Lifestyle disorders

Diseases orchestrating by disturbances in lifestyle are known as lifestyle disorders (LSD). Abuses like alcohol, drug, smoking, bad food habits, lack of physical activity, untimed food intake, disturbed circadian clocks, shift jobs etc. are accountable for initiations of LSDs (Boden-Albala *et al.*, 2000). People with sedentary lifestyle devouring high calorie diet and lacking adequate amount of physical activity are highly susceptible to LSDs. With changing scenario and initialization of industrialization, economic liberalization, invasion of mass media, food and drug availabilities, increasing packed foods, people are drawn towards a comfy life with bare minimum physical activities and unhealthy way of living. Western countries, especially the developed ones show huge amount of population getting LSDs, the trend is gradually setting-in in developing countries as well, including India (Mukhra *et al.*, 2018). The risk factors for LSDs can broadly be classified into the following

(i) Modifiable risk factors; that include ill habits like alcohol consumption, tobacco chewing, physical inactivity etc.

(ii) Non-modifiable risk factors; which cannot be altered based on will that includes age, gender, genetic makeup etc.

Over a period developing countries have observed marked increase in individuals falling victim of LSDs. The most common LSDs include heart disease, stroke, obesity, type II diabetes and chronic liver diseases including Nonalcoholic fatty liver diseases (NAFLD). Cases of liver diseases are increasing around the globe at an alarming rate with NAFLD being high on the charts.

Liver physiology

Liver is the second largest organ of the body, followed by skin. It weighs about 1.5 kg and holds about a pint of blood which is roughly 13% of the total blood volume. Physiologically liver consists of two main lobes. Each lobe has 8 segments that comprises of about 1000 small lobes also called lobules. Liver is a complex organ engaged in wide variety of functions by virtue of it being at the juncture of several metabolic processes. According to a survey conducted by Johns Hopkins institute liver has been attributed to be engaged in about 500 vital functions going on in the body. Right from production of bile, proteins, cholesterol to conversion of glucose to glycogen and back to detoxification liver does plentiful work for smooth physiological functioning (Boyer *et al.*, 1990).

Incessant steady increase is found in liver diseases across the globe. Liver diseases almost accounts for about 2 million deaths per year worldwide. US recognizes liver diseases as second leading cause of mortality whereas cirrhosis is attributed to rank eleventh for befalling mortality globally (Asrani *et al.*, 2018). These statistics add value in exploring new therapeutic and clinical interventions along with modifying the existing strategies for addressing the said disease.

Liver possesses the highest regenerative capacity amongst all the other organs. This regenerative capacity gets compromised based on type and intensity of insults. Liver injuries are broadly classified into acute and chronic liver injuries based on the duration or persistence. Acute liver insults are surmountable, providing immediate medications or eliminating the causative agent a complete restitution of normal liver with perfect architecture and functioning can be regained. Hepatitis,

coagulopathy, jaundice and hepatic encephalopathy are types of ALI with degeneration and necrosis being their hallmark characteristics. On contrary, the chronic liver diseases are marked by gradual liver damage over extended period of time. NAFLD, Progressive fibrosis and Nonalcoholic steatohepatitis (NASH) is the hallmark of chronic liver injury and can eventually result in liver failure or hepatocellular carcinoma.

Diverse causes of liver Injuries and Hepatotoxicity

Hepatotoxicity simply means liver damage owing to consumed toxicants, mainly in form of drug/chemicals. Overload of toxic chemicals and drug result in damaging the liver by creating prolonged hepatotoxicity.

Liver injuries can be of multiple types based on the causative agents. Virus induced liver injury or viral hepatitis is major form of liver hepatitis. There are five types of hepatitis namely, hepatitis A, B, C, D and E caused by HAV (hepatitis A virus), HBV (hepatitis B virus), HCV (hepatitis C virus), HDV (hepatitis D virus), HEV (hepatitis E virus) respectively. HAV and HEV are transmitted orally through contaminated food, drinks or by coming in contact with any infected object causing acute liver failure. HBV, HCV and HDV usually spread through infected blood and other body fluids.

Autoimmune reactions also result in causing liver injury. Like autoimmune hepatitis that causes liver inflammation and a severe form might also cause liver failure. This is more prevalent in females as compared to males (Manns *et al.*, 2010).

Primary biliary cholangitis is a liver disorder wherein bile ducts are the major target of immune attack. Bile ducts carry bile for digestion, damaged ducts ooze out bile inside the liver that develops scars. This is also more common in women than men. In primary sclerosing cholangitis the bile ducts are scared and eventually blocked. Bile builds up inside the liver, making functioning of liver a difficult task, a severe form might culminate to liver cancer. This is more prevalent in male than female (Beery *et al.*, 2014).

Drug-induced liver injury (DILI) is a major public health problem. Liver impairment in detoxification mechanism results in DILI. Acetaminophen (APAP) is an analgesic and an antipyretic drug which is converted to a toxic substance N-acetyl-p-benzoquinone imine (NAPQI) by cytochrome p-450, but such toxic metabolites get detoxified through conjugation with glutathione (GSH) (Jaeschke *et al.*, 2005). However, in condition of APAP overdose, NAPQI is secreted in access which depletes GSH levels and lead to liver damage. Drug induced liver injury is mainly subdivided into two categories: Intrinsic DILI and Idiosyncratic DILI.

1) Intrinsic DILI is dose dependent which directly affects the liver and causes hepatotoxicity within few days.

2) Idiosyncratic DILI is a caused due to an adverse drug reaction that is independent of administrated drug dose, route or time duration.

Mitochondrial dysfunction, oxidative stress, and bile acid homeostasis is mostly affected in response to Intrinsic and Idiosyncratic drug induce liver injury.

Another major cause of liver injury is alcoholic abuse. Binge alcohol consumption can cause scarring of the tissue. If the condition remains unaddressed for longer duration it can be lethal. Alcoholic liver diseases (ALD) are of three types; fatty liver, alcoholic hepatitis and liver cirrhosis. Fatty liver is the first stage of ALD, also known as steatosis. It is characterized by excessive fat accumulation in hepatocytes. This impairs smooth liver functioning and same is reversible if alcohol consumption is stopped.

Second stage of ALD is alcoholic hepatitis. It is characterized by liver inflammation and hepatocyte necrosis. This stage eventually progresses into absolute liver damage if alcohol consumption is not stopped. Jaundice is the most common symptom at this stage. Other common symptoms include nausea, vomiting, loss of appetite, weight loss, abdominal tenderness, fatigue and weakness.

Highly advanced and the last stage of ALD is liver cirrhosis. There is permanent scarring of liver tissue in this stage and is a severe and irreversible medical condition. Fluid accumulation in abdomen, spleen enlargement, bleeding from veins are the major symptoms of this pathological condition.

Further liver can also run into malfunction with excessive fat accumulation in absence of alcohol consumption. Hepatic steatosis is witnessed in both the cases, of binge alcohol consumption or in absolute alcohol-free physiology. Hepatic steatosis occurring in complete absence of alcohol is called non-alcoholic steatohepatitis (NASH). This is an irreversible condition and serious measures need to be taken for the same. Major concern is lack of availability of precise diagnostics and therapeutics. In prolonged period of NASH there is formation of excessive

fibrous connective tissue that stiffens the organ. The ultimate stage of liver damage is cirrhosis which develops after scarring of liver tissue.

