

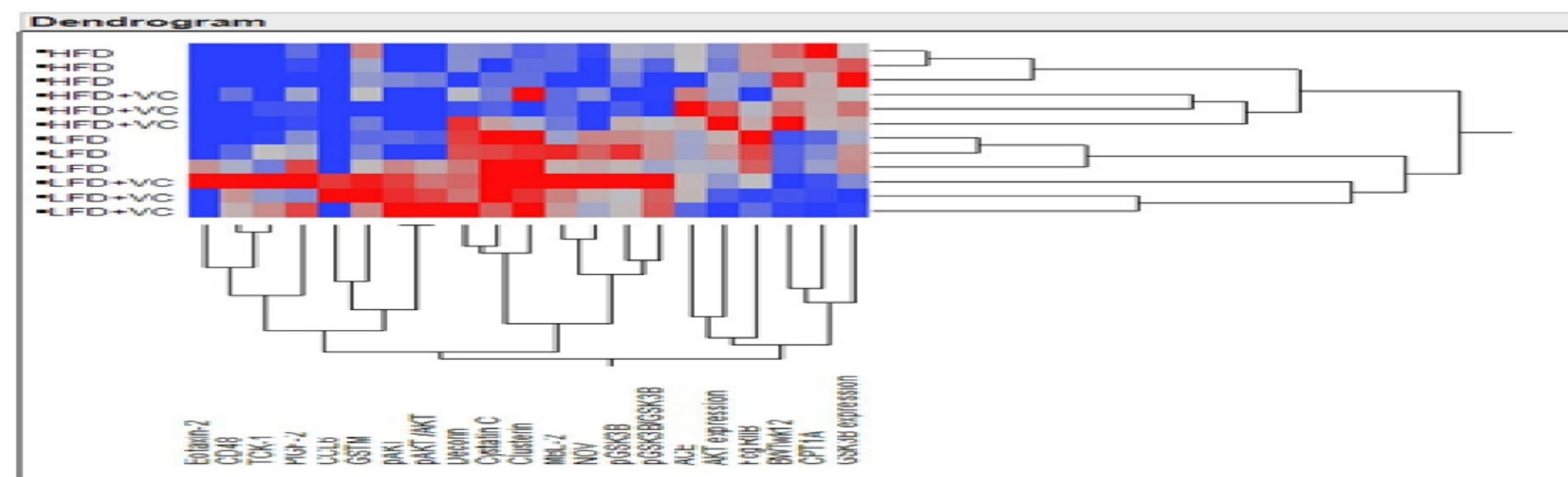
## Objective

Alterations in physiological processes in pancreas have been associated with various metabolic problems and fatty disease syndromes, such as inflammation, fibrosis, necrosis, abdominal obesity, and steatosis. The alterations of normal pancreatic functions can result from environmental exposures, such as chemicals and diets, which can impact susceptibility to metabolic problems and fatty diseases. It has been reported that environmental vinyl chloride (VC) exposure significantly increased steatosis in mice fed a high-fat diet (HFD) but not low-fat diet (LFD). However, little is known about molecular basis such as toxicity pathways and biomarkers and toxic mechanisms underlying the VC-induced environmental susceptibility, steatosis and other fatty disease induced by HFD. The present study was undertaken to examine the protein responses to VC exposure in pancreas tissues of C57BL/6J mice fed LFD or HFD, with focus on the investigation of the changes at expression and/or phosphorylation levels of some protein biomarkers of fat metabolisms, and key regulators of steatotic pathways such as cell proliferation and differentiation, fatty acid oxidation, energy metabolism, cell adhesion and migration, oxidative stress, and inflammation. Our hypothesis is that the protein alterations may modify the environmental susceptibility of mouse to the existing steatotic pathways and adverse outcomes induced by HFD after the exposure to VC.

