MASTER'S THESIS
Faculty of Science and technology (TNW)

Metabolic Reprogramming of Macrophages Using Liposomal Itaconate to Resolve (NASH) Inflammation

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Lastly, I want to thank my family and friends for supporting me during this time.

Abstract

Non-alcoholic fatty liver disease (NAFLD) and its progressive form non-alcoholic steatohepatitis (NASH) is globally becoming the leading cause liver morbidity. The pathophysiology of NASH is multifactorial, however innate immunity is a major contributing factor. It is evident that there is an increased infiltration of inflammatory macrophages in NASH. During inflammation, macrophages switch their metabolic mode to fulfil the high energy requirement, primarily to a glycolytic phenotype from an oxidative phenotype. This metabolic reprogramming produces intermediate metabolites that support the macrophage phenotype reprogramming in response to external stimuli. There is a lot of potential in using these metabolites to create an immunomodulatory effect. One such metabolite of interest is itaconate. This project utilizes the anti-inflammatory effects of itaconate and its derivative 4 octyl itaconate to mitigate NASH inflammation. Itaconate is an intermediate metabolite of the tricarboxylic acid cycle (TCA). Major downside of itaconate is that it is membrane nonpermeable while 4 octyl itaconate is highly hydrophobic. Furthermore, itaconate or its derivatives are rapidly cleared from the body via renal excretion thus limiting their application for NASH. To overcome these challenges, this project proposes for the first time the development of liposomal sodium itaconate formulation which not only allows cell permeability but also provide liver macrophage targeting due to their physical chemical characteristics. In addition to this 4 octyl itaconate solid lipid nanoparticle formulation was also developed to enable IV administration and to enhance pharmacokinetic. A head-to-head comparison of these formulation for their anti-inflammatory effects was performed. The results show that liposomes are an efficient delivery carrier for sodium itaconate as it showed significant downregulation of pro-inflammatory cytokines iNOS, IL-1β and IL-6 in inflammatory murine macrophages compared to free sodium itaconate. 4 octyl itaconate lipid nanoparticles also showed significant downregulation of pro-inflammatory cytokines. Both formulations indicated a macrophage polarization mechanism, especially sodium itaconate liposomes, as they show upregulation of the anti-inflammatory marker IL-10. In conclusion, a successful formulation was prepared, and it showed a promising therapeutic effect against inflammation, proving to be an innovative approach to mitigate chronic inflammation associated with NASH.

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List of abbreviations

Abbreviation	Full form
4OI	4 octyl itaconate
4OI LNP	4 octyl itaconate lipid nanoparticle
Aa	Amino acid
ALD	Alcoholic liver disease
AMPK/FGF21/PPARδ	5' adenosine monophosphate-activated protein kinase/ Fibroblast growth factor 21/ Peroxisome proliferator activated receptor gamma
ATF3	Activating transcription factor 3
BMDM	Bone marrow derived macrophages
BSA	Bovine serum albumin
CAD	Cis aconitate decarboxylase
CCL4	Calcium tetrachloride
CDNA	Complimentary deoxyribonucleic acid
COX-1 & COX-2	Cyclooxygenase -1 & -2
DAMP	Damage associated molecular patterns
DMI	Dimethyl itaconate
DMSO	Dimethyl sulfoxide
DPPC	Dipalmitoylphosphatidylcholine
DSPE-PEG	1, 2-Distearoyl-sn-glycero-3-phosphoethanolamine-Poly(ethylene glycol)
FADH	Flavin adenine dinucleotide
FAO	Fatty acid oxidation
FAS	Fatty acid synthesis
FMNH	Flavin mononucleotide
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
HCC	Hepatocellular carcinoma
HIF-1α	Hypoxia inducible factor 1 alpha
HO1	Heme oxygenase 1
HPLC	High performance liquid chromatography
IFN-γ	Interferon- gamma
IL-1β	Interleukin-1 beta
IL-6	Interleukin-6
iNOS	Inducible nitric oxide synthase
IRG1	Immune responsive gene 1
IκBζ	Inhibitor of nuclear factor kappa B zeta
KEAP1	Kelch like ECH-associated protein 1
LPS	Lipopolysaccharide
MDB	Mallory-Denk bodies
MPS	Mononuclear phagocyte system
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
Na-ITA	Sodium itaconate
Na-ITA_Lip	Sodium itaconate liposome

NASH	Non-alcoholic steatohepatitis
NED	N-1-naphthylethylenediamine dihydrochloride
NO	Nitric oxide
NQO1	Nicotinamide adenine dinucleotide plus hydrogen quinone oxidoreductase 1
Nrf2	Nuclear factor erythroid 2 related factor
NSAID	Non-steroidal anti-inflammatory drugs
PAMP	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PDI	Polydispersity index
PPP	Pentose phosphate pathway
qPCR	Quantitative polymerase chain reaction
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPMI1640	Roswell park medium institute1640
SDH	Succinate dehydrogenase
T_C	Transition temperature
TCA	Tricarboxylic acid cycle
TEM	Transmission electron microscope

1. Introduction

1.1. Non-alcoholic steatohepatitis: pathogenesis

Liver diseases are rapidly becoming a major health concern. Accumulation of excessive fat is the common factor underlying the two most common and emerging causes of chronic liver disease, alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). Today NAFLD represents the most common cause of chronic liver disease in developed countries with a global prevalence of 25% among adults. [1] (Fig.1) Since the last decade the prevalence of NAFLD has been increasing by 0.7% each year.[2]

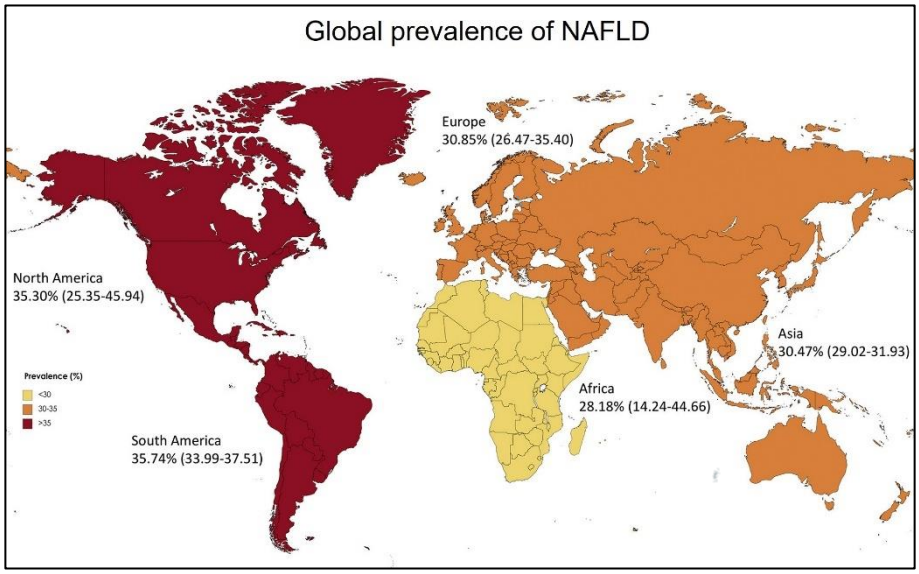

Figure 1: Global prevalence of NAFLD *diagnosed by ultrasound categorized by continent in 2019, by a systemic review and meta-analysis.* [2]

Non-alcoholic fatty liver disease has a spectrum ranging from fatty liver to steatohepatitis, fibrosis, and cirrhosis. (Fig.2) The first step in the development of NAFLD is represented by fat accumulation in the liver, a condition that is commonly associated with features of metabolic syndromes, such as obesity, and type 2 diabetes. Simple steatosis aka fat accumulation is considered a more benign condition but the progress to NASH is associated with a higher risk of cirrhosis and hepatocellular carcinoma (HCC) development. Certain factors such as triglycerides, cytokines released by adiponectin, trigger the inflammation thereby fueling the transition from NAFL to NASH. Non-alcoholic steatohepatitis (NASH) is a significant form of chronic liver disease. It can be defined as the liver manifestation of a metabolic disorder and is the most severe form of NAFLD. NASH is a progressive disease and a prime cause of liver-related mortality globally.[3], [4] The prevalence of NAFLD is closely related to the prevalence of obesity and both these processes are linked to insulin resistance.

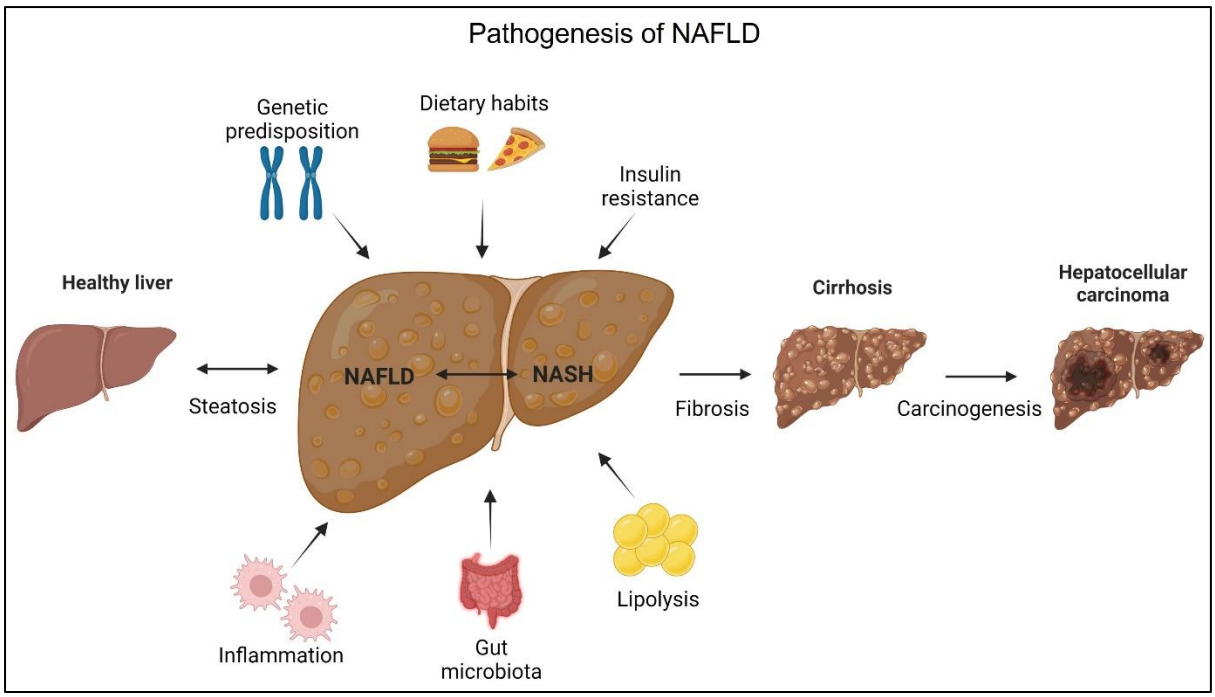

Figure 2: Development and progression of non-alcoholic fatty liver disease. *The first step is the accumulation of fat in the healthy liver which is defined as the first hit. Then progresses to NASH due to certain factors such as excessive lipid accumulation, reactive oxygen species, increased infiltration of immune cells (monocytes), which is deemed the second hit. This further develops into fibrosis, liver cirrhosis, and/or hepatocellular carcinoma. Created with Biorender.com*

Liver biopsy remains the gold standard for the diagnosis of NAFLD. As discussed above, inflammation is a major driver of NAFLD to NASH, there is spectrum of histological lesions that associates with hepatocyte injury, inflammation, and fibrosis.[5] However, liver biopsy comes with challenges such as it is expensive and it represents only a minute area of the organ, also the diagnosis from the biopsy can be subjective to the pathologist.[6]

1.2. Pathogenic drivers for NASH progression: chronic inflammation and oxidative stress

Due to the inefficiency to remove excess lipids, the liver is overloaded with triglycerides in the form of lipid droplets in hepatocytes leading to hepatic steatosis. Steatosis makes the liver more susceptible to inflammatory cytokines, oxidative stress, and mitochondrial dysfunction. In addition, excessive fat intake during obesity enhances liver uptake of fatty acids and glycerol from the circulation, which makes the liver exhibit insulin resistance.[7] Systemic insulin resistance is the major driver of hepatic steatosis in NAFLD. Lipotoxicity of accumulated lipids along with activation of the innate immune system are major drivers of NASH. Innate and adaptive immune mechanisms are the main drivers of inflammation that recognize damage- and pathogen-associated molecular patterns (DAMPS and PAMPS) and contribute to the progression of the inflammatory cascade.[8]

Although inflammation is important in the eradication of pathogens; unresolved, chronic inflammation that occurs when the causative agent is not removed, is detrimental to the host, resulting in tissue damage, and fibrosis.[9] Therefore, inflammation can be considered a major target in NASH. Targeting inflammation can be a promising resolution strategy as NASH is characterized by robust recruitment of immune cells into the liver where they become

activated and can release pro-inflammatory cytokines. Liver inflammation can have triggers originating outside and inside the liver, such as in adipose tissue and gut and lipotoxicity.[10]

The liver has one of the largest resident populations of macrophages, natural killer cells, and dendritic cells, all of which are key components of the innate immune system. As mentioned previously the innate immune system recognizes cell damage and pathogens through PAMPs and contributes to the progression of the inflammation associated with NASH . Activated macrophages produce inflammatory cytokines and chemokines that contribute to injury and inflammatory necrosis in hepatocytes.[11], [12]

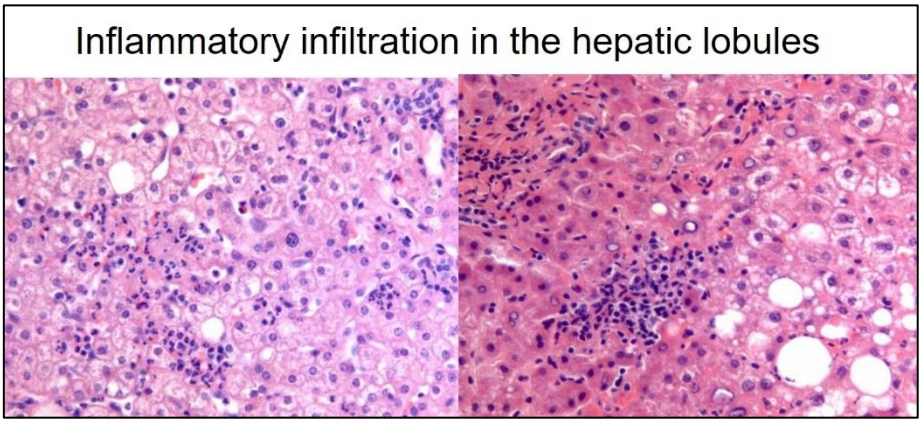

Figure 3: Increased infiltration of inflammatory macrophages is a characteristic of NAFLD. *Mononuclear inflammatory infiltration in the hepatic lobules and/or the portal veins. Neutrophils are usually observed around ballooned hepatocytes. This leads to the initiation of chronic inflammation, which is the main driver of NASH.[Images adapted from*[13]]