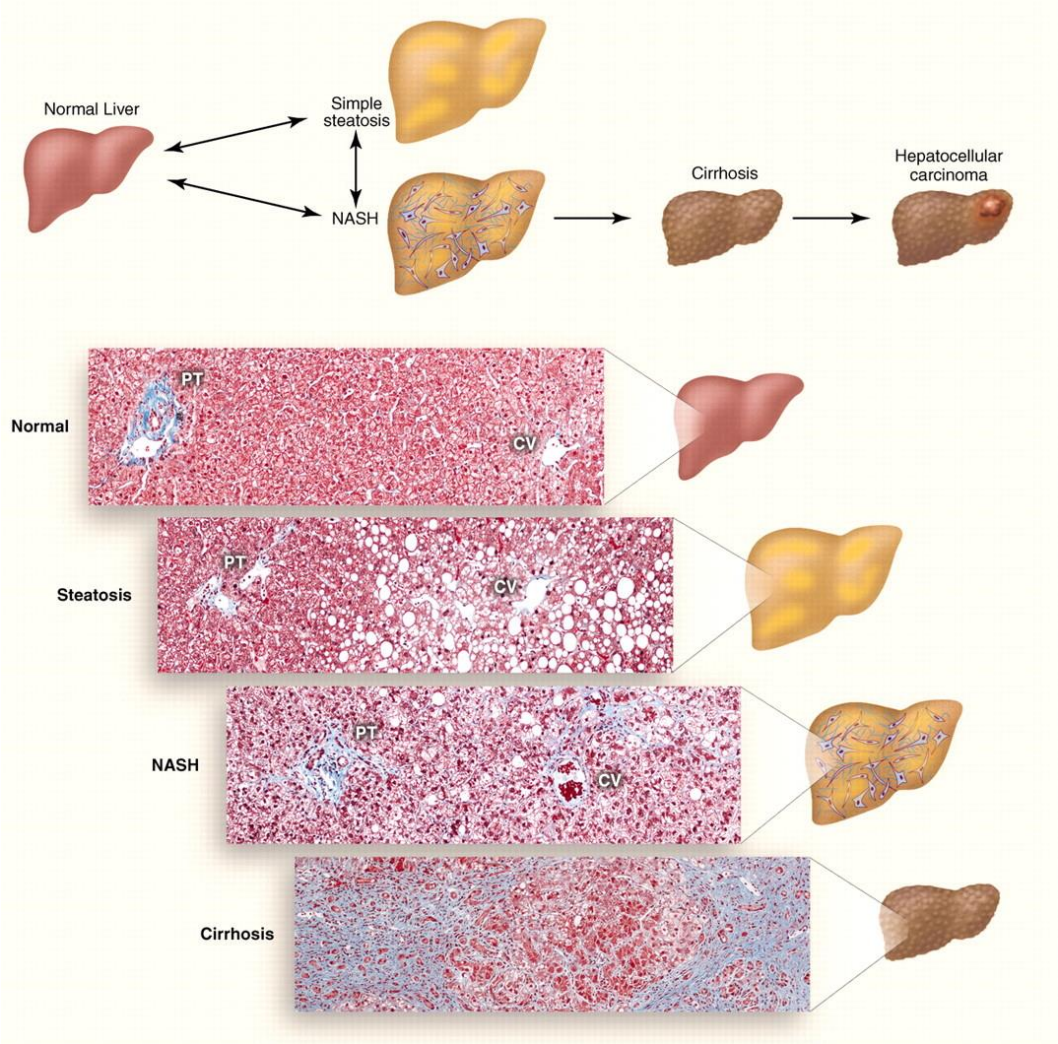


Fig A: Histological representation of liver at different pathological condition; Normal, Steatosis, NASH and Cirrhosis (Cohen *et al.*, 2011).

Nonalcoholic steatohepatitis (NASH): A growing concern globally

Ludwig first coined the term NASH in 1980 describing pathophysiological condition of fatty liver in non-alcoholic individuals (Ludwig *et al.*, 1980). NASH is characterized by liver inflammation and accumulation of lipids in non-alcoholic individuals. Prolonged NASH condition leads to liver cirrhosis. It took an elongated period of time till NASH gained attention and clinical importance. Asymptomatic nature of the disease is one of the major reasons as to why NASH diagnosis, treatment and scaling prevalence lacks clarity (Dabhi *et al.*, 2008). There exists a persistent increment in number of individuals being diagnosed with NASH in Asian countries. People suffering from obesity, insulin resistance (IR) are at high risks of getting NASH (Amarapurkar *et al.*, 2007).

NAFLD is one of the important causes of liver diseases that is the consequential effect of global pandemic of obesity. Global prevalence of NAFLD is about 24% with highest cases reported from South America (31%) and the middle east (32%), followed by Asia (27%), USA (24%) and Europe (23%) and the least observed in Africa (14%) (Lonardo *et al.*, 2016). Prevalence of NAFLD in USA when diagnosed with ultrasonography was 24.13%, on contrary diagnosis with other non-invasive methods culminated into giving 21% of score, suggesting that diagnosis of NAFLD solely based on blood testing or noninvasive method can lead to under-reporting of its true prevalence (Younossi *et al.*, 2016). Overall, prevalence of NAFLD is observing a constant increment from 15% in 2005 to 25% in 2010 which holds true for the cases of NASH as well, where the rates of increment have almost doubled from 33% to 59.1% (Lonardo *et al.*, 2016). With lack in availability of

therapeutants for NASH, it is now considered as second most common condition demanding for liver transplant after hepatitis C (Anstee *et al.*, 2013).

NAFLD has not only been restricted to obese individuals, the cases with non-obese individual suffering from NAFLD is also increasing over the years. There are several factors responsible for NAFLD in non-obese individuals viz. (Anstee *et al.*, 2015, Schneider *et al.*, 2014, Sherif *et al.*, 2016)

- Consumption of high fructose and high fat (HFHF) diet along with dual etiology (high fat diet consumption along with alcohol).
- Metabolically obese with lean phenotype.
- Genetic causes: PNPLA3 variants; congenital defects of metabolism like familial hypobetalipoproteinemia, lysosomal acid lipase deficiency.
- Endocrine disorders such as Polycystic Ovarian Syndrome (PCOS), hypothyroidism or deficiency in growth hormone.
- Drug related causes, effective by amiodarone, methotrexate or tamoxifen.

