ORIGINAL RESEARCH ARTICLE

DOI: 10.26479/2018.0405.01

POTENTIAL THERAPEUTIC TARGETS OF NAFLD IN OFFSPRINGS WITH RESPECT TO MATERNAL WESTERN TYPE DIET

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ABSTRACT: Maternal diet strongly influences development of fetal health status. High fat diets can result in metabolic dysfunctions and even cause hepatic steatosis in the offspring. Nonalcoholic fatty liver disease (NAFLD) can result in liver cirrhosis and cancer. Absence of any approved and effective treatment strategy of NAFLD and incompletely understood pathogenesis is posing great challenges for its management. One major factor for this disease progression is maternal obesity and according to World Health Organization (WHO) worldwide 1.6 billion adults are overweight (BMI 25 kg/m²) and 400 million are obese (BMI 30kg/m²). These alarming data demand more specific therapeutic targets for NAFLD even more urgently than ever before. The mainstay of management continues to be dietary and lifestyle changes tailored to the individual patient, but these modifications have always been difficult to maintain and this approach alone could not cope up with the disease epidemic. However, understanding of pathogenesis and progression of NAFLD are evolving and many novel therapeutic targets are being evaluated. Moreover, the link of maternal diet and offspring health is even more poorly delineated. In the present study previously published datasets of this condition from GEO database have been reanalyzed by applying a series of R programs based novel bioinformatics approaches. This powerful systematic analysis has led to the identification of certain potential NAFLD therapeutic targets, which were further validated by annotation through pathway analysis tools.

KEYWORDS: NAFLD, therapeutic targets, FGNet, R/Bioconductor package, FEA, network-based analysis

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Peer review under responsibility of Life Science Informatics Publications
2018 Sept – Oct RJLBPCS 4(5) Page No.1
1. INTRODUCTION

Maternal physiological health in pregnancy is known to be associated with unborn baby by involvement of mechanisms and pathways linking maternal-fetal relationships [1]. Maternal nutritional status has important influence on development and modification of fetus. It has also been shown to bear consequences on the health of offspring, changing their responses to environmental challenges and thus their predisposition to the disease [2]. David Barker [3] first popularized the concept of fetal origins of adult disease (FOAD) or “Barker hypothesis”. Accordingly, events during early development such as low birth weight and exposure to stress, both nutritional and non-nutritional, during critical periods of development might ultimately result in a diseased state. The Metabolic syndrome, vascular dysfunction, and/or vascular atherogenic lesions have been linked to maternal diet and fetal nutrition rich in saturated fat [4-7]. Gestational energy-rich diets can programme hepatic steatosis or even non-alcoholic steatohepatitis in the offspring in rodent models validating the close interrelationship between maternal diet and fetal liver physiology [8-14]. Likewise, non-alcoholic fatty liver disease (NAFLD) accounting for 75% of all chronic liver diseases is one of the most important causes of liver disease worldwide. The global prevalence of NAFLD is currently estimated to be 24% worldwide, but the highest rates being found in South America (31%), Middle East (32%), followed by Asia (27%), USA (24%) and Europe (23%), while least common in Africa (14%) [15]. In India, NAFLD prevalence has been documented to range between 9-32% [16]. Being hepatic manifestation of obesity and metabolic syndrome, NAFLD encompasses a spectrum of conditions ranging from steatosis, inflammation, fibrosis and an increased risk of progression to liver cirrhosis followed by development of hepatocellular carcinoma [17]. Although, so far, no safe and effective drug therapy currently seems to be available, yet some treatment regimens do exist. Peroxisome proliferator-activated receptors (PPARs) agonist has been considered for treatment of all the aspects of NAFLD [18]. Intestinal hypoxia-inducible factor (HIF)-2α has been shown to play an essential role in controlling fatty liver disease and lends a potential therapeutic approach to treat NAFLD [19]. Micro particles (MPs), small membrane-bound particles with a specific antigenic composition released by hepatocytes and immune cells during lipotoxicity can be attractive new targets for NAFLD treatment. Finally, incretins such as glucagon-like peptid-1 (GLP-1) and glucose-dependent insulin tropic polypeptide (GIP) also hold great promise to become part of the potential targets to treat NAFLD [20]. Thus, due to limited current interventions, there is a consistent need to identify such novel targets for development of new and efficient therapies for NAFLD. Owing to such a conundrum, we have exploited a systems biology approach to find out the potential therapeutic targets of NAFLD and delineate the molecular mechanism with respect to maternal western-type diet intake. Since the introduction of microarrays public databases viz.GEO and Array express, a large body of data has been accumulated of millions of samples and is also readily available. The present study focuses on the analysis of two previously
published such datasets from GEO database. In these datasets we have analyzed 45,282 genes differentially expressed in mouse offspring samples exposed prenatally to a western-style diet (rich in energy, fat, cholesterol) or a low-fat diet and then a post-weaning western diet or a low-fat diet [21]. The data submitted at GEO did reveal some of the interesting novelties for maternal diet influences on offspring health. The same dataset on re-analysis by latest robust bioinformatics approach of R programming has led to even more intriguing novel target discoveries. The Functional enrichment analysis (FEA) performed by R based program packages of these targets has generated functional network. The networks were further correlated with molecular pathways to derive novel biomarkers for NAFLD. It is believed that, owing to conservation of molecular pathways across species and further advances in computational exploration of intricate circuitries of those in mouse model will definitely facilitate a better understanding of their regulation in humans as well.