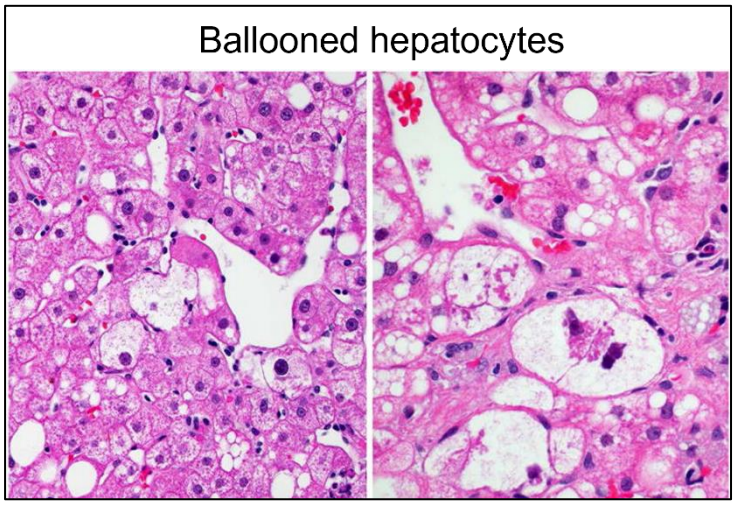

Figure 4: Ballooned hepatocytes are a characteristic of injured hepatocyte. *The ballooned hepatocyte is the characteristic of an injured hepatocyte. Ballooned hepatocytes may or may not have Mallory-Denk Bodies. Ballooning including inflammation is the sign of progression to NASH and fibrosis.[Images adapted from*[14]]

The liver is composed of small division termed the liver lobules. The liver lobules are categorized into three metabolic zones. The metabolic zones, Zones 1, 2 and 3; Zone 1 hepatocytes encircle portal tracts that are exposed to nutrient- and oxygen-rich blood. Zone 3 hepatocytes are located around the central veins and are responsible for glycolysis, lipogenesis, and biotransformation reactions. Zone 2 hepatocytes are located between zones 1 and 3. From histopathology, it can be inferred that inflammatory infiltrates can be seen in

hepatic lobules or the portal tract.[13] (Fig.3) Clusters of mononuclear cells including T cells and macrophages infiltrate into hepatocytes. Neutrophils may surround the ballooned hepatocytes. The ballooned hepatocytes are generally larger than the surrounding hepatocytes and have distinctive cytoplasm that is irregularly stranded and clumped. Ballooned hepatocytes containing Mallory-Denk Bodies (MDB) significant ballooning is a major sign of progression from NASH and further to fibrosis and cirrhosis. [14], [15] (Fig.4)

Hence it can be concluded that NASH is a result of inflammation and hepatocyte injury. It is increasingly becoming a burden to end stage liver disease, therefore there has been significant increase in the number of studies investigating new drug for more effective management of NAFLD.

1.3. NASH pharmacotherapy: an ambiguous domain

There are presently no approved pharmacologic agents for the treatment of NAFLD and NASH, but numerous agents are currently being investigated in phase II and III clinical trials. The current methods of treatment focus primarily on dietary and lifestyle modifications, as obesity can be traced as the root cause of these liver morbidities. To provide a more therapeutic impact, targeting a causative agent is preferred. As discussed above NAFLD/NASH is an emerging global epidemic and the major cause of chronic liver disease and liver-related mortalities. The identification of triggers of inflammation is central to understanding the mechanisms in NASH development and progression and in designing targeted therapies that can halt or reverse the disease. Therefore, targeting the inflammation can serve as a potential strategy to resolve NASH.

The prominent anti-inflammatory drugs available in the market can be divided into steroids and non-steroidal anti-inflammatory drugs (NSAIDs)

Glucocorticosteroids or corticosteroids are a class of drugs structurally like the endogenous hormone cortisol, they exert a highly potent anti-inflammatory effect.[16] The anti-inflammatory effects are mediated by either genomic or non-genomic mechanisms. They have high bioavailability and hence are prone to off-targets; therefore, the use of corticosteroids for the management of systemic inflammation results in more complex effects. They provide a global effect than a targeted effect, hence leading to adverse effects upon continuous usage.[17] The study by T Kuo et al and Lillegraven Siri et al. all lead to the conclusion that glucocorticosteroids increase serum glucose levels through an increase in hepatic glucose production and changes in insulin production and resistance.[18], [19] Hence, glucocorticosteroids bring out more detrimental effects in NASH than blocking the inflammation.

NSAIDs function by inhibition of cyclooxygenase enzymes (COX-1 and COX-2) and reduce pain and inflammation by restraining the formation of prostaglandins. Due to the reduction of prostaglandins production in the gastrointestinal mucosa, NSAIDs can cause gastric damage and compromise cardiovascular safety.[20] Moreover NSAIDs are not potent enough to tackle chronic inflammation. As the regular use over an extended period, the potential side effects may increase.

There has been a significant increase in the number of studies investigating new drugs for more effective management of NAFLD. However, for now no drugs have been approved for treating NASH and treating this disease remains a major unmet clinical need. Multiple phase

II and III clinical trials are ongoing, some drugs are described in Table 1. These drugs target almost all steps in the pathogenesis of NAFLD and NASH, aiming to improve insulin resistance, glucose, and lipid metabolism, to inhibit lipogenesis and delivery of lipids to the liver, and to influence apoptosis, inflammation and fibrogenesis.[21]

Agent	Mechanism of action	Treatment/intervention	Patients	Phase	ClinicalTrials.gov identifier
Elafibranor	Dual PPARα and PPARδ agonist	Elafibranor (120 mg) *vs.* placebo	NASH (NAS>4) and F2/F3	3	NCT02704403
Saroglitazar	Dual PPARα and PPARγ agonist	Saroglitazar (1,2 or 4 mg) *vs.* placebo	NASH and/or NAFLD (biopsy or noninvasive)	2	NCT03061721
Lanifibranor	pan-PPAR agonist	Lanifibranor (800 mg and 1,200 mg) *vs.* placebo	NASH (biopsy)	2	NCT03008070
Liraglutide	GLP-1 receptor agonist	Liraglutide (0,6–3 mg) *vs.* exercise + diet *vs.* bariatric surgery	NASH (biopsy or noninvasive)	3	NCT02654665
Semagultide	GLP-1 receptor agonist	Semagultide (0.1, 0.2, 0.4 mg) *vs.* placebo	NASH (biopsy)	2	NCT02970942
Dapagliflozin	SGLT inhibitor	Dapagliflozin (10 mg) *vs.* placebo	NASH and DM (biopsy)	3	NCT03723252
Pegbelfermin	FGF21 analog	Pegbelfermin (3 doses) *vs.* placebo	NASH and F3 (biopsy)	2	NCT03486899
Cenicriviroc	CCR2/CCR5 antagonist	Cenicriviroc (150 mg) *vs.* placebo	NASH and F2/F3 (biopsy)	3	NCT03028740
Tropifexor	FXR agonist	Tropifexor monotherapy *vs.* combination with cenicriviroc	NASH with F2/F3 (biopsy)	2	NCT03517540
Resmetirom	Thyroid hormone receptor β agonist	Resmetirom (80 mg, 100 mg) *vs.* placebo	NASH (NAS>4)	3	NCT03900429
Firsocostat	Acetyl-CoA carboxylase inhibitor	Firsocostat *vs.* fenofibrate, cilofexor, selonsertib	NASH (F2/F3 and F4/cirrhosis)	2	NCT02781584, NCT03449446

***Table 1**: Drugs in phase 2 and 3 clinical trials* [21]

These drugs have a distinctive signature and targets hence target a plethora of causative agents of NASH as described before. The drug Elafibranor showed a resolution of NASH but no improvement in fibrosis was observed. Another drug Firsocostat showed a significant reduction of hepatic de novo lipogenesis in a 12-week study but unfortunately failed to meet the efficacy threshold by the FDA.[22] Therefore, more potent alternatives or combination strategies are desired.

Considering inflammation as a potential target in NASH, drugs for tackling chronic inflammation are the need of the hour. An innovative approach for therapeutic effects can be by targeting the immune metabolism as many studies suggest that targeting the metabolic pathways of immune cells is a potent approach to regulate immune responses, providing a novel and effective therapy for inflammation.[23]

1.4. Targeting immune metabolism: an innovative anti-inflammatory strategy

Immunometabolism has emerged as a major mechanism central to innate and adaptive immunity. Immunometabolism refers to the changes that occur in intracellular metabolic pathways in immune cells during activation.[24] Early observations of induction of pro-inflammatory cytokines in obese adipose tissues contributed to metabolic disorders the way for metabolism and immunity was paved and the links to these two were studied extensively thereafter.[25] It is well known that macrophages are essential innate immune cells that contribute to host defense during infection and inflammation.[26] During inflammation, macrophages switch their metabolic mode to fulfil the high energy requirement. Inflammatory macrophages release a high level of pro-inflammatory cytokines, such as IL-6, IL-1β and TNF-α. There is a switch to glycolysis from oxidative phosphorolysis in inflammatory phenotype macrophages (M1 phenotype) to accommodate the high energy requirement due

to the high secretion of pro-inflammatory cytokines and this is termed the Warburg effect. [27] (Fig.6) Inflammatory macrophages significantly increase the glucose uptake to fuel aerobic glycolysis and, producing increased lactate and ATP production.[28] There are 5 major pathways used by macrophages to provide energy including glycolysis, Tricarboxylic acid cycle (TCA), Pentose phosphate pathway (PPP), Fatty acid synthesis (FAS), and Fatty acid oxidation (FAO) and amino acid (Aa) metabolism. These pathways are highly interconnected and are tightly regulated in macrophages. (Fig.5) In addition to generating energy, macrophages also produce intermediates metabolites that support their phenotype reprogramming in response to external stimuli. These intermediate metabolites also play a significant role in cellular and molecular signaling.[26]

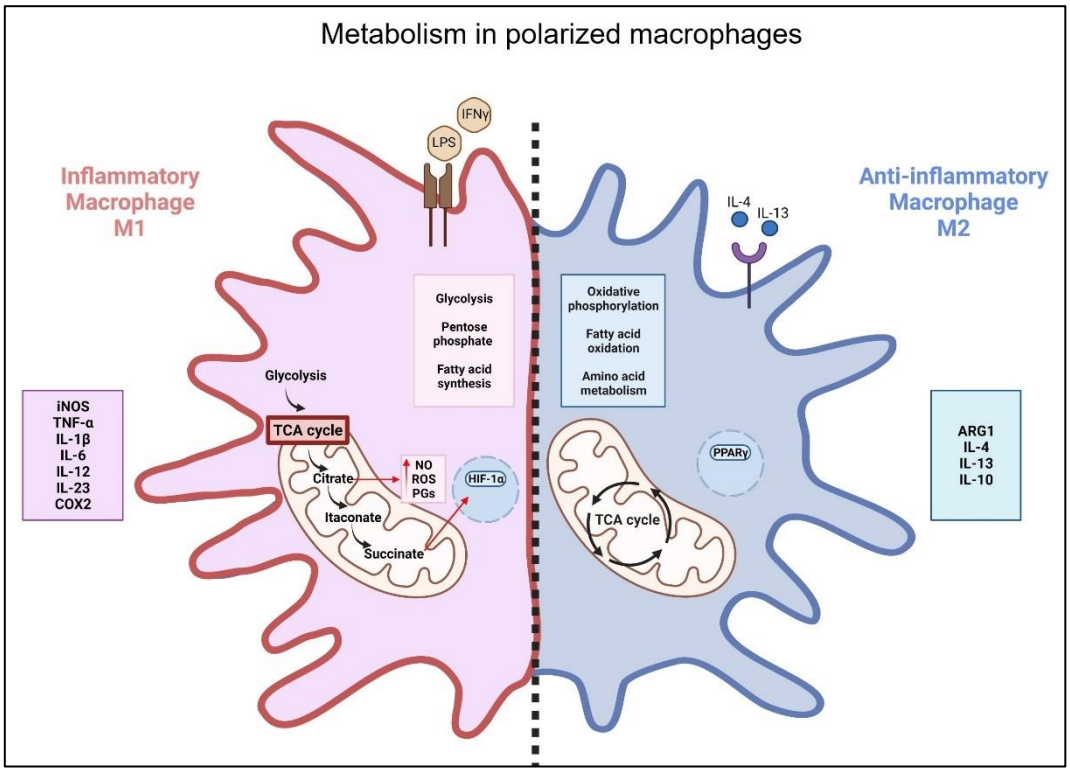

Figure 5: Metabolic reprogramming in macrophage repolarization. *Inflammatory macrophages characteristically increase glucose uptake to fuel aerobic glycolysis resulting in lactate production. The HIF1α transcription factor also becomes activated and can drive production of pro-inflammatory cytokines. Anti-inflammatory macrophages are not metabolically active and utilize an intact TCA cycle and OXPHOS. These key metabolic differences between differentially activated macrophages are widely accepted. Blocking oxidative metabolism not only blocks the M2 phenotype but also drives the macrophage into an M1 state. Similarly, forcing oxidative metabolism in an M1 macrophage potentiates the M2 phenotype. Created with Biorender.com*

As discussed above the metabolic requirement for the two phenotypes of macrophages are different due to the energy demands, hence it is an established concept that the use of small molecules and intermediated metabolites can be used to manipulate the repolarization of macrophages.[23] The TCA cycle provides a highly efficient means of generating ATP, hence the prochoice for metabolism for the M1 inflammatory phenotype. (Fig.6) The Warburg effect previously mentioned leads to metabolic remodeling in the metabolic levels of the TCA cycle. The TCA cycle occurs in the mitochondrial matrix, for a full turn of the cycle to be completed, a series of eight enzymatic reactions is required. However, in response to LPS or LPS in combination with IFN-γ, a reprogramming of the TCA cycle is observed, which includes the accumulation of certain metabolites. These metabolites either can be pro-inflammatory or

anti-inflammatory.[29] The TCA cycle is disrupted in two locations, at isocitrate dehydrogenase and at succinate dehydrogenase. As a result, citrate and succinate accumulate and drive such functions as fatty acid and NO synthesis, antimicrobial itaconate production, and HIF1α activity to promote further glycolysis and inflammatory cytokine production.[28] (Fig.7) Overall, studies have shown that citrate and succinate have pro-inflammatory properties, whereas itaconate, α-ketoglutarate, and fumarate are linked more with anti-inflammatory effects. Of all the metabolites, fumarate, particularly the prodrug dimethyl fumarate, has been established as a therapeutic agent as it is in the clinical trial for Multiple sclerosis.[30] Another metabolite in study is itaconate. Itaconate shows an anti-oxidative, anti-inflammatory, and anti-bacterial properties. [31]

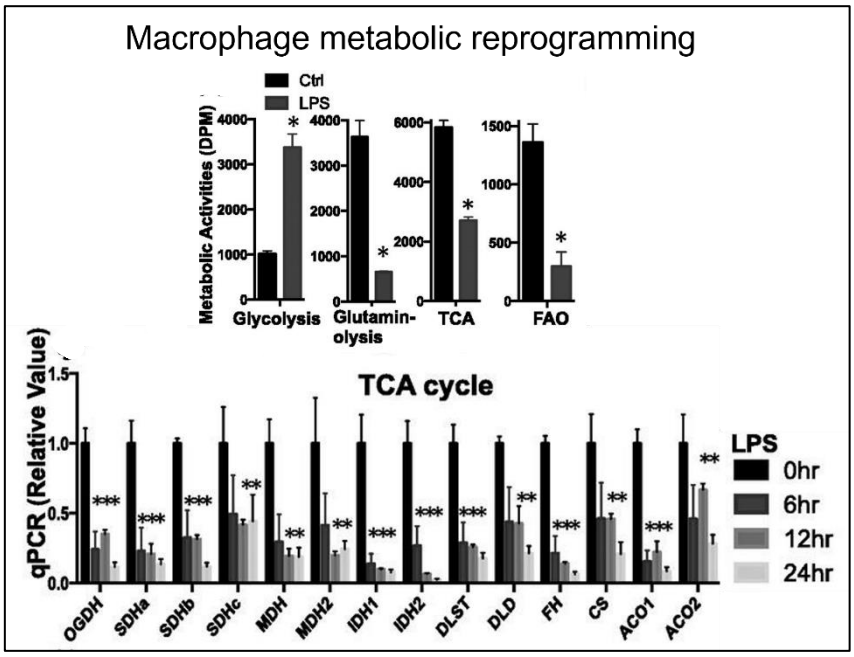

Figure 6: Proinflammatory stimulation differentially drives macrophage metabolic reprogramming. *A high rise in glycolysis is seen in LPS activated BMDM and dysregulated TCA cycle. The gene expression of TCA cycle metabolic genes at different stimulation time with LPS. It is observed there is a downregulation in SDH and IDH, which are the two breakpoints in TCA cycle of M1 macrophages.*[32]

Hence glycolytic metabolism is prominent in M1 macrophage and oxidative metabolism is prominent in M2 macrophage. Blocking oxidative metabolism not only blocks the M2 phenotype but also drives the macrophage into an M1 state. Similarly, forcing oxidative metabolism in an M1 macrophage potentiates the M2 phenotype.[33] Therefore, the prospect of changing the phenotype of an inflammatory cell into an anti-inflammatory one by targeting a specific enzyme of a metabolic pathway in exciting and may show success in an event of chronic inflammation.

