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Lipidomic fatty acid profile and global gene expression pattern in mammary gland of rats that were exposed to lard-based high fat diet during fetal and lactation periods associated to breast cancer risk in adulthood

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ABSTRACT

The persistent effects of animal fat consumption during pregnancy and nursing on the programming of breast cancer risk among female offspring were studied here. We have previously found that female offspring of rat dams that consumed a lard-based high-fat (HF) diet (60% fat-derived energy) during pregnancy, or during pregnancy and lactation, were at a reduced risk of developing mammary cancer. To better understand the unexpected protective effects of early life lard exposure, we have applied lipidomics and nutrigenomics approaches to investigate the fatty acid profile and global gene expression patterns in the mammary tissue of the female offspring. Consumption of this HF diet during gestation had few effects on the mammary tissue fatty acids profile of young adult offspring, while exposure from gestation throughout nursing promoted significant alterations in the fatty acids profile. Major differences were related to decreases in saturated fatty acids (SFA) and increases in omega-6 polyunsaturated fatty acids (PUFAs), monounsaturated fatty acids (MUFAs) and conjugated linolenic acid (CLA) concentrations. In addition several differences in gene expression patterns by microarray analysis between the control and *in utero* or *in utero* and during lactation HF exposed offspring were identified. Differential dependency network (DDN) analysis indicated that many of the genes exhibited unique connections to other genes only in the HF offspring. These unique connections included *Hrh1–Ythdf1* and *Repin1–Elavl2* in the *in utero* HF offspring, and *Rnf213–Htr3b* and *Klf5–Chrna4* in the *in utero* and lactation HF offspring, compared with the control offspring. We conclude that an exposure to a lard-based HF diet during early life changes the fatty acid profile and transcriptional network in mammary gland in young adult rats, and these changes appear to be consistent with reduced mammary cancer risk observed in our previous study.

1. Introduction

Consumption of fat is increasing world-wide [1]. From 1995–1997 to 2001–2003 the percentage of energy intake from total fat increased almost 2% globally and important contributors to this dietary change are vegetable oils and animal fat [2]. Of concern is the increase in animal fat intake as it is high in saturated fatty acids (SFA) [2], which is associated with many diseases, such as cardiovascular diseases, diabetes and neurodegenerative diseases [3–5].

Abbreviations: CO, female rats exposed to control diet [AIN-93G] that has soy oil as fat source during gestation and lactation; DDN, differential dependency network; G, female rats exposed to HF diet during gestation; GL, female rats exposed to HF diet during gestation and lactation; HF, lard-based high-fat; PCA, principal component analyses.

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However, the effects of dietary fats, including saturated fats on breast cancer risk are not clear yet [6–8]. Specifically for animal fat, contradictory effects were reported. In some studies, high consumption of animal fat, represented by red meat and dairy products, has been related to modestly elevated breast cancer risk [6,9,10]. On the other hand, many other epidemiologic studies have not observed an association between animal fat and SFA consumption and breast cancer risk [7,8,11]. Through a lipidomic analysis it was observed that patients with breast cancer present different fatty acids profile in the serum and erythrocyte membrane than cancer-free women, including changes in palmitic acid, stearic acid, linoleic acid and total free fatty acids, suggesting that altered lipid metabolism is an important feature in breast cancer [12,13].

The amount and type of dietary fat consumption during gestation and/or lactation periods is another factor that can modulate breast cancer risk in the offspring [14,15]. Previous animal studies have shown that i*n utero* exposure to diets rich in corn oil, containing high levels of omega-6 polyunsaturated fatty acids (PUFAs), increased the susceptibility to mammary carcinogenesis in adulthood [16,17]. In addition, maternal consumption during gestation and lactation of menhaden (*n*-3 PUFA), canola (*n*-3 and *n*-6 PUFAs and monounsaturated fatty acids [MUFAs]) or olive oil (MUFA)-based diets decreased chemically induced breast cancer development in the offspring [18–20]. It is suggested that early life exposure to dietary fat can modify the susceptibility to breast cancer in adult life through persistent modulation of gene expression [21,22]. Nutrigenomics analysis of global gene expression is an useful strategy to identify functionally relevant pathways involved in breast cancer susceptibility, modulated by nutritional exposures early in life [23].

We have previously shown that female offspring of rat dams consuming high amounts of lard (60% fat-derived energy) during pregnancy, or during pregnancy and lactation, were at a reduced risk of developing mammary cancer [24]. In the group exposed to lard-based high fat (HF) diet only *in utero* all aspects of mammary tumorigenesis were reduced (latency to tumor appearance, tumor incidence, multiplicity and burden) compared with the control group. Offspring of dams consuming the lard-based HF diet both during pregnancy and lactation exhibited only reduced mammary tumor multiplicity, suggesting a lower protection [24]. To better understand these unexpected protective effects by early life lard exposure, we have applied a lipidomics and nutrigenomics approach to investigate the fatty acid profile and global gene expression patterns in the mammary tissue of adult female offspring of dams that consumed a lard-based HF diet during gestation or gestation and lactation periods.

2. Material and methods

2.1. Experimental protocol

The mammary tissues used in the present study were obtained from our previously completed study [24]. Briefly, before mating female Sprague–Dawley rats were divided into 3 groups ($n = 20$ dams per group): (a) CO (female rats exposed to control diet [AIN-93G] that had soy oil as fat source during gestation and lactation; controls); (b) G (female rats exposed to lard-based HF diet during gestation and AIN-93G during lactation), and (c) GL (female rats exposed to lard-based HF diet during gestation and lactation). The HF diet is based on AIN-93G [25] with added lard replacing some carbohydrates. CO and HF diets contained 10% and 60% of total energy from fat, respectively. The distribution of fatty acids in the HF diet was 37%, 38% and 24% of SFA, MUFA and PUFA, respectively, while in the CO diet, the distribution of the same fatty acids was 17%, 27% and 55%. During lactation, the diet of dams from G group was switched to AIN93-G, while the diets of dams from CO and GL groups remained AIN93-G and HF, respectively. As previously described [24] caloric intake was not different ($P > 0.05$) among the dams in any of the diets groups (CO, G and GL) during gestation and lactation, and body weight gain during pregnancy and body weight at the end of lactation were also not different among them. After weaning all female offspring consumed commercial laboratory chow (Nuvital, Brazil). The 7-week-old female offspring were euthanized using CO_2 chamber and the mammary gland stored at −80° for further analysis. The experiments were approved by the Ethics Committee on Animal Experiments of the Faculty of Pharmaceutical Sciences, University of São Paulo (Protocol Number 283). The animal experiment has been described in detail before [24].

