Insulin resistance that is often associated with abdominal obesity is a factor of the metabolic syndrome and is implicated in the development of type 2 diabetes. Insulin resistance together with β-cell dysfunction leads to the appearance and gradual progression of type 2 diabetes. Type 2 diabetes, which accounts for almost 90% of the total diabetic population, stems from decreased responsiveness of the body to insulin (insulin resistance), accompanied by the failure of pancreatic β-cells to secrete insulin to counteract this insulin-resistant state. Obesity is invariably associated with diabetes and a parallel increase in the occurrence of both is evident across all populations. Obesity-induced insulin resistance is thereby characterized by a loss of insulin sensitivity mediated by factors released from adipocytes, mainly free fatty acids and proteins, termed adipocytokines, which act to control various metabolic functions with well-described physiological effects. One such adipocytokine is tumour necrosis factor-α (TNFα), which has been identified as a significant contributor to insulin resistance, and its levels have been reported to be increased significantly in obese diabetic individuals and in several animal models of obesity. TNFα is believed to interfere with several intermediates of the insulin signaling cascade and therefore cause insulin resistance in the major insulin target tissue i.e adipocyte, skeletal muscle and liver. Being a major insulin tissue, the liver plays a significant role in glucose homeostasis, as it can alternate between cycles of glucose output and its inhibition to maintain normal circulating glucose levels; it is this precisely regulated cycle that is disturbed under conditions of insulin resistance and type 2 diabetes. The current literature on diabetes and its associated pathologies especially in the liver is described in Chapter 1.

In addition to making the liver insulin resistant, the proinflammatory cytokine, TNFα, is a crucial mediator of the pathogenesis of several diseases, more so in cases involving the liver wherein it is critical in maintaining liver homeostasis since it is a major determinant of hepatocyte life and death. Gene expression profiling serves as an appropriate strategy to unravel the underlying signatures to envisage such varied responses and considering this, gene transcription profiling was performed in control
and TNFα treated HepG2 cells (human hepatoma). In chapter 2 we did whole genome oligonucleotide expression arrays (Human Genome U133 plus 2.0, Affymetrix) to identify genes altered by TNFα in HepG2 cells incubated in the absence and presence of TNFα (0.5nM, 12 h). TNFα treatment significantly (p< 0.05) altered the expression of 140 genes in HepG2 cells of which 67 were up-regulated and 73 were down regulated. These altered genes were then classified into functional categories of biological processes using Gene Ontology Tree Machine (GOTM) and Database for Annotation, Visualization and Integrated Discovery (DAVID). From our dataset, genes that clearly mapped onto a single GO term were assigned as such and for others that mapped onto more than one GO term, we put genes in the term where there was experimental validation available and when not, we put them in a particular GO class considering their functional closeness to other members of that class. All the 67 up-regulated and 73 down-regulated genes were separately grouped into molecular functions using the GO Tool Box. Interestingly, molecular functions that emerged specifically as being altered among the up-regulated genes were those of lipid binding and ligase activity while those of transcription repressor and enzyme inhibitor activities were specific to the down-regulated genes. Also, for genes up-regulated by TNFα, several GO terms related to lipid and fat metabolism like keto-steroid reductase activity, cholate-CoA ligase activity, squalene monooxygenase activity, oxidoreductase activity were significantly (p<0.01) over represented indicating an overall favor of genes involved in fat metabolism within the hepatocyte by TNFα. GO terms involved with immune responses like IgE receptor activity, immunoglobulin receptor activity and IgE binding were highly over represented in the down-regulated set indicating a compromise in immune defense mechanism(s). TNFα induced up- and down- regulated genes were clustered hierarchically on the basis of their normalized expression intensities and genes with the same GO functional term and within the same cluster were selected for further promoter analysis. Genes from three GO terms i.e. “Metabolism”, “Signal Transduction” and “Regulation of Biological Process” that clustered together were taken for promoter analysis. A 5kb upstream sequence from the transcription start site of each of these genes was retrieved and the binding sites of transcription factors within this region were determined using Over-represented Transcription Factor Binding Site Prediction Tool (OTFBS) tool.
Significant transcription factors consistently occurring in the promoters of genes altered by TNFα and clustering together with common GO terms included SREBP-1, CEBPα, MEF2 and AREB6. All these are in several ways implicated in cellular metabolic processes and immune response, two phenomena that are modulated by TNFα. Surprisingly, binding sites of several members of the forkhead family of transcription factors that are not well characterized were also significantly found to be overrepresented in our dataset. These included FOXJ2, FOXD3 and the fork head homolog-3 (HFH-3). To validate the transcription factors that were found to possess binding sites in the promoters of genes that were altered by TNFα, electrophoretic mobility shift assay (EMSA) was performed for two of the candidate transcription factors namely, SREBP-1 and FOXJ2. The binding of SREBP-1 and FOXJ2 to their respective DNA binding elements was increased in the presence of TNFα.

