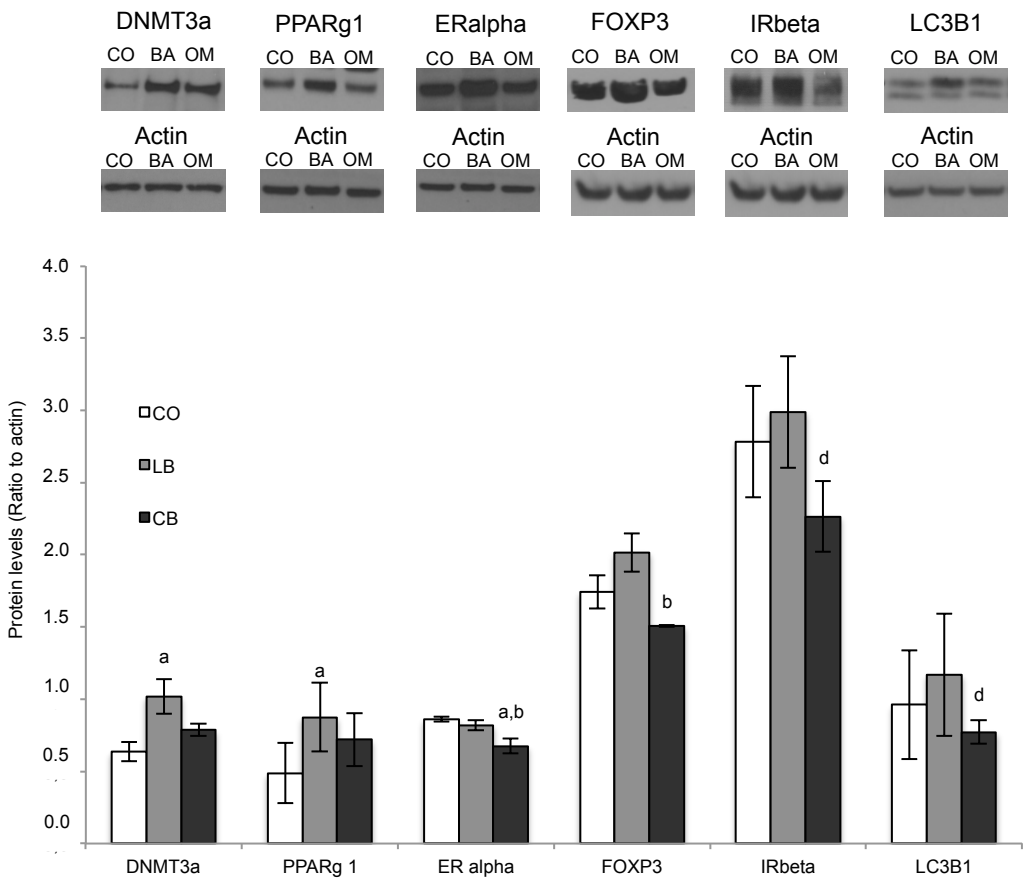

Figure 6: Global protein expression. Western blot analysis of DNMT3a, PPARg1, ER alpha, FOXP3, IRbeta, LC3B1 expression in mammary gland of 50 days old female offspring from control diet (CO), lard (LB) and corn oil (CB) fed males. Statistically significant difference ($p \leq 0.05$) compared to CO[a] and LB[b], according to ANOVA followed by LSD test. Marginal difference ($p \leq 0.06$) compared to LB[d], according to T-test. The data are expressed as mean ± SEM (n=5 per group).

Discussion

Breast cancer is a major health problem and altered cellular lipid metabolism contributes to the onset of cancer, as well as to its progression [22]. We have previously demonstrated that a paternal diet can influence female offspring susceptibility to breast cancer, and that effect could be mediated through miRNA alterations in both males' sperm and female offspring's mammary gland [21], we now postulate that changes in father's fat pad's lipidomic profile could be transferred to female offspring, and this could be due to further epigenetic alterations.

In the present study we showed that male rats consuming lard based high fat diets had increased SFA and MUFA in all abdominal, retroperitoneal and retroepididymal fat pads than control or corn oil fed male rats. Studies showed that accumulation of visceral fat in men is associated with cardiovascular disease and metabolic syndrome [23,24], moreover the accumulation of SFA in the abdominal adipose tissue has been linked to the coronary risk associated with abdominal obesity [25]. On the other hand male rats consuming corn oil based high fat diets had increased PUFA in all abdominal, retroperitoneal and retroepididymal fat pads than control or lard fed male rats. The amount of unsaturated fatty acids has been inversely related to the amount of visceral adipose tissue [26], which is consistent with findings from our previous work.

There is a close correlation between daily intake of PUFA and its content on the adipose tissue, while consumption of SFA and MUFA isn't necessarily associated with its content on the adipose tissue, probably because SFA and MUFA are derived from both diet and endogenous synthesis [27]. Interestingly, female offspring that consumed only commercial diet displayed similar fatty acid profile on theirs fat pads, with the female

offspring of lard fed male rats showing increased SFA and decreased PUFA, while the female offspring of corn oil fed male rats showed increased PUFA. The total adipose PUFA and n-3 PUFA were inversely relate to breast cancer risk [28], which is consistent with the data from our previously reported. Futhermore, our results shows that the major difference between lard based, corn oil based and control diets was due to palmitic (C16:0), stearic (C18:0), palmitoleic (C16:1N9c), oleic (C18:1N9c) and linoleic (C18:2N6c) fatty acids. Study has shown that palmitic, stearic and linoleic are potential biomarkers of breast cancer, indicating that breast cancer is possibly associated with an underlying metabolic disorder that affects fatty acid metabolism [5]. Although the definite mechanisms underlying the effects of the accumulation of specific fatty acids is still not clear, some findings suggests that they are key modulators to breast cancer susceptibility. Palmitic acid concentration in erythrocytes, for example, was directed associated breast cancer risk, while total n-3 PUFA was associated with significantly lower risk [29], and Stearic acid is present in higher concentration in breast cancer tumors compared to normal tissues [30]. Generally, most of SFAs and MUFAs have a predisposition to be directed associated, while n-3 and n-6 PUFAs (except α-linoleic acid) are inversely associated, with postmenopausal breast cancer risk [31].

Additionally to the altered adipose tissue fatty acid profile, certain degree of epigenetic variability have been identified as contributing factors for cancer and interestingly these variability have also been found in infertile men [32]. Our data show that male rats fed a corn oil based diet had lower levels global DNA methylation levels in their testicles than control diet fed male rats, while lard fed male rats had higher global DNA methylation levels in their epididymis than control diet fed male rats. Sperm DNA

hypomethylation has been associated with low sperm motility and increase subcellular reactive oxygen species that may be responsible for the impeding ability of DNMT3a to set proper methylation marks, which can also contribute to the hypomethylation phenotype [33]. On the other hand, workers exposed to radiation showed sperm DNA hypermethylation that was associated with defective chromatin condensation that resulted in morphological abnormal spermatozoa [34]. Although these paternal global DNA methylation patterns weren't present in the female offspring, other epigenetic and metabolics changes could have been triggered by it. It has been shown that mice consumption of high-fat diets can induce hypomethylation of the male testicle, as well as alteration to miRNA expression profile, which are potential signals that program offspring health and could initiate the transmission of impaired metabolic health of two subsequent generations [35].

Further epigenetic changes were observed in the female offspring. Histones modifications are major regulators of breast cancer development [36]. Researchers recognized consistent methylation patterns that frequently precede breast cancer progression, among them low levels of H3K9ac, H3K4me and H4K20Tme in carcinomas of worse prognostic subtypes (i.e. basal carcinomas and HER-2-positive tumors) [37]. Histones H3K9Dme and Tme are repressive marks that are decreased in both *in vitro* and *in vivo* breast cancer models [38]. Patients with lower expression of the H3K27Tme had shorter overall survival time [39] and lower levels of H6K16ac are associated with large tumor size [37]. These are all line with our findings demonstrating that although both female offspring of lard and corn oil fed male rats had lower levels of these marks compared to female offspring of control diet fed male rats, the female offspring of lard

fed males had even lower levels, which could explain the female offspring's different health outcomes.

These epigenetic alterations in the female offspring mammary gland were accompanied by protein alterations. Female offspring of lard fed male rats, that displayed the highest susceptibility to breast cancer had higher PPARgamma1 and DNMT3a protein levels than female offspring of control diet fed male rats. PPAR gamma 1, a subunit of the peroxisome proliferator-activated receptor gamma (PPAR gamma), is responsible for stimulating cell growth and inhibit apoptosis [40]. DNMT3a is a DNA methyltransferase, that is involved in invasion, metastasis and poor prognostic of breast cancer, as well as in the hypermethylation of ERalpha proteins [41]. The Estrogen Receptor alpha (ERalpha) is a major regulator of cell cycle, proliferation and apoptosis, being expressed in almost 70% of all breast cancers [42]. ERalpha levels were lower in the mammary gland of female offspring of corn oil fed male rats, that also showed lower susceptibility to breast cancer.