2. MATERIALS AND METHODS

Data Sets

All the microarray data in this work was downloaded from NCBI (National Center for Biotechnology Information) Gene Expression Omnibus (GEO) database (www.ncbi.nlm.nih.gov/geo). The dataset are from the experiment where female mice were fed either a western (W) or low-fat diet (L) before and during gestation and lactation. At weaning male off-springs were fed either western or low fat diet and different groups, WW, WL, LW, LL were assigned as mentioned below.

Group 1: Maternal western diet effect on post-weaning low-fat diet fed offspring liver

(Dataset: GDS5342, Series: GSE44901, Platform: GPL6887); Samples: GSM1093606, GSM1093607, GSM1093610, GSM1093611, GSM1093620, grouped as Experimental (WL); GSM1093603, GSM1093613, GSM1093614, GSM1093616, GSM1093617, GSM1093618 grouped as Control (LL).

Group 2: Maternal western diet effect on post-weaning western diet fed offspring liver

(Dataset: GDS5293, Series: GSE44901, Platform: GPL6887); Samples: GSM1093600, GSM1093602, GSM1093604, GSM1093609, GSM1093615, GSM1093619 grouped as Experimental (WW); GSM1093599, GSM1093601, GSM1093605, GSM1093608, GSM1093612 grouped as Control (LW). All the data series are already normalized [21].

For the selected datasets, GDS5342 and GDS5293, 11 samples in each dataset were present and 45282 genes were analyzed. The target tissue in each of these studies was offspring liver. After differentiating both the datasets into groups i.e. GROUP 1 and GROUP 2, their samples were divided into control and experimental categories on the basis of their diet pattern as shown in Table 1 (sample subsets of Datasets: GDS5342 (blue boxes) and GDS5293 (green boxes) were grouped into control (green) and experimental (red)). From the 4 groups described in table 1, (LL,LW, WL, WW), GROUP 1 has 5 Experimental (WL: parent mice fed energy-rich-western diet and male offspring fed low-fat-control diet) and 6 Control samples (LL: parent mice as well as male offspring fed low-fat-
control diet) and GROUP 2 has 6 Experimental (WW: parent mice as well as male offspring fed energy-rich-western diet) and 5 Control samples (LW: parent mice fed low-fat-control diet and male offspring fed energy-rich-western diet). This division of samples into various groups based on their diet will be the key factor for identifying the role of diet in any variations of phenotypic development of the offspring.

