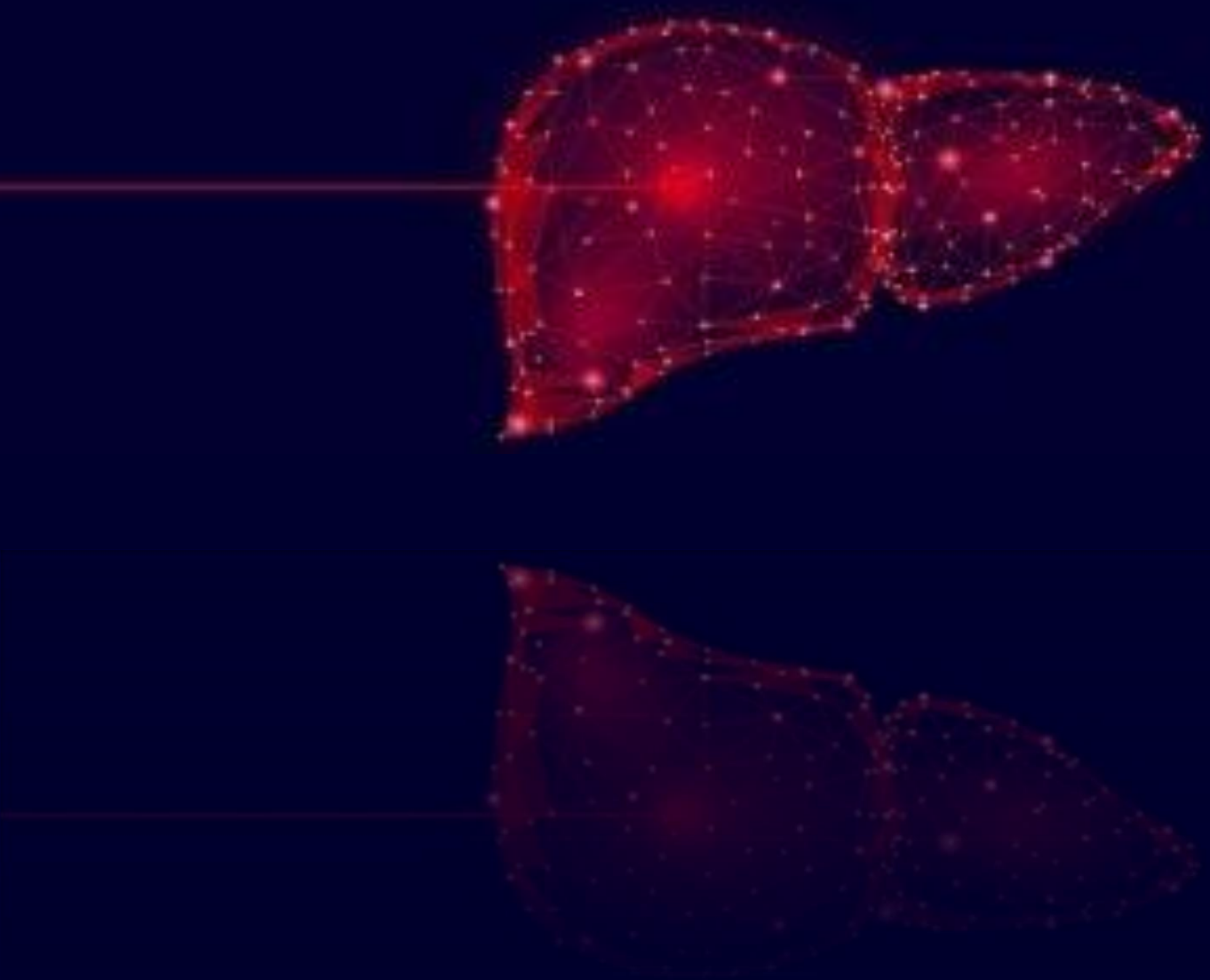
Summary



Summary

Over the past few decades, the proportion of populations that is overweight or obese has greatly increased. It is undoubtedly related to changes in lifestyle that affects the diet and physical activity. Today, about half of the adult population in developed countries is overweight or obese. Obesity is preventable cause of illnesses, which if uncontrolled contributes to premature mortality and metabolic complications. In addition to the increased risk of cardiovascular disease and type II diabetes mellitus, morbid obesity seems to be a significant risk factor for several malignancies, including Non-alcoholic fatty liver disease (NAFLD). The increased body mass index (BMI) and other metabolic disorder such as T2D, obesity and higher cholesterol is associated with an increased risk of NAFLD development.