Female offspring of corn oil fed male rats also had lower FOXP3, IRbeta and LC3B1 protein levels. FOXP3 is a forkhead box transcription factor, that has an important role in the inflammatory response and is an indicator of poor prognostic in ER+ breast cancers [43]. IRbeta is the beta subunit of the insulin receptor and it is responsible for downregulation of the Phosphatase and tensin homolog (PTEN) protein, that is involved in tumor suppression in breast cancer [44]. The ligh chain 3B, subunit 1 (LC3B1) protein is higher in breast tumors [45] and is important for its role in autophagy, that plays a significant role in cancer progression, as well as for its association with proliferation, invasion and metastasis [46].

Altogether, the findings in the present study emphasizes the impact that the paternal diet may have on the female offspring mammary gland and subsequent breast cancer susceptibility. We showed that the consumption of animal or plant based high fat diets could cause differences in the adipose tissue fatty acids profile of the male rats and also of the female offspring that consumed only commercial diet. Further, there were also alterations in the epigenetic and protein levels, that could alter the susceptibility to breast cancer. More studies are needed in order to fully understand the impact of paternal nutrition on female health outcome, however it seems that the diet fatty acids play a major role in it.

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5. FINAL CONSIDERATIONS

To the best of our knowledge, these are the first results indicating that the paternal high fat diet's fatty acid profile is a major determinant of the female offspring breast cancer risk. Further, data show that epigenetic mechanisms (i.e. miRNAs and histones modifications) and altered protein expression could have a key role in regulating this paternal programming effect.

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7. Attachments

UNIVERSIDADE DE SÃO PAULO

FACULDADE DE CIÊNCIAS FARMACÊUTICAS
Comissão de Ética no Uso de Animais - CEUA

Ofício CEUA/FCF/105/2012

CERTIFICADO

A Comissão de Ética no Uso de Animais da Faculdade de Ciências Farmacêuticas da Universidade de São Paulo Certifica que o Projeto **"Nutrição paterna e programação fetal do câncer: influência de dieta rica em ácidos graxos saturados na suscetibilidade da prole à carcinogênese *mamária.*"** (Protocolo CEUA/FCF/381), de responsabilidade da pesquisadora **Camile Castilho Fontelles,** sob a orientação do Prof. Dr. Thomas Prates Ong, está de acordo com as normas do Conselho Nacional de Controle de Experimentação Animal - CONCEA e foi APROVADO em reunião de 05 de novembro de 2012.

São Paulo, 06 de novembro de 2012.

Prof. Dr. Jollson de Oliveira Martins
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Paternal programming of breast cancer risk in daughters in a rat model: opposing effects of animal- and plant-based high-fat diets

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Abstract

Background: Although males contribute half of the embryo's genome, only recently has interest begun to be directed toward the potential impact of paternal experiences on the health of offspring. While there is evidence that paternal malnutrition may increase offspring susceptibility to metabolic diseases, the influence of paternal factors on a daughter's breast cancer risk has been examined in few studies.

Methods: Male Sprague-Dawley rats were fed, before and during puberty, either a lard-based (high in saturated fats) or a corn oil-based (high in n-6 polyunsaturated fats) high-fat diet (60 % of fat-derived energy). Control animals were fed an AIN-93G control diet (16 % of fat-derived energy). Their 50-day-old female offspring fed only a commercial diet were subjected to the classical model of mammary carcinogenesis based on 7,12-dimethylbenz[a]anthracene initiation, and mammary tumor development was evaluated. Sperm cells and mammary gland tissue were subjected to cellular and molecular analysis.

Results: Compared with female offspring of control diet-fed male rats, offspring of lard-fed male rats did not differ in tumor latency, growth, or multiplicity. However, female offspring of lard-fed male rats had increased elongation of the mammary epithelial tree, number of terminal end buds, and tumor incidence compared with both female offspring of control diet-fed and corn oil-fed male rats. Compared with female offspring of control diet-fed male rats, female offspring of corn oil-fed male rats showed decreased tumor growth but no difference regarding tumor incidence, latency, or multiplicity. Additionally, female offspring of corn oil-fed male rats had longer tumor latency as well as decreased tumor growth and multiplicity compared with female offspring of lard-fed male rats. Paternal consumption of animal- or plant-based high-fat diets elicited opposing effects, with lard rich in saturated fatty acids increasing breast cancer risk in offspring and corn oil rich in n-6 polyunsaturated fatty acids decreasing it. These effects could be linked to alterations in microRNA expression in fathers' sperm and their daughters' mammary glands, and to modifications in breast cancer-related protein expression in this tissue.

Conclusions: Our findings highlight the importance of paternal nutrition in affecting future generations' risk of developing breast cancer.

Keywords: Paternal diet, Breast cancer, High-fat diet, Female offspring

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Background

Breast cancer is a global public health problem, with nearly 1.7 million new cases diagnosed in 2012, representing 25 % of all cancers in women worldwide [1]. Its incidence is projected to rise significantly over the next 20 years despite current efforts to prevent the disease [2]. Although the precise reason for this growth is still not clear, it has been suggested that modern women's lifestyles, including postponing first pregnancy and having fewer children, can explain the increase [3].

Nutritional habits, such as adoption of Western dietary patterns, are also linked to increased breast cancer risk [4]. These patterns are characterized by low consumption of fruits and vegetables, increased energy intake, and decreased energy expenditure, leading to obesity, as well as increased intake of saturated fatty acids (SFA), n-6 polyunsaturated fatty acids (PUFA), and *trans*-fatty acids and decreased intake of n-3 polyunsaturated fats [5, 6]. While the majority of epidemiological studies on nutrition and breast cancer risk have been focused on women's diet in adulthood, accumulating epidemiological and experimental evidence highlights early life experiences, including nutrition, as relevant factors for later breast cancer risk determination [7]. The developmental origins of this cancer have been considered predominantly from a maternal perspective, with emphasis placed on the impact of high fat or energy intake during gestation and lactation on female offspring mammary gland development and later breast cancer risk [8, 9].

Although males contribute half of the embryo's genome, only recently has interest begun to be directed toward the potential impact of paternal experiences on the health of offspring [10]. While experimental studies have shown that paternal malnutrition may increase the susceptibility of offspring to metabolic dysregulation, obesity, and cardiovascular diseases [11, 12], the influence of paternal factors on daughter's breast cancer risk has been examined in few studies. Among them, epidemiological studies show an association between higher paternal education level, older age, and smoking with increased rate of breast cancer in the daughters [13, 14].

Unlike the female production of germ cells that takes place predominantly in early life [15], male production of germ cells starts in utero, with mature sperm cells being produced throughout the entire reproductive life of the male [16]. Because spermatogenesis can be dramatically influenced by environmental factors, including malnutrition, obesity, and an exposure to toxic compounds, the father's health during preconception is now acknowledged as a critical factor in the context of the developmental origins of health and disease [17]. In addition to embryogenesis, gametogenesis comprises intense epigenetic (DNA methylation, histone modification, and microRNA [miRNA or miR] expression) remodeling [18, 19]. Thus, epigenetically inherited increased disease risk could be transmitted through the female as well as the male germline [20]. Specific windows within which male gametes would be especially prone to environmentally elicited epigenetic deregulation include prepuberty and the reproductive phase [21].

Given the marked increase in dietary fat intake over the past three decades [22], as well as the notion that different kinds of dietary fats can lead to different health outcomes [23], we designed this study to investigate whether, in rats, consumption of high levels of animal- or vegetable-based fats by fathers would affect their daughters' risk of breast cancer. We also investigated the underlying cellular and molecular mechanisms. We fed male Sprague-Dawley rats, before and during puberty, either a lard-based (high in SFA) or a corn oil-based (high in n-6 PUFA) high-fat diet (60 % of fat-derived energy). Control animals were fed a control AIN-93G diet containing soybean oil as a fat source (16 % of fat-derived energy). Male rats were mated with female rats that were consuming a commercial diet. We show that paternal consumption of these high-fat diets elicited opposing effects, with animal fat increasing and vegetable oil decreasing breast cancer risk in the offspring. These effects could be linked to alterations in miRNA expression in fathers' sperm and their daughters' mammary glands, as well as to modifications in breast cancer-related protein expression in this tissue. These novel data show that paternal high-fat diets influence their female offspring's susceptibility to mammary cancer, with consumption of lard increasing and corn oil reducing daughters' mammary cancer risk. Thus, paternal diet before conception sets a stage for a daughter's risk of developing breast cancer.