We then wanted to identify the interacting network among the altered genes in the context of other biological pathways. TNFα altered genes were analysed using the Ingenuity Pathway Analysis (IPA) software that generates networks, associated biological functions and canonical pathways by overlaying them onto a global molecular network developed from information contained in the Ingenuity knowledge base. Five highly significant networks with scores above 20 were obtained from the set of TNFα induced altered genes. These scores, derived from p values, indicate the likelihood of the focus genes belonging to a network versus those obtained by chance alone thereby eliminating the probability of their occurrence in a network to be due to noise. One of the significant pathways was that of biosynthesis of steroids. Three genes of this pathway namely, SQLE, FDPS and EBP were upregulated. TNFα increased cholesterol accumulation in HepG2 cells. In our study, SQLE emerged the most significant mediator of the effects of TNFα on cholesterol synthesis.

This chapter represents a composite analysis of the effects of TNFα in HepG2 cells that encompasses the altered transcriptome profiling, the functional analysis of the up- and down- regulated genes and the identification of conserved transcription factor binding sites. These could possibly determine TNFα mediated alterations mainly the phenotypes of hepatic steatosis and fatty liver associated with several hepatic pathological states.
Consequent to assessing the global effect TNFα in HepG2 cells, in Chapter 3, the specific effects of TNFα on gluconeogenesis is described. Initially, we determined whether the cells were insulin unresponsive in our study at the dose and period of TNFα preincubation prior to insulin treatment used. Akt is one of the most important insulin signaling intermediates, and is well known to be activated by insulin, an effect that is equally well known to be prevented in cells preincubated with TNFα prior to insulin incubation. Insulin significantly stimulated Akt phosphorylation relative to the control (P < 0.001), and this effect was decreased significantly on TNFα pretreatment (P < 0.01). One of the notable features of inhibition of insulin signaling in the liver is attenuation of the insulin-mediated inhibition of gluconeogenic gene expression.

As the transcription factor Foxa2 has been implicated, in part, in the regulation of gluconeogenic genes, we studied the effects of TNFα and or insulin on its cellular status in hepatocytes, followed by an assessment of its occupancy on the phosphoenolpyruvate carboxykinase (PEPCK) promoter. In cells pretreated with TNFα (1 nM, 24 h), followed by insulin incubation, Foxa2 was found to be mainly localized in the nucleus (P < 0.05) and was significantly (P < 0.01) less detected in the cytosol, relative to cells incubated in the presence of insulin alone. Foxo1 also showed an almost complete nuclear localization in cells pretreated with TNFα prior to insulin incubation, whereas, in cells incubated in the presence of insulin alone, it was exclusively localized in the cytosol. These results imply that, in the presence of TNFα, wherein cells are rendered insulin insensitive, insulin-mediated nuclear exclusion and inactivation of Foxa2 are prevented, with the result that it is primarily localized in the nucleus. Thus, although TNFα alone does not alter the levels of Foxa2 within the cell, it attenuates insulin- stimulated Foxa2 nuclear exclusion, possibly by blunting insulin signaling within the cell. As we observed a predominant localization of Foxa2 in the nuclei of cells pretreated with TNFα prior to insulin incubation, and considering its possible involvement in the regulation of gluconeogenic enzymes, we analysed the Foxa2 occupancy of the promoter of gluconeogenic genes, mainly PEPCK, it being the rate-limiting enzyme. When compared with the control, insulin caused a significant (P < 0.01) decrease in Foxa2 occupancy of the PEPCK promoter. This decrease was significantly (P < 0.01) attenuated in cells preincubated in the
In cells incubated in the presence of TNFα alone, Foxa2 did not show any significant change in its occupancy on the PEPCK promoter after normalization with the input DNA and comparison with the control. All of these results indicate that preincubation with TNFα significantly abrogates the insulin-mediated decrease in Foxa2 occupancy of the PEPCK promoter. As we had observed, an increase in gluconeogenic gene transcript levels in TNFα-pretreated cells as a result of a decrease in the effects of insulin, mediated in part, by the transcription factor, Foxa2, we sought to determine the effect(s) of this on glucose production from HepG2 cells, the ultimate phenotype that, together with glucose uptake, regulates the circulating glucose level within the body. The incubation of HepG2 cells with insulin inhibited glucose release by almost threefold when compared with the control (P < 0.01); pretreatment with TNFα prior to insulin incubation significantly attenuated this inhibition (P < 0.001), i.e. in the presence of TNFα, the extent of inhibition of hepatic glucose output by insulin was markedly attenuated. Our results indicate that Foxa2, is at least in part responsible for the attenuating effect of TNFα on insulin action on PEPCK expression and glucose production in HepG2 cells.