## Methods

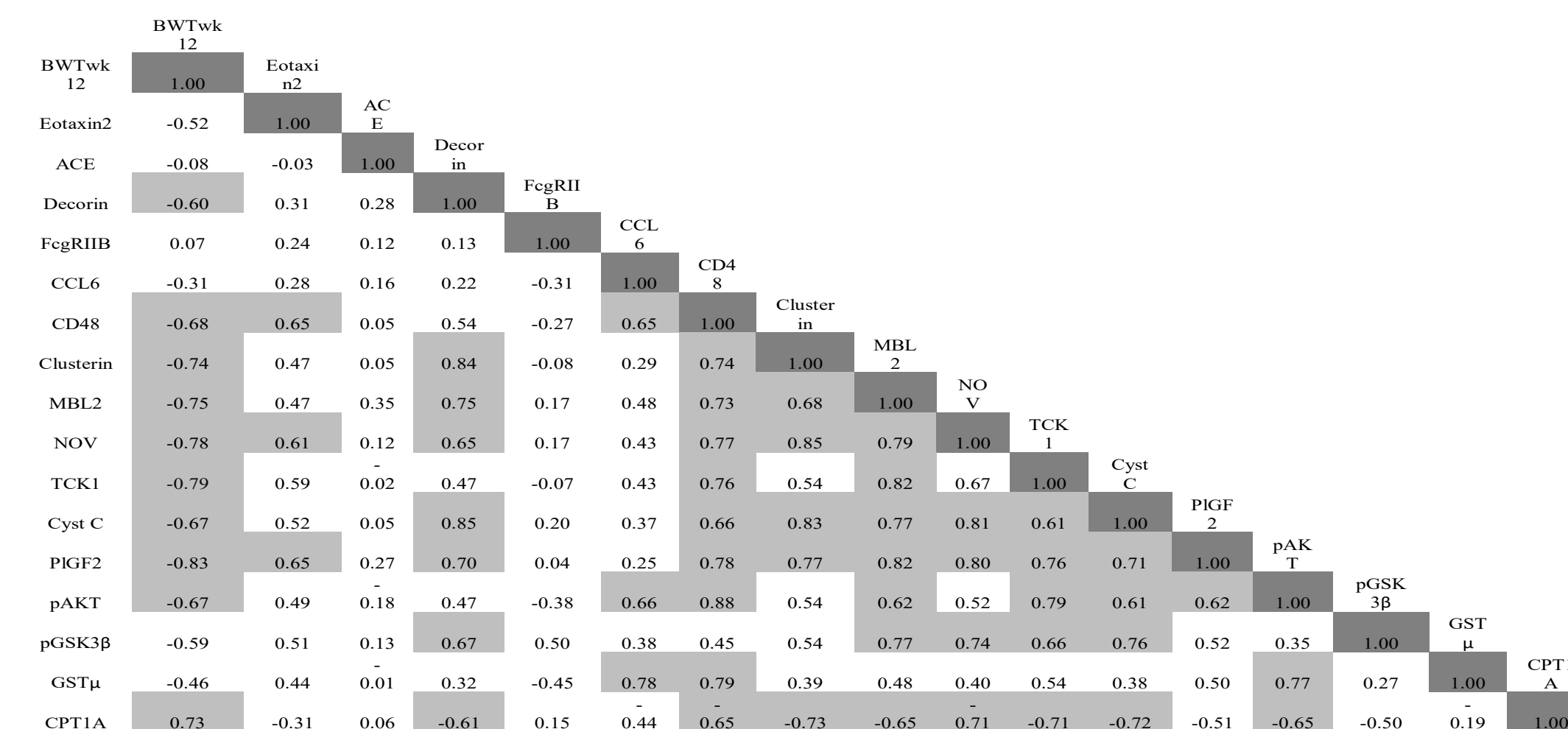
Six-week-old C57BL/6J mice (Jackson Laboratory) fed LFD or HFD (Envigo Teklad Diets, Madison) were exposed to VC (targeted concentration: 0.1 ppm) by air inhalation in chambers for 6 hours per day, 5 days per week for 12 weeks. The pancreas tissue samples were homogenized using polytron for 20 seconds in the buffer containing 150 mM NaCl, 20 mM Tris pH 7.7, 1mM EDTA, 1 mM EGTA and 1% Triton X-100. After incubation in ice for one hour, the homogenized tissue samples were then centrifuged at 10,000 g for 10 minutes. The supernatants containing total tissue proteins of pancreas were analyzed for identification of changes at expression and/or phosphorylation levels of protein biomarkers using a combination of 200 Mouse cytokines array (QAM-CAA-4000, Raybiotech, Norcross GA) and Western blot. Pancreas tissues from four groups of mice treated with LFD, HFD, LFD+VC, LFD+VC were used for the study. The accession numbers, relative fold changes, and IDs of the differentially altered proteins were tabulated and imported into ingenuity pathway analysis (IPA) for identification of top canonical pathways, toxlists, molecular and biological functions, biomarkers, and protein interaction networks that were regulated by the identified proteins.