Table 1: Grouping Of Sample Subsets of Dataset: GDS5342 and GDS5293

<table>
<thead>
<tr>
<th>Samples</th>
<th>Diet Protocol</th>
<th>Title</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSM1093606</td>
<td>prenatal western diet, post-weaning low-fat diet</td>
<td>WL1</td>
</tr>
<tr>
<td>GSM1093607</td>
<td></td>
<td>WL2</td>
</tr>
<tr>
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<td>GSM1093611</td>
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<td>WL4</td>
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<td>GSM1093620</td>
<td></td>
<td>WL5</td>
</tr>
<tr>
<td>GSM1093603</td>
<td>prenatal low-fat diet, post-weaning low-fat diet</td>
<td>LL1</td>
</tr>
<tr>
<td>GSM1093613</td>
<td></td>
<td>LL2</td>
</tr>
<tr>
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<tr>
<td>GSM1093616</td>
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<tr>
<td>GSM1093618</td>
<td></td>
<td>LL6</td>
</tr>
<tr>
<td>GSM1093600</td>
<td>prenatal western diet, post-weaning western diet</td>
<td>WW1</td>
</tr>
<tr>
<td>GSM1093602</td>
<td></td>
<td>WW2</td>
</tr>
<tr>
<td>GSM1093604</td>
<td></td>
<td>WW3</td>
</tr>
<tr>
<td>GSM1093609</td>
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<td>GSM1093619</td>
<td></td>
<td>WW6</td>
</tr>
<tr>
<td>GSM1093599</td>
<td>prenatal low-fat diet, post-weaning western diet</td>
<td>LW1</td>
</tr>
<tr>
<td>GSM1093601</td>
<td></td>
<td>LW2</td>
</tr>
<tr>
<td>GSM1093605</td>
<td></td>
<td>LW3</td>
</tr>
<tr>
<td>GSM1093608</td>
<td></td>
<td>LW4</td>
</tr>
<tr>
<td>GSM1093612</td>
<td></td>
<td>LW5</td>
</tr>
</tbody>
</table>
Data Analysis:

R and R studio: R is available as Free Software under the terms of Free Software Foundation’s GNU General Public License. R studio version 1.1.447 [22] was used in the present study for the data analysis. R packages used for the analysis are available at following two repositories;

Comprehensive R Archive Network (CRAN) is global repository of open-source packages extending capabilities of R. The CRAN packages used in the present study are ggplot2 and readxl function. We have used the latest readxl package version (1.1.0) [23]. The ggplot2 version (2.2.1) [24], was used for data analysis.

Bioconductor repository provides tools for analysis and comprehension of high-throughput genomic data. It has 1560 software packages. The current release of Bioconductor is version 3.7 [25]. The packages of Bioconductor used are: Biobase, GEOquery, Limma, FGNet. Biobase contains standardized data structures to represent genomic data. We have used biobase version 2.38.0 [26]. The NCBI Gene Expression Omnibus (GEO) is a public repository of microarray data. GEOquery (version used 2.46.15) [27] is the bridge between GEO and Bioconductor. LIMMA (version used 3.34.9) [28] is a library for analysis of gene expression microarray data, especially the use of linear models for analyzing designed experiments and assessment of differential expression. Functional Gene Networks (FGNet) (version used 3.12.0) [29] is an R/Bioconductor package that generates gene networks derived from the results of functional enrichment analysis (FEA) and annotation clustering. In addition to building the functional network, FGNet also provides a distance heat map and a bipartite network of functionally overlapping genes. The application includes an interface to directly perform FEA queries using different external tools: DAVID, GeneTerm Linker, TopGO or GAGE; and a graphical interface to facilitate the use. We have used the TopGO version 2.30.1 [30] in the FGNet for the data analysis.
Procedural Algorithm:
The procedure used for data analysis, via R studio on microarray datasets is explained in the following steps:

1. **Selection of up-regulated and down-regulated genes:**
   After importing series matrix .xlsx file into R studio, mean values and expression ratio values of control and experimental samples were calculated for both the Datasets (GDS5342 and GDS5293). The Data consists of the mean expression values for 45282 transcripts from 11 liver samples. The 2 fold threshold was applied to select list of up and down-regulated genes for functional enrichment analysis [31,32].

2. **Hypothesis testing:**
   The goal of statistical analysis of microarray data is to test hypothesis via limma package of bioconductor that some genes are differentially expressed. P-values were calculated from the data obtained after analyzing. The analysis was on the basis of P-Values set at p<0.05 [33].

3. **Plots generation:**
   To visualize the microarray data, Scatter plots were obtained for analyzing maternal western diet effect on post-weaning low-fat diet fed offspring and maternal western diet effect on post-weaning western diet fed offspring.

4. **Functional enrichment analysis (FEA):**
   It was performed with differentially expressed genes from Step 1. The results obtained by applying FGNet package on HTML file of TopGo [31] via FGNet GUI (Graphical User Interface). The functional network was built based on analysis of all the clusters provided by the FEA tool. The clusters provided by FEA tool were used to generate a genes adjacency matrix with the number of common clusters. The network produced was provided as an igraph object for further analysis [29, 34-36].