2.2. Fatty acid lipidomics analysis

Lipids were extracted from the 4th mammary gland obtained from 7-week-old female offspring of CO, G or GL groups ($n = 4$ per group) using Folch method. About 50 mg of tissue was macerated with liquid nitrogen, mixed with 1 mL of chloroform:methanol (2:1); 25 μL of PMSF; 10 μL of C19 standard diluted in dichloromethane and incubated overnight at 4 °C. After the incubation, water was added (5× volume), tissue was homogenized for 50 min and incubated again overnight at 4 °C. The methanolic phase was then collected. Derivatization of fatty acids to methyl esters (FAMEs) was carried out according to the protocol described by Ichihara and Fukubayashi (2010) [26] with some modifications. The samples were injected (1 μl) in split mode (5:1) at 250 °C in the GC/MS system (Agilent 6890 coupled to an Agilent 5973 Mass Selective Detector). The column was a DB-5MS capillary column (30 m × 0.25 mm inner diameter, 0.25 μm film thickness; Agilent). The FAMEs were identified by comparison of their retention time to their respective standards, and a mass spectral survey was performed using the NIST Mass Spectral Library (2008). The analyses were performed in triplicate for each sample and each FAME was quantified based on curves made with authentic standards (Supelco, Inc). Supplementary Table 1 provides a list of all lipids that were analyzed in the mammary gland.

2.3. Analysis of global gene expression in female offspring's mammary gland

Total RNA was extracted from the 4th mammary glands obtained from 7-week old female offspring from CO, G or GL groups ($n = 5$ per group) using RNeasy Lipid Tissue Mini Kit (QIAGEN, USA) according to the manufacturer's protocol. RNA quality was confirmed using an Agilent 2100 Bioanalyzer and RNA 6000 LabChips, from which the RNA integrity numbers (RIN) were calculated. High quality total RNA was labeled and hybridized to Affymetrix Rat Genome 230 2.0 GeneChip using the manufacturer's protocols. Expression data were normalized by the probe logarithmic intensity error (PLIER) method as implemented in Affymetrix expression console 1.2 (http://www.affymetrix.com/). Student *t*-test *P*-values and fold changes were calculated by comparing 30,000 transcripts and variants between CO × G, CO × GL and G × GL, with $P \leqslant 0.05$ (univariate, two tailed) and fold change $\geqslant 1.5$ identified as differentially expressed genes. Principal component analyses (PCA) was obtained with the data set to summarize the variation of gene expression under HF exposure on pregnancy, and pregnancy and lactation. For differential dependency network (DDN) analysis MATLAB and Cytoscape were used for analysis and visualization, respectively [27]. The parameters used for DDN analysis were $K = 1$, P value cutoff = 0.01, threshold = 0.25. Bio Function analysis of all significantly differentially

expressed genes among groups was performed by Ingenuity pathway analysis (IPA).

2.4. Validation of differently expressed genes in female offspring's mammary tissue

Expression levels of target genes were measured using a 7900HT Fast Real-Time PCR System (Applied Biosystems) by Relative Standard Curve Method as described before [24]. *Gapdh* was used as the reference gene to normalize target gene expression. Primers were designed using IDT tool primer design (Supplementary Table 2). Results are expressed as mean ± S.E.M, and all analyses were conducted with STATISTICA 8.0 (Statsoft, USA). One-way analysis of variance (ANOVA) followed by Duncan's post hoc was applied. For all data analysis, $P \leqslant 0.05$ was applied as the threshold for statistical significance.

3. Results

3.1. Mammary tissue lipid profile of 7-week old female offspring of CO, G and GL groups

The lipid profile of the mammary tissue clearly differentiated the female offspring of GL dams from CO and G offspring (Fig. 1a). These differences were mostly seen in the following fatty acids: SFAs stearic, myristic, lauric, behenic, heneicosylic and pentadecylic acid; MUFAs myristoleic, *cis*-13-octadecenoic, heptadecenoic and oleic; omega-3 PUFAs docosahexaenoic, eicosapentaenoic and linolenic acids; and omega-6 PUFAs dihomo-γ-linolenic acid, arachidonic, linoleic, eicosadienoic; and the conjugated linoleic acid (CLA) 10-*trans*, 12-*cis*- octadecadienoic (Fig. 1b). In addition, less notable differences were observed between the female offspring of G and CO dams (Fig. 1a), mainly in the SFAs lauric, myristic, heneicosylic, behenic, and lignoceric acids and omega-3 PUFA docosahexaenoic acid (Fig. 1b).

According to ANOVA test, 15 fatty acids showed different mammary gland concentrations among the CO, G and GL female offspring groups (Fig. 2). More specifically, compared with CO group, the G female offspring group presented higher ($P \leqslant 0.05$) mammary gland levels of arachidonic acid (*n*-6) and lower ($P \leqslant 0.05$) levels of SFAs lauric and myristic fatty acids. The offspring of GL dams, in turn, presented higher ($P \leqslant 0.05$) levels of stearic acid (SFA), myristoleic, *cis*-10-heptadecenoic, *cis*-13-octadecenoic, oleic and *cis*-11-eicosenoic acids (MUFA), dihomo-γ-linolenic acid, linoleic, arachidonic and eicosadienoic acids (omega-6), and *trans*-10, *cis*-12-octadecadienoic (CLA) fatty acids than the CO group. They also had lower ($P \leqslant 0.05$) levels of SFAs lauric, myristic, heneicosylic and behenic fatty acids (Fig. 2). Compared with the G group, the offspring from GL group presented higher ($P \leqslant 0.05$) levels of stearic acid (SFA), myristoleic, *cis*-10-heptadecenoic, *cis*-13-octadecenoic and *cis*-11-eicosenoic acids (MUFA), dihomo-γ-linolenic acid, linoleic and eicosadienoic acids (omega-6), and *trans*-10, *cis*-12-octadecadienoic (CLA) fatty acids, as well as lower ($P \leqslant 0.05$) levels of lauric and myristic (SFA) fatty acids (Fig. 2).

3.2. Comparison of global gene expression in the mammary tissue of female offspring of CO and G dams

We identified 51 genes as significantly differentially expressed in the mammary tissue between the female offspring from CO and G groups (Fig. 3a). Compared with the CO, offspring from the G group presented 25 downregulated genes and 26 upregulated genes. PCA of the differentially expressed genes separated the experimental samples from the CO and G groups into two distinct clusters (Fig. 3b). The differentially expressed genes are involved in important pathways related to carcinogenesis such as *cell growth and proliferation*, *cellular function and maintenance*, *cellular development*, *cell-to-cell signaling and interaction*, *cell signaling*, *nucleic acid metabolism*, *cell death and survival* (Fig. 3c; Supplementary Table 3). Genes involved in *carbohydrate metabolism* were also significantly differentially expressed between the CO and G groups.