Methods

Experimental design

This study was approved by the ethics committee on animal experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo (protocol number CEUA/FCF/381). Twenty-one-day-old male rats were divided into three groups ($n = 20$ rats per group): control rats (those fed the control AIN-93G diet, with 16 % of total calories provided by lipids), lard-fed males (exposed to a high-SFA diet, with 60 % of total calories provided mainly from lard), and corn oil-fed males (exposed to n-6 PUFA diet, with 60 % of total calories provided mainly from corn oil). At 12 weeks of age, all male rats were switched to a chow diet and mated by housing one male with one female per cage. Pregnant female rats and their offspring consumed only commercial laboratory chow (Nuvital Nutrientes, Colombo, Brazil). Body weight and food intake were recorded two or three times per week.

Determination of the diets' lipid profiles

The lipid profiles of the control, lard, and corn oil diets were determined according to the methods published by the Association of Official Analytical Chemists [24]. Fatty acids were esterified to fatty-acid methyl esters according to the method reported by Hartman and Lago [25], and their composition was analyzed with a gas chromatograph (GC 17A/Class GC 10; Shimadzu, Kyoto, Japan) equipped with a flame ionization detector and a SUPELCOWAX® 10 fused silica capillary column (30 mm × 0.25 mm inner diameter; Sigma-Aldrich, St. Louis, MO, USA). The temperature was set at 170 °C, raised to 225 °C at a rate of 1 °C/minute, and held for 25 minutes. The temperatures of the vaporizer and detector were 250 °C and 270 °C, respectively. Helium was used as the carrier gas (1 ml/minute). Identification of the fatty acids was performed by comparison of the retention times with the standard mixture of fatty-acid methyl esters. Each determination was performed in duplicate using two different samples for each diet.

Insulin tolerance test

The tests were performed at 0800 h after the rats were fasted for 12 h, according to the method described by Takada et al. [26]. The insulin load (75 mU/100 g body weight) was injected as a bolus, and the blood glucose levels were determined at 0, 3, 6, 9, 12, and 30 minutes after injection in male rats and their 50-day-old female offspring. The AUC was calculated according to the trapezoid rule [27].

Mature spermatozoa collection and purification

Control diet and lard- and corn oil-fed male rats were killed once females were pregnant, and the caudal epididymis was dissected for sperm collection. The cauda and vas deferens from male rats were collected, punctured, and transferred to tissue culture dishes containing M2 medium (M2 medium with HEPES, without penicillin and streptomycin, sterile-filtered, suitable for rat embryo; Sigma-Aldrich), where it was incubated for 1 h at 37 °C. Spermatozoa samples were washed with PBS and then incubated with somatic cell lysis buffer (SCLB; 0.1 % SDS, 0.5 % Triton X-100 in diethylpyrocarbonate water) for 1 h, according to a protocol described by Goodrich et al. [28]. SCLB was rinsed off with two washes of PBS, and the purified spermatozoa sample (at least 95 % purity as assessed by microscopy) was pelleted and used for miRNA extraction.

Determination of daily sperm production

The right testis was maintained at −20 °C until processing to determine the daily sperm production. The technique proposed by Robb et al. [29] is based on the resistance of elongated spermatids present in phases 17–19 of spermatogenesis to intense mechanical stress due to the high compaction of chromatin.

Sperm morphological analyses

According to the method of Seed et al. [30], the epididymis was previously frozen at −20 °C, underwent incision, and was subsequently immersed in PBS to promote the dissemination of gametes to the aqueous medium. Then the obtained solution was placed on slides for examination by light microscopy. Two hundred sperm per animal were analyzed microscopically at × 400 magnification.

Mammary gland harvesting

Abdominal mammary glands of female offspring of control diet- and lard- and corn oil-fed male rats ($n = 6$ per group) were collected on postnatal day 50 and used for preparing mammary whole mounts and miRNA and protein extraction.

Analysis of mammary gland morphology and development

Whole-mount preparations of the fourth abdominal mammary gland from 50-day-old female offspring ($n = 5$/group) were obtained, and the epithelial elongation and number of terminal end buds (TEBs) were determined as described by de Assis et al. [31].

Mammary tumor induction

Mammary tumors were induced in 50-day-old female rat offspring ($n = 24$ rats/group) by administration of 7,12-dimethylbenz[a]anthracene (DMBA, 50 mg/kg body weight; Sigma-Aldrich). The carcinogen was dissolved in corn oil and administered by oral gavage. Animals were examined for mammary tumors by palpation twice per week. Latency of tumor appearance, the number of animals with tumors, and the number of tumors per animal (multiplicity) were evaluated. The tumor volume was calculated with tumor measures of length (a), height (b), and width (c) taken with a caliper rule once per week since tumor appearance and throughout the experiment. The formula $(1/6 \times 3.14) \times (a \times b \times c)$ was used to calculate the tumor volume, as described by Spang-Thomsen et al. [32]. The tumor growth rate was calculated using the measured volumes of each tumor at a given week (d) and the subsequent week (e) of appearance using the formula $[(e - d)/d] \times 100$. Those animals in which tumor burden approximated 10 % of total body weight were killed. All others animals were killed 19 weeks after carcinogen administration.

Analysis of mammary gland and tumor cell proliferation and apoptosis in female offspring

Cell proliferation was evaluated in mammary glands (ducts and lobules) and tumors from 50-day-old female offspring ($n = 4$/group) by Ki-67 immunohistochemistry.

After being harvested, mammary tissue was directly fixed in 10 % buffered formalin, embedded in paraffin, and sectioned. Sections were then deparaffinized in xylene and hydrated through graded ethanol. Antigen retrieval was performed with 10 mM citrate buffer, pH 6, for 20 minutes in a pressure cooker. Peroxidase blocking was performed with 10 % H_2O_2 for 10 minutes, and nonspecific binding was blocked for 1 h with 1 % skimmed milk in PBS. Sections were incubated overnight with anti-rat Ki-67 primary antibody (Abcam, Cambridge, UK) at a 1:50 dilution. After washes, sections were incubated with the LSAB 2 System-HRP kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions, stained with 3,3′-diaminobenzidine in chromogenic solution (Dako) for 10 minutes, washed, and counterstained for 1.5 minutes with hematoxylin. Cell proliferation was quantified by assessing the number of Ki-67-positive cells among 1000 cells. The slides were evaluated using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Apoptosis analysis was conducted in mammary glands (ducts and lobules) and tumors from 50-day-old female offspring ($n = 4$/group), according to the method described by Elmore et al. [33], using ImageJ software. Results are presented as mean number of apoptotic cells per 1000 cells.

microRNA expression profile analysis

Total RNA from paternal sperm and their female offspring's total mammary gland was extracted using the miRNeasy Mini Kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. RNA samples were quantified and stored at −80 °C until use. miRNA arrays were performed at the Genomics and Epigenomics Shared Resources at Georgetown University using Applied Biosystems TaqMan Array Rodent MicroRNA arrays (Life Technologies, Carlsbad, CA, USA) to generate the miRNA expression profiles for each experimental group. The TaqMan® Array Rodent MicroRNA A + B Cards Set v3.0 is a two-card set containing a total of 384 TaqMan® MicroRNA assays per card (Life Technologies). The set enables accurate quantitation of 641 and 373 unique miRNAs for rat. There are three TaqMan® MicroRNA Assay endogenous controls for each species on each array to aid in data normalization [34]. The geNorm algorithm was applied to those endogenous controls to determine the optimal number of stable controls. The geometric mean of these selected controls was used for array normalization. To conduct further statistical analysis, the normalized value was log-transformed to meet the t test requirement. Statistical analysis was conducted using the limma package in R [35]. miRNAs that had a false discovery rate <0.1 were considered as significantly altered and selected for further analysis. Target prediction for miRNAs of interest was conducted using TargetScan (release 6.2). The predicted targeted messenger RNA (mRNA) list was then uploaded to Ingenuity Pathway Analysis (IPA; QIAGEN Silicon Valley, Redwood City, CA, USA) for gene set enrichment analysis. We selected the top canonical pathways for further analysis.