1.5. Itaconate: a metabolite of interest

As stated earlier upon LPS stimulation the Krebs cycle is the predominant pathway for metabolic requirements. This leads to the metabolic reprogramming in the macrophages relating to the production or inhibition of certain metabolites. One such metabolite of interest is itaconate. Itaconate is synthesized by the decarboxylation of cis-aconitate by the protein IRG1/ACOD1 coded by the gene IRG1.[34] (Fig.7)

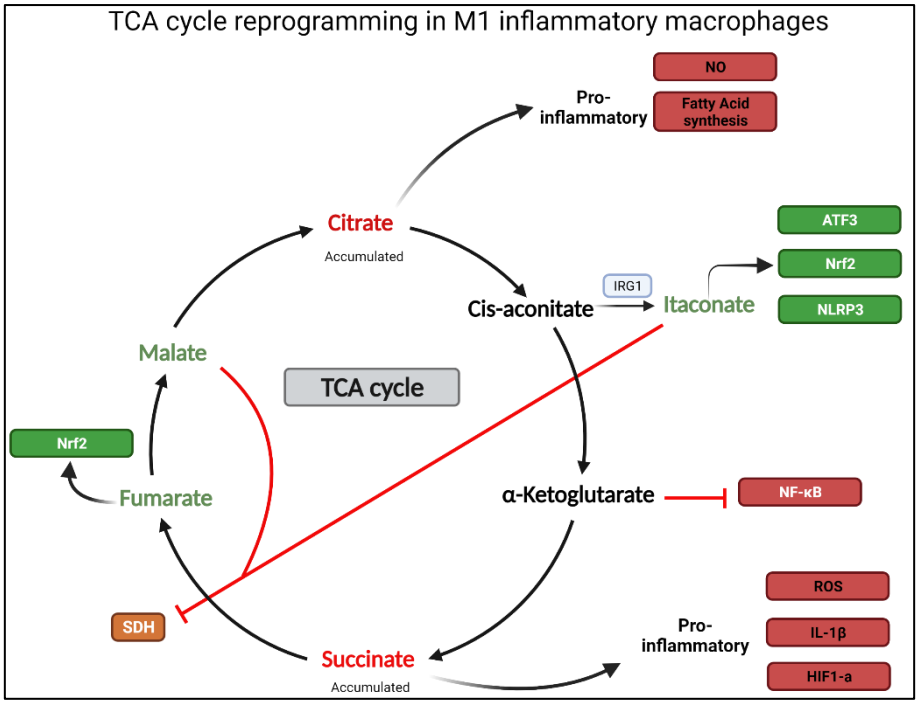

Figure 7: TCA cycle of M1 macrophage. *A dramatic rewiring of the Krebs cycle in macrophages is seen, as identified by the accumulation of the Krebs cycle intermediates which are involved as either pro-inflammatory or anti-inflammatory. Itaconate is a metabolite of the TCA cycle, produced from the decarboxylation of cis-aconitate by the enzyme cis-aconitate decarboxylase. It has been studied to show anti-bacterial and anti-inflammatory effects. TCA-The citric acid cycle IRG1-Immune responsive gene 1, NO-Nitric oxide, ATF3- Activating transcription factor 3, Nrf2-Nuclear factor erythroid 2 related factor, NLRP3-, ROS-, NFκB-, IL-1β, HIF-1α. Created with Biorender.com*

Itaconate is a progressively studied metabolite and it has been proved to show anti-bacterial properties, and immunoregulatory/immunomodulatory properties by showing anti-inflammatory properties and it is electrophilic.

Itaconate follows different pathways to show anti-inflammatory effects. Notably Inhibition of succinate dehydrogenase (SDH); Inactivation of Kelch like ECH-associated protein 1 (KEAP1); Inactivation of inhibitor of nuclear factor kappa B zeta (IκBζ).[35] (Fig.8) Itaconate has been shown to inhibit SDH. Succinate oxidation by SDH induced the production of pro-inflammatory factors, notably IL-1β, and suppressed the production of anti-inflammatory factors. Succinate oxidation also leads to the generation of reactive oxygen species (ROS). Itaconate directly inhibits the enzymatic activity of SDH thereby increasing succinate accumulation and limiting ROS production. Itaconate inactivates KEAP1 by alkylating certain cysteine residue and liberates the antioxidant transcription factor nuclear factor erythroid 2-related factor 2 (NFE2L2 or NRF2), which is free to initiate transcription of antioxidant genes such as those encoding Nicotinamide adenine dinucleotide plus hydrogen quinone oxidoreductase 1 and Heme oxygenase 1 (NQO1 and HO-1). Activation of an antioxidant program in this manner, reduced hypoxia-inducible factor 1α (HIF1α)-dependent IL-1β expression, dependent on ROS. Itaconate selectively shuts down IκBζ-driven secondary transcriptional responses to inflammatory stimuli in BMDMs through an Activating transcription factor 3 (ATF3)-dependent mechanism and thereby inhibits IL-6 production. Activation of Nrf2 and ATF3 are the hallmarks of electrophilicity.[36]

Application of itaconate and its derivative under inflammation reduced nitric oxide, reactive oxygen species, and pro-inflammatory cytokines upon exogenous addition. [31] (Fig.9) (Fig.10)

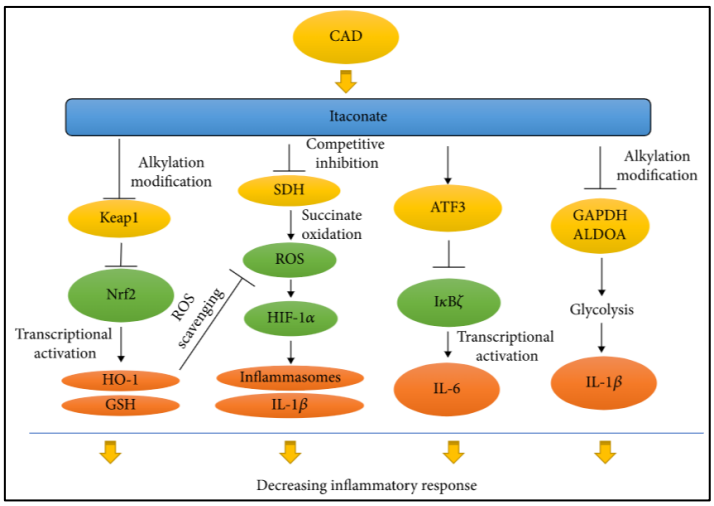

Figure 8:The relevant signaling pathway in itaconate-induced anti-inflammatory and antioxidative effects. *Increasing itaconate activates the Nrf2 pathway via alkylation Kelch-like ECH-associated protein 1 (Keap1), which induces the transcription of various Nrf2-dependent antioxidant and anti-inflammatory genes. Itaconate can also inhibit succinate dehydrogenase (SDH) and reduce ROS generation and, consequently, IL-1β secretion by activation. Itaconate promotes the transcription of activating transcription factor 3 (ATF3), which directly inhibits IκBζ expression and leads to decreasing IL-6.* [35]

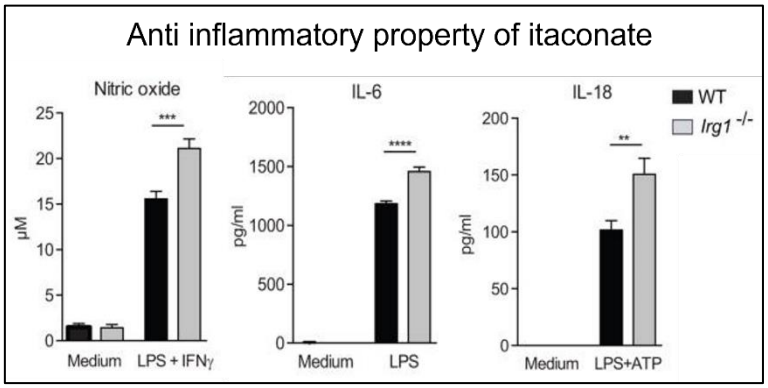

Figure 9: Increased inflammatory response in Irg1 knockout macrophages. *Irg1 is the gene responsible for itaconate production. The study by Lampropoulou, Vicky, et al. show the NO levels in supernatants of WT and BMDM stimulated with LPS + IFN-γ (24 hr), IL-6 levels in supernatants of LPS-activated (24 hr) WT and Irg1−/− BMDM and mature IL-1β levels in supernatants of WT and Irg1−/− BMDM stimulated with LPS (4 hr) and ATP in macrophages lacking itaconate.*[31]

The major drawback of this metabolite is that due to its chemical properties, a charged itaconate molecule can hardly cross the plasma membrane and the lack of evidence for either a transporter or a cell-surface receptor, is the reason why in, in-vitro experiments extracellular itaconate is applied in the milli molar range to cross the plasma membrane or it is considered necessary to structurally alter itaconate into a membrane-permeable form. (Fig. 10) Hence, further studies brought out derivatives of itaconate through the esterification of a carboxyl group. Membrane permeability is provided by esterification of a carboxyl group to reduce the negative charge of itaconate, but this modification also substantially increases

the electrophilicity of the compound. The well-researched and most potent derivatives are dimethyl itaconate and 4-octyl itaconate. In the most recent studies, it is demonstrated that itaconate derivatives have significantly different immunological properties compared to itaconate, most probably due to higher electrophilicity and distinct metabolism. (Fig.10) There is evidence of Nrf2 and ATF3 activation/upregulation but the influence of itaconate derivatives on SDH is unclear.[36]

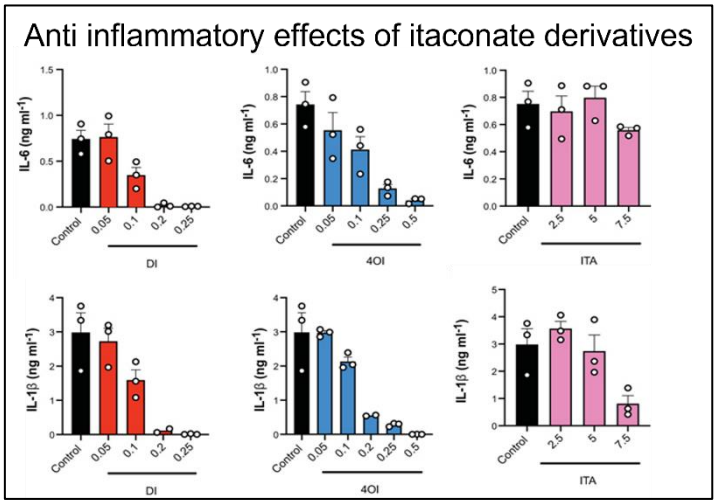

Figure 10: Anti-inflammatory effects of itaconate and derivatives*. Different concentration of itaconate (ITA) and its derivatives dimethyl itaconate (DI) and 4 octyl itaconate (4OI) show the anti-inflammatory effects against IL-6 and IL-1β in LPS activated BMDM. It is inferred that electrophilicity of itaconate derivative play an important role in therapeutic property. It is clear from the high downregulation of IL-6 and IL-1β by DI.*

Hence, we realize the therapeutic role of Itaconate in inflammatory diseases, due to its active inhibition of pro-inflammatory cytokines. We have seen that cells secrete itaconate at low levels and there is evidence that itaconate is catabolized in the liver. Thus, altering intracellular itaconate levels may be an attractive strategy to mitigate patient outcomes of inflammatory diseases. It was previously considered that the IRG1 gene is expressed in monocytes and dendritic cells, but recent studies show that IRG1 is expressed in non-immune cells as well. From the study by Yi, Zhongjie, et al we see that hepatocytes rapidly up-regulate IRG1 in response to ischemia-reperfusion injury.[37]

1.5.1. Itaconate and its derivatives: role in liver disease and metabolic reprogramming

Derivatives of itaconate in recent times have been in research against liver diseases. As mentioned previously oxidative stress, inflammation, and apoptosis are the main characteristics of chronic hepatic diseases. Itaconate and the derivatives of itaconate have been observed to show anti-inflammatory, antioxidant, and anti-bacterial properties and hence are a prime candidate for mitigating hepatic diseases. The study by Li Ruidong et al. indicates the antioxidant and anti-inflammatory properties of 4 octyl itaconate in CCL_4 induced liver damage. Treatment of OI mitigated CCl_4-induced hepatocyte death by reducing oxidative stress and the inflammation response *in vivo* and *in vitro*.[38] In another study by Li Ruidong et al. 4 octyl itaconate protected mice from LPS/D-GalN- induced acute liver failure. [39] IRG1 gene encoding the protein ACOD1 responsible to produce itaconate has been found to play a role in protecting against hepatic ischemia-reperfusion injury in the study by Yi Zhongjie et al. The study highlights that IRG1 is expressed in non-immune cells as well, hepatocytes rapidly up-regulate IRG1 in response to hypoxia/reoxygenation. Also in the

study, 4 octyl itaconate ameliorated hepatic ischemia/reperfusion injury *in-vivo* and rescued hepatocytes from injury resulting from hypoxia/reoxygenation *in-vitro*.[40] An interesting study by Park Seung Yeon et al. investigates the effects of dimethyl itaconate on the impairment of insulin signaling and inflammation in palmitate-treated C2C12 myocytes. Dimethyl itaconate alleviated insulin resistance through the AMPK/FGF21/PPARδ. [41]

The primary mechanism of action highlighted in these studies is the activation of Nrf2 which is a master regulator of intracellular redox hemostasis.[42] Decreased levels of Nrf2 in liver macrophages from humans and mice with NASH was observed in the study by Wang Peng et al.[43] (Fig.11) Furthermore it is inferred from the study by Azzimato, Valerio, et al. that IRG1 is highly downregulated in obese insulin resistance patients.[44] (Fig.12)