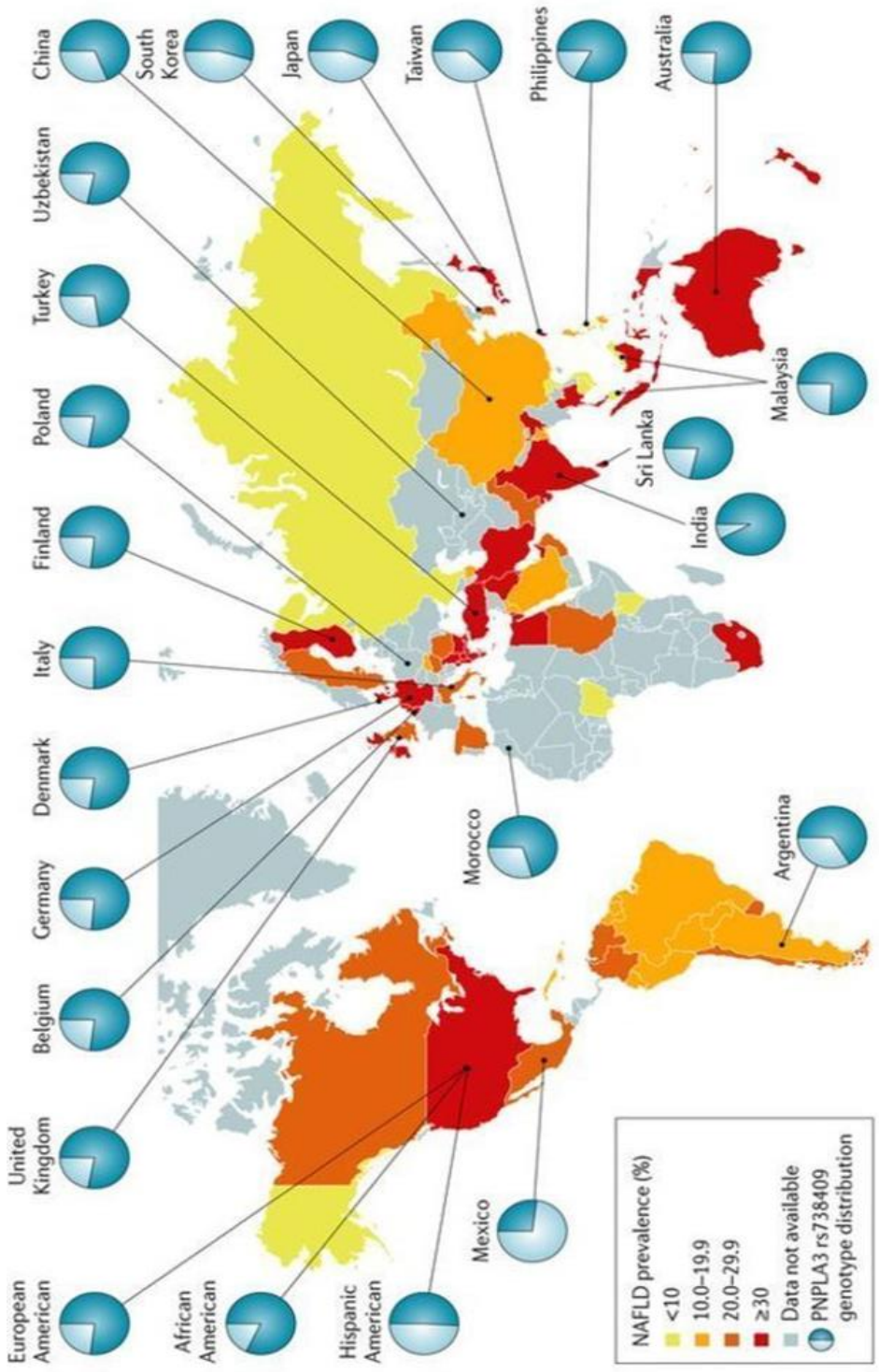


Fig B: Prevalence of NAFLD across the globe (Younossi *et al.*, 2018).

Lean NAFLD accounts for 20% population in India (Kumar *et al.*, 2013), 18.3% in China (Fan *et al.*, 2005), 15.2% in Japan (Nishioji *et al.*, 2015), 22.4% in south Korea (Kwon *et al.*, 2012),19.3% in Hong Kong which surmounts up to one third of that of obese individuals suffering from NAFLD.

21% of the individuals suffering from NAFLD were observed to have NASH in the US. The corresponding occurrence of comorbid conditions associated with NASH in these individuals has been reported to be: obesity in 82%; type 2 diabetes mellitus (T2DM) in 48%; hyperlipidemia in 82%; the metabolic syndrome in 76%; and hypertension in 70% of the population (Sherif *et al.*, 2016). NASH is not only prevalent and increasing in adults but also taking a serious hike in pediatric population. About 10% population in age group of 2-19 years shows NASH conditions. About 20% of pediatric population suffers from NAFLD in US, 44% in Italy and 75 % in China (Elizabeth *et al.*, 2019). The increment in cases of NAFLD and NASH rises an alarming situation specially owing to lack of precise diagnostic and therapeutic availabilities.

Pathophysiology of NASH

American Association for the Study of Liver Diseases (AASLD) describes NAFLD as fatty liver development in patients with alcohol intake less than 20 g per day (Neuschwander-Tetri *et al.*, 2003). NAFLD is histologically divided into nonalcoholic fatty liver (NAFL) and more severe condition succeeding it, NASH. The development of NASH is the result of a complex interaction between the environment, represented in the case of NAFLD by a sedentary lifestyle and

excessive intake of calories and individual predisposition that affects both the development of excess adiposity and its consequences. If these physiological conditions prevail for longer period of time, they advance into life threatening hepatic cirrhosis and hepatocellular carcinoma (Nakajima, 2012). Hepatocellular damage, inflammation and fibrosis are major characteristics of NASH. It is a multifactorial disorder wherein several overlapping physiological events simultaneously affect the organ ailment. Considering which, Day and James proposed a "two-hit" hypothesis explaining the pathophysiology of NASH, which was widely accepted (Day *et al.*, 1998). Herein, 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 and the subsequent vulnerability of the liver for second hits wherein inflammatory changes are key events leading to NASH and/or fibrosis (Cortez-Pinto *et al.*, 2006).

Second hit incorporate several sets of factors that involves as complex interactions between hepatocytes, stellate cells, adipose cells, Kupffer cells, inflammatory mediators and reactive oxygen species (ROS) driving NAFLD state to NASH. The cause of progression of NAFLD to NASH is yet unclear but several animal studies tend to suggest that the driving force is the formation of harmful adducts as by-products of fatty acid oxidation by mitochondria, peroxisomes or microsomes (Tilg *et al.*, 2010).

Further hepatic injury inflicted by oxidized by-products results into development of liver fibrosis. Multiple cells, cytokines and miRNAs are involved in

development of fibrosis and cirrhosis. Defenestration and capillarization of liver sinusoidal endothelial cells are major factors contributing to hepatic dysfunction in liver cirrhosis. Hepatic stellate cells get stimulated, when activated kuffer cells destroys hepatocytes. Physiologically a constant cycle of apoptosis and regeneration of hepatocytes is functional that gets disturbed owing to perturbed hostile environment. These repetitive events contribute to pathogenesis of cirrhosis.

Fatty Acid metabolism in steatotic Liver

Triglycerides are transported in very low-density lipoproteins or chylomicrons to liver and are degraded to FFA by hepatic lipase. Long chain fatty acids (LCFA) are capable of crossing phospholipid bilayers to enter into the cells and that was thought to be the only way of transport. However, recent investigations reveal a partial LCFA transport via membrane proteins called fatty acid transport proteins (Poirier *et al.*, 1996). Free fatty acid either circulating or generated by adipose tissue lipolysis under fasting conditions circulate in plasma is in a complex with albumin. The efficacy of this complex is found to be predisposed to hepatocellular uptake than the unconjugated form of fatty acid (Weisiger *et al.*, 1981). Several trans-membrane proteins are being associated in the transportation of plasma FFAs to the liver including plasma membrane FA binding protein, fatty acid transporter protein, caveolins and fatty acid translocase (FAT)/CD36. All, six Fatty Acid Transport Proteins (FATP1–6) have been identified in mammalian cells. FATP-1 is an insulin sensitive fatty acid transporter present in all the insulin sensitive tissue. FATP-2 and FATP-5 are expressed in liver and FATP-5 is liver specific transporter.