Analysis of protein levels in mammary glands of female offspring

Protein levels of 50 ng/μl were assessed by Western blot analysis in total mammary glands obtained from 50-day-old female rats ($n = 5$ per group). Total protein was extracted from mammary tissues using radioimmunoprecipitation assay buffer with protease inhibitor (Roche, Basel, Switzerland), glycerophosphate (10 mM), sodium orthovanadate (1 mM), pyrophosphate (5 mM), and phenylmethylsulfonyl fluoride (1 mM). The samples were mixed with the buffer for 5 minutes, then incubated on ice for 30 minutes and centrifuged for 10 minutes at high speed. Protein in the supernatant was quantified using Pierce bicinchoninic acid protein assay reagent (Thermo Scientific, Rockford, IL, USA). Protein extracts were resolved on a 4–12 % gradient denaturing polyacrylamide gel (SDS-PAGE). Proteins were transferred using the Invitrogen iBlot® 7-Minute Dry Blotting System (Life Technologies) and blocked with 5 % nonfat dry milk for 1 h at room temperature. Membranes were incubated with the specific primary antibodies at 4 °C overnight. After several washes, the membranes were incubated with HRP-conjugated secondary antibody (1:5000; Santa Cruz Biotechnology, Dallas, TX, USA) at room temperature for 1 h. Membranes were developed using HyGLO chemiluminescent HRP antibody detection reagent (Denville Scientific Inc., Metuchen, NJ, USA), and exposed to Kodak autoradiography films (Carestream Health, Rochester, NY, USA). The optical density of the bands was quantified using Quantity One software (Bio-Rad Laboratories, Hercules, CA, USA). To control for equal protein loading, expression of the proteins of interest was normalized to the β-actin signal.

Statistical analysis

Results are expressed as the mean ± SEM, and all analyses were conducted with the limma package in R. Multiple-group comparisons were performed using one-way analysis of variance (ANOVA) followed by a least significant difference (LSD) test, and two-group comparisons were performed using Student's t test. Repeated-measures ANOVA was applied for caloric intake data evaluation, and Kaplan-Meier curves and log-rank tests were applied for determining differences in tumor incidence. For all data analyses, $p \leq 0.05$ was applied as the threshold for statistical significance.

Results

Paternal dietary and health parameters

Compared with control diet-fed male rats, the ones that were on the lard- or corn oil-based high-fat diets consumed more ($p \leq 0.05$) SFA (predominantly palmitic [C16:0] and stearic [C18:0] acids), monounsaturated fatty acids (MUFA) (predominantly oleic acid [C18:1n9c]) and PUFA (predominantly linoleic acid [C18:2n6c]) (Fig. 1a). Corn oil-fed male rats consumed less ($p \leq 0.05$) SFA (predominantly palmitic [C16:0] and stearic [C18:0] acids) and MUFA (predominantly oleic acid [C18:1n9c]) and more ($p \leq 0.05$) PUFA (predominantly linoleic acid [C18:2n6c]) than the lard-fed male rats (Fig. 1a). Daily caloric intake was approximately 7 % higher ($p \leq 0.05$) in both lard- and corn oil-fed male rats than in control diet-fed male rats (data not shown). Although lard- and corn oil-fed male rats consumed nearly the same amount of calories per day, lard-fed male rats gained more ($p \leq 0.05$) weight than control diet- and corn oil-fed male rats ($p \leq 0.05$) (Table 1). There was no difference ($p > 0.05$) between control diet- and corn oil-fed male rats regarding weight gain. Both lard- and corn oil-fed male rats had greater ($p \leq 0.05$) abdominal, retroperitoneal, and epididymal fat pad weights than control diet-fed male rats (Table 1). Compared with lard-fed male rats, corn oil-fed male rats had lesser ($p \leq 0.05$) epididymal fat pad weights, but there was no difference ($p > 0.05$) in abdominal or retroperitoneal fat pad weights (Table 1). Further, lard-fed male rats had lesser testicle ($p \leq 0.05$), epididymis ($p \leq 0.05$), and seminal vesicle ($p \leq 0.08$) weights than the control diet- and corn oil-fed male rats (Table 1). There was no difference ($p > 0.05$) between control diet- and corn oil-fed male rats regarding these parameters (Table 1). Lard-fed male rats also had fewer ($p \leq 0.05$) normal sperm cells and lower ($p \leq 0.05$) daily sperm production than control diet- and corn oil-fed male rats (Table 1). There was no difference ($p > 0.05$) between control diet- and corn oil-fed male rats regarding these parameters (Table 1). Both lard- and corn oil-fed male rats had higher ($p \leq 0.05$) fasting glucose levels than control diet-fed male rats (Table 1). There was no difference ($p > 0.05$) between lard- and corn oil-fed male rats regarding this parameter (Table 1). Further, in the insulin tolerance test, lard- and corn oil-fed male rats had higher ($p \leq 0.05$) AUCs than control diet-fed male rats (Fig. 1b), indicating that they were insulin-intolerant. There was no difference ($p > 0.05$) between lard- and corn oil-fed male rats regarding this parameter (Fig. 1b).

Female offspring health parameters

Female offspring of both lard- and corn oil-fed male rats had greater birth weight ($p \leq 0.05$) and greater weight gain ($p \leq 0.05$; $p \leq 0.08$ for offspring of corn oil-fed male rats) than offspring of control diet-fed male rats (Table 1). There was no difference ($p > 0.05$) between female offspring of lard- and corn oil-fed male rats regarding birth weight (Table 1). Female offspring of corn oil-fed male rats had less ($p \leq 0.05$) weight gain than offspring of lard-fed male rats (Table 1). Similarly to the male rats, female offspring of both the lard-fed and the corn oil-fed male rats had greater ($p \leq 0.05$) retroperitoneal fat weights than offspring of control diet-fed male rats (Table 1). There was no difference ($p > 0.05$) between female offspring of lard- or corn oil-fed male rats regarding this parameter (Fig. 1c). Female offspring of lard-fed male rats had higher ($p \leq 0.05$) fasting glucose levels (Table 1) and higher ($p \leq 0.05$) AUCs than female offspring of control diet- or corn oil-fed

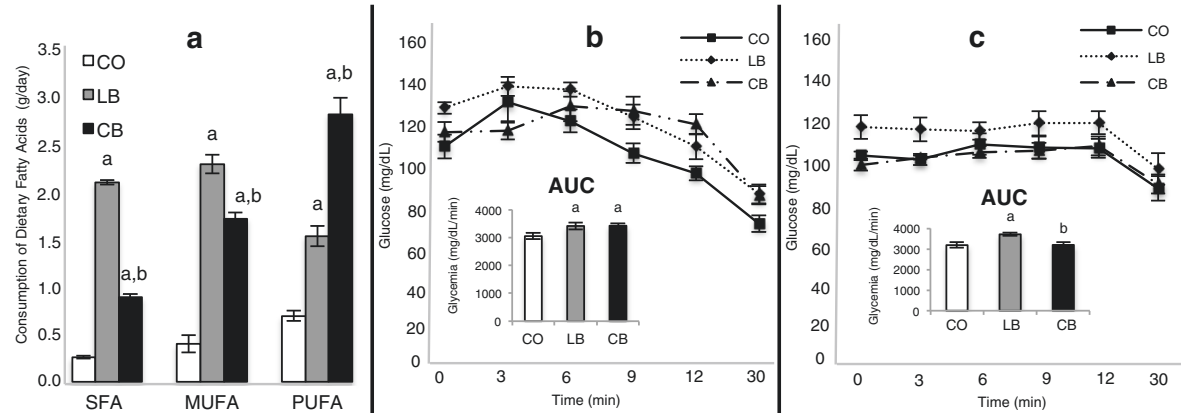

Fig. 1 Paternal fatty-acid consumption and insulin tolerance test. **a** Male rats fed a control diet (CO) or a lard-based (LB) or corn oil-based (CB) high-fat diet consumption of dietary fats (saturated fatty acids [SFA], monounsaturated fatty acids [MUFA], and polyunsaturated fatty acids [PUFA]) ($n = 20$ per group). **b** Insulin tolerance test (ITT) of the CO-, LB-, and CB-fed male rats ($n = 6$ per group). *Inset*: ITT is shown as the AUC. **c** Fifty-day-old female offspring ($n = 6$ per group). *Inset*: ITT is shown as the AUC. Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by a least significant difference test. The data are expressed as mean ± SEM

Table 1 Health parameters of male rats and their 50-day-old female offspring in control diet, lard-based diet, and corn oil-based diet groups