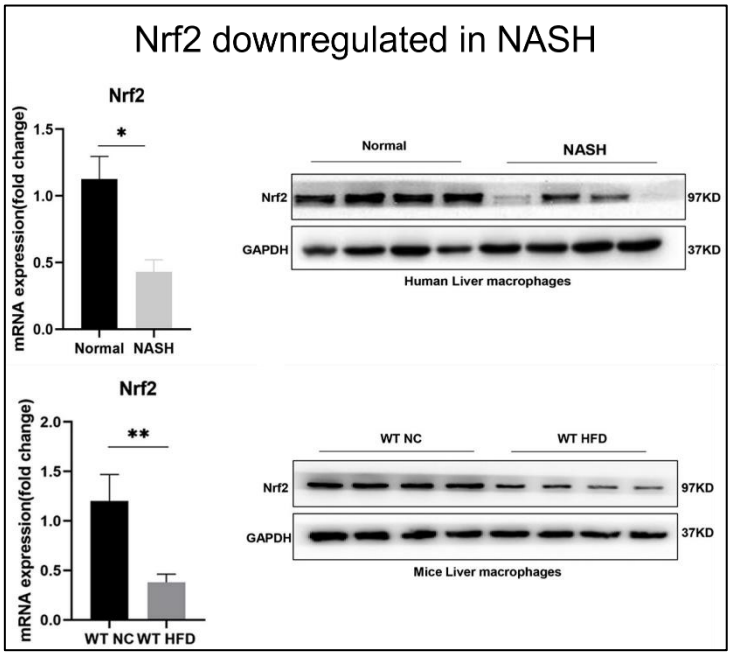

Figure 11: Nrf2 is downregulated in liver macrophages from mouse NASH models and patients with NAFLD. *qPCR and western blotting analysis of Nrf2 in liver macrophages of patients with NAFLD and liver macrophages of mouse NASH models.*[43]

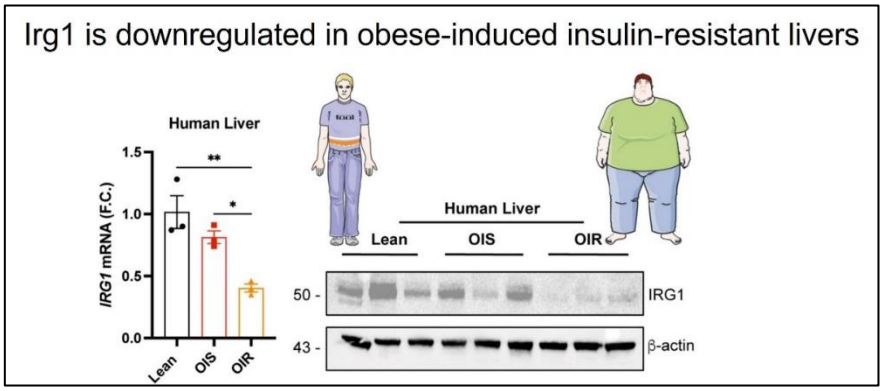

Figure 12: Irg1 is downregulated in obese-induced insulin-resistant livers. *IRG1 in human liver biopsies of lean, obese insulin-sensitive (OIS) and obese insulin-resistant (OIR) individuals showed downregulation in gene and protein expression.*[44]

It is inferred that itaconate is a byproduct of metabolic reprogramming of the inflammatory M1 macrophage, furthermore itaconate is also responsible for the metabolic reprogramming

of M1 macrophage to M2 macrophage. The primary source of energy is obtained through glycolysis in M1 macrophage and glycolysis is reduced and shifts to oxidative phosphorylation (OXPHOS) in M2 macrophages. This reduction in glycolysis is majorly brought by the activation of Nrf2. Hence the activation of antioxidant genes can also result in metabolic reprogramming in macrophages. In the study by Wang Li et al. the role of Nrf2 was studied in chronic obstructive pulmonary disease (COPD) and it was inferred that Nrf2 activation by CPUY192018 results in down regulation of glycolysis. CPUY192018 suppressed basal glycolysis, maximum glycolytic capacity, and glycolytic reserve capacity in mice COPD alveolar macrophage when compared with the glycolytic activity in healthy mice macrophages. Furthermore, CPUY192018 elevated oxidation in mice COPD model with significant increases in ATP production and maximum OCR capacity indicating that CPUY192018 shifts metabolism from glycolysis to mitochondrial oxidation. (Fig.13)

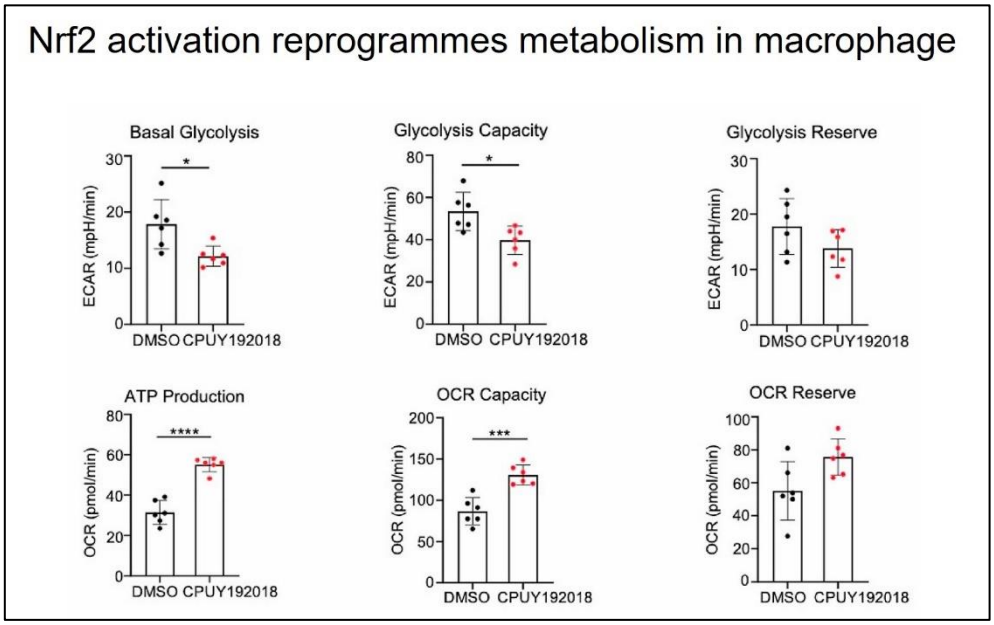

Figure 13: Nrf2 activation reprograms metabolism in macrophages*. The extracellular acidification rate (ECAR) measures glycolysis and oxygen consumption rate (OCR) measures mitochondrial oxidative phosphorylation. Nrf2 activation by CPUY192018 results in down regulation of glycolysis, evident from the reduction in the basal glycolysis. The OCR capacity increases with the activation of Nrf2. The anti-inflammatory macrophages acquire energy through oxidative phosphorolysis hence activation of Nrf2 results in metabolic reprogramming of inflammatory macrophage to anti-inflammatory macrophage.*

In the study by Slocum Stephen L. et al. Nrf2 protected against HFD-induced obesity with lower fasting glucose levels, better glucose tolerance. Based on the lower fasting glucose levels of the Nrf2 activated mice that can potentially be attributed to repressed gluconeogenesis, the mRNA levels of the key gluconeogenic genes phosphoenolpyruvate carboxykinase 1 (Pepck) and glucose-6-phosphatase (G6pase) were assessed by qRT-PCR. The expression of these genes is repressed by about 30% in the Keap1-hypo mice compared to WT on high fat diet, but no difference can be detected between the two genotypes on standard diet.(Fig14)

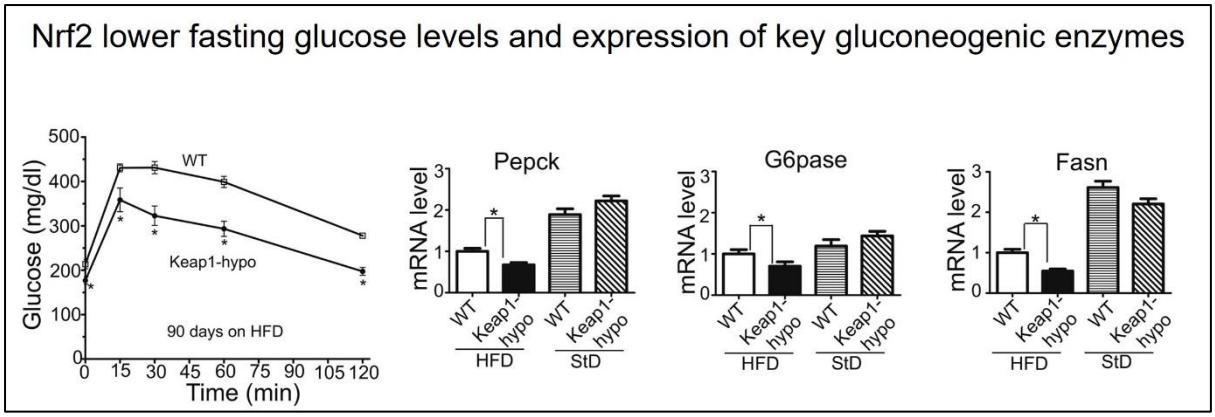

Figure 14: Nrf2 lowered fasting glucose levels and lowered expression of key glucogenic enzymes. *Nrf2 protected against HFD-induced obesity with lower fasting glucose levels, better glucose tolerance. Based on the lower fasting glucose levels of the Nrf2 activated mice that can potentially be attributed to repressed gluconeogenesis*

Therefore, with these data, we can utilize itaconate and its derivatives to mitigate the outcomes of NAFLD and inflammation in NASH. To utilize itaconate's therapeutic effects an efficient method to deliver itaconate is required, because in long run in the case of chronic inflammation, the use of derivatives can lead to immunosuppression. The challenges of delivering itaconate are that it is membrane non-permeable, and it is a small molecule, hence it has low bioavailability. Therefore, encapsulating itaconate in liposomes is a viable option for delivery. Moreover, liposomes could also be used to improve the bioavailability and M1 macrophage specific delivery of 4-OI.

1.6. Liposome and lipid nanoparticles: enhanced drug delivery and efficacy

Nanomedicine is the application of nanotechnology for medical purposes, which include diagnosis, drug delivery, and imaging. Because nanomaterials are similar in scale to biologic molecules and systems yet can be engineered to have various functions, nanotechnology is potentially useful for medical applications. The field of nanomedicine aims to use the properties and physical characteristics of nanomaterials for the diagnosis and treatment of diseases at the molecular level. [45] Nanomedicine is primarily focusing to aid the delivery of therapeutic agents through biological barriers. Nanomaterials are highly malleable; they can be decorated with a wide variety of targeting ligands and can be produced in a range of sizes. Nanomedicine often exhibits greater therapeutic efficacy than conventional small molecular drugs due to their size (10–100 nm), large surface area to volume ratio, and flexibility of surface functionalization. These features provide nanomedicine with low toxicity, improved bioavailability, and enhanced pharmacokinetics and therapeutic effect.[46] Nanomedicines are classified based on the nanomaterials used in the formulations, which are liposomes, polymeric nanoparticles, inorganic nanoparticles, protein-based nanoparticles, and drug nanocrystals.

Liposomes are small artificial vesicles of spherical shape, ranging from 30 nm to several micrometers that can be created from cholesterol and natural non-toxic phospholipids. Due to their size and hydrophobic and hydrophilic character (besides biocompatibility), liposomes are promising systems for drug delivery. They consist of one or more lipid bilayers surrounding aqueous units, where the polar head groups are oriented in the pathway of the interior and exterior aqueous phases. They are composed of natural phospholipids that are biologically

inert and weakly immunogenic they have low inherent toxicity.[47] Due to their amphiphilic nature they can encapsulate hydrophilic as well as hydrophobic drugs, lipophilic drugs are entrapped almost totally in the lipid bilayer and hydrophilic drugs are located entirely in the aqueous compartment.[48]

Liposomes are mainly composed of phospholipids, of two major categories including glycerophospholipids and sphingomyelins. Phospholipid molecules are mainly composed of different polar head groups and two hydrophobic hydrocarbon chains. The polar groups can be zwitterionic or negatively charged. Many types of research have been conducted for the surface modification of liposomes to enhance their properties and increase their applicability to deliver the active pharmaceutical ingredients to the site of action for in-vivo applications.[49] In general, liposome formulations contain a mixture of lipids, cholesterol, and an antioxidant. To prevent oxidation of lipids during storage and to facilitate uniform and molecular dispersion of various lipids among themselves. The release of the drug from the liposome is affected by several parameters. For instance, the presence of cholesterol in liposome formulation will influence the release of the hydrophilic drugs as the amount of cholesterol increases, the release will be rapid. Conversely, the release of the hydrophobic drugs exhibited a reduced release action.[49] Another factor affecting the delivery, or the stability of the lipid layers is the glass transition temperature (Tc) of the phospholipids used. The Tc of phospholipids is a temperature at which phospholipids transit from gel to liquid crystalline state.

Liposomes are the most extensively investigated carriers because of their low immunogenicity, high biocompatibility, and the feasibility of controlling their physicochemical properties and surface modifications. They can improve the stability and prolong the biological half-life of drugs and deliver a medicine specifically to inflammatory sites. As stated previously the innate immune system is the first line of defense against pathogens and infection. One of their key roles is to remove foreign bodies through phagocytosis. This innate property makes the macrophages the primary target for the liposomes. This is validated by many studies, one such is the study by Gawne Peter J. et al. the *ex-vivo* biodistribution of radiolabeled PEGylated liposomal methylprednisolone, has high uptake at inflamed joints of rheumatoid arthritis. (Fig.15)

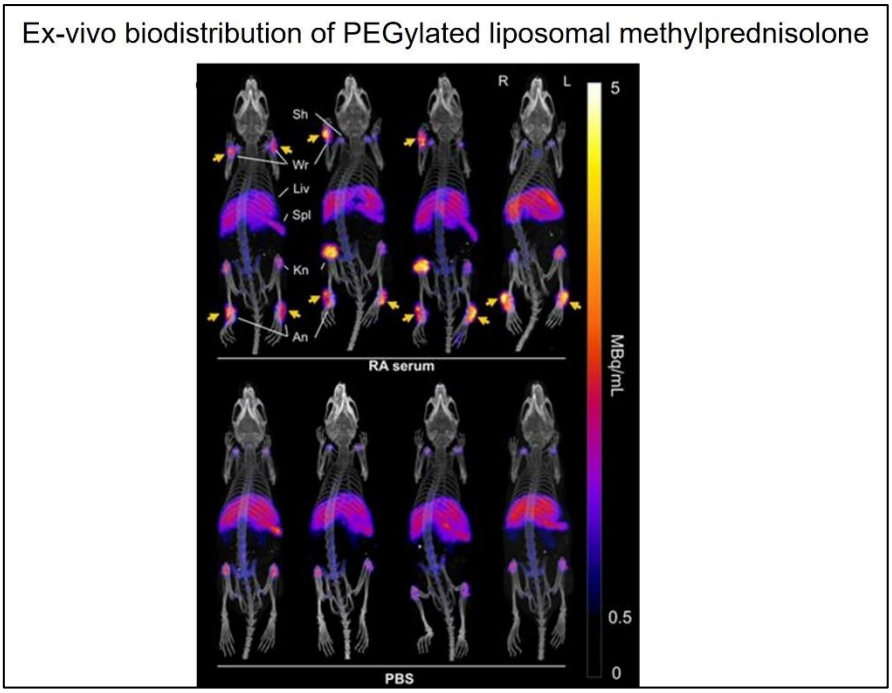

Figure 15: Liposomes targets inflammatory site. *Ex vivo biodistribution of radiolabeled PEGylated liposomal methylprednisolone in rheumatoid arthritis mice models. PET/CT imaging of the mice showed high uptake at inflamed joints (3.6 ± 1.5 % ID; 17.4 ± 9.3 % ID/mL, indicated by the orange arrows.*[50]

Also, they tend to accumulate in the liver, as the liver being a large organ contains high numbers of macrophages.[51] (Fig.16) The accumulation of the liposomes is also aided due to the presence of fenestrated endothelium which accommodates hepatic macrophages and is in direct contact with blood.[52] Thus passive targeting capacity of liposomes is an attractive property to utilize to provide a therapeutic effect of itaconate and its derivative for NASH.