5. **Identification of potential therapeutic targets:**
   Results displayed gene’s adjacency matrices, functional network, distance heat map and intersection networks. All of these were used for identification of common modules and hubs, with respect to maternal western diet effect on post-weaning low-fat diet fed offspring and maternal western diet effect on post-weaning western diet fed offspring.

3. RESULTS AND DISCUSSION
Several studies have explained the molecular basis for fat accumulation, liver injury and fibrosis, yet the gaps exist in understanding of NAFLD pathogenesis and therapeutics [37, 38]. Since currently there are no approved therapies for this ailment making it all the more imperative to find suitable targets than ever before [39]. In the present study, we have re-analyzed two of the previously published datasets from GEO database. We have applied a series of novel bioinformatics approaches by incorporating R programs based evaluations. Systematic analysis of transcriptomic data powered by more sophisticated computational programme viz. functional enrichment analysis has lead to the identification of certain potential therapeutic targets, which have been further validated by annotation of each of these by extensive functional pathway analysis.
Microarray Datasets Analysis

Selection of up-regulated and down-regulated genes:
The comparison of control and experimental subjects with respect to maternal western diet effect on post-weaning low-fat diet fed offspring / western diet fed offspring was performed. The analysis of 45,282 genes in each group through limma package of Bioconductor, confirmed that both groups expressed genes differentially at empirical p-values <0.05. Two fold threshold criteria was applied on the resultant expression ratio for the selection of up or down regulated genes for further analysis. After applying the two fold cut-off, a total of 94 genes were selected showing differential expression with respect to maternal western diet effect on post-weaning low-fat diet fed offspring and a total 293 genes were selected that showed differential expression with respect to maternal western diet effect on post-weaning western diet fed offspring as described in Table 2.

Table 2: Up-Regulated and Down-Regulated Genes for Group 1 (WL, LL) and Group 2 (WW, LW)

<table>
<thead>
<tr>
<th></th>
<th>DataSet</th>
<th>Total genes</th>
<th>Selected genes (Two fold criteria)</th>
<th>Two fold up-regulated genes</th>
<th>Two fold down-regulated genes</th>
</tr>
</thead>
<tbody>
<tr>
<td>GROUP 1</td>
<td>GDS5342</td>
<td>45282</td>
<td>94</td>
<td>44</td>
<td>50</td>
</tr>
<tr>
<td>GROUP 2</td>
<td>GDS5293</td>
<td>45282</td>
<td>293</td>
<td>39</td>
<td>254</td>
</tr>
</tbody>
</table>

Scatter Plots:

(a)

(b)

Figure 1: Scatter plots for group1 (a) and group 2 (b). The plot of intensity is between the mean values of experimental samples on x axis (WL (group1) and WW (group 2)) versus mean values of control samples on y axis (LL (group1) and LW (group 2)).

In figure 1 (a) and (b) a total of 45,282 genes for each group were analysed by scatter plot. The scatter plot displayed all the transcripts that were most dramatically and differentially regulated in these experiments. Most data points were falling on a 45 degree line showing substantial correlation.
between expression values of conditions being compared. The up or downregulated transcripts were positioned off the line. Another feature observed in these plots was that majority of the genes were expressed at basal values and only few were expressed at high level. All the genes which were up or down regulated by greater than two fold values were further analysed for inferential decisions by functional enrichment analysis.

**Functional Enrichment Analysis (FEA)**

Analysis of gene expression data generated from transcriptome or metabolome studies with over thousands of measurements for each sample have led to omics-based research which includes in majority molecular networks. Analysis of interaction networks is very important for detecting the disease-specific biomarkers that show the differential expression during the disease development [40, 41]. The gene expression profiling of 118 genes linked by 866 potential functional associations for breast cancer when combined with functional genomics and proteomic data has helped to identify more reproducible sub-network markers than individual markers [42]. In the present analysis functional enrichment analysis (FEA) and annotation clustering was performed via Functional Gene Networks (FGNet). FGNet built the functional networks based on the groups obtained from clustering genes of common functional terms in GO (Gene Ontology). The clusters, genes and associated terms were validated by an enrichment p-value. FEA analysis also provided functional networks, intersection networks and heat maps.