3.3. Comparison of global gene expression in the mammary tissue of female offspring of CO and GL dams

We identified 101 genes as significantly differentially expressed in the mammary tissue between the female offspring from CO and GL groups (Fig. 4a). Compared with the CO, offspring from the GL

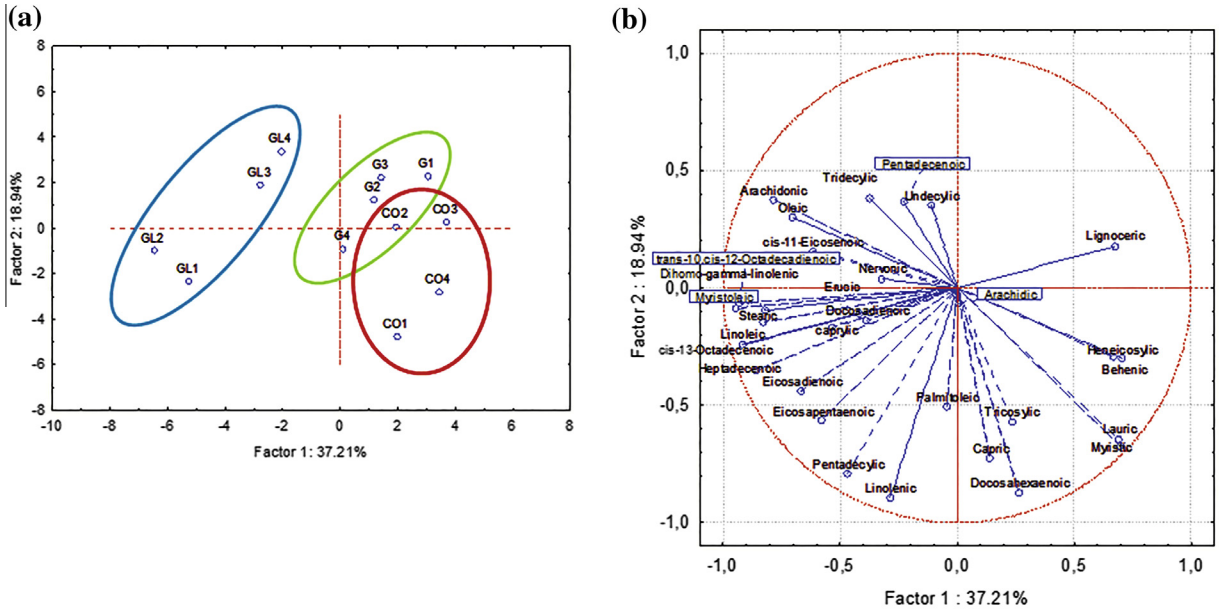

Fig. 1. Principal component analysis of lipid profile by groups (a) and by fatty acids (b). The first principal component (PC1) and PC2 accounted for 37.21% and 18.94% of the variation, respectively. CO: control group; G: *in utero* high fat exposed group; GL: *in utero* and during lactation high fat exposed group.

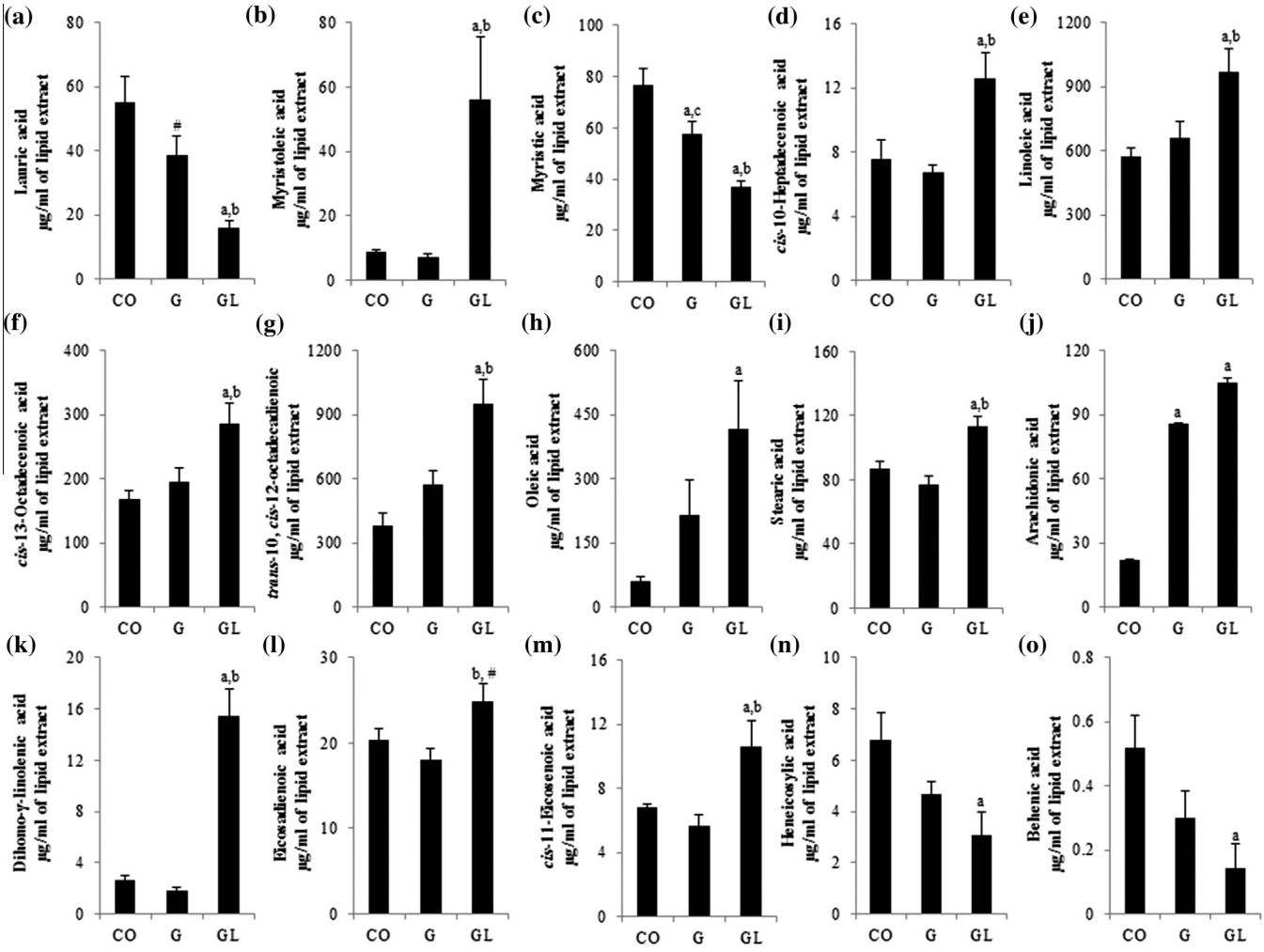

Fig. 2. Mammary lipid profile in 7-week-old female offspring from the control (CO), dams fed high fat diet during gestation (G) and dams fed high fat diet during gestation and lactation (GL) groups. The data are expressed as mean ± SEM ($n = 4$). Statistically significant ($P \leqslant 0.05$) compared with CO[a], G[b] and GL[c]. [#]Marginal ($P < 0.09$) difference compared with CO according to ANOVA followed by the Duncan test.

group presented 46 downregulated genes and 55 upregulated genes. Because GL group offspring presented lower protection against mammary tumor development compared with G group offspring, the total number of differentially expressed genes is not directly linked to mammary cancer risk. Rather, the higher number of changes could just be a reflection of longer exposure to the lard-based HF diet. PCA of the differentially expressed genes separated the samples into two distinct clusters discriminating the GL group from the CO group (Fig. 4b). The differentially expressed genes are involved in important pathways related to development and carcinogenesis such as *cancer*, *tumor morphology*, *cell cycle*, *organ development*, *tissue development*, *cell death and survival* (Fig. 4c, Supplementary Table 4). Genes involved in *lipid and carbohydrate metabolisms* were also significantly differentially expressed between the CO and GL groups.