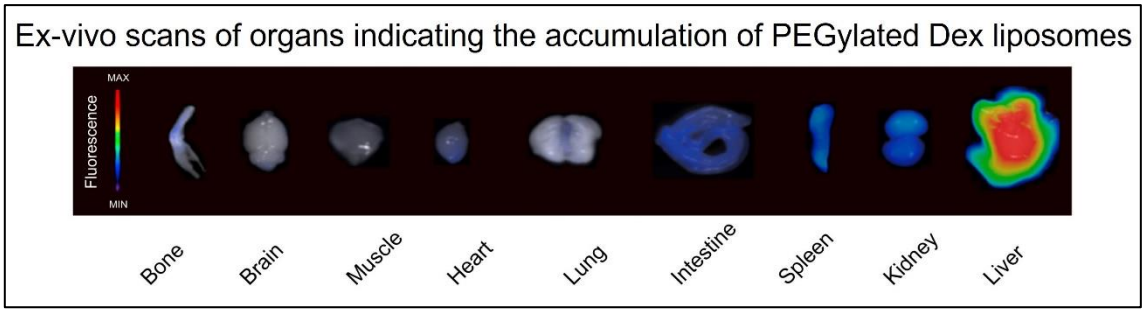

Figure 16: Liposome accumulates majorly in liver*. In the study by Bartneck, Matthias, et al. dexamethasone loaded liposomes are used for the treatment of liver disease. In the ex-vivo scans, fluorescent liposomes (labeled with NBD-PE and Alexa Fluor 750) are majorly accumulated in the liver*[52]

2. Research Aim

2.1. Aim and objective

As discussed above inflammation is the major driver of NAFLD/NASH progression and can be a potential mitigation target. Also, that no successful treatment is available for NASH. Hence the main aim of this project was to mitigate inflammation using the therapeutic effects of a TCA cycle metabolite itaconate. (Fig.17)

Several types of research are ongoing or already established the anti-inflammatory and antioxidant effects of itaconate derivatives *in-vitro* and *in-vivo* for inflammatory diseases as well as in reperfusion injury. But there is still a lag in the usage of the parental product itaconate. Hence, the objective of this project, firstly was to develop a delivery mechanism for sodium itaconate and 4 octyl itaconate through lipid encapsulation and secondly a head-to-head comparison of the two formulations evaluating its anti-inflammatory effects *in-vitro* in RAW264 macrophages. The closest derivative mimicking the parental drug itaconate is 4 octyl itaconate therefore, it was reasonable to choose this itaconate derivative. The major drawback with 4 octyl itaconate is that it is highly hydrophobic and a small molecule. Hence lipid encapsulation would enhance the therapeutic effect of 4 octyl itaconate.

This is the first project proposing lipid encapsulation as a delivery mechanism for itaconate.

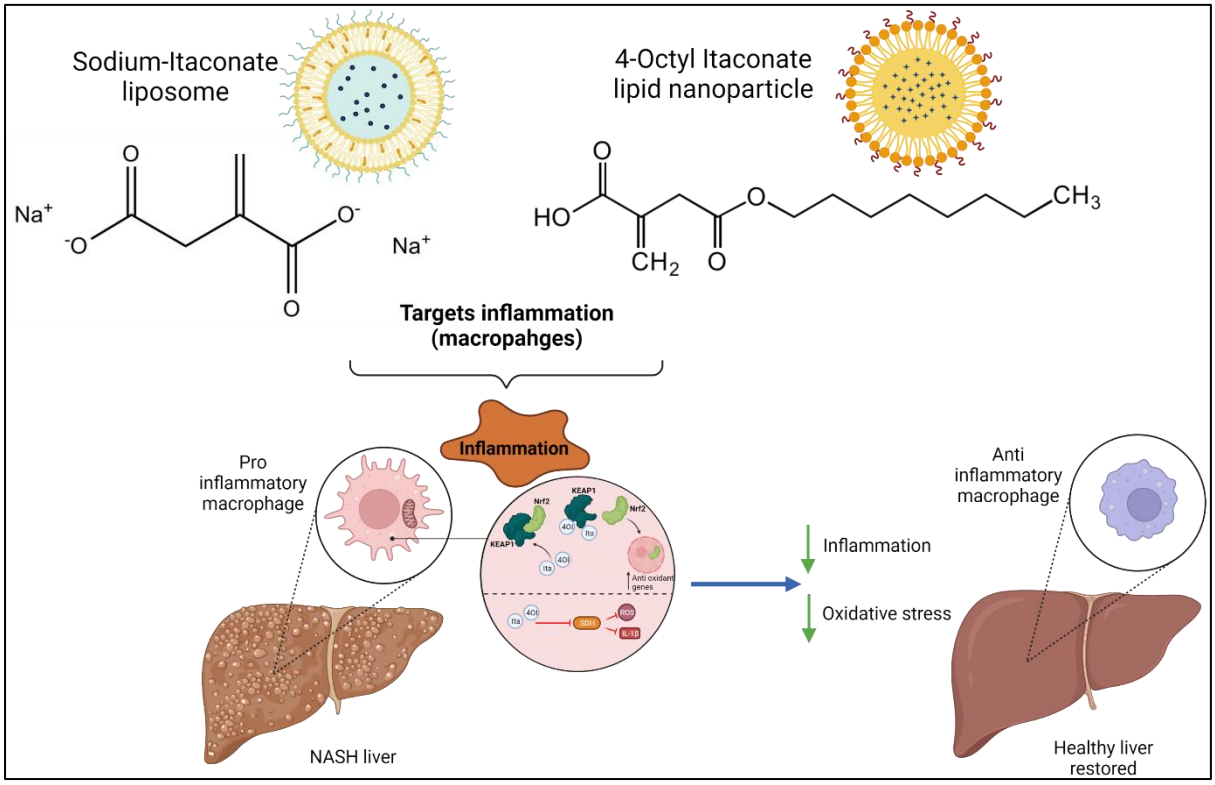

Figure 17: Aim. *The main scope of this project was the development of formulation of lipid encapsulated sodium itaconate and 4 octyl itaconate. These formulations are further evaluated for their anti-inflammatory effects. Created with Biorender.com*

2.2. Strategy

The project will commence with the review of literature for liposome formulation and the properties of sodium itaconate and 4 octyl itaconate. Firstly, sodium itaconate liposome will be prepared and characterized. Then in-vitro studies will be undertaken for the sodium itaconate liposomal formulation in inflammatory murine macrophages. Secondly, 4 octyl itaconate lipid nanoparticle formulation is prepared and characterized and evaluated for its anti-inflammatory effects.

Therefore, the project proceeds in two steps

1. The establishment of lipid encapsulation formulation for sodium itaconate and 4 octyl itaconate and characterized.
2. In-vitro analysis of the prepared formulations for the gene expression of markers in inflammation.

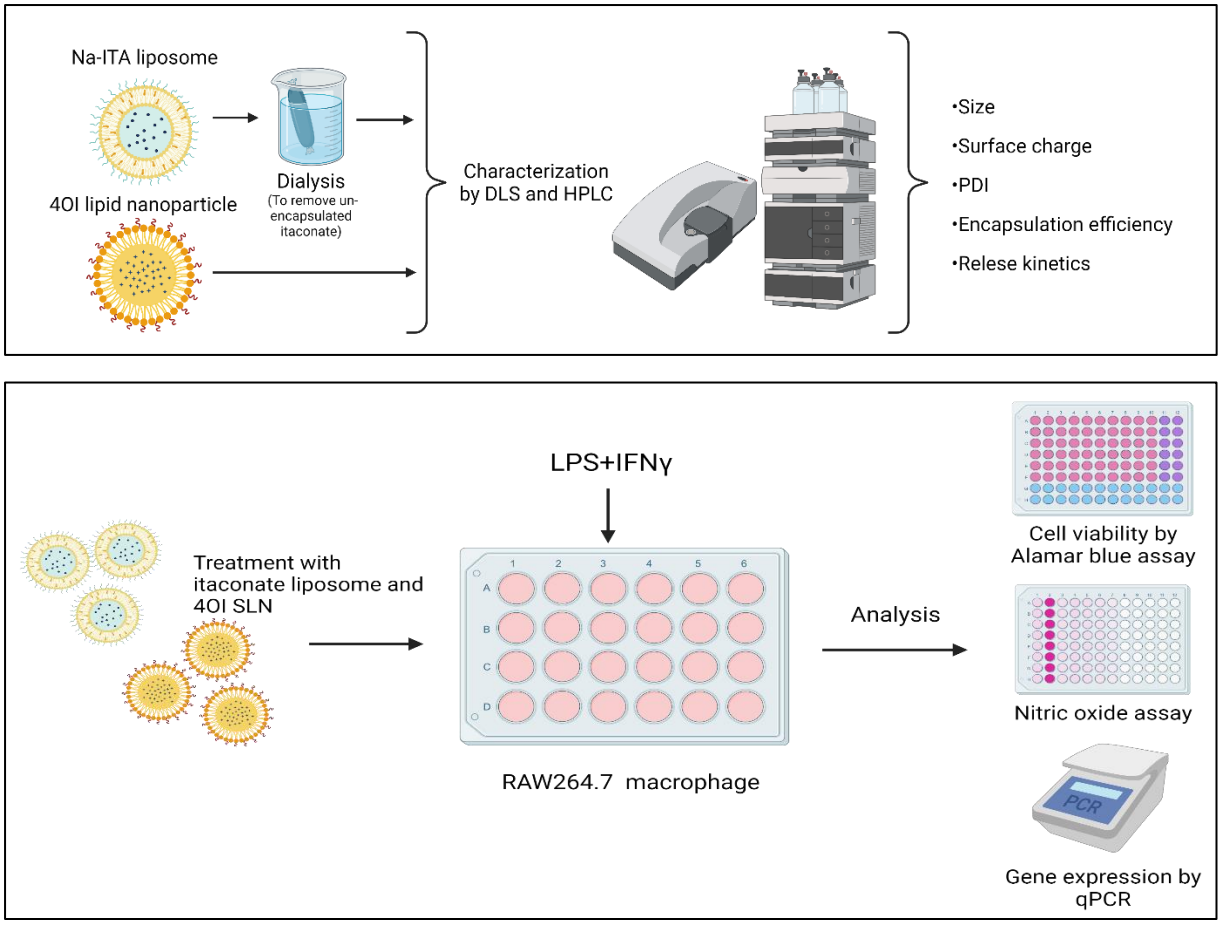

Figure 18: Strategy. *A two-step methodology is followed. First is the development and characterization of the formulations. Second is the evaluation of the anti-inflammatory effects of the formulations. Created with Biorender.com*

3. Materials and Methods

3.1. Preparation and characterization of sodium itaconate liposomes and 4-octyl itaconate lipid nanoparticles

Lipid encapsulation of sodium itaconate and 4 octyl itaconate were prepared by the hot ethanol method.(Fig.19)

The phospholipids DPPC (51mM), DSPE-PEG (3.9mM), and cholesterol (23.9mM) were dispersed in 1ml of ethanol and subjected to heat at 60°C. Sodium itaconate at a concentration of 100mg/ml was dissolved in 5ml of PBS and subjected to heat at 60°C. The warm PBS solution was then filtered into a vial. Under constant stirring, the warm ethanol solution was added swiftly. The dispersion was let to stir and then transferred to an extruder and was then extruded through 200nm polycarbonate membrane (5 times) and 100nm polycarbonate membrane (5times). The liposomes formulation was then dialyzed using cellulose membrane at 14kDa cut off (Sigma dialysis tubing) overnight in PBS to remove the un-encapsulated sodium itaconate.

The phospholipids DPPC (61.9mM), DSPE-PEG (2.7mM), and 4OI (86.6mM) were dispersed in 800µl ethanol and subjected to heat at 60°C also 5ml of PBS was heated to 60°C. The warm PBS was transferred into a vial. Under constant stirring, the warm ethanol solution was added swiftly. The dispersion was let to stir and then transferred to an extruder (Lipofast LF-50, Avestin, Ottawa, Canada) and was then extruded through 200nm polycarbonate membrane (5 times) and 100nm polycarbonate membrane (5 times) (Sartorius lab instrument GmBH&Co., Germany)

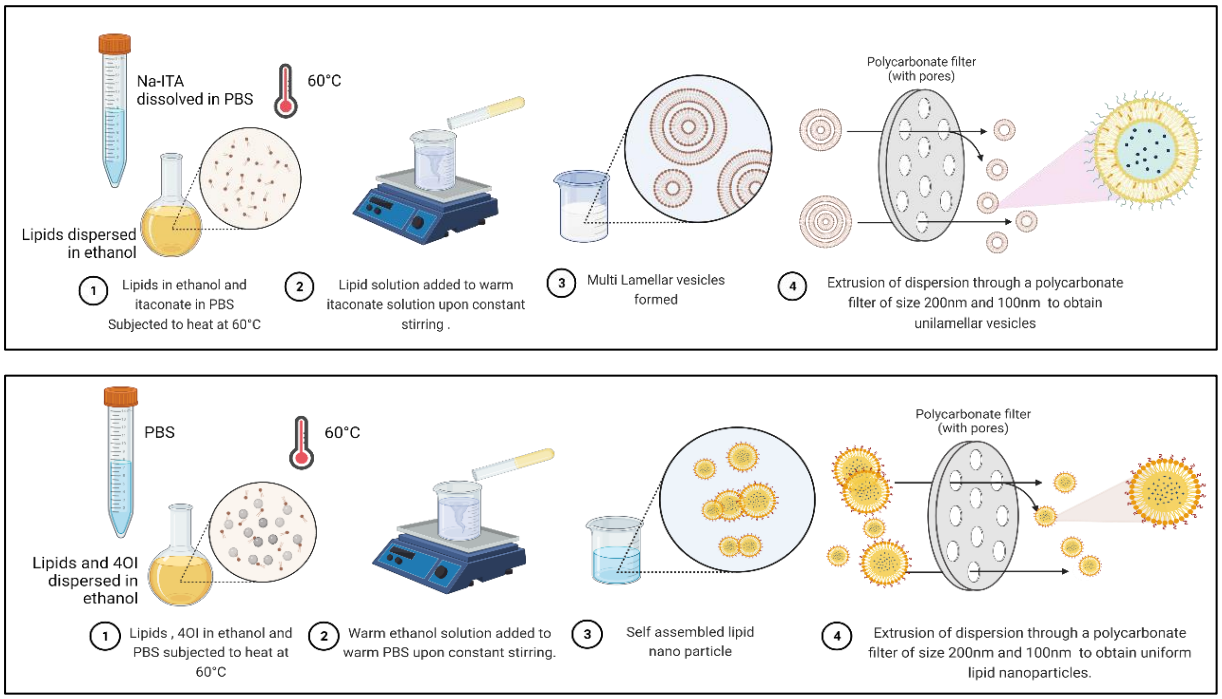

Figure 19: Liposome preparation. *Hot ethanol method is utilized for the self-assembly of the liposome and lipid nanoparticle and then extruded to produce uniform sized nanoparticles.*

Both formulations are stored at room temperature or >25°C as they are highly temperature-sensitive and crystalize under lower temperatures, especially 4 octyl itaconate lipid nanoparticles.