Deletion of FATP-5 resulted into significant reduction in LCFA and triglyceride uptake by hepatocytes (Martius *et al.*, 2014). Along with FATPs, caveolins are also responsible for lipid uptake. Caveolins are the membrane proteins found in caveoles of the membrane that play crucial role in protein trafficking and formation of lipid droplets. In all, there are three types of caveolins, caveolin 1-3. Caveolin-1 contributes majorly, the knockout study revealed significant decrement in hepatocyte lipid uptake (Ikonen, 2008). Out of all the proteins responsible for protein uptake, FAT/CD36 is highly studied. CD36 plays vital role in lipid uptake and cellular metabolism. Basal expression levels of CD36 is low but an increased expression is observed in NAFLD condition (Bieghs *et al.*, 2010). Further, expression of CD36 is also high in hyperinsulinemia, insulin resistance and NASH (Miquilena-Colina *et al.*, 2011). Membrane associated FA-binding protein (FABP) facilitates intracellular-extracellular fatty acid transfer. This family includes twelve FABP proteins and five FABP-5 like proteins. FABPs are identical to mitochondrial aspartate aminotransferase that functions in maintaining the cytoplasmic/mitochondrial nicotinamide adenine dinucleotide NADH/NAD ratio (Alves-Paiva *et al.*, 2018). Amongst FABP isoforms FABP1 is present in liver, also known as LFABP. Absence of LFABP abrogates fasting induced hepatic TG accumulation.

Free fatty acids have multiple fate once they reach to liver:

1) FFAs are converted into complex lipid species, followed by packaging into VLDL.
2) β-oxidation

3) stored as lipid droplets within hepatocytes.

Fatty acids undergo alpha (α), beta (β) and omega (ω) oxidation, wherein alpha and omega oxidation do not yield much energy. β-oxidation is a major source of energy in fed as well as fasting state. β-oxidation of FAs take place in the mitochondria as well as in peroxisome. Peroxisomal *β*-oxidation is mainly responsible for the metabolizing long and very long chain FAs. Whereas medium or short chained fatty acids like palmitic acid (PA), oleic acid (OA), and linoleic acid (LA) are β-oxidized in mitochondria. Peroxisome lacks TCA cycle and hence acetyl Co-A cannot be hydrolyzed to carbon dioxide (CO_2) and water. The first dehydrogenation step of β-oxidation is oxidation of acetyl CoA oxidase (Wanders *et al.*, 1999) that produces H_2O_2 rather than reduced NAD+. As the TCA cycle proteins are absent in peroxisome very less amount of ATP is produced in comparison to mitochondrial β-oxidation. Acyl-CoA oxidase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA de-hydrogenase, and 3-ketoacyl-CoA thiolase are the major enzymes involved in peroxisomal β-oxidation. The reactions further proceed via 2E-enoyl-CoA, a chiral 3-hydroxyacyl-CoA and 3-ketoacyl-CoA intermediates. In contrast, degradation of fatty acids with double bonds typically require the participation of auxiliary enzymes, including Δ3, Δ2-enoyl-CoAisomerase, Δ3, 5, Δ2, 4-dienoyl-CoA isomerase and 2,4-die-noyl-CoA reductase (Voet *et al.*, 1999).

In mitochondrial β-oxidation fatty acids are transported to mitochondrial matrix by carnitine palmitoyl transferase (CPT-1) which act as the rate limiting step in long chain fatty acid oxidation by regulating the entry of fatty acids into mitochondria (Kerner *et al.*, 2000). The end product of β-oxidation is acetyl co-A which is further

metabolized to carbon dioxide and water in TCA cycle to yield energy. In TCA cycle the electrons are transferred to flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide (NAD) resulting to the reduced form of these coenzymes. These reduced forms donated electron to electron transport chain (ETC) to drive ATP synthesis (Van den Berg *et al.*, 2002). The fatty acid molecules are progressively shortened by two carbons that detached the fatty chain to form the end product acetyl co-A by a series of dehydrogenation, hydration, and cleavage reactions.

Lipid overload observed in NAFLD conditions results into increased rate of β-oxidation that has been linked to development of oxidative stress in the cell. Intense overload of FFA and subsequent β-oxidation lead to ROS generation and hepatocellular stress. Hepatic lipid accumulation gets affected by activation/ inhibition of β-oxidation suggesting crucial role of mitochondrial β-oxidation in pathological conditions (Carmiel-Haggai *et al.*, 2005).

α-oxidation operates mainly for catabolism of branched chain fatty acids and takes place in peroxisome. Mainly the fatty acids with methyl (CH3) group at the beta-carbon undergoes this oxidation because this methyl group prevents β-oxidation of the fatty acid. α-oxidation mainly removes one of the carbon unit from α carbon from the carboxylic end in form of CO_2. The reaction requires O_2, Fe^{2+} and Ascorbic acid for efficient functioning. This reaction does not yield any ATP and hence no energy is obtained. Phytanic acid that is derived from phytol present in chlorophyll and animal fat is the substrate for the said oxidation. Phytanoyl Co-A

synthase, Phytanoyl Co-A hydroxylase, Lyase, Aldehyde dehydrogenase are the enzymes involved in α-oxidation.

ω -fatty acid oxidation takes place in ER and is a minor pathway for fatty acid oxidation. Studies suggest that a significant amount of fatty acids are catabolized via ω-oxidation in individuals with NAFLD. (Reddy and Rao, 2006). Major risk factor associated with ω-oxidation is generation of ROS and lipid peroxidation as the rate of consumption of NADPH increases that can reduce oxygen to superoxide or hydrogen peroxide. Microsomal oxidation is performed by the CYP-450 enzymes including CYP2E1 and members of the CYP4A. Higher levels of FFA is proposed factor for up-regulation of CYP enzymes that explains overexpression of CYP2E1 in NAFLD. However knockout studies showed that absence of CYP2E1 had no effect in decrement of diet induced steatohepatitis.

Cellular oxidative stress and NASH

Oxidative stress is accumulation of reactive oxidation species (ROS/RNS) and cellular inability to clear it. Prolonged period of persistent ROS and diminishing quantities of antioxidants within liver initiates progression from steatosis to NASH and eventually culminating into cirrhosis. Mechanistic progression from steatosis of NAFLD to necro-inflammatory state of NASH still lacks scientific clarity. However, increased ROS levels have been attributed to contribute majorly in progression of NASH pathology (Takaki *et al.*, 2013, Tariq *et al.*, 2014). Mitochondrial abnormalities alter the balance between pro-oxidant and antioxidant mechanisms leading to an increase of non-metabolized fatty acids in the cytosol as

a result of the blockade of fatty acid β-oxidation and the consequent induction of ROS production (Fromenty *et al.*, 2004). In addition to the formation of excess ROS, increased microsomal activity and peroxisomal fatty acid oxidation further worsens the oxidative stress in NASH.