Liver is known to be engaged in vital metabolic processes including metabolism of drugs that necessitates highly controlled biochemical reaction. Physiologically it is taken care off, as liver is imparted with highest regenerative capacity amongst all the other organs in the body. Liver tends to get damaged when it fails to combat the insults slashed at it in form of viral infections, alcoholic abuses, drug and chemical toxicant, autoimmune reactions and lipid overloads etc. Liver diseases are broadly classified into acute and chronic liver diseases based on duration and persistence.

Liver diseases are pandemic and calls for major attention looking at the steady rate of increase over the period of time. Rate of NAFLD is shooting up in Indian population with changing in life style and urbanization. About 9-32% of Indian population suffers from fatty liver. Globally the rate has risen from 15% in 2005 to 25% in 2010 and about 30% in 2017. Prevalence rates of NASH are no different

from that of NAFLD, the rates have almost doubled from 33% to 59.1%. This steep increment calls for an immediate attention for fatal, asymptomatic diseases.

Nonalcoholic steatohepatitis (NASH) is majorly characterized by lipid accumulation, inflammation and fibrosis in advance stages. The broad classification of the events taking place in NASH pathology was proposed by Day and James wherein the first hit is marked by triglyceride loading within hepatocytes known as steatosis or fatty liver (NAFLD) that is due to an overflow of free fatty acids (FFA) into the liver and consequent esterification. Subsequently liver is vulnerable for second hits wherein inflammatory changes are key events leading to NASH and/or fibrosis. But lately scientific fraternity has moved on to accepting a multi-hit model according to which several events take place simultaneously or parallelly. The events of complex interactions between hepatocytes, stellate cells, adipose cells, Kupffer cells, inflammatory mediators, and reactive oxygen species (ROS) together act as driving force for onset and progression of NASH pathology. Major event of formation of harmful adducts as by-products of fatty acid oxidation by mitochondria, peroxisomes or microsomes is attributed to be the main reason for transition of NAFLD to NASH.

NASH is a multifactorial disease and hence a multipronged approach shall be employed in order to cure the disease. Major challenges in upfront of the said pathology are its asymptomatic nature, lack of precise diagnostic tests and therapeutants. Investigations have identified some common characteristic exhibited in NASH that includes right upper quadrant pain, fatigue, malaise and hepatomegaly on physical examination. For diagnostics non-invasive tests have

also been developed for identification of steatosis and fibrosis in NASH. However, lack of FDA approved drug still pertains as major challenge to combat the disease. In diseases with high oxidative stress Nuclear erythroid 2-related factor 2 (Nrf2) has been employed as the therapeutic target to maintain redox homeostasis. Nrf2, a transcription factor of the Cap-n-collar basic leucine zipper family (Moi *et al.*, 1994) has been recognized as a key regulator of oxidative stress in different tissue and cell types including hepatocytes. In reaction to oxidative stress, Nrf2 binds to the promoter region of ARE sequences and induce expression of antioxidant and cytoprotective genes (Espinosa-Diez *et al.*, 2015a, Reddy, 2008a). In recent years, Nrf2 activators of natural and synthetic origin have been extensively reported for their antioxidant potential against variety of tissues/cells. Carbon monoxide (CO), an endogenously produced gasotransmitter, is reported as Nrf2 activator. Physiological role of CO as a regulator of cellular function is well investigated in several pathological conditions including vascular inflammation, autoimmune neuroinflammation, sickle cell disease, diabetes, acute hepatitis, colitis and sepsis. CO easily diffuses across cellular and biological membranes and therefore can mediate functional changes rapidly.

Owing to the reported impact of CO on Nrf2 under oxidative stress condition, we have planned our investigation with CORM-A1 on the same target in steatotic liver. Subsequently, the role of CORM-A1 in regulating intracellular antioxidant status, ARE genes and overall mitochondrial function shall be put to a detailed scrutiny. The aim of these experiments is to obtain an insight in improvement of pathophysiology of NASH mediated by CORM-A1 treatment.