The size distribution and zeta potential were determined by dynamic light scattering (Zetasizer Nano ZS, Malvern Instruments, Malvern, UK). Sodium itaconate liposomes were diluted in Milli-Q water (10µl sample + 990µl MilliQ) and an undiluted sample of 4 octyl itaconate lipid nanoparticles was used for size measurement using a disposable capillary cuvette. Laser Doppler electrophoresis with a Zetasizer Nano-ZS (Malvern Instruments, Malvern, UK) was used to evaluate the formulation's ζ-potential. 20ul (2%) of samples were diluted in 980ul (98%) of 0.001 M NaCl solution. Special electrode-based cuvettes were used for ζ-potential measurement.

To determine the encapsulation efficiency of the formulations, 4-octyl lipid nanoparticles were analyzed using HPLC at wavelength 210nm. 4 octyl itaconate lipid nanoparticles samples were distributed in acetonitrile. The run time for each sample was 10 minutes with a retention time of 5 minutes. Sodium itaconate liposomes were analyzed in a UV/VIS spectrophotometer (Shimadzu UV-2450 UV/VIS spectrophotometer) at 240nm. Sodium itaconate liposome samples were distributed in MilliQ water. For both formulations standard were prepared and analyzed accordingly. Next a standard plot was plotted for both formulations and encapsulation efficiency were determined using the formula

$$Percentage\ encapsulation\ efficiency = \frac{Amount\ of\ drug\ encapsulated}{Total\ amount\ of\ drug\ used} \times 100$$

3.2. Release study of sodium itaconate in liposomes and 4-octyl itaconate in lipid nanoparticle

Sodium itaconate liposomes were loaded into a dialysis cellulose membrane tube (SpectraPor® Float-A-Lyzer® Dialysis Device). The dialysis cassettes were suspended in PBS and kept at 37°C at constant shaking. 100µl of the sample was obtained from inside the cassette at intervals (30min, 1hr, 2hr, 4hr, 6hr, and 24hr). The same setup was followed for 4 octyl itaconate except the dialysis cassettes were suspended in BSA (50mg/ml). The 4OI samples were analyzed using HPLC and the sodium itaconate samples using UV/VIS spectrophotometry. The results were plotted with cumulative release percentage in the y axis and time in x axis. Cumulative release percentage was calculated using the formula

$$Cumulative\ release\ percentage = \left[100 - \left\{\frac{Wavelength\ of\ control}{Wavelength\ of\ sample} \times 100\right\}\right]$$

3.3. Cell culture

A mouse macrophage-like cell line of RAW264 cultured in RPMI 1640 1% L-glutamine medium (Lonza, Basel, CH), containing 10% v/v fetal bovine serum and 1% v/v penicillin. Cells were incubated at 37°C in an atmosphere of 5% CO2 and then prepared for each experiment. The macrophages were seeded on a 24-well plate at 2.5x10^5 cells per well per 500µl. After 24 hours the macrophages were stimulated with LPS (100ng/ml) + IFN-γ (10ng/ml) for 24 hours to stimulate M1 macrophage and IL-4 (10ng/ml) + IL-13 (10ng/ml) to stimulate M2 macrophage. The M1 macrophages were treated with different concentrations of the free drug and the formulations. These concentrations were selected based on the experiments found in the literature.[31], [53] DMI (0.125mM, 0.250mM) Na-ITA (5mM, 10mM) Na-ITA-Lip

(0.125mM, 0.250M) 4OI (0.062mM, 0.125mM, 0.250mM) and 4OI LNP (0.062mM, 0.125mM, 0.250mM) (Fig.20)

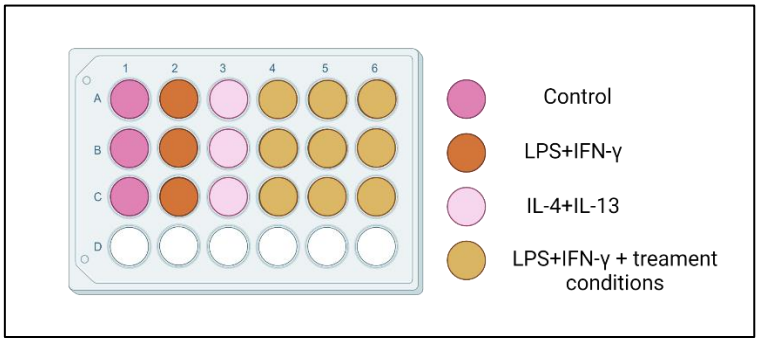

Figure 20: Plate setup in-vitro analysis

3.4. Cell viability assay

The viability of RAW264 macrophages was determined by an Alamar blue assay. RAW264 cells were seeded into 24 well plates overnight, followed by treatment with the above-mentioned compounds. Media was used as a control. After 24 h of treatment, cells were incubated in the medium containing 10% Alamar blue dye (Sigma-Aldrich) at 37°C for 4 h, and the colorimetric change was measured with a microplate reader (PerkinElmer, Groningen) at 570nm, and the percentage viability was calculated using the formula below.

$$Percentage\ viability = \frac{Absorbance\ unit\ of\ sample}{Absorbance\ unit\ of\ control} \times 100$$

3.5. Nitric oxide

The concentration of NO in culture supernatants was determined as nitrite, a major stable product of NO, by the Griess reagent (1% sulfanilamide in 2.5% H3PO4 and 0.1% N-[1-naphtyl]ethylenediamine HCl) as described previously. 100µl of the cell culture supernatant was mixed with an equal volume of Griess reagent in a 96 well plate and incubated for 10 min at room temperature. The absorbance at 540 nm was measured using a microplate reader. (PerkinElmer, Groningen). The percentage NO reduced is calculated by the following formula.

$$Percentage\ NO\ reduction = \frac{Absorbance\ unit\ of\ sample}{Absorbance\ unit\ of\ LPS + IFN - \gamma} \times 100$$

3.6. qPCR

Total cellular RNA was extracted from RAW macrophages using the EURx molecular research laboratory total RNA miniprep kit, according to the manufacturer's instructions. RNA concentration and purity were quantified with the Nanodrop (Nanodrop ND-1000, Wilmington). Complementary DNA (cDNA) was synthesized using the iScript cDNA synthesis kit (Bio-Rad Laboratories) on the Arktik Cycler (ThermoFisher Scientific). Gene expression was measured using Sensimix™ SYBR Green on a CFX384 Touch™ Real-Time qPCR (Bio-Rad Laboratories). Primer sequences are shown in Table2. The Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a reference gene to normalize the data of the target genes. Data were analyzed using the CFX Maestro Software (Bio-Rad Laboratories) according to the standard curve and fold change values calculated using the ΔΔCt method in Excel.

Primer	Sequence
GAPDH	ACAGTCCATGCCATCACTGC GATCCACGACGGACACATTG
IL-6	GGGACTGATGCTGGTGACAA TGGAAATTGGGGTAGGAAGGAC
IL-1β	TGCCACCTTTTGACAGTGATG TTCTTGTGACCCTGAGCGAC
iNOS	GGTGAAGGGACTGAGCTGTT GCTACTCCGTGGAGTGAACAA
IL-10	CCAAGGTGTCTACAAGGCCA AGGAAGAACCCCTCCCATCA

Table 2: Primer sequence

3.7. Statistical analysis and illustrations

Statistical analysis was performed using Prism 8.0.1 (GraphPad Software). Relative values between treatments were compared using the one- or two-way ANOVA comparison test. Data are expressed as mean ± SD. Significance was observed for outcomes with a P-value ≤0.05*, ≤0.01**, ≤0.001***, or ≤0.0001****

Illustrations were created using BioRender.com

4. Results and Discussion

4.1. Preparation and characterization of sodium itaconate liposomes and 4 octyl itaconate lipid nanoparticle.

Liposomes were prepared by the hot ethanol method. Dynamic light scattering was used to determine the size and PDI of the formulation. The PDI determines the homogeneity of the formulations. The sodium itaconate liposomes had an average size of 103nm while the 4 octyl itaconate solid lipid nanoparticle had an average size of 67nm. (Fig.21A; Fig.21B) Looking into the size and the characteristics of the drug we speculated that the sodium itaconate formed liposomes where the sodium itaconate was suspended in the aqueous layer with a bilayer of the phospholipids. 4 octyl itaconate is a hydrophobic drug hence, the phospholipids could have formed a core entrapping the 4 octyl itaconate forming what could be termed a solid lipid nanoparticle.

Zeta potential determines the surface charge of the particles. The particles were found to have a negative charge. (Fig.21C)

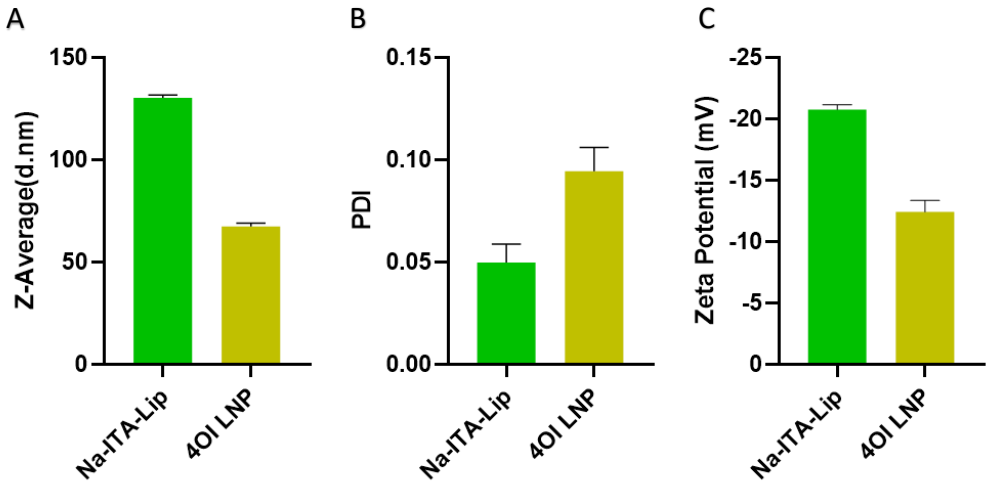

Figure 21: Liposome characterization: *(A) Size: both formulations are within the optimal delivery size for the liver (B) Polydispersity index of the formulation indicating the homogeneity of the size distribution of liposomes and lipid nanoparticles. (C) Surface charge of the liposome and lipid nanoparticle: both formulations show a negative charge. Na-ITA_Lip: sodium itaconate liposome. 4OI_LNP: 4 octyl itaconate lipid nanoparticle.*

Encapsulation efficiency of both formulations were determined through HPLC. Sodium itaconate liposomes had an encapsulation efficiency of 1mg/ml while the 4 octyl itaconate lipid nanoparticles had an encapsulation efficiency of 3mg/ml.

The release of drugs is essential for any nanomedicine. Release kinetics were evaluated for both the formulations for 24 hours. The formulations were loaded into dialysis cassettes that were immersed in either PBS or BSA, under constant shaking at 37°C. The release kinetics of sodium itaconate liposomes was done in PBS. The 4 octyl itaconate lipid nanoparticle were immersed in BSA, as it is a hydrophobic drug, the 4 octyl itaconate is bound between the phospholipids, and the serum proteins play a key role in the release of the drug. The results indicate (Fig.22) a stable release for sodium itaconate liposomes and an exponential release at 2hrs for 4 octyl itaconate lipid nanoparticle, which becomes sustained from 4hrs.

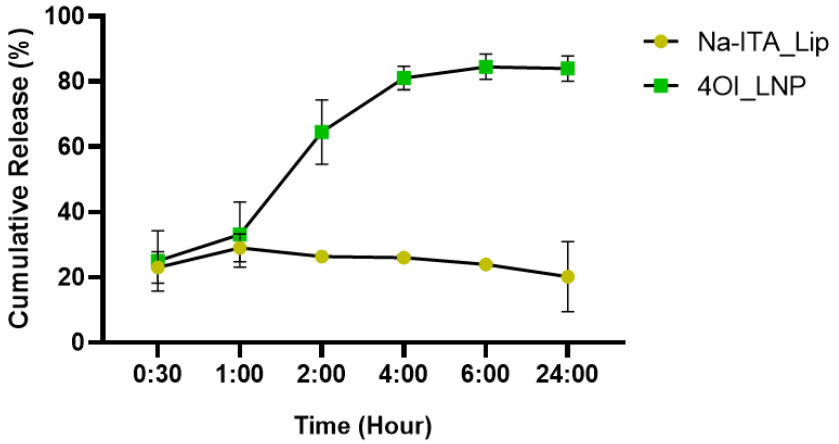

Figure 22: Release kinetics of the two formulations for 24 hours. *A stable release for sodium itaconate liposomes and an exponential release at 2hrs for 4 octyl itaconate lipid nanoparticle, which becomes sustained from 4hrs is observed. Na-ITA_Lip: sodium itaconate liposome. 4OI_LNP: 4 octyl itaconate lipid nanoparticle.*

Formulations were developed successfully for sodium itaconate and 4 octyl itaconate. As mentioned earlier it is considered that lipid encapsulation of sodium itaconate results in a liposome and this is concluded when observing the size and the property of sodium itaconate. It is highly hydrophilic, and it is trapped in the aqueous core of the liposome, also the formulation consists of cholesterol along with DPPC. Cholesterol plays a vital role in the liposome's composition. It is one of the most critical structural components in the mammalian cell plasma membrane. Various vital roles have been attributed to cholesterol, including membrane permeability regulation, elasticity and stiffness, and membrane strength. Cholesterol is the most used sterol in the formulation of liposomes which can prevent liposome aggregation and improve the liposomal membrane's stability.[54] The 4 octyl itaconate lipid nanoparticles have a smaller size when compared to sodium itaconate liposomes. 4 octyl itaconate is highly hydrophobic, also the lipid composition of this formulation does not include cholesterol, hence it was speculated that the lipid DPPC formed a tight core over the drug, resulting in lipid nanoparticles. This needs to be further confirmed with cryo-TEM.