3.4. Comparison of global gene expression in the mammary tissue of female offspring of G and GL dams

Although our previous study found that both the G and GL offspring exhibited lower susceptibility to mammary tumorigenesis than the control CO offspring [24], we identified 100 genes as significantly differentially expressed in the mammary tissues between the female offspring from G and GL groups (Fig. 5a). Compared with the G, offspring from the GL group presented 83 downregulated genes and 17 upregulated genes. PCA of the differentially expressed genes separated the samples between the GL and G groups into two distinct clusters (Fig. 5b). The differentially expressed genes are involved in *cancer*, *tumor morphology*, *cellular development*, *cellular growth and proliferation*, *tissue development* and *DNA replication, recombination and repair* (Fig. 5c; Supplementary Table 5). Genes involved in *lipid and carbohydrate metabolisms* were also significantly differentially expressed between the G and GL groups.

3.5. DDN analysis between CO and G groups

DDN was used to detect statistically significant topological changes in transcriptional networks between CO and G offspring [27]. According to this analysis, the transcriptional network changes in offspring exposed to HF diet *in utero* (G group) (Fig. 6a) comprised of 9 connections (green connections) that exist only in G samples, and 7 connections (red connections) that exist only in CO samples. We then performed RT-PCR analysis to verify that the genes in the connections were differentially expressed and observed that *Hrh1* connecting to *Ythdf1*, and *Repin1* connecting to *Elavl2* were significantly up-regulated in the offspring of dams fed HF during pregnancy (G group) (Fig. 7).

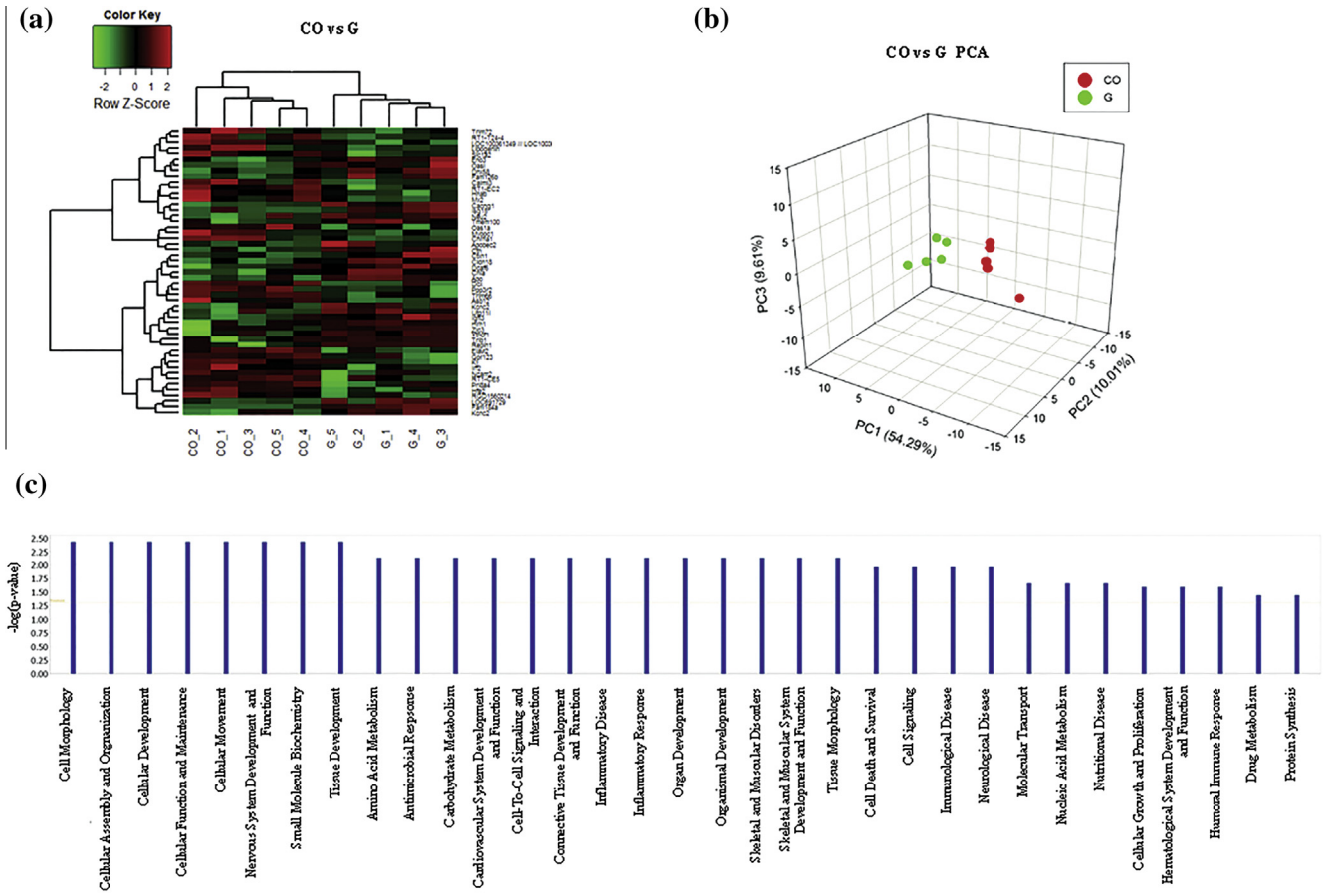

Fig. 3. Genes differentially expressed between the control (CO) and *in utero* high fat exposed (G) groups. (a) Heat map shows scaled individual expression scores for the genes that were significantly differentially expressed. (b) Principal component analysis of CO (red) and G (green) samples using genes differentially expressed between these groups. The first principal component (PC1), PC2 and PC3 accounted for 54.29%, 10.01% and 9.61% of the variation, respectively. (c) Bio Function analysis of the significantly differentially expressed genes between CO and G groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

3.6. DDN analysis between CO and GL groups

According to DDN analysis, the transcriptional network changes in offspring exposed to HF diet *in utero* and nursing (GL group) (Fig. 6b) comprised 2 connections (green connections) that exist only in GL samples and 10 connections (red connections) that exist only in CO samples. We observed for the first time that *Rnf213* and *Htr3b*, as well as, *Klf5* and *Chrna4* are connected in the mammary gland just in the condition of exposure to HF diet in *in utero* and lactation periods.

3.7. DDN analysis between G and GL groups

According to DDN analysis, the transcriptional network changes in offspring exposed to HF diet *in utero* and nursing (GL group) compared with G group (Fig. 6c) comprised 3 connections (red connections) that exist only in G samples and 6 connections (green connections) that exist only in GL samples. Examples of new interactions that were observed only on the latter group include: *Agpat3/Ydjc, Mycbp2/S100g* and *Tmcc1/Ubash3b/Lemd2/Tpo* and another interesting connection that occurred just in G group included *Fhl1/Crkrs*.