The first chapter deals with preventive mechanisms of CORM-A1 against tert-Butyl hydroperoxide (t-BHP) induced oxidative damage to HepG2 cells. In preliminary studies, cell viability was measured using MTT assay. Herein, t-BHP treated HepG2 cells showed significant decrement in cell viability whereas; CORM-A1 co-treatment recorded higher number of viable cells. Further for mechanistic investigation of modus operandi of CORM-A1 in alleviating cellular stress, expression levels of antioxidant genes were measured. Increment in antioxidant's mRNA viz. Nrf2, Keap1, GCLC, GCLM, HO-1 and NQO-1 were observed with CORM-A1 treatment in HepG2 cells. A detailed investigation of nuclear and cytoplasmic fractions of HepG2 cells had revealed CORM-A1 mediated augmented rate of translocation of Nrf2 protein into the nucleus. This event was instrumental in upregulation of HO-1 expression that in turn upregulated ARE genes thus orchestrating cytoprotection in t-BHP induced oxidative stress.

In silico analysis was performed to assess docking of CO and Keap1 protein. Results of the docking interactions were compared with a small molecule (3S)-1-[4-[(2,3,5,6-tetramethylphenyl) sulfonylamino]-1-naphthyl] pyrrolidine-3-carboxylic acid (RA839). 3D visualization of CO binding on Keap1 protein revealed that its binding location was at the base of Nrf2 binding site. This study provided compelling evidence on competitive binding of CO in kelch domain of Keap1 thus inhibiting protein-protein interaction between Nrf2 and Keap1 protein. Overall, the key findings in this study were that CORM-A1 facilitates nuclear translocation of Nrf2, reduces oxidative stress, upregulates ARE genes, prevents GSH depletion and promotes cell viability.

Chapter two focus on hepatoprotective potential of CORM-A1 against APAP induced hepatotoxicity in swiss albino mice wherein; the Nrf2-Keap1 pathway forms the epicenter of our investigation. In the set of experiments performed herein, Male Swiss albino mice (6-8-week-old) were administered with intraperitoneal injection of APAP (300 mg/kg bodyweight). Followed by administration of single dose of (20 mg/kg) CORM-A1 after 1 h of APAP overdose.

Circulating titers of liver functional enzymes AST, ALT and ALP were found to be significantly high following APAP treatment suggesting development of hepatotoxicity. Whereas, CORM-A1 administration accounted for decreased levels of the said enzymes. CORM-A1 was also found to alleviate hepatocyte necrosis caused by APAP as evidenced by histoarchitecture of liver sections stained with H&E. Tissue level oxidative stress is reduced either directly by scavenging ROS or by upregulating antioxidant status. Hence, protective effect of CORM-A1 against hepatotoxicity with changes in Nrf2 and related enzymes was investigated. Our results suggest that CORM-A1 modulate later events such as GSH recovery and activate ARE genes leading to improvement in antioxidant milieu in liver. Activation of Nrf2 contributes in stimulating cellular defense mechanism by upregulation of HO-1, GCLC, GCLM and NQO-1 genes that reduces oxidative stress and maintain the ratio of oxidant: antioxidant in a cell. Herein, APAP induced downregulation of Nrf2 and related genes resulted in GSH depletion. However, significantly high mRNA levels of antioxidant genes in APAP + CORM-A1 treated group corroborates with the increment in GSH levels. APAP-mediated sterile inflammation is initiated and perpetuated by damage-associated molecular patterns

(DAMPs). In this study, treatment with CORM-A1 reduced hepatic cytokine production, possibly via downregulation of NF-κB, thus reducing the sterile inflammation.