Both formulations contain DSPE-PEG at a concentration of 2.5%, we know from the literature that one of the methods of uptake is mainly accomplished through a scavenge function of cells of the mononuclear phagocytic system (MPS). PEG forms a steric barrier around the liposome or lipid nanoparticle. This steric barrier or steric stabilization reduces opsonization and protein interaction thereby providing stealth and hence greatly increasing the blood

circulation time of the liposomes and lipid nanoparticles.[55] Steric stabilization here refers to reduction in particle interactions leading to aggregation and fusion. Such a steric barrier, though, can be constructed not only to reduce particle-particle interactions but also adsorption of various (macro)molecules onto the particle surface, loss of components to other particles, or interactions with cells providing greater liposome stability, especially in biological environments. The formulation used in this project only uses 2.5% w/w, therefore the main role of PEG is to provide steric stabilization and not to greater extent stealth.

The key reason to encapsulate sodium itaconate and 4 octyl itaconate was to enhance their delivery mechanism. Both are small molecules and hence have short circulation time. To achieve optimal therapeutic effect frequent administration is required. Nanoparticle-based drug delivery platforms have emerged as suitable vehicles for overcoming pharmacokinetic limitations associated with conventional drug formulations. Nanoparticles, such as liposomes, have proven advantageous at solubilizing therapeutic cargos, substantially prolonging the circulation lifetimes of drugs. Furthermore, lipid encapsulation allows sodium itaconate to be cell permeable. Given the property of liposomes to significantly increase circulation time, this drug delivery system has been developed to treat inflammatory disease due to the innate ability of liposomes to target the mononuclear phagocyte system.[56] The reticuloendothelial system (RES) is the main site of liposome accumulation following their systemic administration. Primary organs associated with the RES include the liver, spleen, kidney, lungs, bone marrow, and lymph nodes.[57] The liver exhibits the largest capacity for liposomal uptake followed by the spleen. Furthermore, the liver has fenestrations in their microvasculature, that allow liposomes permeate.[58] These properties prove to be a great benefit to this project as the main target are the liver macrophages. The size of both the formulations are within 150nm which allow both to be permeable through the microvasculature and have a prolonged circulation time.[58] The release of drugs is a prime property of nanomedicine. The release kinetics suggests a highly stable formulation for sodium itaconate liposomes as a plateau is observed over 24 hours. 4 octyl itaconate shows a peak at 2 hours and then reaches a plateau thereafter. From studies it is inferred that nanomedicine accumulate in the liver within 2 hours of administration through IV.[59] Hence both the formulations are stable enough to deliver the drug.

This project is the first to encapsulate itaconate to enhance the delivery. From literature it is inferred that due to the cell impermeability of itaconate, derivatives were designed. The derivatives show superior therapeutic effect compared to itaconate; hence no study suggests nano drug delivery methods, but frequent administration of these derivatives may result in immunosuppression. Therefore, this project is the first of its kind to provide a novel method to use the parental product itaconate.

4.2. Sodium itaconate liposome and 4 octyl itaconate lipid nanoparticle do not show cytotoxicity.

Alamar blue cell viability assay was performed to assess the toxicity of the formulation. Percentage cell viability was calculated using the formula given below.

Different concentrations of sodium itaconate liposomes were compared with different concentrations of dimethyl itaconate and free sodium itaconate. (Fig.23A) Different concentrations of 4 octyl lipid nanoparticles were compared with free 4 octyl itaconate. Additionally, empty liposomes and lipids were assessed for viability. (Fig.23B) The assay results show that neither of the formulations is toxic to the cells and the empty liposomes and lipids also do not hinder the viability of the cells.

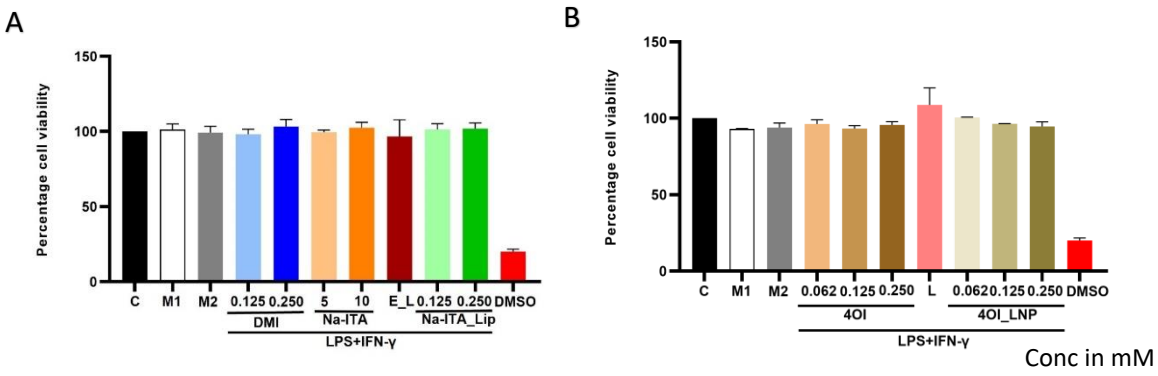

Figure 23: Cell viability result from ABA*. treatment groups DMI, Na-ITA, Na-ITA_Lip, 4OI, and 4OI_LNP show no toxicity to cells. Lipids and empty liposomes at the concentration used in the formulation also show no toxicity. (A) Compared to the control, free drug dimethyl itaconate (DMI), sodium itaconate (Na-ITA) and sodium itaconate liposomes (Na-ITA_Lip) show no significant change. (B) Free 4 octyl itaconate (4OI) and 4 octyl itaconate lipid nanoparticles (4OI_LNP) show no significant change compared to the control. From both graphs, the empty liposomes and lipids also show no significant change compared to the control.*

Alamar Blue is a water-soluble dye that has been commonly used for quantifying in vitro viability of various cells. When added to cell cultures, the oxidized form of the Alamar blue, resazurin enters the cytosol and is converted to the reduced form, resorufin by mitochondrial enzyme activity by accepting electrons from NADPH, FADH, FMNH, NADH as well as from the cytochromes. This redox reaction is accompanied by a shift in color of the culture medium from indigo blue to fluorescent pink, which can be easily measured by colorimetric or fluorometric reading. Alarm blue assay is also a good indicator of metabolic mitochondrial activity. The reduction of resazurin to resorufin is through mitochondrial enzymes, hence a quantitative assay of metabolic activity of cells and an indirect indication of viability of cells. [60]

Sodium itaconate and 4 octyl itaconate are non-cytotoxic as itaconate is endogenously produced. Lipids DPPC, cholesterol and DSPE-PEG have been studied to be non-cytotoxic. The formulations also resulted as non-cytotoxic.

4.3. 4 octyl itaconate lipid nanoparticle suppress nitric oxide

Nitric oxide (NO) is an indicator of inflammation. NO is released because of citrate accumulation, in the event of TCA cycle disruption. NO is considered a pro-inflammatory mediator that induces inflammation due to overproduction in abnormal situations. In inflammatory reactions, pro-inflammatory cytokines lead to the expression of the inducible NO synthase in monocyte/ macrophages, neutrophil granulocytes, and many other cells. [61] The NO assay was performed using the Griess reagent, whereby the reduced product of nitric oxide, nitrite (NO_2^-) is measured.

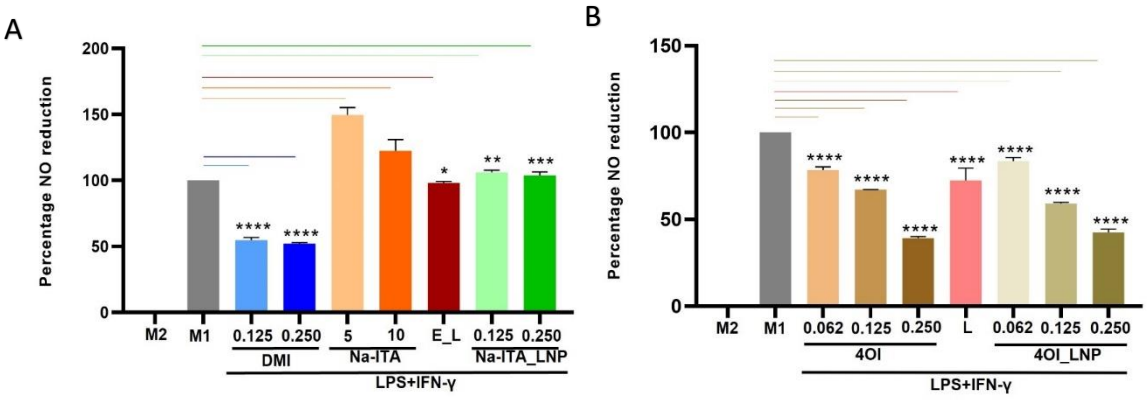

Figure 24: Nitric oxide release assay. *NO reduction percentage of (A) DMI, Na-ITA, and Na-ITA_Lip (B) 4OI and 4OI_LNP compared to LPS+IFNγ treated RAW 264 macrophages. A concentration-dependent effect is observed for the treatment groups. All treatment groups were stimulated with LPS and IFN-γ unless stated otherwise. . From both graphs, the empty liposomes and lipids also show no significant change compared to the control. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 using one way ANOVA with multiple comparison Tukey test.*

The different concentrations of sodium itaconate liposome, dimethyl itaconate, and free sodium itaconate were evaluated to assess the level of NO reduction in macrophages treated with LPS and IFN-γ. The results (Fig.24A) show a clear and significant decrease in NO in the dimethyl itaconate treated cells, which complies with the literature, also free sodium itaconate does not decrease NO production. Upon treatment with the sodium itaconate liposome, no difference or a minute decrease in NO production was observed. Empty liposomes were also evaluated, and it was observed that there is a slightly significant decrease in NO production. Comparing the free sodium itaconate and sodium itaconate liposome, it is observed that the liposome formulation has a significant effect. Different concentrations of 4 octyl itaconate and 4 octyl itaconate lipid nanoparticles were also evaluated in a similar manner. The results indicate a concentration-dependent decrease in NO, for both the free drug and the lipid nanoparticles, which is significant. There is only a slight difference between the effects of the free 4 octyl itaconate and the 4 octyl lipid nanoparticles. It is also observed that the lipids significantly decreased the NO, but it is in accordance with the other treatment groups. (Fig.24B)

Nitric oxide is a major mediator of inflammation. The NO assay was performed using the Griess reagent. It is a colorimetric assay that measures nitrite. NO is an intermediate in the oxidation of L-Arg to nitrite and nitrate in macrophages. The Griess reagent system is based on a chemical reaction that uses sulphanilamide and N-1-naphthylethylenediamine

dihydrochloride (NED) under acidic (phosphoric acid) conditions. The Griess reaction is a two-step diazotization reaction, the nitrite reacts with the sulphanilamide to produce a diazonium ion which then couples with NED to form a chromophore azo product which absorbs strongly at 540nm. [62]

In the earlier research by V Lampropoulou studying the anti-inflammatory effects of itaconate and derivatives, in response to inflammatory stimuli, Irg1 deficiency increased nitric oxide production.[31] It is seen from the results that free sodium itaconate did not decrease the NO compared to LPS IFN-γ activated macrophages because itaconate is cell non permeable. On the contrast sodium itaconate liposomes show no difference in NO reduction. This result needs to be further studied upon to understand the mechanism of NO release by inflammatory macrophages. A correlation must be established between iNOS and NO release to establish the relation between the inhibitory effect of itaconate against nitric oxide.

4.4. Encapsulated itaconate and 4 octyl itaconate show more potent anti-inflammatory effect than the free forms

Chronic inflammation is a prominent characteristic of NASH. Inflammatory cytokines are released by adiponectin. Inefficiency to remove the inflammatory agents results in a prolonged inflammation that progresses to fibrosis. Thus, the balance between pro- and anti-inflammatory cytokines plays a major role in reducing the progression of NASH.

The pro-inflammatory markers IL-6 IL-1β and iNOS along with the anti-inflammatory marker IL- 10 were investigated. One primary aim of this study was to evaluate the potential effect of the liposomal formulation of itaconate on the inhibition of pro-inflammatory cytokines. There is a significant increase in the pro-inflammatory markers IL-6, IL-1β, and iNOS upon stimulation with LPS and IFN-γ. From the graphs (Fig.25) a concentration-dependent effect is seen for the treatment conditions. Sodium itaconate liposome is compared to dimethyl itaconate and sodium itaconate (Fig. 25A,C and E), there is a significant downregulation by dimethyl itaconate followed by the effects by sodium itaconate liposomes. Sodium itaconate shows no therapeutic effect in the downregulation of iNOS, IL-6, and IL-1β. Similarly, there is a significant decrease in IL-6, IL-1β, and iNOS by the 4 octyl itaconate lipid nanoparticles. (Fig.25B,D and F). Compared to 4 octyl itaconate, the 4 octyl itaconate lipid nanoparticles show similar efficacy.

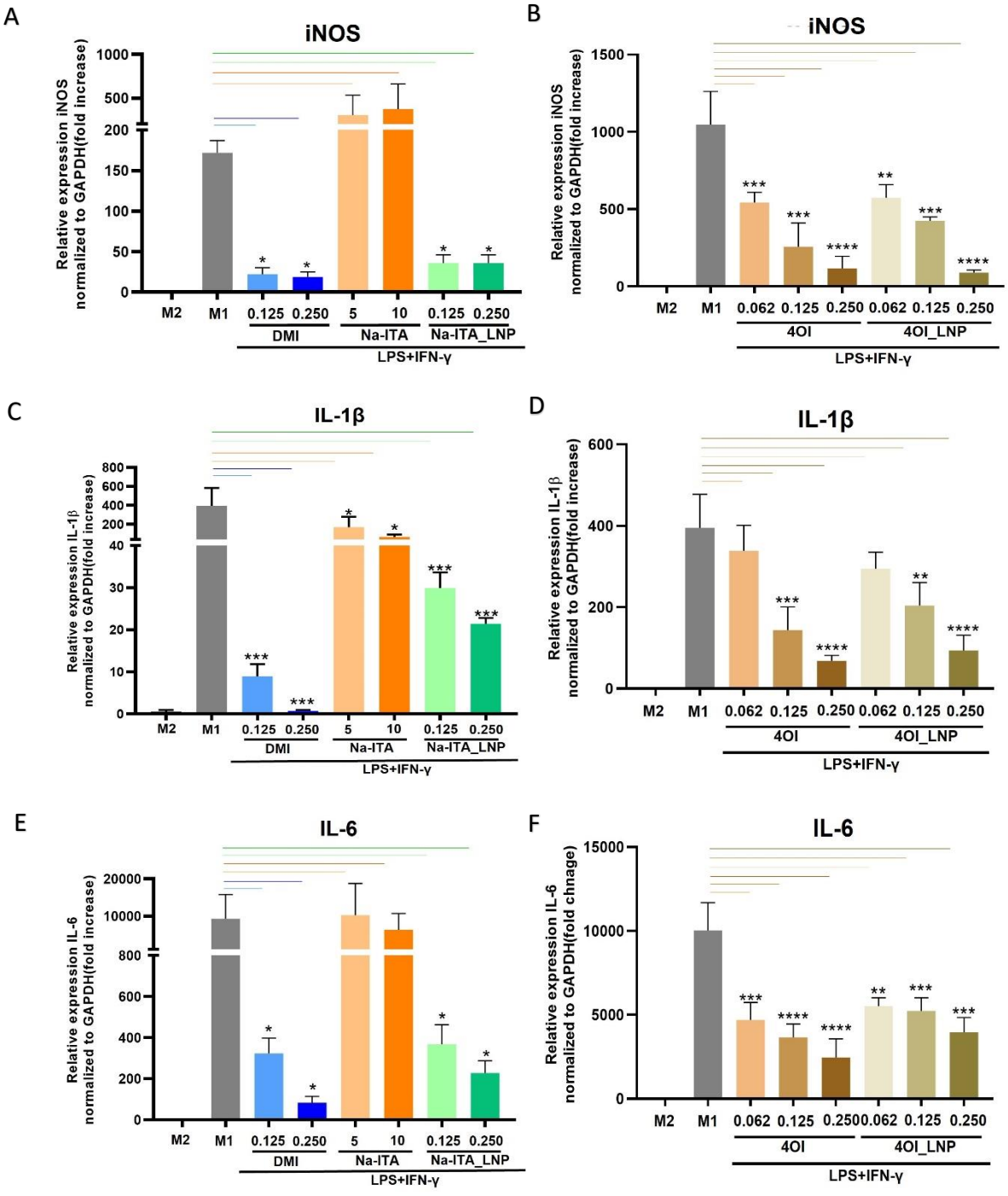

Figure 25: Relative gene expression of pro-inflammatory cytokine upon treatment with different concentrations of DMI, Na-ITA, Na-ITA_Lip, 4OI, and 4OI_LNP*. (A-B) iNOS (C-D) IL-1ß (E-F) IL-6. A dose-dependent effect is observed across the treatment conditions. Sodium itaconate liposomes (A,C,E) and 4 octyl itaconate lipid nanoparticle (B,D,F) show significant downregulation of pro-inflammatory cytokines. Compared to free sodium itaconate, liposomal sodium itaconate showed a more potent effect. The 4 octyl itaconate lipid nanoparticle showed slightly better or similar effects compared to the free form. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 using one way ANOVA with multiple comparison Tukey test.*

Additionally, to investigate the effects of the lipids used, empty liposomes and lipids were also evaluated for anti-inflammatory effects. The amount used was equivalent to the concentration used for 0.25mM of both the formulations.