3.8. Verification of differential gene expression

We then determined if any of the genes identified in the microarray analysis were differentially expressed by RT-PCR. For that purpose, six genes that were differentially expressed in the microarray analysis were randomly chosen to be studied: *Hrh1*, *Repin1*, *Stra6*, *Tlr1*, *Crkrs* and *Pam*. In the offspring of G group, *Hrh1* ($P \leqslant 0.05$) and *Repin1* ($P = 0.08$) were upregulated, compared with the control group, while in the offspring of GL group a marginal increase in *Stra6* ($P = 0.09$) was seen (Fig. 7). *Crkrs* was not affected, compared with the offspring of CO group, but the offspring of GL group exhibited significantly lower expression of this gene than the G group (Fig. 7). Expression of *Pam* and *Tlr1* in the offspring of G and GL groups presented no statistical significance ($P > 0.05$).

4. Discussion

We have previously shown that increased maternal consumption of animal fat during pregnancy, or pregnancy and lactation, reduced female offspring's susceptibility to mammary cancer [24]. In the present study we applied lipidomics and nutrigenomics approaches to better understand how early life animal fat exposure might provide protection against later development of breast cancer.

Although the effects on breast cancer programming in offspring of dams consuming different types of fatty acids during pregnancy have been characterized, no information is available on the impact of such exposures on the lipidomic profile in the offspring's mammary glands. According to our results, exposure to a lard-based HF diet during gestation affected the mammary tissue fatty acid

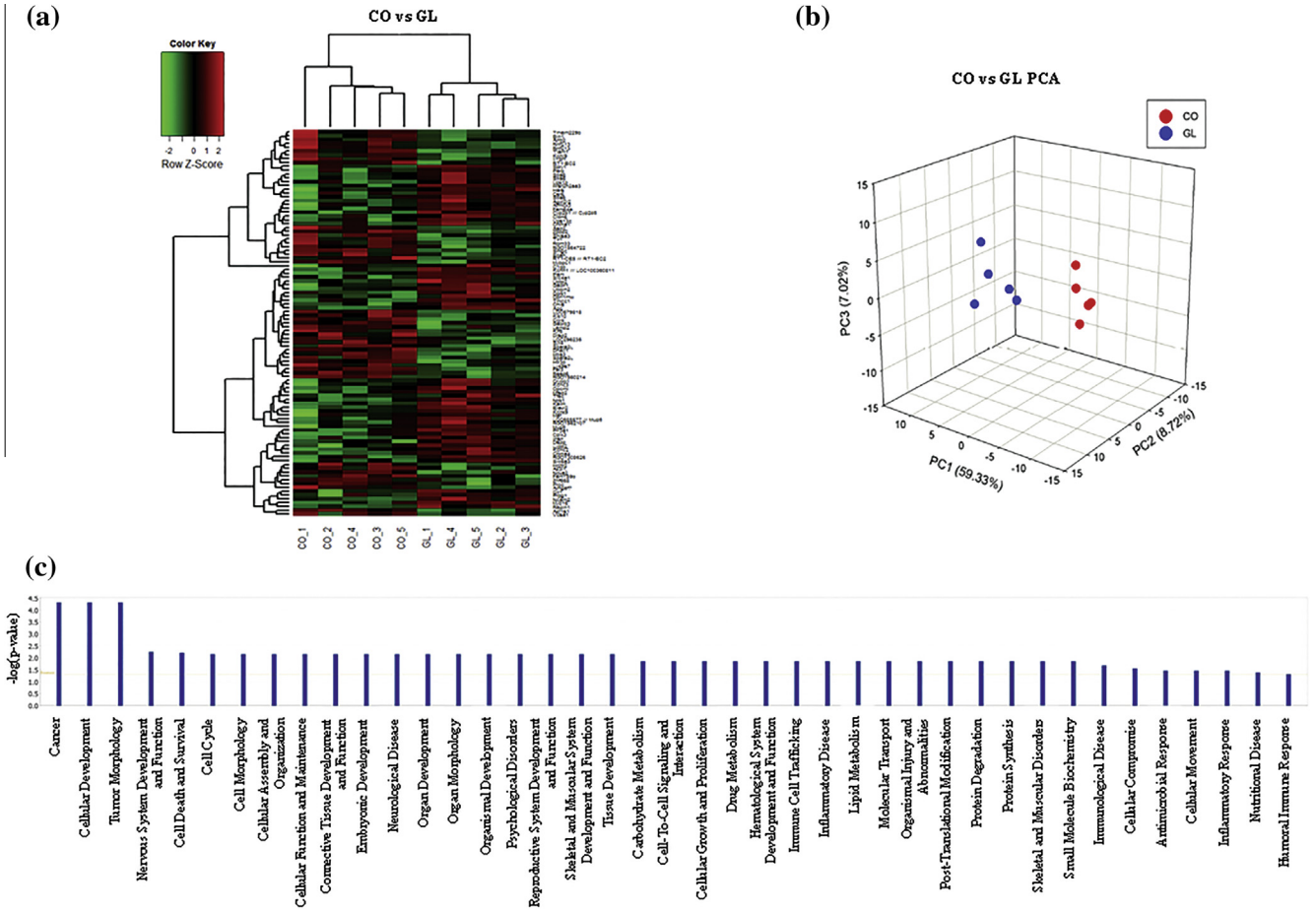

Fig. 4. Genes differentially expressed between the control (CO) and *in utero* and during lactation high fat exposed (GL) groups. (a) Heat maps show scaled individual expression scores for the genes that were significantly differentially expressed. (b) Principal Component analysis of CO (red) and GL (blue) samples using genes differentially expressed between these groups. The first principal component (PC1), PC2 and PC3 accounted for 59.33%, 8.72% and 7.02% of the variation, respectively. (c) Bio Function analysis of the significantly differentially expressed genes between CO and GL groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

profile of adult offspring, and even more drastic changes were seen in the mammary glands on the offspring of dams consuming HF diet during both pregnancy and lactation. Since we previously [24] observed that the protection against breast cancer was stronger in the offspring exposed to the lard-based HF diet only *in utero* compared with the offspring exposed to this diet both *in utero* and lactation, the number of differences in the fatty acid profile is not directly linked to protection against breast cancer. Rather, it is consistent with the longer exposure to this diet.

Our findings of reduced SFA levels in rats with reduced mammary cancer risk are consistent with reports of some clinical studies that have linked increased serum and adipose tissue SFA concentrations to higher breast cancer risk [12,28]. Increased levels of MUFA (oleic, *cis*-10-heptadecenoic, *cis*-13-octadecenoic and *cis*-11-eicosenoic acids) and the CLA *trans*-10, *cis*-12-octadecadienoic acid may be associated with the programming effect of early life exposure to a lard-based HF diet and subsequent decreased susceptibility to mammary carcinogenesis in adulthood. Oleic acid is the main component of the Mediterranean diet and it has been shown to possess protective actions against breast cancer [29]. Results from a clinical study indicated that increased levels of oleic acid in the adipose breast tissue were associated with a decreased risk for the disease [30]. Olive oil-based diet consumption for 5 weeks prior and throughout gestation and lactation decreased chemically-induced mammary cancer development in the offspring [19]. Many *in vitro* and *in vivo* studies link high levels of CLA to reduced breast cancer risk, and *trans*-10, *cis*-12-octadecadienoic acid has been proposed to acts as a chemopreventive agent against breast cancer [31]. Increased levels of omega-6 PUFA in the mammary tissue, in contrast, are not in agreement with findings showing that total omega-6 PUFA serum and adipose tissue (buttock) levels are positively associated with breast cancer [12,32]. Higher adipose tissue concentrations of linoleic and arachidonic acids were also associated with increased breast cancer risk [29,33]. In addition, maternal consumption during gestation of corn oil, a source of omega-6 PUFA, increased susceptibility to mammary carcinogenesis among female offspring [16]. These earlier studies have not taken into consideration the possibility that in combination with reduced levels of saturated fatty acids and increased levels of MUFA, high *n*-6 PUFA levels in the mammary tissue may not affect breast cancer risk.