Studies with acute lethal dose of APAP (600 mg/kg) were conducted to assess the efficacy of CORM-A1 in improving overall survival. APAP causes 50% mortality within first 4 h followed by 100% mortality by 12 h. However, CORM-A1 treatment had delayed the onset of toxic phase by improving (30%) survival at 4 h stage and an improved survival (50%) at the end of 12 h. These results when compared with NAC treatment were found to be more effective in negotiating toxicity because, unlike NAC, CORM-A1 was found to be effective even in the later stages as it had accounted for an overall higher percentage of survival. This is an important observation and first report on hepatoprotective potential of CORM-A1. Overall, it can be concluded that CORM-A1 can modulate experimentally induced hepatotoxicity by upregulation of Nrf2 and related genes, reducing inflammation and preventing GSH depletion resulting in improved survival rate. These results also underline the importance of slow releasing molecule, CORM-A1 in intracellular signaling and negotiating hepatotoxicity.

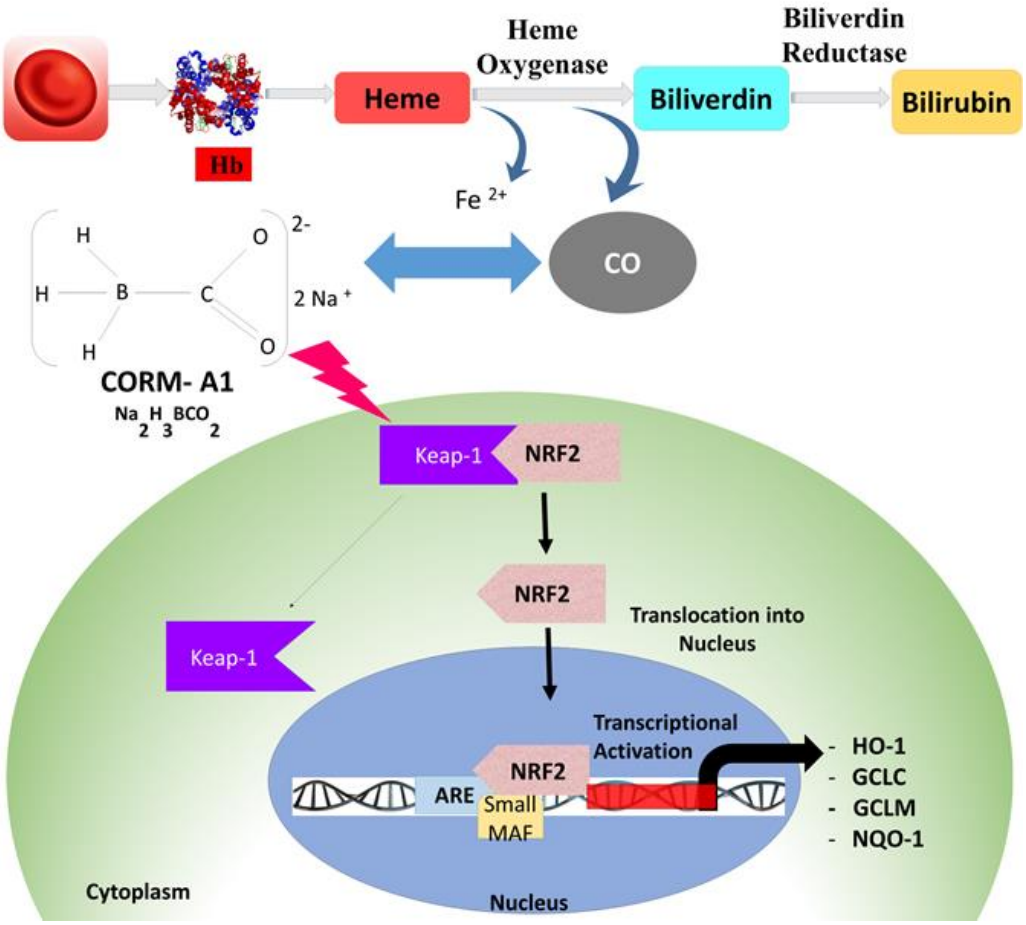


Fig.G. Summary- Chapter-1&2; indicating underlying mechanisms of CORM-A1 mediated improvement in APAP induced hepatotoxicity and role of Nrf2-ARE pathway.

In the third chapter, therapeutic potential of CORM-A1 against experimentally induced NASH model was studied wherein, the previous findings formed the basis of our investigation. In this study, high fat and high fructose (HFHF) fed C57BL/6J mice and palmitic acid treated HepG2 cells were used as experimental models of NASH and CORM-A1 mediated corrective changes in regulatory genes governing cellular lipid metabolism and inflammation in NASH were investigated.