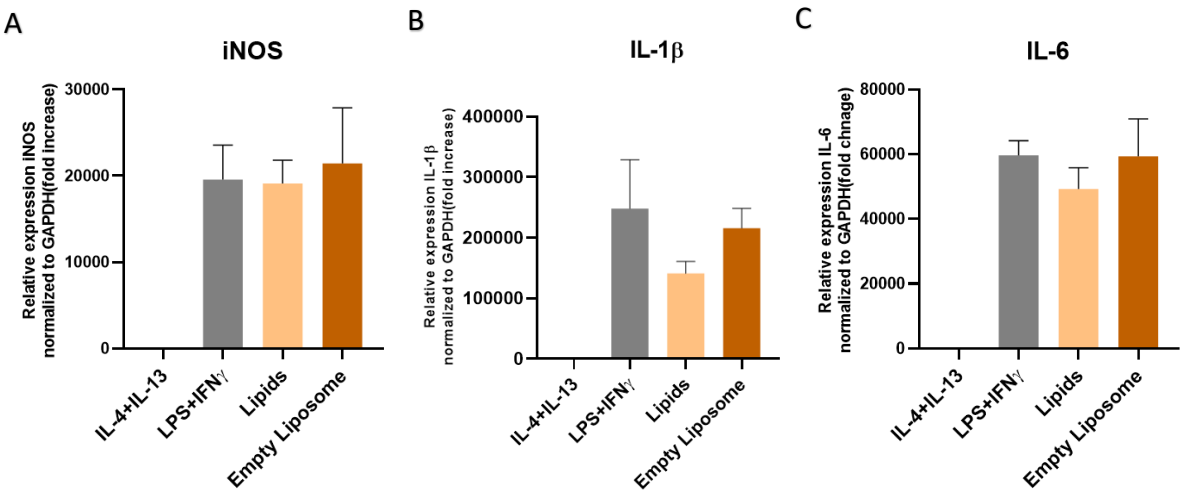

Figure 26: Relative gene expression of pro-inflammatory cytokine upon treatment with the concentration of lipids and empty liposomes used for the formulations. *Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 using one way ANOVA with multiple comparison Tukey test.*

An expected result is observed with iNOS, as there is no significant decrease observed. A decrease is observed in IL-6 and IL-1β but is not significant. (Fig. 26)

In the pathogenesis of NASH, the cytokines represent as central mediators which promote injury and inflammation that may finally lead to end-stage liver diseases. The study by Bocsan, Ioana Corina, et al. showed a significant high plasmatic level of IL-6, IL-1β and TNF-α in patients with NASH compared to control groups. This resulted in systemic inflammation in NASH patients. A negative correlation between pro-inflammatory cytokines and IL-10 is observed in the same study as well as in other studies.[63]

There is a significant upregulation in the gene expression of the pro-inflammatory cytokines IL-6, IL-1β, and iNOS in RAW macrophages upon activation by LPS and IFN-γ. From literature it is evident that itaconate does not produce a notable effect in the downregulation of pro-inflammatory cytokines, the main reason could be that the chemical property of itaconate makes it cell membrane non-permeable. Hence research has been using esterified derivatives of itaconate. Electrophilic stress plays a major role in the immunological impact of itaconate and its derivatives. A study by Bambouskova et al showed that the endogenous itaconate contributes to the activation of Nrf2 and ATF3.[64] Dimethyl itaconate is the most electrophilic of the derivatives followed by 4 octyl itaconate[64], [65] however, the magnitude of electrophilicity of itaconate is yet to be explored. In the study by Swan, Amanda et. al. we can understand that itaconate behaves distinctly different from the derivatives due to its electrophilic properties and it has been suggested itaconate serves as an immunoregulatory rather than a purely immunosuppressive metabolite.[53]

From the results, we see a distinguished immunological impact of the sodium itaconate liposomes compared to sodium itaconate. Sodium itaconate liposomes significantly downregulate the pro-inflammatory cytokines IL-6, Il-1β, and iNOS. Hence a conclusion can be achieved that the sodium itaconate was delivered to the cells and liposomes are an excellent delivery mechanism. The biological pathways followed by itaconate and cited from literature are through the Nrf2 pathway and an ATF3-dependent mechanism.[36], [53] Further experiment is required to confirm the expression of Nrf2 and ATF3 to confirm the immunological effect of itaconate. We see that sodium itaconate liposome downregulates iNOS but the NO reduction percentage result does not correlate with the same. Nitric oxide production is dictated by the iNOS protein. Further experimentation is required to establish a relation between the gene expression of iNOS with the NO level in the cell medium and the degradation of the iNOS protein under chronic inflammation. 4 octyl itaconate is a cell-permeable derivative of itaconate and has a thiol reactivity closest to itaconate and hence is suggested as the best alternative for itaconate. Literature also suggests that 4 octyl itaconate can de-esterify to itaconate intracellularly.[65] A formulation was devised to encapsulate 4 octyl itaconate because 1) it is a hydrophobic drug and hence lipid encapsulation was a better strategy for delivery and 2) dimethyl itaconate is hydrophilic and a small molecule and has a high rate of leakage from liposome, which was previously tested by us.

4.5. Sodium itaconate liposome highly upregulates IL-10

An interesting observation was noticed for the anti-inflammatory marker IL-10 . Both sodium itaconate and sodium itaconate liposome significantly express IL-10, compared to the derivatives itaconate.(Fig.27A) A significant increase in IL-10 is observed in 0.25mM 4 octyl itaconate lipid nanoparticles. (Fig.27B) Empty liposomes and lipids do not show any expression of IL-10. (Fig.28) This is expected as lipids have not been studied to show significant anti-inflammatory effects.

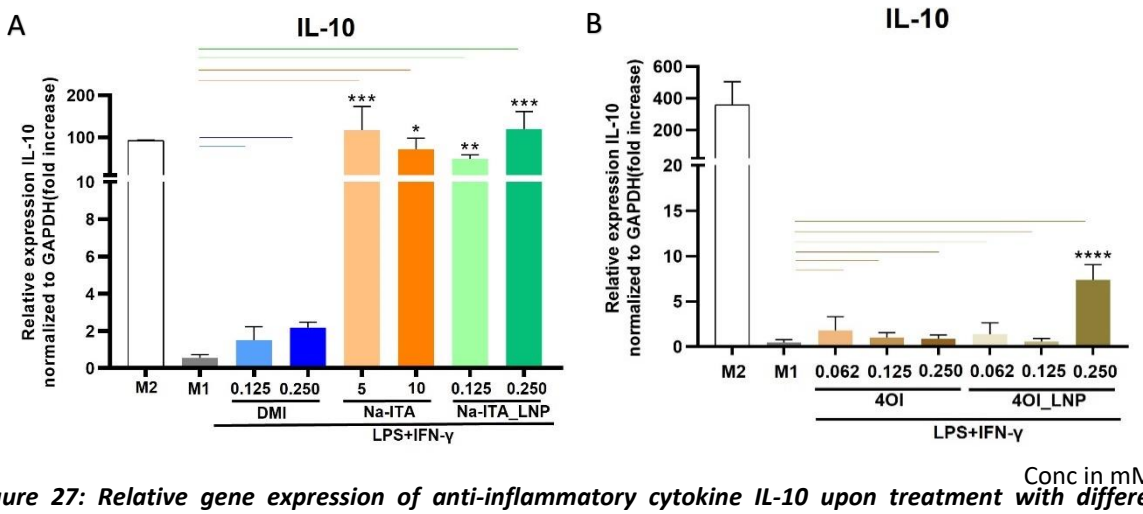

Figure 27: Relative gene expression of anti-inflammatory cytokine IL-10 upon treatment with different concentrations of DMI, Na-ITA, Na-ITA_Lip, 4OI, and 4OI_LNP. *A dose-dependent effect is observed across the treatment conditions. Sodium itaconate liposomes and sodium itaconate showed a significant upregulation of IL-10 compared to the derivative dimethyl itaconate. The highest concentration of 4 octyl itaconate lipid nanoparticles showed a significant upregulation of IL-10 compared to the derivative 4 octyl itaconate. Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 using one way ANOVA with multiple comparison Tukey test.*

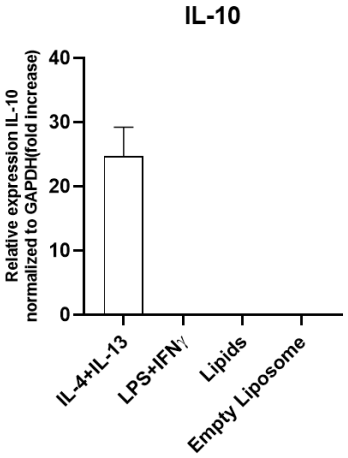

Figure 28: Relative gene expression of anti-inflammatory cytokine upon treatment with the concentration of lipids and empty liposomes used for the formulations. *Data are presented as means ± SD. *P < 0.05, **P < 0.01, ***P<0.001 and ****P<0.0001 using one way ANOVA with multiple comparison Tukey test.*

This is an interesting result as sodium itaconate and sodium itaconate liposome, both showed an upregulation in IL-10 under inflammatory conditions. The serum IL-10 progressively decrease with the level of steatosis.[63], [66]–[68] The balance between pro- and anti-inflammatory cytokines plays a major role in reducing the progression of NASH to cirrhosis. More insight is required to conclude this prominent effect of itaconate, but it has been suggested in the literature that itaconate weakly inhibits IκBζ.[53] The study by Hober, Sebastian, et al. shows that IκBζ is indeed an activator of IL-10,[69] but further research is required to understand this unique result. Similarly, an upregulation in IL-10 is observed in 0.25mM 4 octyl itaconate lipid nanoparticles. Both formulations show promising anti-inflammatory effects and it is evident that the balance between pro- and anti-inflammatory cytokines plays a major role in reducing the progression of NASH.

5. Conclusion

The pathophysiology of NASH involves altered metabolism, hepatic inflammation, and fibrogenesis. Hepatic activation of immune cells is centrally involved in the pathogenesis of NASH progression to fibrosis.[59], [70] Hence, resolving NASH is a multipoint system. Till date no pharmacotherapy is approved for NASH. Liver fibrosis represents a common stage of CLD with a major impact on the human population, and there is an eager need for novel treatments to block and reverse the underlying pathological process. Unfortunately, the current drugs in the clinical trial for NASH are failing to show an optimal therapeutic effect. This primarily due to the systemic effect the drug provides rather than a targeted effect, this brings adverse side effects whilst resolving NASH pathogenesis. Immunometabolism provides a promising solution to the immune suppression brought by the conventional drugs. Varied external stimuli leads to a change in metabolism of immune cells bring a drastic change in the metabolic pathways chosen by the cells to provide energy. This change or reprogramming can lead to accumulation of certain metabolites. These metabolites bring an immunomodulatory change, either inflammatory or anti-inflammatory. One metabolite of interest is itaconate, a metabolite of the TCA cycle produced due to inflammatory stimuli to macrophages. Dismally, these metabolites are small molecules and are cleared at a faster rate. Nanomedicine paves an interesting path for this. With their innate property of targeting liver and macrophages, they can be deemed as a superior choice for pharmacotherapy of NASH. Nanomedicine increases systemic bioavailability, controlled release of drug and lessens adverse effect. [71] Liposomes are promising drug delivery mechanism, due to their physical and chemical properties.

In a word, this project developed a lipid encapsulation formulation for sodium itaconate liposomes and 4 octyl itaconate and evaluated the same to show a favorable anti-inflammatory effect. From literature we infer that itaconate is cell non permeable and hence show no effect upon exogenous addition. Therefore, derivatives were developed to mitigate this issue. Unfortunately, itaconate and its derivatives are in fact small molecules and need continual administration, as they can have high clearance rate. Furthermore, the derivatives are highly electrophilic and repeated administration would result in immunosuppression. Therefore, lipid encapsulation was the golden choice for delivery of itaconate and its derivatives. These formulations provide a great scope in the future for the treatment of liver disease and other chronic inflammatory diseases.

6. Future Perspectives

The *in-vitro* results from this study establish a promising anti-inflammatory effect. That was the key scope of this project. Secondarily, the mechanism behind the therapeutic effects of the sodium itaconate liposomes and 4 octyl itaconate lipid nanoparticles. From the literature, it is evident that the main anti-inflammatory pathway for itaconate and its derivatives is through the activation of Nrf2, inhibition of IκBζ through activation of ATF3, and the inhibition of SDH Therefore, the next step in the project would be to investigate the protein expression of Nrf2, ATF3 and SDH to demonstrate the therapeutic effects. The current study utilizes the treatment conditions under M1 stimulated macrophages, it would be an interesting idea to evaluate the formulations in M2 macrophages and check the anti-inflammatory markers to have a perspective of the polarization ability of the formulation. The ability of itaconate to polarize macrophages is still being researched hence little is known about it and would be interesting to study as the result of upregulation of IL-10 by sodium itaconate liposome and sodium itaconate is established in this study. Another aspect to check would be whether itaconate acts through a surface receptor because it is seen that free sodium itaconate also upregulates IL-10 but does not downregulate the pro-inflammatory cytokines.

These formulations have been evaluated *in-vivo* for NASH mouse models and further *in* and *ex vivo* tests are to be performed to demonstrate the therapeutic effects to further establish the therapeutic effects of the formulated nanomedicine. The primary scope of this project was to tackle the inflammation in NASH, but it can be broadened to check the anti-fibrotic effect of the formulations, as a study establishes the protective effects of itaconate in pulmonary fibrosis.[72] The extended *in-vivo* study can then include the study of mitigating long-term inflammation.

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