**Functional Networks**

The analysis of functional association amongst finally selected genes showing differential expression (two fold cut off for up-regulated and down-regulated) in response to maternal western diet effect on post-weaning low-fat diet fed offspring (94 genes) and maternal western diet effect on post-weaning western diet fed offspring (293 genes), was performed via functional network (figure 2 & 3). Edges linked the genes that were in the same clusters. FGNet used the force-directed Fruchterman–Reingold algorithm to place the nodes within the same clusters. The Fruchterman–Reingold algorithm considers a force between any two nodes and worked on the basis that attractive force is analogous to the spring force and the repulsive force is analogous to the electrical force [43]. Genes included in more than one cluster were colored white. In total, 38 clusters were generated arising from the clustering of 76 genes in reference to maternal western diet effect on post-weaning low-fat diet fed offspring (figure 2) and 95 clusters were generated arising from the clustering of 203 genes in reference to western diet effect on post-weaning western diet fed offspring (figure 3). For the ontology analysis, a functional network illustrates the gene-gene interaction and the functional clusterization [34]. Recently, potential biomarkers were derived from functional connectivity data of Alzheimer’s disease [44] and a second novel brain network was proposed via functional network organization of human brain [45].
Figure 2: Functional network of 76 genes in reference to maternal western diet effect on post-weaning low-fat diet fed offspring (WL, LL). Genes with more than one cluster are coloured white while each cluster is coloured differently.

Figure 3: Functional network of 203 genes in reference to maternal western diet effect on post-weaning western diet fed offspring (WW, LW). Genes with more than one cluster are coloured white while each cluster is coloured differently.

**Intersection Networks and Heat Maps**

High-throughput data can be analyzed by gene categorization and pathway analysis, but it fails to give the completely satisfactory results for identification of genes that are at the interface among pathways with a very large number of functional links. This limitation can be overcome by generating intersection networks or bipartite networks [46-48]. The FGNet used the intersection network graphs to highlight hub genes emerging from the different clusters in the case of maternal western diet effect on post-weaning low-fat diet fed offspring (figures 4&5) WL, LL as well as for maternal western diet effect on post-weaning western diet fed offspring (figures 6&7) WW, LW. In all of these figures clusters are represented by squares and genes are represented by circles.
provides evidence that only the significant (hub genes) are functionally related to various clusters. Genes with more than one cluster are shown as white nodes while genes included in single cluster are colored. This intersection network also facilitated the identification of multifunctional genes. In order to depict the distances between the significant clusters representing many samples, many genes, or both Heat maps were generated. From the dendrogram above the distance matrix in the heatmap we could also decipher the distance between any two clusters by looking at the height at which the two groups split into two. Heatmaps have been widely used in genomics and other high throughput fields [49,50](figures 8 & 9). The distance matrix was calculated based on the pair wise binary distance in the adjacency matrix of common clusters. The distance matrix represented the similarity between gene groups, representing the closest clusters. The analysis of finally selected genes that show differential expression with respect to maternal western diet effect on post-weaning low-fat diet fed offspring is shown in figure 8, in case of refereeing the maternal western diet effect on post-weaning western diet fed offspring in figure 9.

Figure 4: Intersection network 1 in reference to maternal western diet effect on post-weaning low-fat diet fed offspring (WL, LL).

Figure 5: Intersection network 2 in reference to maternal western diet effect on post-weaning low-fat diet fed offspring (WL, LL)
Figure 6: Intersection network 1 in reference to maternal western diet effect on post-weaning western diet fed offspring (WW, LW).

Figure 7: Intersection network 2 in reference to maternal western diet effect on post-weaning western diet fed offspring (WW, LW).