In our study, HFHF diet was fed to C57BL/6J mice to mimic fructose and fat rich foods consumed by humans. Fructose feeding facilitates transition of NAFLD to NASH in HFD fed C57BL/6J mice wherein, significant increment in body weight, abdominal fat and liver: body weight ratio was recorded. In our study, significantly elevated titer of AST, ALT, ALP and pathological score of liver section of HFHF fed mice confirmed hepatocyte damage and steatotic changes. These set of changes were lower/not seen in liver and serum of CORM-A1 treated mice. Further, CORM-A1 was also found to regulate lipid metabolizing genes in liver and improve fasting blood glucose and glucose tolerance in liver of HFHF fed mice. Also, significant decrement in mRNA expression of inflammatory genes (IL-1β, IL-6 and TNF-α) in CORM-A1 treated group was observed. Studies with Palmitic acid (PA) treated HepG2 cells had recorded improved cell viability and lowered lipid accumulation in presence of CORM-A1 implying towards its cytoprotective role. Overall, we can surmise that CORM-A1 was effective against experimentally induced NASH in HFHF fed C57BL/6J mice or PA treated HepG2 cells by regulating expression of lipid metabolizing genes.

The chapter four deals with investigation of underlying mechanisms of CORM-A1 in regulating and improving pathology of NASH. This study is further subdivided into **A** and **B** sections. Herein, in section **4-A** we have investigated the role of Nrf2 and antioxidant signaling in improving the pathology of NASH with CORM-A1 administration.

Liver of CORM A-1 treatment accounted for lowered levels of Nrf2 protein in cytosol and significantly higher levels in nucleus as compared to HFHF fed mice. Further, the mRNA expression and protein content of HO-1 showed non-significant changes but CORM A-1 treatment resulted in a 2-fold increment. Conversely, Keap1 protein was significantly reduced in CORM A-1 treated group. Supportive evidence was obtained in from of increased mRNA expression of ARE genes viz GCLC, GCLM and NQO-1 in CORM-A1 treated mice. These set of findings demonstrate that CORM-A1 induced Nrf2 activation and subsequent upregulation of antioxidant genes in HFHF fed mice.

In vitro studies with PA treatment to HepG2 cells resulted in heightened levels of intracellular ROS as evidenced by CellROX (green) and by DHE (red) staining but CORM-A1 co-supplementation resulted in significant decrement in red and green florescence respectively. Translocation of Nrf2 protein content (Cytoplasm to nucleus) was further confirmed in HepG2 cells wherein, CORM-A1 treatment accounted for significantly higher Nrf2 protein in nucleus as compared to PA treated cells. Keap1 protein (negative regulator of Nrf2) showed a significant decrement in PA and CORM-A1 treated groups. Co-immunoprecipitation (co-IP) results revealed time depended decrement in Keap1 protein content after treatment

with CORM-A1 to HepG2 cells. Increase in HO-1 mRNA and protein was recorded in PA treated cells whereas, more pronounced effect in mRNA and moderate nonsignificant increment in protein was noted in CORM-A1 co-supplemented group. Other ARE genes viz. GCLC, GCLM and NQO-1 were also studied wherein, GCLM mRNA levels was decreased significantly following PA treatment. But, GCLC and NQO-1 mRNA levels in the same group showed non-significant changes. CORM-A1 co-supplementation to PA treated HepG2 cells was marked by significantly elevated mRNA levels of GCLC, GCLM and NQO-1. Overall, Chapter 4A summarizes the mechanism of anti-NASH property of CORM-A1 in both in vivo and in vitro experimental models.

Chapter 4-B comprises of focused study on mitochondria and its energetics under experimentally induced NASH and subsequent effect of CORM-A1 treatment. CORM-A1 accounted for improved mitochondrial biogenesis and functions in HFHF fed mice and in PA treated HepG2 cells. Regulators of mitochondrial fission (Drp-1) and mitochondrial DNA (TFAM) showed moderate to significant increment in mRNA levels in liver of HFHF fed and CORM-A1 treated mice respectively. Functional status of mitochondria was accessed by total ATP production in liver cells wherein, CORM-A1 treatment was found to be highly beneficial. Mitotracker staining further provided evidence on CORM-A1 induced improvement in mitochondrial mass. Also, mtDNA content showed a CORM-A1 mediated increased mitochondrial copy number. Further, MitoSOX and JC1 staining showed decreased ROS levels and improved membrane potential respectively in CORM-A1 treated HepG2 cells.