	CO	LB	CB
Male rats			
Weight gain	363.1 ± 6.4 g	398.2 ± 12.0 g[a]	369.4 ± 7.8 g[b]
Fat weight			
Abdominal fat	1.4 ± 0.1 g/100 g body weight	2.7 ± 0.2 g/100 g body weight[a]	2.6 ± 0.2 g/100 g body weight[a]
Retroperitoneal fat	0.8 ± 0.1 g/100 g body weight	1.9 ± 0.1 g/100 g body weight[a]	1.7 ± 0.2 g/100 g body weight[a]
Epididymal fat	1.2 ± 0.1 g/100 g body weight	2.7 ± 0.1 g/100 g body weight[a]	2.0 ± 0.2 g/100 g body weight[a,b]
Reproductive organs			
Testicle	0.45 ± 0.01 g/100 g body weight	0.40 ± 0.01 g/100 g body weight[a]	0.45 ± 0.01 g/100 g body weight[b]
Epididymis	0.14 ± 0.00 g/100 g body weight	0.13 ± 0.00 g/100 g body weight[a]	0.14 ± 0.00 g/100 g body weight[b]
Seminal vesicle	0.33 ± 0.01 g/100 g body weight	0.29 ± 0.02 g/100 g body weight[c]	0.33 ± 0.02 g/100 g body weight[d]
Sperm morphology (% normal)	66.4 ± 2.8 %	50.1 ± 3.0 %[a]	70.9 ± 2.9 %[b]
Daily sperm production, *n*/testicle/day	$31 \pm 1.9 \times 10^6$	$23 \pm 0.9^a \times 10^6$	$29 \pm 0.8^b \times 10^6$
Fasting glycemia	100.8 ± 3 mg/dl	124.6 ± 4 mg/dl[a]	116.1 ± 3 mg/dl[a]
50-day-old female offspring			
Birth weight	8.0 ± 1.5 g	8.8 ± 1.0 g[a]	9.1 ± 1.5 g[a]
Weight gain	138.9 ± 1.5 g	147.0 ± 1.2 g[a]	142.1 ± 1.1 g[b,c]
Retroperitoneal fat	0.9 ± 0.1 g/100 g body weight	1.1 ± 0.0 g/100 g body weight[a]	1.2 ± 0.1 g/100 g body weight[a]
Fasting glycemia	106.4 ± 2.0 mg/dl	112.5 ± 1.9 mg/dl[a]	106.3 ± 1.2 mg/dl[b]

Abbreviations: CB rats fed a corn oil-based high-fat diet and their offspring, *CO* rats fed a control diet and their offspring, *LB* rats fed a lard-based high-fat diet and their offspring

Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by least significant difference test. Marginal difference ($p \leq 0.08$) compared with [c]CO and [d]LB, according to *t* test. The data are expressed as mean ± SEM ($n = 20$ per group)

male rats (Fig. 1c). There was no difference ($p > 0.05$) between female offspring of control diet- and corn oil-fed male rats regarding these parameters (Table 1 and Fig. 1c).

Female offspring mammary gland morphology

Mammary gland morphology was assessed on the basis of mammary whole mounts obtained from 50-day-old female offspring. Both elongation of the mammary epithelial tree (Fig. 2c) and the number of TEBs (Fig. 2d) were higher ($p \leq 0.05$) in female offspring of lard-fed male rats than in female offspring of control diet- and corn oil-fed male rats. There was no difference ($p > 0.05$) between female offspring of control diet- and corn oil-fed male rats regarding these parameters (Fig. 2c and d).

Female offspring mammary gland tumors data

Mammary tumors in the female offspring were induced by administering the carcinogen DMBA. Female offspring of lard-fed male rats had increased mammary tumor incidence ($p \leq 0.05$) compared with offspring of both control diet- and corn oil-fed male rats (Fig. 3a). There was no statistical difference ($p > 0.05$) between female offspring of control diet- and corn oil-fed male rats regarding tumor incidence. Female offspring of corn oil-fed male rats exhibited longer ($p \leq 0.05$) tumor latency and lower tumor multiplicity ($p \leq 0.05$) than female offspring of lard-fed male rats (Fig. 3b and d). Compared with female offspring of control diet-fed male rats, female offspring of lard- and corn oil-fed male rats did not show differences ($p > 0.05$) regarding tumor latency and multiplicity. Further, female offspring of corn oil-fed male rats showed less ($p \leq 0.05$) tumor growth in the first week of tumor appearance than offspring of both control diet- and lard-fed male rats (Fig. 3c). There was no statistical difference ($p > 0.05$) between offspring of control diet- and lard-fed male rats regarding tumor growth. Additionally, there was no statistical difference among groups in the tumor growth rate for the remaining experimental weeks.

Female offspring mammary gland and tumor cell proliferation and apoptosis

Female offspring of lard-fed male rats exhibited an increased ($p \leq 0.06$) number of proliferative cells (Fig. 4b) and a decreased ($p \leq 0.05$) number of apoptotic cells (Fig. 4e) in mammary gland lobules compared with female offspring of control diet- and corn oil-fed male rats. There was no difference ($p > 0.05$) between female offspring of control diet- and corn oil-fed male rats regarding these parameters (Fig. 4b and e). Further, there was no difference ($p > 0.05$) in cell proliferation and apoptosis in mammary gland ducts among female offspring of all groups. Female offspring of both lard- and

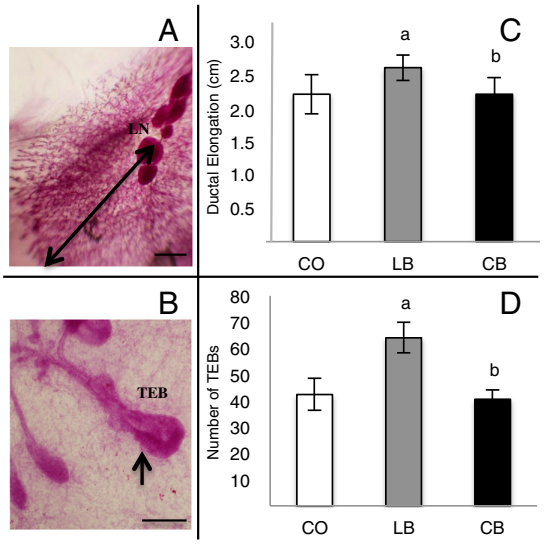

Fig. 2 Mammary gland development of 50-day-old female offspring of control diet (CO)-, lard (LB)-, and corn oil (CB)-fed male rats. **a** Histological depiction of the fourth abdominal mammary gland showing ductal elongation, indicated by *arrow*. **b** Terminal end buds (TEBs), indicated by *arrows*. **c** Ductal elongation. **d** Number of TEBs. All values are expressed as the mean ± SEM. Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by a least significant difference test. The data are expressed as mean ± SEM ($n = 5$ per group)

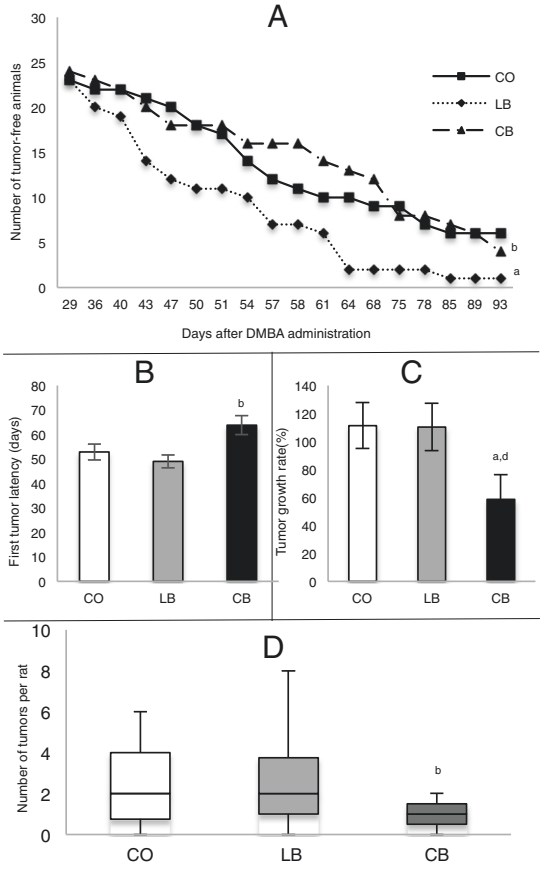

Fig. 3 Mammary tumorigenesis in female offspring of control diet (CO)-, lard (LB)-, and corn oil (CB)-fed males. **a** Number of tumor-free rats. **b** Number of days before the appearance of the first tumor. **c** Tumor growth rate in the first week of appearance. **d** Total number of tumors per rat (multiplicity). Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by a least significant difference test. Marginal difference ($p \leq 0.08$) compared with [d]LB, according to *t* test. The data are expressed as mean ± SEM ($n = 24$ per group). *DMBA* 7,12-dimethylbenz[a]anthracene

corn oil-fed male rats exhibited a decreased ($p \leq 0.05$) number of apoptotic cells (Fig. 4f) in mammary tumors compared with female offspring of control diet-fed male rats. There was no difference ($p > 0.05$) between female offspring of lard- and corn oil-fed male rats regarding this parameter (Fig. 4f). In addition, there was no difference ($p > 0.05$) among groups regarding cell proliferation in the mammary tumors (Fig. 4c).

miRNA expression profile in fathers' sperm cells and in their daughters' mammary glands

To compare the outcomes of the distinct paternal high-fat diets on the basis of miRNA expression, Applied Biosystems TaqMan Rodent MicroRNA arrays were used to generate the miRNA profile for lard- and corn oil-fed fathers' sperm cells, as well as for their respective daughters' mammary glands. The microarray data are deposited in the Gene Expression Omnibus (GEO) public repository under accession number [GEO:GSE77012]. Corn oil-fed male rats had 89 downregulated ($p \leq 0.05$) miRNAs in the sperm compared with lard-fed male rats (Fig. 5a). Furthermore, female offspring of corn oil-fed male rats had 21 downregulated ($p \leq 0.05$) and 2 upregulated ($p \leq 0.05$) miRNAs in their mammary glands compared with female offspring of lard-fed male rats (Fig. 5b). There were three miRNAs that were downregulated in both the sperm and the mammary glands of the corn oil-fed fathers and their daughters, respectively: miR-1897-5p, miR-219-1-3p, and miR-376a#. IPA (Additional file 1: Table S1) indicated that these miRNAs could regulate signaling pathways associated with key physiological processes such as growth hormone, phosphatase and tensin homolog (PTEN), and prolactin signaling, as well as disease processes such as Huntington's disease, cardiac hypertrophy, type 2 diabetes mellitus, and breast cancer.