Figure 8: Heatmap visualization for distance analyses of all 38 clusters consisting of genes that show differential expression with respect to maternal western diet effect on post-weaning low-fat diet fed offspring liver (WL, LL). It reveals the proximity and similarity between the clusters. The values on the x and y axis are the cluster numbers. Distance is calculated based on values (pairwise binary distance in the adjacency matrix of common clusters), represented on the side bar. The blue shade represents the closest gene clusters (proximity between clusters increases as the colour turns dark blue) while the red shade illustrates the distance between the gene clusters (proximity between clusters decrease as the colour turns dark brown).
Figure 9: Heatmap visualization for distance analyses of all 95 clusters consisting of genes that show differential expression with respect to maternal western diet effect on post-weaning western diet fed offspring liver (WW, LW). It reveals the proximity and similarity between the clusters. The values on the x and y axis are the cluster numbers. Distance is calculated based on values (pairwise binary distance in the adjacency matrix of common clusters), represented on the side bar. The blue shade represents the closest gene clusters (proximity between clusters increases as the colour turns dark blue) while the red shade illustrates the distance between the gene clusters (proximity between clusters decrease as the colour turns dark brown).

Analyzing the Network

It is difficult to obtain information from a network with hundreds or thousands of nodes and links, unless the information is presented in a scale-specific context. However, cartographic representation helps to extract the information from complex networks. This method helps to find modules (clusters) and classify important nodes (or genes) according to their pattern of within- and between-module connections. Node betweenness approach, first proposed by Freeman [51], represents total number of edges between the nodes. By analyzing the connectivity patterns of the nodes, hubs (genes highly connected in the network) can be identified [52, 53]. Most of the networks are scale-free network and the majority of the nodes have one or two links but a few nodes can have a large number of links. Interestingly, researchers have found that these type of networks show robustness because according to power-law distribution where majority of nodes have only few links and these nodes with small connectivity will be selected with much higher probability [54]. Since these highly connected hub or nodes are central to the network’s architecture, reports suggested role of hub genes in humans and mice [54-56]. The loss of a ‘genetic hub’ has been shown to greatly influence multiple functionally unrelated genes and in many otherwise unlinked pathways that may affect susceptibility to unrelated genetic diseases [57]. The network analysis graph represents the values of normalized nodes degree and node. Betweenness for each node in the global network (common clusters) and
within each cluster (intra-cluster) with respect to percentage of total nodes in a network in reference to maternal western diet effect on post-weaning low-fat diet fed offspring (figure 10) and maternal western diet effect on post-weaning western diet fed offspring (figure 11). The normalized node degree graph generated by FGNet showed total 76 nodes for a functional network after analyzing the finally selected genes that show differential expression in reference to maternal western diet effect on post-weaning low-fat diet fed offspring. Amongst these 76 nodes, Cluster 35 has a value of 45, which is the highest recorded value as compared to other nodes in figure 10. FGNet generated total 203 for a functional network after analyzing the finally selected genes that show differential expression in reference to maternal western diet effect on post-weaning western diet fed offspring. Amongst 203 nodes, Cluster 102 has a value of 70, which is the highest recorded value as compared to other nodes in figure 11. The node betweenness graph measures the total number of edges between each node and global network as well as other clusters (figures 10&11). Inter-modular hubs represented the nodes with betweenness within the top 75% in the global network, while Intra-modular hubs represented the nodes with betweenness within the top 75% in each cluster sub-network.

Figure 10: Network analysis of FEA results in reference to maternal western diet effect on post-weaning low-fat diet fed offspring liver (WL, LL). Percentage of total nodes is represented on y-axis while normalized node degree and node betweenness are shown on x-axis. The graph contains the values that are normalized nodes degree and node betweenness for each node in the global network (common clusters) and within each cluster (intra-cluster) with respect to percentage of total nodes in a network.
Figure 11: Network analysis of FEA results in reference to maternal western diet effect on post-weaning western diet fed offspring liver (WW, LW). Percentage of total nodes is represented on y-axis while normalized node degree and node betweenness are shown on x-axis. The graph contains the values that are normalized nodes degree and node betweenness for each node in the global network (common clusters) and within each cluster (intra-cluster) with respect to percentage of total nodes in a network.

Outcomes of network analysis in reference to maternal western diet effect on post-weaning low-fat diet fed offspring liver (WL, LL)

For the potential inter-modular hubs and top betweenness in whole network most common hubs identified were for Cyp2b23, Cxcl1, Osgin1, Ly6d, 8430408G22Rik, Csad, Serpina6, Cyp3a11, Dbp, Foxq1, Clec2d, F11, Mup2, Mup4, Mup5, Rgs16, S100a11, Slc6a13, Wfdc2 genes. Potential intra-modular hubs for the maternal western diet effect on post-weaning low-fat diet fed offspring liver are illustrated in table 3 with the clusters, hub genes and total number of hub genes in each cluster. Among these Mup2, Mup5, Mup4, Clec2d genes were identified as the most common ones. Out of these total 38 clusters, cluster no.29 is the most important cluster as it has the largest number of hub genes, 10 hub genes, (table 3) and its various Functional gene ontology characterization are biological processes, molecular functions and cellular components.