Endoplasmic reticulum stress and NASH

Endoplasmic reticulum (ER) is a membranous network that runs in continuation of nucleus and is engaged in numerous amounts of work right from synthesis, folding and trafficking of a number of secretory trans-membrane and lysosomal proteins (Kaufman, 2002). ER is also important for lipid synthesis, carbohydrate metabolism, drug detoxification and is crucial organelle owing to its sensitivity to cellular homeostasis (Fu *et al.*, 2012). The mechanisms by which fatty acids contribute to liver injury are not completely deciphered. However, ER stress i.e. disruption in ER homeostasis is the proximal event in fatty acid induced liver injury which culminate into disruption of several cellular events (Pineau *et al.*, 2009). The signaling pathway activated by disruption of ER homeostasis and the unfolded protein response (UPR) is linked to lipid and membrane biosynthesis, insulin action, inflammation and cellular apoptosis (Zhang *et al.*, 2014). ER is the main site of lipid biosynthesis, it produces structural phospholipid and cholesterol along with non-structural triglycerol and cholesterol esters. Hence activity of such organelle is ought to be crucial in any disease related to lipid contents and metabolism. Induction of ER stress has been proposed as a potential mechanism enhancing hepatic steatosis via cellular energy status (Lake *et al.*, 2013). Oxidative stress

generated primarily in NASH causes lipotoxicity in the cell where ER is the principal target organelle (Han *et al.*, 2016). Further, it is learned that saturated fatty acids (SFA) are more potent in causing ER stress than unsaturated fatty acids (FAs). SFAs do not convert to triglycerides easily, thus they are left freely circulating which culminate into their absorption by ER and eventually disturb ER homeostasis (Mantzaris *et al.*, 2011). Palmitic acid (PA) altered ER lipid membrane composition resulting in higher degree of saturation that precedes apoptosis (Zhang *et al.*, 2011).

Studies also unveils association of ER and mitochondria in conditions of liver disorder. On imposing PA stress to the cells ER passes the calcium flux to mitochondria resulting into mitochondrial permeability transition (MPT) (Demaurex *et al.*, 2003). Collective falling off of mitochondria, ER, proteins (increased amount of unfolded proteins because of ER stress) and cellular oxidative stress results in failure of activation of downstream recovery pathways along with activation of c-jun-N-terminal kinase (JNK) resulting in cellular inflammation and apoptosis (Swanton *et al.*, 2003, Szegezdi *et al.*, 2006). Considering the functional status, ER is attributed to play a significant role in NASH pathophysiology.

Mitochondrial stress and dysfunction in NASH

Mitochondria addresses to the cellular need of energy, the organelle is involved in several processes including ATP production, β-oxidation, maintaining cellular redox status etc. (Bjørndal *et al.*, 2018). Mitochondrial dysfunction in NASH has been addressed as one of the major cellular event promoting the pathology

(Pessayre *et al.*, 2005). The major event getting affected is ATP production (Cortez-Pinto *et al.*, 1999), mainly because of alterations in the abundance and activity of oxidation and the phosphorylation (OXPHOS) proteins (e.g., complex I, III and V) (Pérez-Carreras *et al.*, 2003); decreased ATP levels causes ER stress that further culminates into increased load of unfolded proteins. The resultant UPR activation is linked to the initiation of de novo lipogenesis pathways that further aggravates steatosis. Moreover, incorrect protein folding, e.g., in Apo B, an essential protein for very-low density lipoprotein (VLDL), may impair lipid export from the liver and exacerbate steatosis in mice (Uchiyama *et al.*, 2006). Likewise, disturbed protein folding leads to failure of activation of several cellular defense mechanisms because of deficit in protein availability. Chronic activation of UPR results in hepatocyte death via activation of CHOP-dependent signaling pathway (Willy *et al.*, 2015). Uncoupling of the OXPHOS and increased free radical production and lipid peroxidation causes cellular injury (Ruiz-Ramírez *et al.*, 2016). High cellular ROS causes lipid peroxidation of mitochondrial membranes leading to impaired mitochondrial function and perpetuate the ROS generation. Persistent oxidative stress triggers production of inflammatory cytokines, initiate inflammation and fibrogenic response. In NASH patients, depletion of mitochondrial GSH (mtGSH) is linked to the higher accumulation of cholesterol (Gan *et al.*, 2014) that may be caused by the impaired transport of mtGSH from the cytosol to the mitochondria due to cholesterol-induced alterations in membrane permeability. Increased mitochondrial cholesterol accumulation is related with the progression of steatosis to steatohepatitis. Prolonged cholesterol accumulation and ROS culminates into

opening of mitochondrial permeability transport pore (MPT) and that seems to play a critical role in hepatocyte death (Yin *et al.*, 2015). High cholesterol has also been shown to activate TNF-α and Fas induced hepatocyte apoptosis.

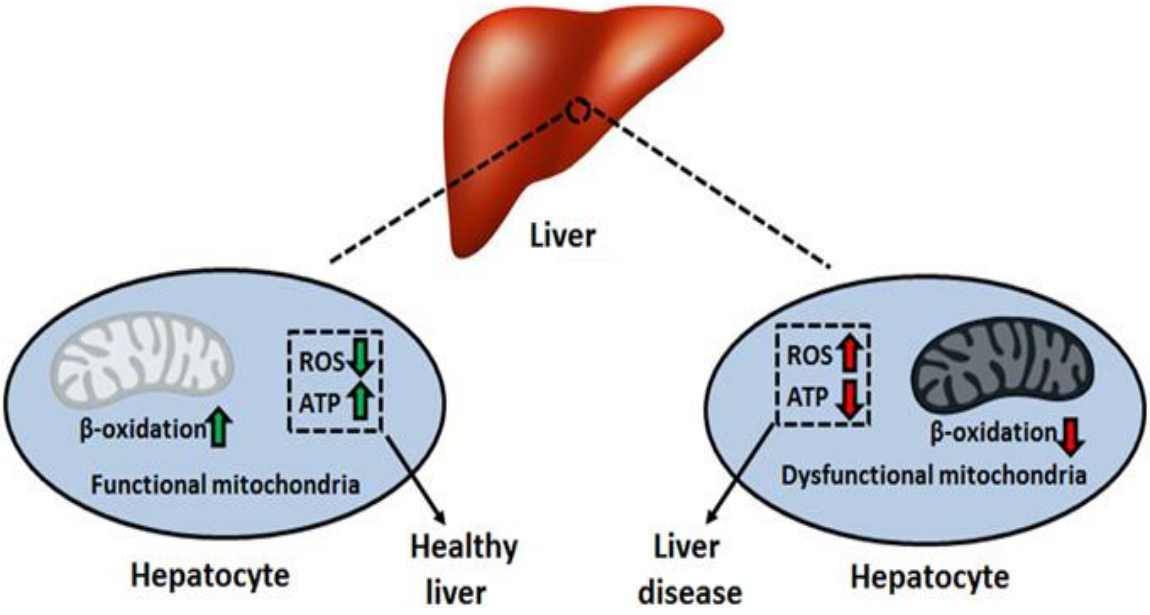


Fig C: Physiological status of hepatocytes in healthy and NASH liver (Auger *et al.*, 2015).

Inflammatory changes in liver and progression of NASH

Inflammation in the body is vital response of immune system against infection, injury or any sort of damage. Lipid accumulation and resultant inflammatory response precedes steatosis in NASH (Tiniakos *et al.*, 2010). A lot of research is been employed in addressing the delinquent inflammatory response. TNF-α resides at the juncture of several pathways, especially the ones leading to inflammation. Investigations reveal utility of metformin, an antidiabetic drug, to inhibit hepatic TNF-α expression and thus improving steatosis (El-Lakkany *et al.*, 2016). TNF-α inhibition by infliximab is also proven to be beneficial against alcoholic hepatitis (Koca *et al.*, 2008). There are several other cellular targets that have been ventured into for defending inflammation. Loss of Kupffer cells also leads to hepatic steatosis probably via decreased interleukin-10 (IL-10) release (Leroux *et al.*, 2012) and several others. Also, there is involvement of a diverse ailment including toxic lipids, nutrients and other gut-derived and adipose-derived signals in representing inflammatory insults. Overall in the pathological system hepatic steatosis may be considered as ‘‘bystander phenomenon’’ subsequent to inflammatory attacks.