Few studies have evaluated the effects of early life nutrition on global gene expression in adulthood, especially in the context of programming for later susceptibility to develop breast cancer. Exposure to high fat diets (butter, olive and safflower-based oils; 39% of energy from fat) *in utero* and through 50 days of age significantly altered the expression of genes associated with increased cell proliferation (Cyclin B1 and Cyclin A2), in the mammary glands of adult rats [22]. Pups fed either low-fat omega-3PUFA (16% energy from fat) or high-fat omega-3 PUFA diet (39% energy from fat) during prepubertal period (between postnatal days 5 and 24) showed decreased and increased carcinogen-induced mammary

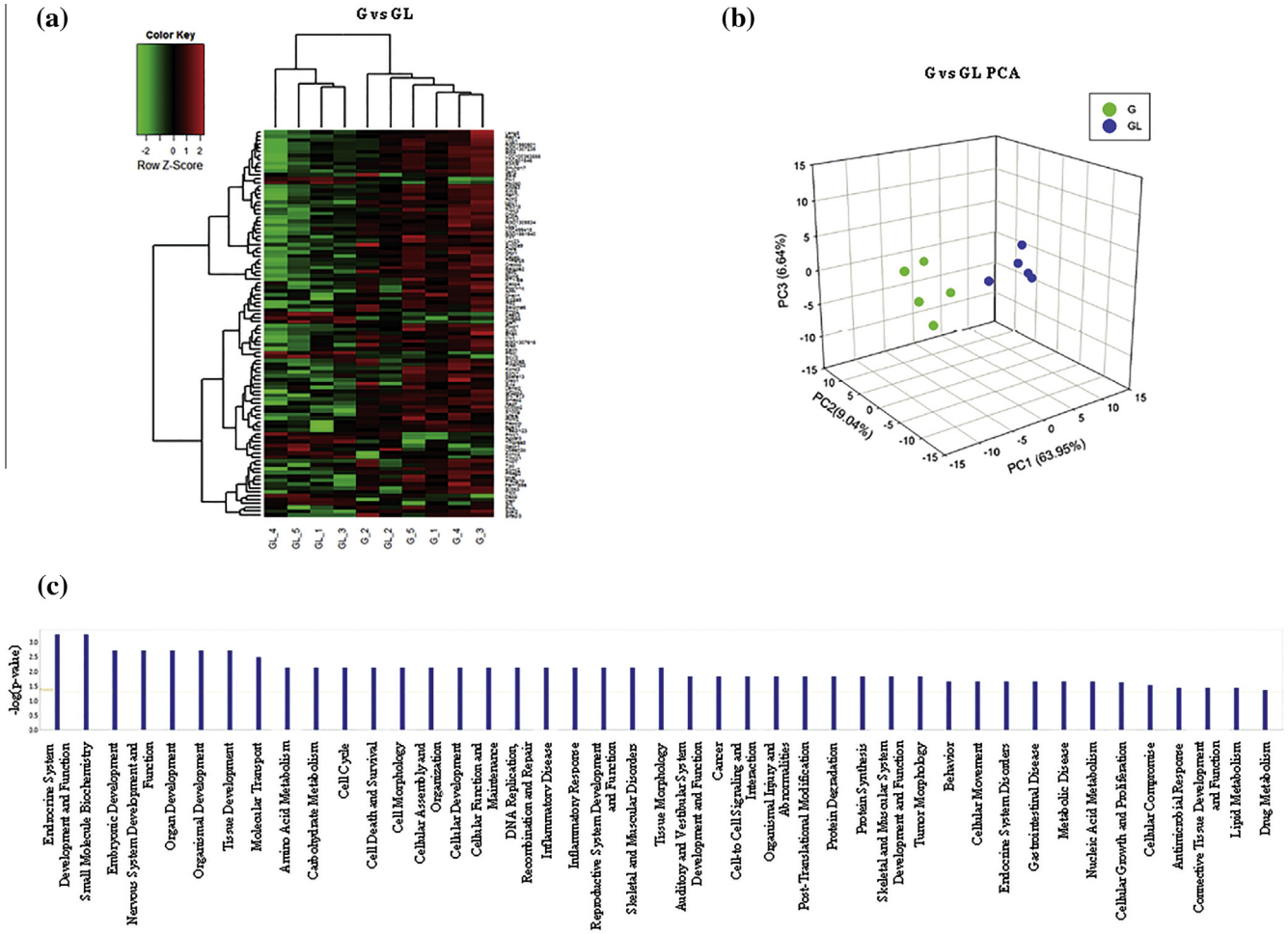

Fig. 5. Genes differentially expressed between the *in utero* (G) and *in utero* and during lactation (GL) high fat exposed groups. (a) Heat maps show scaled individual expression scores for the genes that were significantly differentially expressed. (b) Principal Component analysis of G (green) and GL (blue) samples using genes differentially expressed between these groups. The first principal component (PC1), PC2 and PC3 accounted for 63.95%, 9.04% and 6.64% of the variation, respectively. (c) Bio Function analysis of the significantly differentially expressed genes between G and GL groups. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

tumorigenesis, respectively [34]. The protective effects were associated with upregulation of antioxidant genes, including thioredoxin and heme oxygenase in mammary glands [23]. In the present study, alterations in gene expression were also evaluated in the normal mammary gland, before breast cancer initiation, to investigate if the risk of developing this disease can be predicted from a gene signature.

We attempted to verify changes in the expression of multiple genes identified in the microarray analysis, but not all were differentially expressed when assessed using RT-PCR assay. Among the genes that were confirmed were *Hrh1* and *Repin1* that were upregulated in the mammary glands from offspring of dams fed HF lard diet during pregnancy. Histamine receptor 1 (*Hrh1*) is involved in liberating arachidonic acid from phospholipids and in the development of various aspects of the antigen-specific immune responses [35]. Elevated expression of *Hrh1* has been associated with reduced tumorigenicity of human ovarian cancer cell (OV90) [36]. Replication initiator 1 (*Repin1*) regulates the expression of genes involved in adipogenesis, lipid droplet formation and fusion, and glucose and fatty acid transport in adipocytes [37]. Future studies are needed to determine if changes in the expression of these two genes that both affect lipid metabolism are causally related to reduced breast cancer risk in the offspring exposed to lard-based HF during fetal development.