Table 3: Potential intra-modular hubs in reference to maternal western diet effect on post-weaning low-fat diet fed offspring liver (WL, LL) (Top Betweenness within each cluster).

Cluster number 29 has the highest number of genes (Mup5, Cxcl1, Mup4, Csad, S100a11, S100a13, Mup2, Cyp2b23, Cyp3a11, Hsd17b11)
Outcomes of network analysis in reference to maternal western diet effect on post-weaning western diet fed offspring liver (WW, LW)

In this group, the potential inter-modal hubs and top betweenness in whole network most common hubs were identified for Cfp, Csad, Ly6d, Tmem176a, Nudt18, Lgals3bp, Orm1, Orm2, Ly6a, Ear2, Aqp8, Col4a1, Aldh1a7, Cd52, Tagln2, Tmsb10, Wfde2, Mup5, Nptn, Rnase4, Cyth4, Ear3, Isoc2a, Tmem50a, Uap1ll1, Cib3, Cox6c, Gsta4, Mrpl54, Serpina7, Sr xn1, Acot1, Ccl9, Cyp4a31, Paqr7, Spon2, Cyp4a14, Gls2, Laptm5, Ms4a6d, Pnpla7, Retsat, Sl100a11, Tmem86a while most common genes identified were Ms4a6d, Tmem86a, Cib3, Ear3, Mup5, Ear2, Cd52, Orm1, Orm2, Cfp, Aldh1a7, Lgals3bp, Serpina7, Col4a1, Thr sp. Potential intra-modal hubs for the maternal western diet effect on post-weaning western diet fed offspring liver are illustrated in table 4. Out of these total 95 clusters, cluster no. 114 is the most important cluster as it has the largest number of hub genes, 10 hub genes, (table 4) and its various functional gene ontology characterization are biological processes and cellular components only.

**Table 4: Potential intra-modal hubs in reference to maternal western diet effect on post-weaning western diet fed offspring liver (WW, LW) (Top Betweenness Within Each Cluster).**

Cluster number 114 has the highest number of genes (Ms4a6d, Tmem86a, Ear2, Cd52, Lgals3bp, Tmem176a, Ly6a, Palmd, Cfp, Nptn)

<table>
<thead>
<tr>
<th>Cluster (Numbers)</th>
<th>Number of Genes per cluster</th>
</tr>
</thead>
<tbody>
<tr>
<td>15, 25, 32, 33, 44, 56, 72</td>
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</tr>
<tr>
<td>8, 9, 10, 11, 12, 17, 18, 19, 22, 24, 26, 27, 28, 30, 34, 37, 38, 41, 52, 64, 65, 70, 76, 83, 87, 100, 115, 139, 148, 158, 160</td>
<td>3</td>
</tr>
<tr>
<td>3, 5, 6, 7, 14, 23, 29, 58, 59, 61, 63, 66, 74, 85, 94, 102, 113, 130, 137</td>
<td>4</td>
</tr>
<tr>
<td>4, 31, 77, 166, 168</td>
<td>5</td>
</tr>
<tr>
<td>69, 162, 178</td>
<td>6</td>
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<td>81</td>
<td>7</td>
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<td>126, 128</td>
<td>8</td>
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<td>167</td>
<td>9</td>
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<td>114</td>
<td>10</td>
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Common Hubs and their Expression Level in Control and Experimental Conditions