**Figure 1:** Hierarchical clustering of differentially expressed and phosphorylated proteins in pancreas mice treated with LFD and HFD with or without VC. Blue areas represented downregulation and red ones represented upregulation of proteins. As shown in Fig 1, different treatment groups (LFD, LFD+VC, HFD, or HFD+VC) were distinctively separated (left side of the Figure), which suggested that protein expression and phosphorylation patterns in pancreas proteome of mice treated with LFD, LFD+VC, HFD, or HFD+VC are different, and HFD-induced protein change is similar as the one induced by HFD+VC, as compared to those induced by LFD and LFD+VC. The lower portion of the dendrogram depicted seven main clusters and each cluster usually consisted of two proteins. These clusters included the cluster of TCK-1 and PIGF2, Eotaxin 2 and CD48, GST $\mu$  and pAKT, cystatin C and clusterin. The pair of proteins found in the same cluster might suggest some functional correlations with each other in coordinating similar key events in pancreas. For example, cystatin C might coordinate with clusterin in tissue regeneration. Most of the proteins which were clustered together had a correlation efficiency with  $p \geq 0.6$ . The correlation of CPT1A with 8 proteins and GSK3 $\beta$  with MBL2, NOV, and TCK-1 suggested the importance of CPT1A and GSK3 $\beta$  in the regulation of the susceptibility of mouse to VC exposures in context of the existing steatotic pathways induced by HFD.