Diagnosis of NASH: The enigma and available therapeutic options

There are several challenges yet need to be addressed when it comes to pathology of NASH. One of the major and the most critical is diagnosis of NASH pertaining to its asymptomatic nature. Though investigations have identified some common characteristic exhibited in NASH that includes right upper quadrant pain, fatigue, malaise and hepatomegaly on physical examination. Along with that the non-invasive tests have also been developed for identification of steatosis and fibrosis in NASH. Further, analysis of most common and classical parameters of measurement of Alanine Transaminase (ALT), Aspartate Transaminase (AST) and γ-glutamyltranspeptidase (γGT) levels is also employed for diagnosis purpose. In addition to these tests, recently clinicians have also started using imaging techniques such as ultrasonography, Magnetic Resonance Imaging (MRI) and Computed Tomography (CT) for diagnosing. However, liver biopsy remains the gold standard method for the diagnosis of severity of NASH. The doors to introduction and development of NASH specific diagnostics still remains wide open (Chalasani *et al.*, 2012, Lewis *et al.*, 2010).

Lipid accumulation and overall circulating lipid levels are vital target for therapy of NASH. Physical exercise and diet improvement help in reducing the overall magnanimity of the disease. The modest weight loss of about 3% results into overall improvement of 10% in steatosis (van der Windt *et al.*, 2018). For improvement in inflammation and regression of pathology, higher amount of weight loss is required with calorie deficit diet. Further, several lipid lowering drugs have been employed for efficient management of hyperlipidemia and obesity. But these drugs barely

affect NASH pathology (Nakamoto *et al.*, 2009), pertaining a major challenge for pharmaceutical industry. However, on other hand herbal medicines are gaining popularity because of minimal side effects and effective management of obesity, NASH, hyperlipidemia and insulin resistance (Jadeja *et al.*, 2014). Herbal medicines are also being given as a combinational therapy for effective control over pathology along with pathophysiology. *Alisma orientalis, Acanthopanax senticosus, Sida rhomboidea Roxb*, etc. are being studied for effective control of NASH and have proven to be efficient (Hong *et al.*, 2006). In intend of scavenging the free radicals, multiple approaches have been employed. Vitamin E has great antioxidant potential, it can easily donate hydrogen atom from hydroxyl group to neutralize free radicals and prevent lipid peroxidation. However, when tested experimentally, multiple clinical trials failed in exploiting significant effect of vitamin E on transaminases, inflammatory conditions and fibrotic pathology (Sanyal *et al.*, 2010). N-acetylcystine (NAC), a precursor of GSH is also a potent antioxidant. NAC has been studied independently and in combination with drugs like metformin as therapeutants in NASH (De Oliveira *et al.*, 2008). S-adenosylmethionine and betain are known to restore GSH levels along with reduction of transaminases, hepatic steatosis and fibrosis. Also, probucol, viusid and silibinin are other couple of antioxidants evaluated for their anti-NASH potential (Pappachan *et al.*, 2017).

Other pharmaco-therapeutic options available, include drugs targeting specific cellular proteins. Peroxisome proliferator-activator receptors (PPARs) agonists, are one such therapeutants (Tanaka *et al.*, 2006). PPARs are the nuclear receptors that

play important role in several metabolic pathways like β-oxidation, lipid transport, gluconeogenesis and are present in wide number of tissues. PPAR agonists like fibrates are successfully used in treating hypertriglyceridemia but failed in alleviating NASH. Further dual PPAR α/δ agonist, elafibranor was successful in resolving NASH in phase IIb randomized double-blind placebo controlled trial (Ratziu *et al.,* 2016). Extensively used PPARγ agonists as insulin sensitizers in the form of Thiazolidinediones are effective in the treatment of NASH. A dual PPARα/γ agonist, saroglitazar which is approved to treat diabetic dyslipidemia in India also shows an effective decrement in ALT levels in subjects with NAFLD and biopsy-proven NASH (Jain *et al.,* 2018).

Lipid altering agents form another big class of therapeutics for NASH. Deficiency of Stearoyl-CoA desaturase (SCD), an enzyme that catalysis rate limiting step in synthesis of monosaturated fatty acid is known to reduce hepatic steatosis (Narce *et al.,* 2012). Obese subjects suffering from NASH tend to have higher levels of SCD than lean ones. Aramchol is a SCD-1 inhibitor and is under evaluation in multicenter phase IIb trial against NASH (Allen *et al.,* 2018). Statins are highly popular for their effective plasma lipid lowering abilities. Statins are HMG-CoA reductase inhibitors that are widely used in primary and secondary prevention of cardiovascular diseases. Dyslipidemia is common etiology of both the pathologies, statins are tried as therapeutants for NASH as well.

Further there are incretin-based therapies where hormones like Glucagon-like peptide 1 (GLP-1) are exploited for their potential of having wide metabolic functionalities including delayed gastric emptying, appetite suppression, enhanced

liver glucose uptake and peripheral insulin sensitivity. GLP-1 receptor agonists, exenatide and liraglutide showed beneficial effects in alleviating NASH. It lowers ALT levels, helps reducing weight and cures other metabolic syndromes. Dipeptidyl peptidase 4 (DPP-4) inhibitors act on the enzyme DPP-4 that is known to rapidly degrade GLP-1. Thus, these medications are expected to prolong the action of GLP-1 (Aso *et al.,* 2012).

There is other class of medication that focuses on oxidative stress, cell injury, inflammation and apoptosis in hepatocytes. TNF-α hold the center for several pathways mediating hepatocyte cell injury and caspase-regulated programmed cell death in NASH and hence stands up to be a potent target for therapeutic implication. Pentoxifylline (PTX) a methylxanthine derivative imparts inhibitory effect on phosphodiesterase as well as on TNFα and hence could modulate the functions of other inflammatory cytokines (Lee *et al.,* 2008). Several inflammatory chemokines like CCL2 (MCP1), CCL5 (RANTES) are released by the organ that attracts macrophages to the site. Cenicriviroc is a CCR2/CCR5 antagonist that controls the recruitment and downline events of the pathology (Friedman *et al.,* 2018).

Another class of drug is antifibrotics. Liver fibrosis is the strongest predictor of mortality in NAFLD patients. Simtuzumab is a monoclonal antibody that targets LOXL2 that is a key matrix enzyme in collagen formation and is abundantly expressed in liver. This drug is under clinical trial for both cirrhotic and non-cirrhotic patients (Ikenaga *et al.,* 2017). GR-MD-02 is a Galectin-3 inhibitor that currently is in two phase II clinical trials in NASH patients with fibrosis/cirrhosis (Harrison *et al.,* 2016). Gelatin-3 is expressed predominantly in immune cells that

binds to galactose residues. It is an essential protein in liver fibrogenesis and thus a good target of the inhibitor. Inspite of all the trials and tests till date, there is no FDA approved treatment for NASH and hence therapeutic interventions yet remain open for researchers to dive in.