In addition to these mechanisms responsible for attenuation of insulin signaling, miRNAs that are small non-coding RNAs have also emerged as critical regulator of insulin signaling. They act by binding to their complementary sequence on the 3’UTR of target genes and cause translation repression. In a step towards decoding the regulatory role of miRNAs during this hepatic pathophysiology, in chapter 4 we assessed the status of miRNA in the livers of diabetic (db/db) and control (db/+). To determine the status of miRNAs in the livers of control (C7BL/KsJ db/+ vs. db/db (C57BL/KsJ db/db) mice, we performed a miRNA microarray analysis in these groups of animals. A set of 14 miRNAs was found to be differentially expressed in the livers of diabetic db/db mice of which 12 miRNAs were up regulated and 2 were down regulated, suggesting an altered miRNA signature in the livers of db/db mice that might play an important role in determining the pathophysiology of the diabetic liver.

One of the miRNAs that was significantly upregulated was miR-29a. In chapter 5 we attempted to explore the role of elevated levels of miR-29a on insulin inhibition of
PEPCK expression and to unravel the intermediary mechanisms involved. Insulin signaling within the cell initiates by binding to its membrane receptor that is subsequently transmitted to downstream mediators phosphatidylinositide-3-kinase and then to the Ser/Thr kinase, Akt. In HepG2 cells, overexpression of miR-29a led to significant inhibition of insulin stimulated phosphorylation of Akt in a dose dependent manner. However the total levels of Akt remained unchanged at all treatments. This above results of miR-29a inhibiting only insulin stimulated Akt phosphorylation without altering total Akt levels prompted us to look for direct targets of mir-29a in intermediates upstream of Akt and in doing so we found using TargetScan that the p85α regulatory sub unit of PI3K harbors binding sites for mir-29a at positions 331–337 of its 3’UTR that is conserved across species. Over expression of mir-29a significantly decreased the p85α subunit protein level that was prevented in the presence of the specific miR-29a inhibitor. This was further confirmed by the Luciferase reporter assay where in miR-29a significantly inhibited the luciferase activity of the reporter vector containing the PI3Kp85 wild type 3’UTR that was prevented by the miR-29a inhibitor. This inhibition was not observed with the reporter containing the PI3Kp85 mutated 3’UTR (two nucleotides in the miRNA binding site were changed using site directed mutagenesis kit) that indicates the specificity of the miR-29a PI3Kp85 3’UTR interaction. These implicate that miR-29a directly targets the PI3Kp85 3’UTR and that the counteracting effects of miR-29a on insulin stimulated Akt phosphorylation in fact begins with direct inhibition of PI3Kp85 by miR-29a that translates downstream to prevent insulin stimulated Akt phosphorylation even in the presence of unaltered total Akt levels.

Considering that the major downstream pathway of insulin action in the liver culminates into inhibition of gluconeogenic genes, we consequently determined the effect of these conditions on the status of PEPCK in HepG2 cells. As compared to control, insulin led to significant inhibition (p < 0.001) of PEPCK gene expression. Interestingly, in cells overexpressed with miR-29a, this insulin mediated inhibition of PEPCK expression was markedly prevented (p < 0.05). miR-29a prevented insulin inhibition of PEPCK expression and this was associated with inhibition of PI3Kp85α levels and attenuation of insulin effect on Akt phosphorylation. To correlate the
decrease in PI3Kp85α and increase in PEPCK levels in \textit{in vivo} diabetic milieu, we assessed the protein levels of PI3Kp85α and PEPCK in the liver tissue of normal and diabetic db/db mice. The protein levels of PI3Kp85α are significantly (p< 0.05) decreased in the db/db mice liver while that of PEPCK are significantly (p< 0.001) elevated. These results thus show the role of miR-29a in counteracting insulin action on PEPCK gene expression by primarily targeting PI3Kp85α and abrogating downstream insulin signaling in HepG2 cells.

Results presented here put forth the detailed global analysis of the action of TNFα in HepG2 cells by encompassing the altered gene expression profile. This identified genes and transcription factors that are possibly critical among other significant liver phenotypes, hepatic steatosis and fatty liver that are crucially associated with several hepatic pathological states. We have also demonstrated the role of TNFα in the regulation of gluconeogenesis and determined the underlying molecular mechanisms. Our results indicate that transcription factor, Foxa2, is at least, in part, responsible for the attenuating effect of TNFα on insulin action on PEPCK expression and glucose production in HepG2 cells. In addition, further studies add on to show that miR-29a levels are elevated in the diabetic liver and miR-29a targets the p85α subunit of PI3K and therefore participate in abrogating insulin action on PEPCK expression.