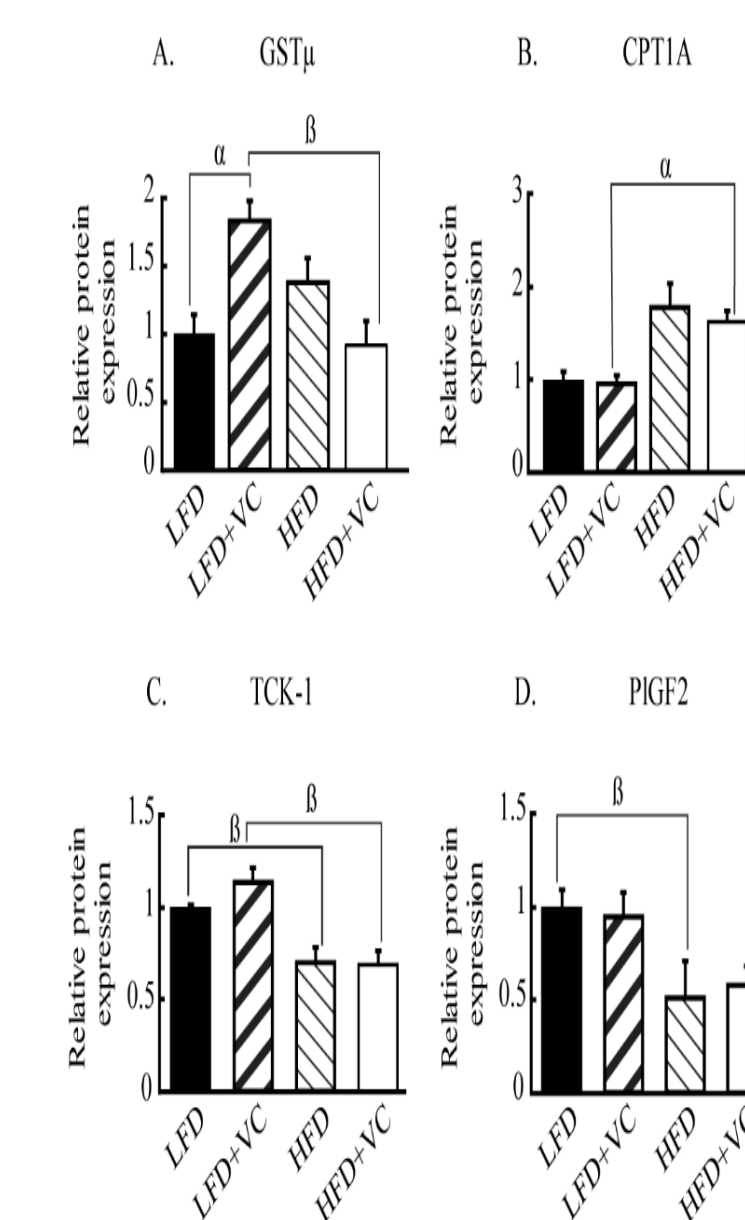


## Results

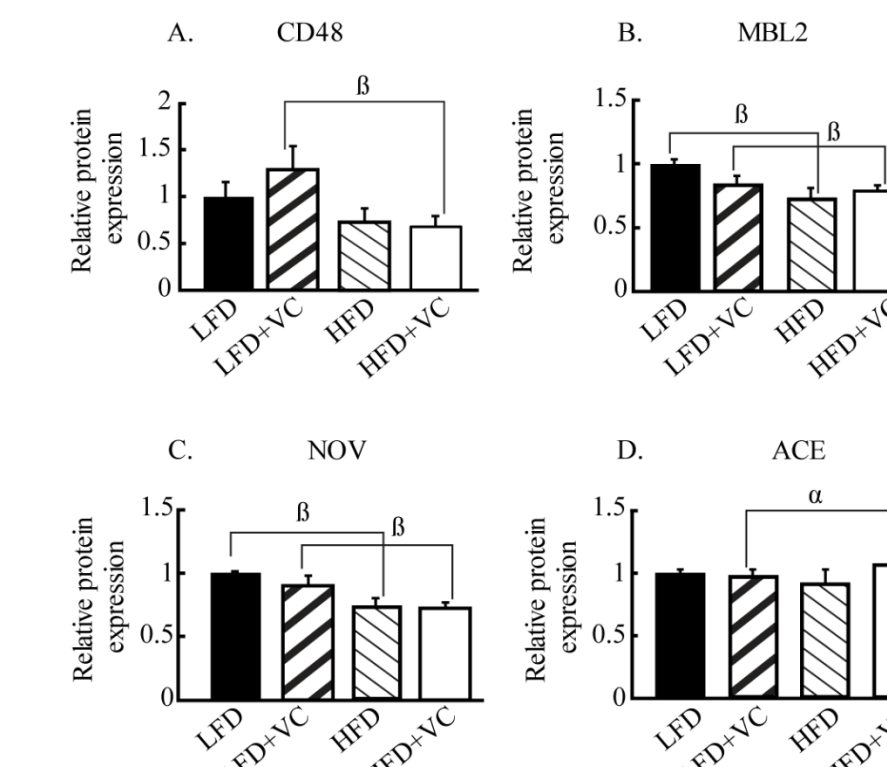
**Figure 2:** Pearson analysis of correlations of the differentially expressed or phosphorylated proteins with mouse body weights. Values which are negative show an inverse correlations, and those which are positive are direct correlations. The correlations considered significant are shown in light gray and have a correlation  $p$  value  $\geq 0.6$ . Pearson correlation analysis was employed to identify protein changes that were critical to steatotic pathways induced by HFD and environmental susceptibility induced by VC. In pancreas of mice fed HFD and VC, Decorin, CD48, clusterin, MBL2, NOV, TCK-1, cystatin C, PIGF2, pAKT, and pGSK3 $\beta$  were negatively, and CPT1A were positively correlated with mouse body weights, suggesting these small group of proteins played important roles in pancreas pathology mediated by HFD and/or VC. The information could help to gain insight into toxic mechanisms through which HFD and/or VC induced steatosis or other health outcomes in pancreas.