By comparing the hubs genes, derived from functional analysis of finally selected genes that show differential expression with respect to maternal western diet effect on post-weaning low-fat diet fed
offspring liver (WL, LL) as well as maternal western diet effect on post-weaning western diet fed offspring liver (WW, LW)(tables 3&4), four genes found to be common in both the groups were Csad, Ly6d, Wfdc2 and Mup5. While analyzing the maternal western diet effect on post-weaning low-fat diet fed offspring , Csad, Ly6d and Wfdc2 expressions were found to be low under the WL condition (parental western diet, post-weaning low-fat diet) while high in LL condition (parental low-fat diet, post-weaning low-fat diet). But Mup5 expression was high during the WL condition (parental western diet, post-weaning low-fat diet) and lower during the LL condition (parental low-fat diet, post-weaning low-fat diet). While analyzing the maternal western diet effect on post-weaning western diet fed offspring liver, Csad, Ly6d and Wfdc2 expressions were found to be high in WW condition (parental western diet, post-weaning western diet) while low during LW condition (parental low-fat diet, post-weaning western diet) but Mup5 showed the opposite result by representing the low expression in WW condition (parental western diet, post-weaning western diet) and high in LW condition (parental low-fat diet, post-weaning western diet).

Figure 12: Consolidated information of Differential Gene Expression.

Functional Annotations and Pathways involved for Common Hubs
Based on the cumulative data of the present study four gene targets were zeroed in. These could be suggested as the potential target genes which might be getting skewed in their expression in the event of NAFLD [figure 12]. These target genes are Csad, Ly6d, Wfdc and Mup5. It is noteworthy to state that all these genes have never been identified as targets in NAFLD thus represent the novel targets. Csad (cysteine sulfinic acid decarboxylase) is involved in various biological processes. It catalyzes the decarboxylation process of cysteinesulfinate to hypotaurine that helps in the biosynthesis of taurine [58]. It plays role of a rate limiting enzyme in taurine biosynthesis. In our studies, down regulation in prenatal western diet in comparison to prenatal low fat diet is strongly suggestive of potential decrease in taurine levels which might contribute to NAFLD development in offspring, as taurine has been shown to play a beneficial role in regulating NAFLD. This further finds strong support in the similar reduction in expression data seen in post-weaning western diet.
fed offsprings, where as in WW group with prenatal western diet and post-weaning western diet showed over expression of this gene. The over expression of Csad stimulated by hepatocarcinogenesis has also been shown to result in autoantibody production in rats [59]. These fluctuating levels of Csad in the presence of western diet are strongly indicative of imbalance in taurine metabolism due to western diet. Ly6d is one of the genes that has already been reported to show significant association with the degree of hepatosteatosis. In the present study also we have observed this gene being influenced for regulation by prenatal and post weaning western diet. Additionally, cells without Ly6d (lymphocyte antigen 6 complex, locus D) has been shown to exhibit full lymphoid potential and early thymic seeding activity, while on the other hand, those with Ly6d become B-cell progenitor cells [60, 61]. Wfnc2 (WAP four-disulfide core domain 2), the third target of this study, functions as a protease inhibitor in many family members and has also been shown to be involved in inflammatory responses and host defense. Wfnc2 activity has been shown to be increased in chronically inflamed lungs with cystic fibrosis [62]. This gene is expressed in pulmonary epithelial cells, and it’s over expression has also been recorded in ovarian cancers. The expression profile of this gene was also being strongly influenced by prenatal and post weaning diets. As per the previous literature reports plausibly this gene might be involved in inflammation process here also. Finally Major urinary proteins (Mups) are important for rodent scent communication and sexual behavior. The encoded protein also has notable chances for involvement in sperm maturation [58]. Mup5 (Major urinary protein 5) has been involved in both chemical and metabolic signals that coordinate sexual behavior as well as nutrient metabolism [63].

4. CONCLUSION
In the absence of any existing approved medications for the treatment of NAFLD, lifestyle changes are considered as the most effective therapeutic strategy. Since the optimal diet to treat NAFLD is not known and obesity is increasing continuously, novel pharmacotherapy is urgently needed. Numerous therapeutic targets have been added to the pipeline for increasing the promise of successful treatment of NAFLD. Based on the data analysis of differentially expressed genes with respect to maternal diet-type intake, we have shown that Csad, Ly6d, Wfnc2 and Mup5 have never been associated directly with NAFLD. The variation in the expression level of these genes in response to maternal diet quality poses these as potential therapeutic targets.

ACKNOWLEDGEMENT
Computational facilities Support provided by Centre for Systems Biology and Bioinformatics (UIEAST), Panjab University, Chandigarh is duly acknowledged.

CONFLICT OF INTEREST
We have no conflicts of interest to disclose.
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Peer review under responsibility of Life Science Informatics Publications
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