Stra6 and *Pam* genes were upregulated and *Tlr1* was downregulated in the mammary glands of animals that were exposed to HF diet both *in utero* and during nursing compared with control rats, although the differences did not reach statistical significance. Stimulated by retinoic acid 6 (*Stra6*) induces cellular internalization of retinol and is associated with anti-cancer actions of retinoids [38]. *Stra6* is upregulated by DNA damage and plays a role in mediating p53-induced cell death [38]. These actions of *Stra6* are consistent with lower mammary tumor multiplicity in the offspring of dams consuming HF diet during pregnancy and lactation [24]. Peptidylglycine alpha-amidating monooxygenase (PAM) is the rate-limiting enzyme in the modification of secretory peptides through C-terminal α-amidation [39]. It has a catalytic role by producing amidated product peptides and a non-catalytic role by affecting cytoskeletal organization, vesicular trafficking and secretion and it is sensible to ambient copper levels [40]. Although PAM is expressed in MCF-7 and ZR-75-1 human breast cancer cell lines [41], its role in cancer remains to be established. Toll-like receptor 1 (*Tlr1*) is member of a family of genes that mediates the production of cytokines necessary for the development of effective immunity. In general, higher expression of TLRs promotes inflammation and cancer cell survival [42]. Reduced expression of *Tlr1* in the HF exposed GL offspring could be related to their reduced mammary tumorigenesis.

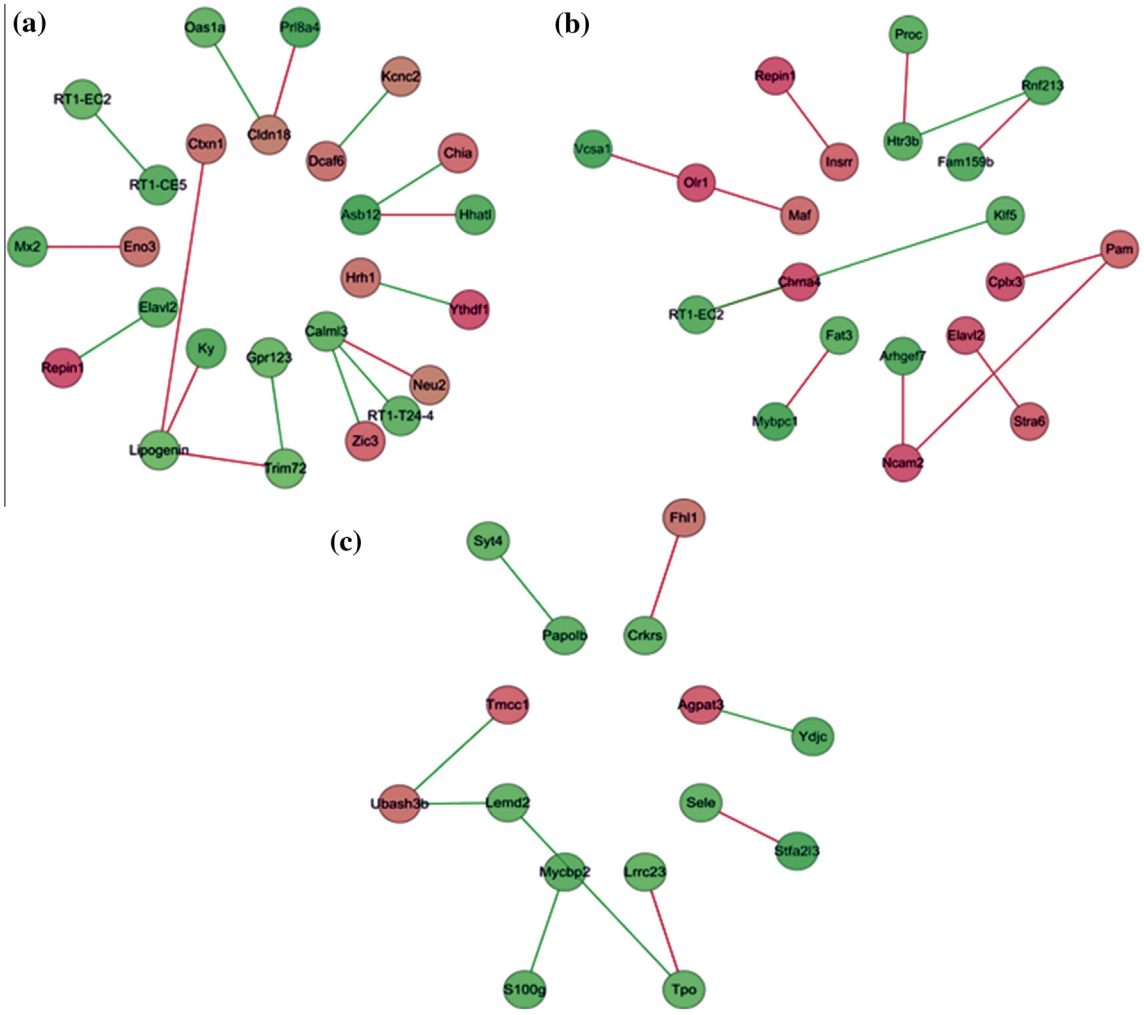

Fig. 6. Differential dependency network analysis (DDN) of significantly differentially expressed genes between control (CO) and *in utero* high fat exposed (G) groups (a); CO and *in utero* and during lactation high fat exposed (GL) groups (b); G and GL groups (c). Lines in the network indicate regulatory relationships between genes. The red lines represent the regulatory relationships that only exist under CO (a and b) and G (c) conditions. The green lines represent the regulatory relationships that only exist under G (a) and GL (b and c) conditions. Green circle means that the gene is downregulated and red circle means that the gene is upregulated in G compared to CO (a), in GL compared to CO (b) and in GL compared to G (c). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

To help us to understand how the observed signaling changes might explain the reduced susceptibility to breast cancer among offspring of dams fed lard-based HF diet during pregnancy, or during pregnancy and lactation, the microarray data were subjected to DDN analysis. This analysis is a tool to detect unique connections in transcriptional networks present for example only in an experimental or control group [27]. In the present study, DDN analysis suggested new connections between *Hrh1* and *Ythdf1* and between *Repin1* and *Elavl2* in the mammary glands of G offspring exposed to a HF diet only during fetal development. Both *Hrh1*and *Ythdf1* were upregulated in mammary gland of rats exposed to HF *in utero* compared with controls. As described above, *Hrh1* is involved in regulating omega-6 fatty acids release and immune functions [35,43]. *Ythdf1* modulates RNA degradation in a N6-methyladenosine (m^6A) dependent manner [44]. Targets of this protein family include genes that are involved in cell metabolism, death and survival [44], and linked to body fat mass and obesity [45].

Repin1 was also upregulated, but its signaling partner, *Elavl2* (also called HuB) was down-regulated in the G offspring. As described above, *Repin1* regulates fatty acid transport and is proposed to serve as a target to inhibit obesity and reverse insulin resistance [36]. The Hu proteins, in turn, are RNA-binding proteins that are involved in RNA metabolism, including in translation, and stability [46]. It's connection to cancer is not known, but another Hu protein Elavl1/HuR allows survival of tumor cells by activating tumor-promoting genes, such as VEGF [47]. It remains to be determined whether the connections between *Hrh1* and *Ythdf1* and between *Repin1* and *Elavl2* are related to reduced mammary tumorigenesis in the offspring of dams fed-lard-based HF diet during pregnancy.