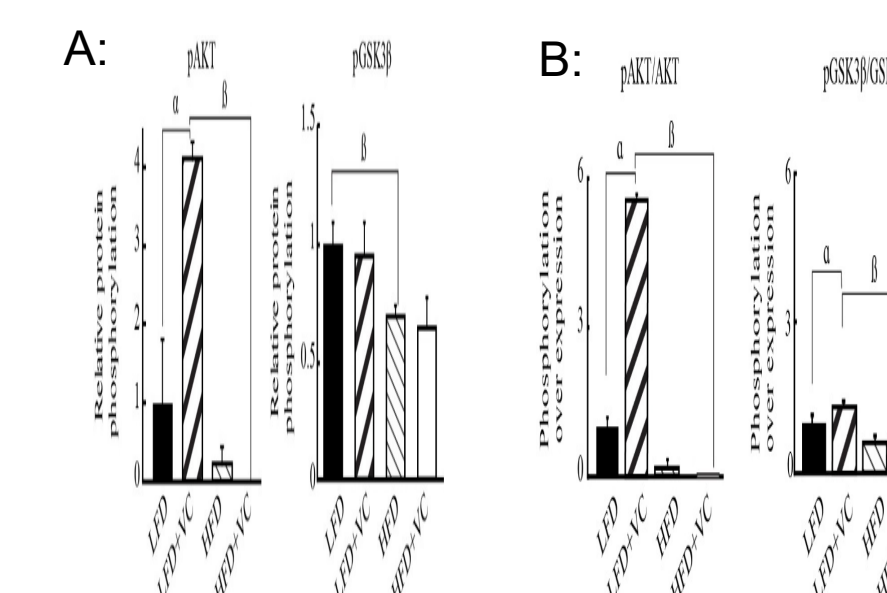
**Figure 3:** Changes at protein expression levels of 4 identified proteins involved in oxidative stress (A), energy metabolism (B), fatty acid oxidation (C), and angiogenesis (D). The expression levels were expressed by means with their standard errors. Protein changes between two compared experimental groups was considered significantly higher ( $\alpha$ ) or lower ( $\beta$ ) when  $p < 0.05$ . Data demonstrated that a variety of biological events and pathways were altered in the treated rats. As shown in Figure 3B, CPT1A expression was increased, but not significantly in pancreas of mice fed HFD as compared to pancreas of mice fed LED or LED+VC. However, CPT1A expression was significantly increased in pancreas of mice fed HFD+VC, which suggested that VC modified or enhanced HFD-induced CPT1A expression and possibly fatty acid oxidation activity regulated by CPT1A. VC treatment also dysregulated energy homeostasis and impaired mitochondrial function, even in mice fed LED (Figure 3A).