Gasotransmitter as a therapeutant: Carbon monoxide (CO)

Gasotransmitters are gaseous signaling molecules that are produced endogenously. CO, H_2S and NO are gasotransmitters naturally being produced in the body. Carbon monoxide (CO) is the colorless and odorless gas that is toxic in nature. Structurally, CO has covalent triple bond which imparts chemical stability to the molecule. CO is not readily water soluble. Its solubility in water is about 3.54mL/100mL ~44.3 ppm by mass. Extra-physiologically CO is produced as byproduct of incomplete combustion of fuels (Piantadosi, 2002), higher amount of CO in environment is considered to be dangerous as it binds to hemoglobin forming carbon monoxide hemoglobin (COHb). Enthalpy of COHb is about 210 to 250 times higher than that of oxygen bound to hemoglobin (OHb), resulting into inefficient and decreased transportation of O_2 in physiological system (Ryter *et al.*, 2006).

Physiologically, CO is endogenously produced by degradation of heme by heme oxygenase (HO). Heme oxygenase is present is three isoforms; HO-1 (inducible) and HO-2 and HO-3 (constitutive). It works as the prosthetic group of various proteins including several enzymes of oxidoreductase nature. HO transforms heme to biliverdin that is further converted to antioxidant bilirubin (Wu *et al.*, 2005). A healthy individual produce about 20 μmol of CO/h (Johnson *et al.*, 2003).

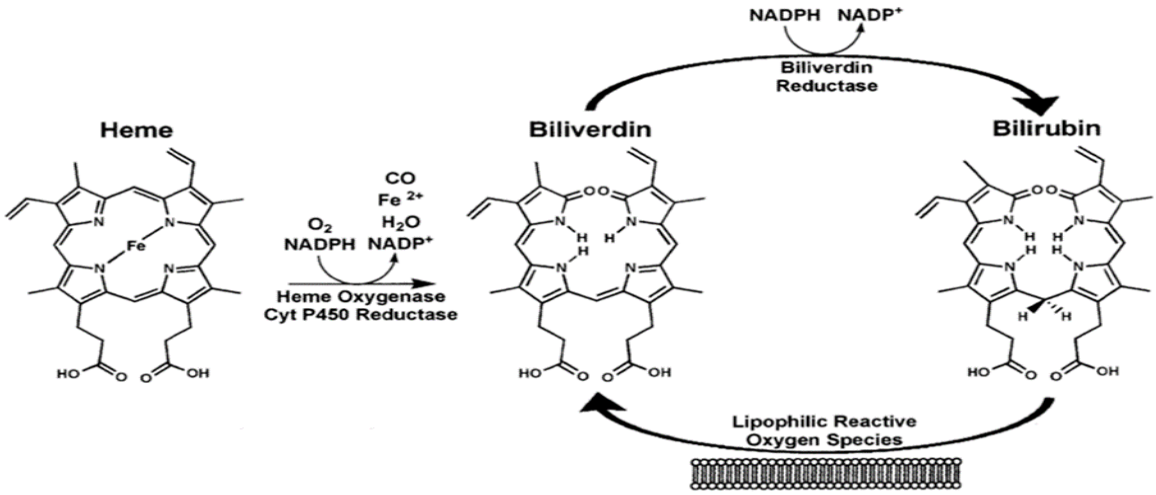


Fig D: Metabolic pathway representing endogenous CO production via heme degradation.

If pathological levels of CO increases beyond the range it can induce both acute and chronic health hazards. CO is considered as a 'silent killer' due to its strong affinity for haemoglobin and as an environmental pollutant (Motterlini *et al.*, 2010). But it is important to pay heed to the fact that every mammalian cell expresses HO enzymes and thus possess the potential to generate CO. CO has crucial physiological roles in the system. Experimental investigations pertaining to the role of CO were conducted using HO-1 deficient models. But it turned out that the *Hmox1* knockout mouse is embryonically lethal (Poss *et al.*, 1997). More than 95% of all fertilizations resulted into death. The mice that do survive exhibits a shortened life span. These models showed organ anomalies and dramatic sensitivity to any sort of stress.

Even a mention of CO as plausible therapeutant draws great attention and intrigues people, though other gasotransmitters like NO and H_2S are already in use as therapeutants. NO is routinely uses as a therapeutants in infants suffering from pulmonary hypertension (Bloch *et al.*, 2007). Whereas H_2S is in early stage of clinical development targeted as an pharmaceutical agent (Szabó, 2007). In comparison to NO and H_2S, CO turns out to be with higher potential because of its chemical nature. It is highly unlikely for CO to react indiscriminately with multiple intracellular targets like other gasotransmitters. CO is stable and possesses high affinity towards transitional metals (Boczkowski *et al.*, 2006). CO targets metals in their particular redox state making it highly specific and potent molecule to be exploited for targeted therapy.

Carbon monoxide releasing molecules (CORM's)

Carbon Monoxide Releasing Molecules (CORMs) are constructed and have gained wide popularity amongst scientific fraternity and clinicians. The concept of using chemically bound form of CO with gradual physiological release was developed by Motterlini and co-workers about a decade ago. This concept eventually turned out to be a big success and opened wide scopes for utilizing CO as a therapeutants. They coined the term CORM, as CO was released by these molecules in a controlled manner (Motterlini *et al.*, 2002). Haloalkanes, aldehydes, oxalates and silacarboxylic acids along with several other organic compounds were highly explored for their potential as CORM but it all failed because of their undesirable release rates and toxicological profiles (Mann, 2010, Romao *et al.*, 2012). Subsequently carbonyl complexes were explored widely, mainly because of amendable binding of CO to transition metal centers. CORM-1 and CORM-2 are already commercialized metal carbonyl compounds. They are insoluble in water and require photolysis to release CO. But CORM-1 is highly toxic in nature. There was still a need for developing a molecule that could enact with ease at physiological level, which was finally satisfied with discovery of CORM-3. It was a water-soluble molecule and was highly investigated in biological systems but a major flaw that pertained to its failure was rapid release of CO. Release of CO from CORM-3 is $t_{1/2}$ <1 min at 37°C, pH= 7.4 (Clark *et al.*, 2003), hence it took the backseats, leaving search for bio-compatible and bio-efficient CORMs open. This was fulfilled by discovery of CORM-A1.