Protein expression in female offspring mammary gland

Since miR-1897-5p, miR-219-1-3p, and miR-376a# can directly or indirectly modulate several targets (shown in Additional file 1: Table S1), we decided to perform

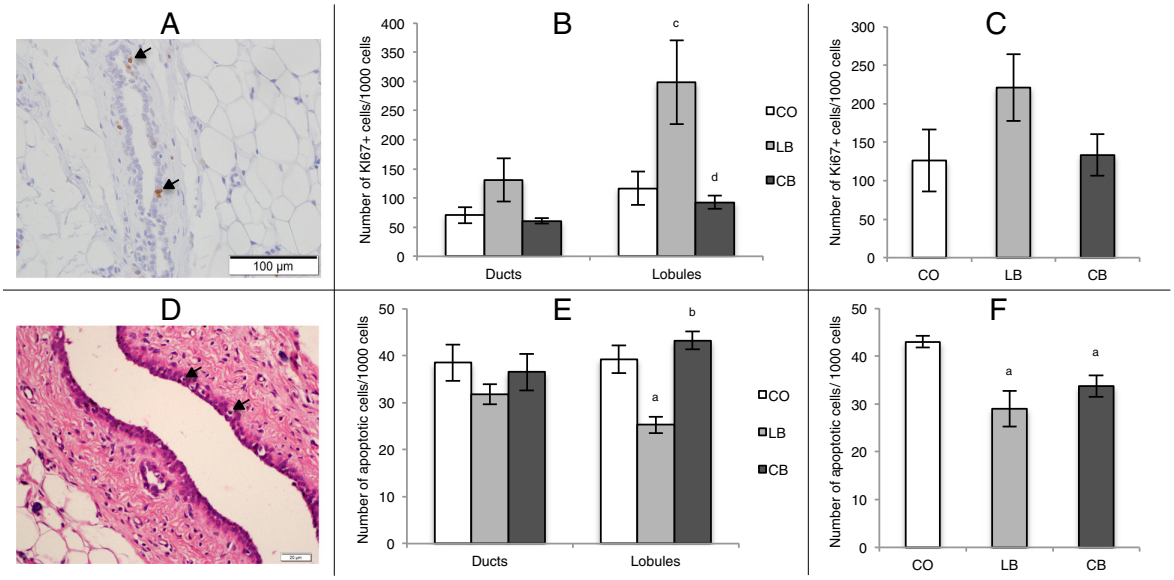

Fig. 4 Quantification of cell proliferation and apoptosis in mammary gland and tumors. **a** Photomicrograph (×20 original magnification) of Ki-67 immunostaining (cells indicated by *arrows*). **b** Quantification of cell proliferation in the mammary gland ducts and lobules of 50-day-old female offspring of control diet (CO)-, lard (LB)-, and corn oil (CB)-fed male rats. **c** Quantification of cell proliferation in mammary tumors of female offspring from CO-, LB-, and CB-fed male rats. **d** Photomicrograph (×40 original magnification) showing apoptotic cells (indicated by *arrows*). **e** Quantification of apoptosis in the mammary gland ducts and lobules of 50-day-old female offspring of CO-, LB-, and CB-fed male rats. **f** Quantification of apoptosis in mammary tumors of female offspring of CO-, LB-, and CB-fed males. Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by a least significant difference test. Marginal difference ($p \leq 0.06$) compared with [c]CO and [d]LB, according to *t* test. The data are expressed as mean ± SEM ($n = 4$ per group)

Western blot analysis of the following proteins linked to breast cancer: CCAAT/enhancer-binding protein beta (Cebpβ), caspase 3 (Casp3), insulin-like growth factor 1 receptor (Igf1r), protein kinase D1 (Pkd1), and transforming growth factor, beta receptor I (Tgfβr1). On the one hand, there was no difference ($p > 0.05$) among female offspring of the control diet-, lard-, and corn oil-fed male rats regarding Cebpβ, Casp3, and Igf1r levels (data not shown). On the other hand, female offspring of the corn oil-fed male rats had higher ($p \leq 0.05$) Pkd1 levels in the mammary glands than female offspring of lard-fed, but not control diet-fed, male rats (Fig. 6). There was no difference ($p > 0.05$) between female offspring of control diet- and lard-fed male rats regarding this protein (Fig. 6). In addition, Tgfβr1 levels were significantly increased in the offspring of lard-fed male rats (Fig. 6) compared with offspring of both control diet-fed ($p \leq 0.05$) and corn oil-fed ($p \leq 0.06$) male rats. There was no difference ($p > 0.05$) between female offspring of corn oil-fed and control diet-fed male rats regarding this protein (Fig. 6). Interestingly, both proteins are involved in regulating epithelial-to-mesenchymal transition (EMT): Pkd1 inhibits this process [36], and Tgfβr1 promotes it [37].

We further explored if Tgfβ and key regulators of its activity were altered by measuring protein levels of v-akt murine thymoma viral oncogene (Akt), cofilin (Cfl), v-raf leukemia viral oncogene (c-Raf), extracellular signal-regulated kinase 1/2 (Erk1/2), phosphorylated mitogen-activated protein kinase 8 (p-Jnk), mitogen-activated protein kinase 4 (Mkk4), mechanistic target of rapamycin (Mtor), mitogen-activated protein kinase 14 (p38), phosphorylated Smad family member 3/Smad family member 3 (p-Smad3/Smad3) ratio, and Harvey rat sarcoma virus oncogene (Ras). Tgfβ protein expression was higher ($p \leq 0.05$) in the mammary glands of the female offspring of lard-fed male rats than in the offspring of control diet- and corn oil-fed male rats. There was no difference ($p > 0.05$) between female offspring of corn oil- and control diet-fed male rats regarding this protein (Fig. 6). Further, the levels of Akt and p-Jnk were higher ($p \leq 0.05$) in the female offspring of lard-fed male rats than in female offspring of control diet-fed male rats (Fig. 6). There was no difference ($p > 0.05$) in female offspring of corn oil-fed male rats and the offspring of control diet- and lard-fed male rats regarding these proteins (Fig. 6). Female offspring of corn oil-fed male rats had lower levels of Mtor, Mkk4 ($p \leq 0.06$), and p-Smad3/Smad3 ($p \leq 0.05$) than female offspring of lard-fed, but not control diet-fed, male rats (Fig. 6). There was no difference ($p > 0.05$) between female offspring of control diet- and lard-fed male rats