Mitochondrial respiration was assessed by seahorse XF extracellular flux analyzer as oxygen consumption rate (OCR) whereas; the glycolytic activity was assessed as resultant lactic acid production and extracellular release (ECAR). Results of mitochondrial function had revealed improved basal respiration in PA + CORM-A1 treated HepG2 cell and significant increment in indices of proton leak thus justifying higher ATP production.

The extracellular acidification rate (ECAR) is indicative of glycolytic activity in absence of mitochondrial respiration. ECAR revealed higher indices of glycolytic reserves in PA+ CORM-A1 treated HepG2 cells. Thus, results obtained in our study imply towards a healthy state of mitochondria and less dependency on non-mitochondrial respiration. Overall findings of the study suggest multipronged effects of CORM-A1 that benefits hepatocytes in sustaining lipid stress in multiple ways. Hence this study on CORM-A1 mediated improvement of mitochondrial function and favorable changes orchestrated via Nrf2 in steatotic liver is the first report that establishes the anti-NASH potential of CORM-A1.

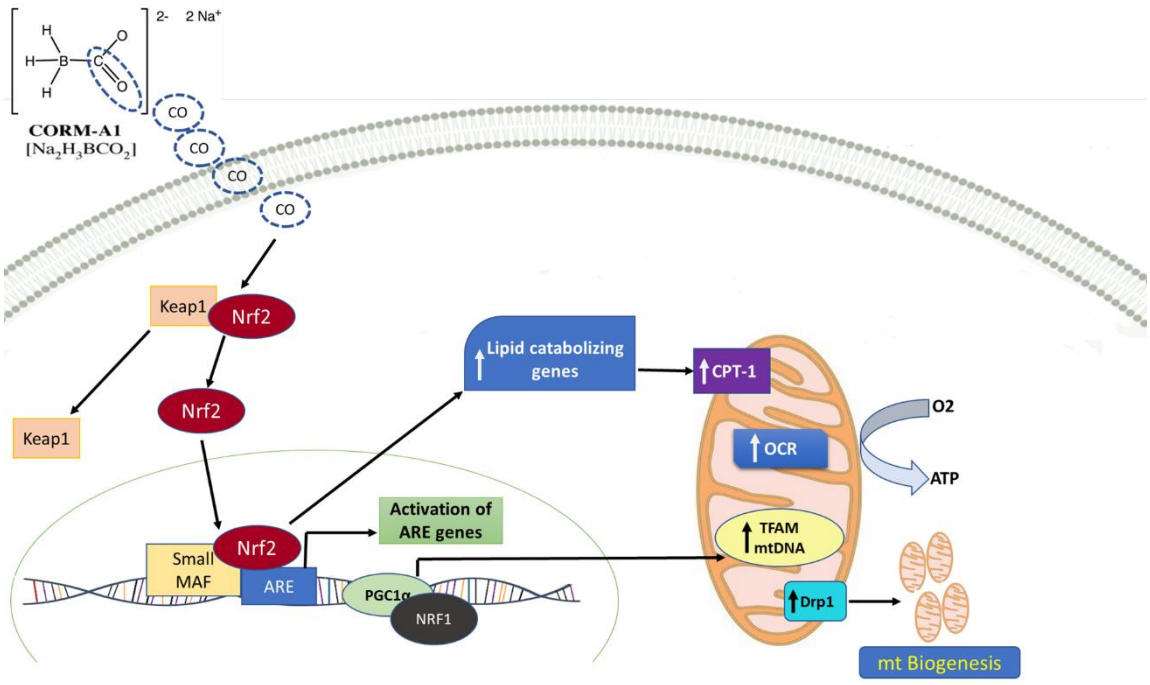


Fig.H. Summary-Chapter-3,4A&4B; indicating underlying mechanism of CORM-A1 mediated improvement in NASH.