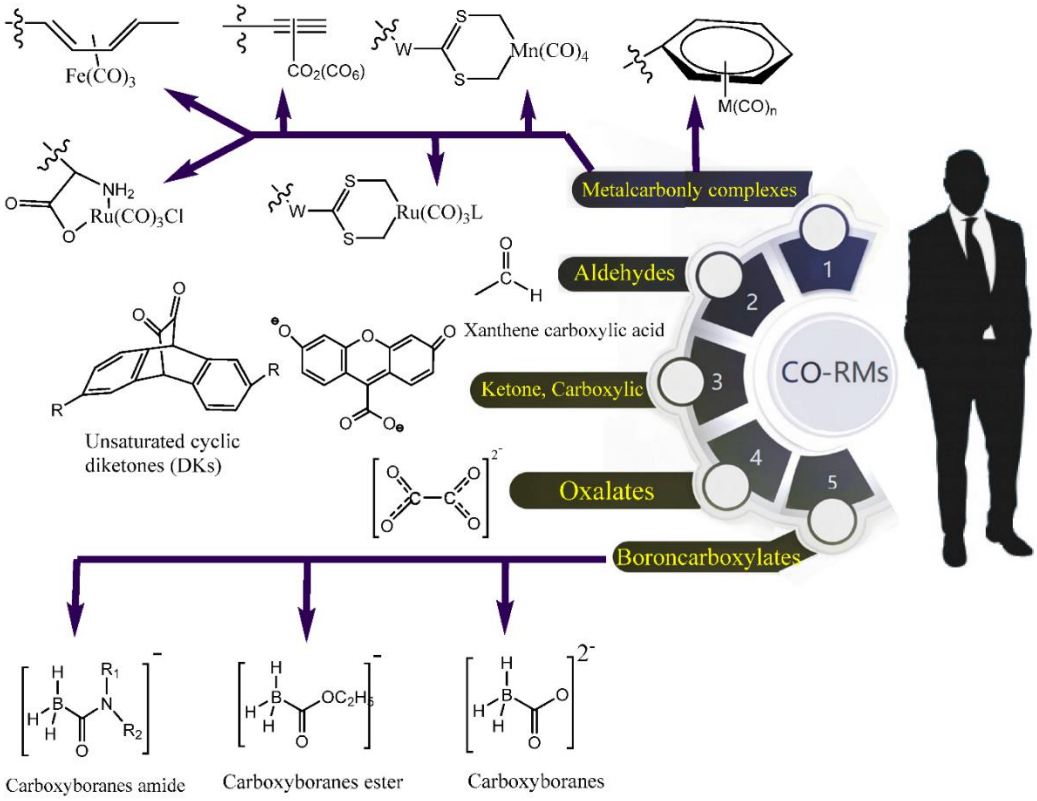
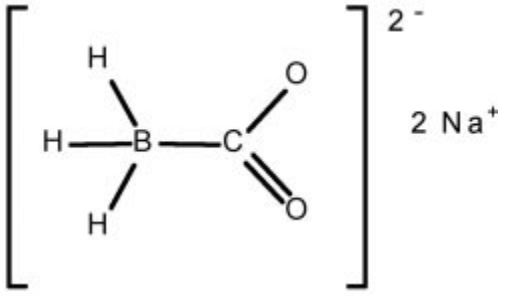


Fig E: Various CO-releasing molecules (CORMs) formulation associates with different functional groups (Faizan *et al.*, 2019).

Carbon monoxide releasing molecule-A1 (CORM-A1): The key player in our study

After a series of discoveries, CORM-A1 was formulated that fulfilled all the criteria required for biological studies. It is highly water-soluble compound that possess boron core, chemically named as sodium boranocarbonate $Na_2[H_3BCO_2]$ and otherwise known as CORM-A1. Unlike the conventional transition metal carbonyl, CORM-A1 has a carboxylic group that is converted into CO during hydrolysis. It is a slow releaser of CO at 37°C with a half-life of $t_{1/2}$ = 21 min (Motterlini *et al.*, 2005). CORM-A1 has widely been investigated for its therapeutic potential against several pathological conditions including LSDs (Motterlini *et al.*, 2010).



CORM-A1
$[Na_2H_3BCO_2]$

Fig F: chemical structure of CORM-A1 with boron base.

CORM-A1: Physiology and pharmacokinetics of CO

CO is not metabolized, instead it binds to the targets reversibly. This property makes pharmacodynamics and pharmacokinetic studies simpler and by well-defined respiratory physiology and oxygen dissociation curves. CO diffusion across the circulatory system is limited pertaining to lack of partial pressure changes in blood. However, it has complete access to the entire tissue enabling it to affect the cellular functions. Extermination of CO from mammalian system happens only through exhalation via lungs and no other metabolism. Tolerable CO load is 3 mg per kg for a 1 h inhalation where COHb reaches a peak of 14.3% which is the upper limit. Post CO instillation COHb levels were identical in rodents as in humans with maximal amount present in liver, spleen and heart. The reason being that maximal erythrocyte turnover along with large heme pool occur in the said organs (Dercho *et al.*, 2006). Preclinical toxicology tests suggest no undesirable effect of CO at concentrations of CORMs that were effective in rodent model. Efficacious concentrations of CO in animal model is about COHb levels <18–20% and more so over no adverse effects were not observed in a clinical trial evaluating CO to prevent lung inflammation. (ClinicalTrials.gov identifier: NCT00094406).

CORM-A1: Therapeutic potentials and the basis of our study

Anti-inflammatory properties of CO are widely studied in several type of animal models. Chronic pathologies like cardiovascular disease, diabetes, cancer and obesity along with innate body response in acute conditions share similar underlying consequence of inflammation and hence it is rational to imply towards

CO as a therapeutant (Sawle *et al.*, 2005). CO is known to reduce the production of pro-inflammatory cytokines like TNF-α and IL-1β and furthers the release of anti-inflammatory cytokines like IL-10. When tested in mice model of cerebral malaria, where inflammation in the brain drives much of the morbidity and mortality, CO completely prevented brain edema with 100% survival of the animal model (Pamplona *et al.*, 2007). In models of carrageenan induced inflammation, CO administration results in reduced leukocyte rolling, adhesion and neutrophil migration into the inflammatory site of the mesenteric microcirculation with a mechanism partially dependent on GC activation (Freitas *et al.*, 2006, Urquhart *et al.*, 2007). In a sepsis model, administration of CORM-2 or CORM-3 resulted in a reduced migration of polymorphonuclear leukocytes and an increased survival rate in mice (Mizuguchi *et al.*, 2009). Further CO has also been tested in models of osteoarthritis that is major inflammatory related disorders (Ferrandiz *et al.*, 2008). CORM-3 administration suppresses manifestations of collagen induced arthritis. Mechanistically CO reduces the levels of PGE-2 and many other inflammatory mediators in the joint that results in a better preservation of cartilage tissue and bone structures. CORMs are also found to be effective in vascular inflammation (Watkins, 2010), autoimmune neuroinflammation (Chora *et al.*, 2007), sickle cell disease (Beckman *et al.*, 2009, Watkins, 2010), diabetes (Di Pascoli *et al.*, 2006) and acute hepatitis (Zuckerbraun *et al.*, 2003).

CO has found to be very effective in vascular disorders mainly because of easy mode of delivery. The delivery just includes unloading from hemoglobin directly to the endothelium and smooth muscle. Short-term administration of CO is known

to be protective against vascular injury. Intravenous injection of CO-saturated saline produces vasodilatation and improved microvascular hemodynamics possibly via increased cardiac output and local cGMP content. Clinicians recommend CO as beneficial molecule in preventing arteriosclerotic lesions that occur following aorta transplantation (Otterbein *et al.*, 2003). This was tested experimentally where aortic transplantation in HO-1 deficient mice resulted in 100% mortality but CORM-2 treatment improved survival by about 62%. CORM-3 when induced intravenously before reperfusion, reduces infract size, fibrillation and tachycardia (Chen *et al.*, 2009). CORMs exploit potassium channels for orchestrating cardioprotective effect, as minor amounts of CORMs are lost in the presence of inhibitors of mitochondrial ATP dependent potassium channels. CO is also known to prevent intimal hyperplasia by arresting hyperproliferative vascular smooth muscle cells (Zheng *et al.*, 2009). CO is also known to increase mobilization and recruitment of bone-marrow derived progenitor cells to the denuded vessel for enhancing reendothelialization (Watkins, 2010). In vascular endothelial cells effect of CO is highly dependent on NOS and NO that involves modulation of RhoA and AKT signaling pathways. Whereas in vascular smooth muscle cells the effect is independent of NO and involves cGMp and p38 mitogen activated protein kinases. CO and CORMs have been widely investigated in a variety of biological system.