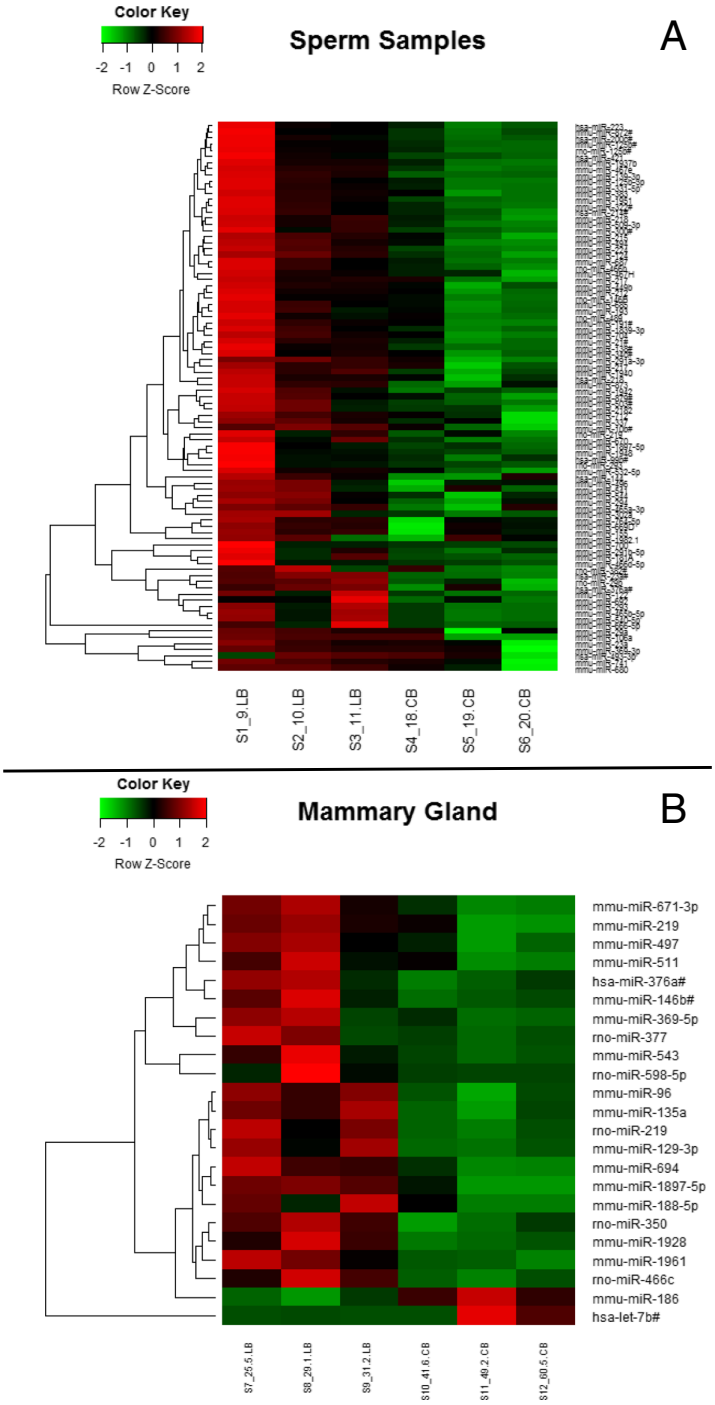

Fig. 5 Heat map of microRNA (miRNA or miR) expression profiles. **a** Heat map of miRNA expression profile in sperm of the male rats from control diet-, lard-, and corn oil-fed male rats. **b** Heat map of miRNA expression profile in normal mammary tissue of 50-day-old female offspring ($n = 3$ per group)

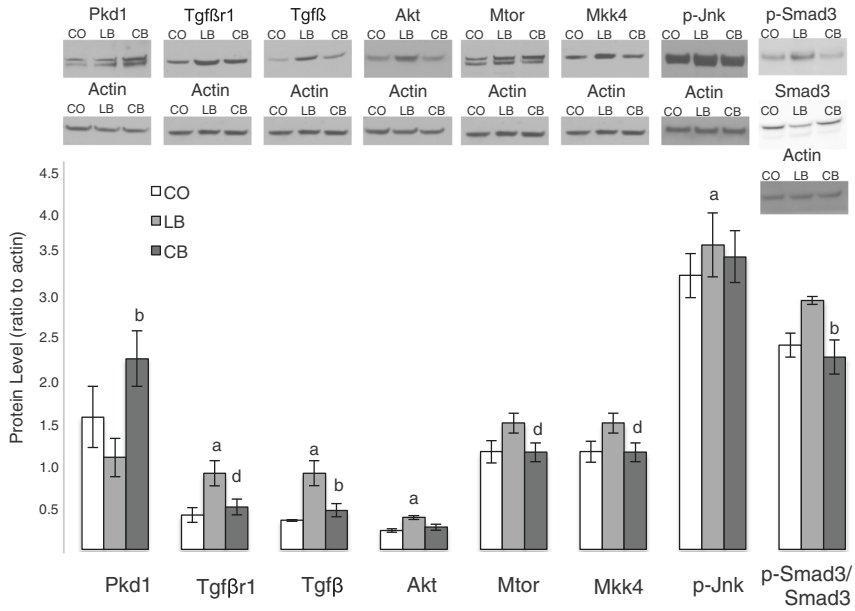

Fig. 6 Protein alterations associated with microRNA (miRNA) expression. Western blot analysis of protein kinase D1 (Pkd1), transforming growth factor, beta receptor I (Tgfβr1), transforming growth factor beta (Tgfβ), v-akt murine thymoma viral oncogene (Akt), mechanistic target of rapamycin (Mtor), mitogen-activated protein kinase kinase 4 (Mkk4), phosphorylated mitogen-activated protein kinase 8 (p-Jnk), and phosphorylated Smad family member 3/Smad family member 3 (p-Smad3/Smad3) ratio protein expression in mammary glands of 50-day-old female offspring of control diet (CO)-, lard (LB)-, and corn oil (CB)-fed male rats. Statistically significant difference ($p \leq 0.05$) compared with [a]CO and [b]LB, according to analysis of variance followed by a least significant difference test. Marginal difference ($p \leq 0.06$) compared with [d]LB, according to *t* test. The data are expressed as mean ± SEM ($n = 5$ per group)

regarding these proteins (Fig. 6). In addition to promoting EMT, all these altered proteins are involved in increasing cell survival, growth, migration, and invasion.

Discussion

Breast cancer is a complex disease with a multifactorial etiology [38]. It is increasingly evident that in utero environment can program later susceptibility to breast cancer [39]. The findings of our present study suggest that breast cancer risk can be determined even earlier through diet-induced changes in paternal germ cells before conception. Our study shows that, compared with female offspring of control diet-fed fathers, offspring of lard-fed fathers did not differ in tumor latency, growth, or multiplicity. However, female offspring of lard-fed fathers had increased elongation of the mammary epithelial tree, number of TEBs, and tumor incidence compared with both control diet- and corn oil-fed fathers, showing that paternal exposure to a lard-based high-fat diet containing SFA increased their daughters' mammary cancer risk. TEBs are considered sites of tumor initiation [40], and increased epithelial elongation reflects rapid epithelial growth [41]. Additionally, female offspring of lard-fed fathers showed increased cell proliferation and decreased apoptosis in the mammary gland lobules compared with female offspring of both control diet- and corn oil-fed fathers. Altogether, these findings support the view that altered mammary gland development represents a potential underlying mechanism of increased breast cancer risk [42].

Compared with female offspring of control diet-fed fathers, female offspring of corn oil-fed fathers had decreased tumor growth. There was no difference in tumor incidence, latency, or multiplicity between female offspring of control diet- and corn oil-fed fathers. In addition, female offspring of corn oil-fed fathers had longer tumor latency, decreased tumor growth, and decreased multiplicity compared with female offspring of lard-fed fathers. These data show that paternal exposure to a corn oil-based high-fat diet containing n-6 PUFA had an effect opposite that of a lard-based high-fat diet and reduced their daughters' mammary cancer risk.

Although male rats that were fed the lard-based and corn oil-based high-fat diets consumed the same amount of calories, lard increased the body weight and size of epididymal fat pads more than corn oil did. Thus, different fatty acids can have distinct effects on adipose accumulation, as already shown by others [43]. Our results further show that lard, but not corn oil, elicited detrimental effects on male reproductive parameters (fewer normal sperm cells and lower daily sperm production).

This is in line with earlier human and animal data showing that SFA disrupt testicular metabolism and sperm quality, while PUFA are essential for sperm cell membrane fluidity and flexibility as well as fertilization [44]. Excessive epididymal fat in lard-fed males may have been detrimental to spermatogenesis, as epididymal tissue is an essential depot for spermatogenesis [45]. The adverse effects of lard may not be mediated through increased insulin resistance. Although a correlation between insulin resistance and impaired sperm production has been reported in rats fed a diet high in SFA [46], as also found in the present study, a corn oil-based high-fat diet also impaired insulin tolerance but did not affect male reproductive parameters. We propose that impaired sperm quality and function in lard-fed fathers could be associated with disruption in metabolic programming and increased breast cancer risk among their daughters.

The impact of obesity in fathers leading to metabolic dysfunction in their female offspring was previously observed in rodent studies [10], and it was also seen in our present study. Female offspring of both lard- and corn oil-fed fathers exhibited increased body weight and adiposity. However, only female offspring of lard-fed fathers displayed an impaired insulin response, indicating that the type of dietary fatty acids consumed represents a key factor in metabolic programming through the male germline.