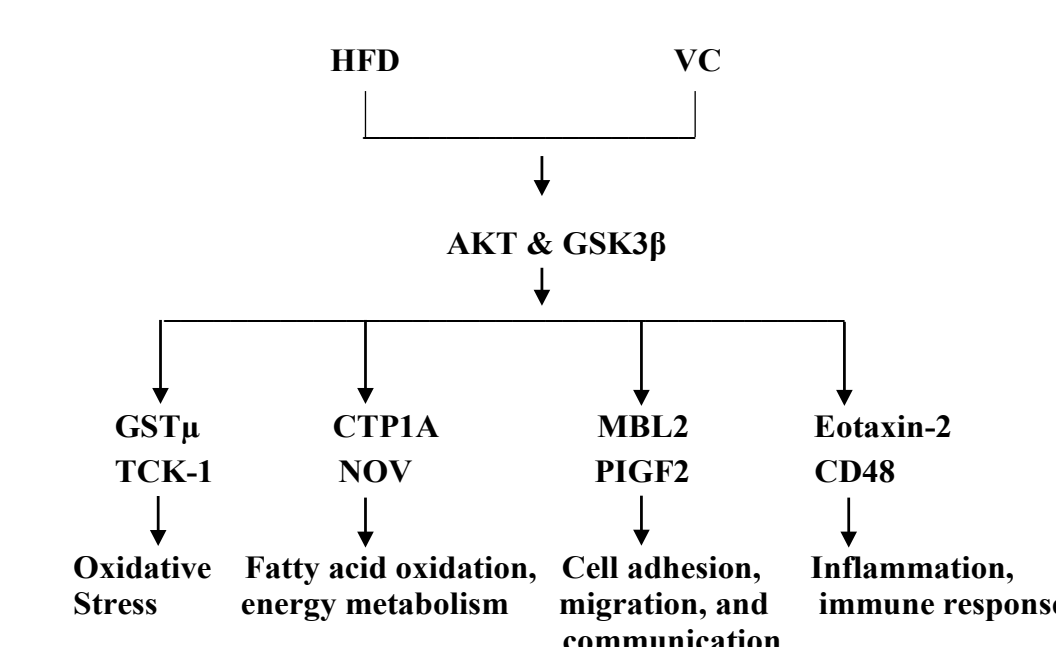
**Figure 4:** Expression of 4 representative cytokines involved in immune responses (A), anti-inflammatory response (B), cell adhesion and migration (C), and metabolic regulation (D) in pancreas of mice treated with LFD and HFD with or without VC. The expression levels are expressed by means with their standard errors. Protein changes between two compared experimental groups was considered significantly higher ( $\alpha$ ) or lower ( $\beta$ ) when  $p < 0.05$ . As shown in the Figure 4A to 4D, VC significantly decreased expression of CD48 (A), MBL-2 (B), NOV (C), and increased expression of ACE (D) in mice fed HFD, which might dysregulate energy homeostasis, cell communication and interaction, inflammatory and immune activities. The data also suggested that VC treatment may cause a variety of metabolic stresses that sensitize the treated mice more susceptible to fatty disorders and/or steatosis caused by HFD.



**Figure 5:** Western blot analyses of expression and phosphorylation levels of AKT and GSK3 $\beta$  in pancreas of mice treated with LFD and HFD with or without VC. (A): Phosphorylation levels of AKT and GSK3 $\beta$ . (B) Ratio of phosphorylation/expression of AKT and GSK3 $\beta$ . Values were means with their standard errors (SE). Protein changes between two compared experimental groups were considered significantly higher ( $\alpha$ ) or lower ( $\beta$ ) when  $p < 0.05$ .  $\beta$ -actin was used as internal control to normalize protein expression level. As shown in Figure B, VC significantly increased phosphorylation of AKT in mice fed LED, but significantly decreased phosphorylation of AKT in mice fed HFD. GSK3 $\beta$  showed the similar phosphorylation patterns as AKT.



**Figure 6:** A proposed protein interaction map to decipher key toxicity pathways underlying the susceptible effects or activities induced by VC in mice fed HFD. Considering all the proteomic results obtained from this study using protein array, multiplexed ELISA, Western blot, and statistical correlation of protein changes with mouse body weights, we proposed this core protein and toxicity pathway map to describe the interplay of these proteins and pathways associated with the toxic responses of pancreas in mice treated with HFD and VC. The diagram was generated by taking considerations of the connectedness of these proteins as hub nodes in the protein networks of pancreas of mice treated with LFD, HFD, LFD and VC, and HFD and VC as well, which were identified by IPA (data not shown).



## Conclusions

- A small group of proteins such as CPT1A, AKT, and GSK3 $\beta$  that proteins may play important roles in HFD-mediated steatotic events and VC-mediated environmental toxicity was identified.
- These identified proteins may alter VC-mediated susceptibility of mouse to fatty disorders or steatosis induced by HFD in the context of protein biomarker and biochemical pathways.
- Oxidative stress, fatty acid oxidation, energy metabolism, cell adhesion, migration and communication, inflammation, and immune response are critical to VC-mediated environmental susceptibility to the existing steatotic pathways and adverse outcomes induced by HFD.
- An efficient proteomic approach to identify key pathways regulators and protein biomarkers to enhance exploration of exposure and toxicity effects of the interactions of susceptibility factors was established.

This poster does not reflect EPA policy. No conflicts of interest to declare.