We also observed new connections that occur only in the offspring of dams fed a HF lard diet during pregnancy and lactation, compared with the control group, such as interactions between *Rnf213* and *Htr3b*, as well as among *Klf5* and *Chrna4*. *Rnf213* and *Htr3b* were both downregulated in the GL group. *Rnf213* encodes a protein with ring finger domain and it is involved in chromosomal arrangement with c-Myc and ALK in anaplastic large cell lymphoma, and with SLC26A11 in chronic myeloid leukemia [48–50]. *Htr3b* is a serotonin receptor expressed in the central and peripheral nervous system, but it can also be expressed in other cells [51]. Its relation to cancer process has not been studied, but *Htr3b* is an effective target in preventing chemotherapy-induced nausea and vomiting [52]. *Klf5* was downregulated and *Chrna4* was upregulated in the GL offspring. Kruppel-like factor 5 (*Klf5*) is a transcription factor that regulates cell proliferation by down-regulating tumor suppressor p21 and stimulating cyclin D1

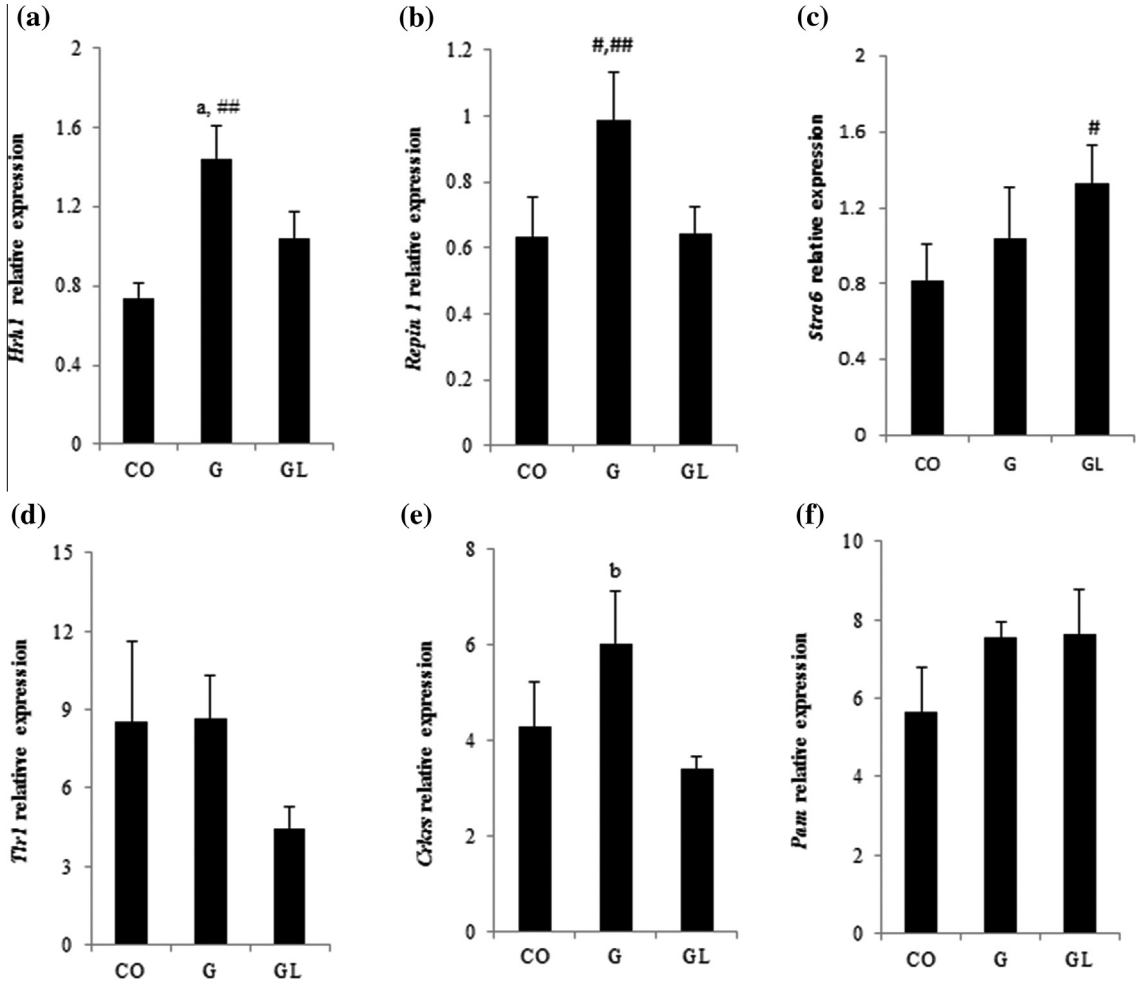

Fig. 7. Relative expression of *Hrh1* (a), *Repin 1* (b), *Stra6* (c), *Tlr1* (d), *Crkrs* (e) *Pam* (f) genes. The data are expressed as mean ± SEM ($n = 5$). Statistically significant differences ($P \leqslant 0.05$) compared to [a]CO and [b]GL; and marginal differences compared with [#]CO ($P = 0.084$; *Repin1* and $P = 0.09$; *Stra6*) and [##]GL ($P = 0.067$; *Hrh1* and $P = 0.078$; *Repin 1*) according to ANOVA followed by the Duncan test or Student *t* test (*Stra6* and *Crkrs*).

[53,54]. Further, elevated expression of *Klf5* is proposed to be a biomarker of poor breast cancer prognosis [55]. *Chrna4* encodes a nicotinic acetylcholine receptor which participates in the predisposition for preneoplastic lesions and emergence of lung carcinomas [56]. On the other hand, silent polymorphism in exon 5 in *Chrna4* is associated to high levels oxidative DNA damage [57]. Additional studies are needed to evaluate the significance of these connections and the possible effects on mammary tumorigenesis.

We also investigated differences in gene signaling connections in the mammary glands of offspring exposed to HF diet only *in utero* or both *in utero* and during lactation. In the first group, *Fhl1* (down-regulated) and *Crkrs* (upregulated) formed a connection. *Fhl1* inhibits estrogen receptor α (ER-α) phosphorylation and causes repression of translation and transcription of ER-responsive genes [58]. *Crkrs* regulates the expression of DNA damage response genes, including BRCA1 [59]. The GL group presented specific gene networking among *Agpat3/Ydjc*, *Mycbp2/S100g* and *Tmcc1/Ubash3b/Lemd2/Tpo*. Among their functions are regulation on biosynthesis of phospholipids (*Agpat3*), carbohydrate metabolism (*Ydjc*), mTOR signaling (*Mycbp3*) and organization of endoplasmic reticulum membrane (*Tmcc1*). At present time we can only conclude that these differences in gene expression are seen, but it is not known whether they are causally involved in explaining differences in mammary tumorigenesis among offspring exposed to HF lard diet during fetal period or *in utero* and postnatally during nursing.

We conclude that an exposure to a lard-based HF diet during early life permanently changes the fatty acid profile and transcriptional network in the mammary gland, but more studies are needed to determine a causality between the lipidomic and gene expression changes and the observed reduced susceptibility to breast cancer in adulthood [24]. Timing of exposure to HF diet differentially impacted all these outcomes, reinforcing the concept of different developmental susceptibility windows.

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Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cbi.2015.06.035.

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