Epigenetic modifications that are necessary for achieving reproductive capacity of male gametes include DNA methylation, histone retention, and expression of noncoding RNAs such as miRNAs [47]. Because miRNAs can modulate the expression of hundreds of mRNAs that affect embryonic development as well as the establishment of the offspring's epigenome [48], they have been proposed to mediate paternal programming effects on the offspring [49]. The epididymis has been implicated as the site of the alterations of miRNA signatures occurring during the maturation of sperm cells, and therefore an increase in epididymal fat pad size could potentially impact inheritance of miRNA signatures and/or the developmental trajectory of the offspring [50]. The impact of high-fat-diet-induced male obesity on the miRNA profile in mature spermatozoa has been examined in rodent studies [51]. In a study by Fullston et al. [52], males fed a high-saturated-fat diet exhibited changes in miRNAs in the testes and mature spermatozoa that target mRNA associated with spermatogenesis, embryonic development, and metabolic diseases in the offspring. We provide further evidence that paternal nutrition can impact the sperm miRNA profile and possibly the subsequent mammary gland miRNA profile, which in turn targets genes implicated in breast cancer and other diseases.

Some of the miRNAs that were differentially expressed in the lard- and corn oil-fed fathers' germ cells also were differentially expressed in their daughters' mammary glands, although the daughters were never directly exposed to the high-fat diets. When we compared lard-fed fathers and their daughters, we observed three miRNA that were significantly altered in both the sperm and mammary glands of corn oil-fed fathers and their daughters: miR-1897-5p, miR-219-1-3p, and miR-376a#. Since miRNAs can modulate gene expression by inhibiting the translation of mRNA or by directing their degradation [53], we focused on determining if the expression of the top potentially targeted proteins (Additional file 1: Table S1) was altered in the daughters of lard- or corn oil-fed fathers. Among them, we highlight Pkd1 and Tgfβr1. PKD1 is a serine/threonine kinase that is expressed in ductal epithelial cells of the mammary gland, maintains the epithelial phenotype, and prevents EMT [54]. Inhibition of PKD1 can lead to pathological conditions such as cancer [55]. Thus, our finding of increased Pkd1 levels in the mammary glands of corn oil-fed fathers' offspring, compared with female offspring of lard-fed fathers, is in line with their lowest susceptibility to breast cancer. In addition, compared with female offspring of control diet- and corn oil-fed fathers, another miRNA target, Tgfβr1, was increased in the daughters of lard-fed fathers that displayed the highest susceptibility to mammary cancer. Tgfβr1 expression is related to promotion of breast carcinogenesis through multiple mechanisms, including enhancing EMT [56]. We assessed the protein levels of up- and downstream signaling partners of Tgfβr1. Female offspring of lard-fed fathers showed higher protein levels of Tgfβ than female offspring of both control diet- and corn oil-fed fathers, as well as higher protein levels of Akt and p-Jnk than control diet-fed fathers. In addition, female offspring of corn oil-fed rats had lower levels of Mtor, Mkk4, and p-Smad3/Smad3 than female offspring of lard-fed fathers. These proteins collectively play important roles in cell survival, growth, migration, and invasion [57, 58]. These findings indicate that mechanisms other than miRNAs contribute to changes in gene expression in the daughters' mammary tissue following paternal exposure to a lard-based high-fat diet.

Because fathers, mothers, and their daughters tend to share the same nutritional habits [59], it is important to further investigate if paternally programmed breast cancer risk is affected by maternal and female offspring's fat intake. Maternal intake of a high corn oil diet during pregnancy increases female offspring's mammary cancer risk [8], while intake of lard has opposite effects [9]. In addition, because obesity-induced altered sperm miRNA expression in the fathers can be normalized through exercise or dietary intervention (consumption of balanced diet), which then improves the metabolic health of female offspring [60], the efficacy of a similar intervention in reducing daughters' breast cancer risk should be investigated.

Conclusions

In the present study, we show that paternal intake of a lard-based high-fat diet rich in SFA increases female offspring's mammary cancer risk, as indicated by the increased elongation of the mammary epithelial tree, number of TEBs, and tumor incidence in female offspring of lard-fed fathers compared with female offspring of both control diet- and corn oil-fed rats. However, if the paternal fat source is corn oil that is high in n-6 PUFA, these male rats' offspring's mammary cancer risk is reduced, as indicated by decreased tumor growth in female offspring of corn oil-fed fathers compared with female offspring of both control diet- and lard-fed fathers, as well as by longer tumor latency and decreased tumor multiplicity compared with female offspring of lard-fed fathers. Altered miRNA expression in fathers' sperm and daughters' mammary glands may at least underlie these effects, but other epigenetic changes are likely to be involved. Our findings highlight the importance of paternal nutrition in affecting future generations' risk of developing breast cancer.

Additional file

Additional file 1: Table S1. Canonical IPA analyses of the target pathways and molecules modulated by altered miRNA from father's sperm and 50-day-old female offspring mammary glands from lard-fed (LB) and corn oil-fed (CB) males. (DOC 31 kb)

Abbreviations

Akt, v-akt murine thymoma viral oncogene; ANOVA, analysis of variance; Casp3, caspase 3; CB, rats that were fed a corn oil-based high-fat diet and their offspring; Cebpβ, CCAAT/enhancer-binding protein beta; Cfl, cofilin; CO, rats that were fed a control diet and their offspring; c-Raf, v-raf leukemia viral oncogene; DMBA, 7,12-dimethylbenz[a]anthracene; EMT, epithelial-to-mesenchymal transition; Erk1/2, extracellular signal-regulated kinase 1/2; Igf1r, insulin-like growth factor 1 receptor; ITT, insulin tolerance test; LB, rats that were fed a lard-based high-fat diet and their offspring; LSD, least significant difference; miRNA or miR, microRNA; Mkk4, mitogen-activated protein kinase kinase 4; mRNA, messenger RNA; Mtor, mechanistic target of rapamycin; MUFA, monounsaturated fatty acid; p38, mitogen-activated protein kinase 14; p-Jnk, phosphorylated mitogen-activated protein kinase 8; Pkd1, protein kinase D1; p-Smad3/Smad3, phosphorylated Smad family member 3/Smad family member 3 ratio; PTEN, phosphatase and tensin homolog; PUFA, polyunsaturated fatty acid; Ras, Harvey rat sarcoma virus oncogene; SCLB, somatic cell lysis buffer; SFA, saturated fatty acid; TEB, terminal end bud; Tgfβr1, transforming growth factor, beta receptor I

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Authors' contributions

CCF conceived of the study; supervised animal work, tissue collection, and molecular analysis; and drafted the manuscript. LNG assisted with animal studies, tissue collection, and molecular assays and helped to revise the manuscript. MPR assisted with animal studies, tissue collection, and molecular assays and helped to revise the manuscript. FdOA assisted with animal study design and data analyses and helped to revise the manuscript. LJ performed miRNA data analysis and helped to revise the manuscript. JI assisted with tumor and statistical analyses and helped to revise the manuscript. VCP conducted male reproductive parameter analysis and helped to revise the manuscript. IAdC conducted analysis of the diets' lipid profiles and helped to revise the manuscript. LHC provided intellectual input and helped to draft the manuscript. SdA provided intellectual input and helped to draft the manuscript. TPO conceived of the study, oversaw the research, and drafted the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing financial or non-financial competing interests.

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Data de Aprovação da Banca:	
Data Máxima para Defesa:	
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Histórico de Ocorrências:	Primeira Matrícula em 29/08/2012

Aluno matriculado no Regimento da Pós-Graduação USP (Resolução nº 5473 em vigor de 18/09/2008 até 19/04/2013).
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Carga

Sigla	Nome da Disciplina	Início	Término	Horária	Cred.	Freq.	Conc.	Exc.	Situação
FBA5728-3/7	Aprimoramento Didático	19/03/2013	15/04/2013	60	4	100	A	N	Concluída
HNT5759-2/1	Nutrigenômica no Contexto das Doenças Crônicas não Transmissíveis (Faculdade de Saúde Pública - Universidade de São Paulo)	16/05/2013	21/06/2013	60	4	100	A	N	Concluída
NHA5701-5/3	Fundamentos Biológicos da Nutrição Humana Aplicada (Curso Interunidades: Nutrição Humana Aplicada - Universidade de São Paulo)	05/08/2013	08/09/2013	75	5	80	A	N	Concluída
BIO5725-3/2	Genética e Câncer (Instituto de Biociências - Universidade de São Paulo)	31/10/2013	29/11/2013	90	6	90	A	N	Concluída
FBA5712-6/1	Fisiologia da Nutrição I	19/05/2014	29/06/2014	90	6	100	A	N	Concluída

	Créditos mínimos exigidos		Créditos obtidos
	Para exame de qualificação	Para depósito de tese	
Disciplinas:	0	25	25
Estágios:			
Total:	0	25	25

Créditos Atribuídos à Tese: 167

Conceito a partir de 02/01/1997:

A - Excelente, com direito a crédito; B - Bom, com direito a crédito; C - Regular, com direito a crédito; R - Reprovado; T - Transferência.

Um(1) crédito equivale a 15 horas de atividade programada.

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