Studies on Adiponutrin and Microsomal Triglyceride Protein in Non-alcoholic Fatty Liver Disease patients

Thesis submitted for the award of the degree of

Doctor of Philosophy

in

Cell Biology and Molecular Genetics

Under the faculty of Allied Health and Basic Sciences

by

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2022

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ACKNOWLEDGEMENT

First and above all, I praise God, for providing me this opportunity and granting me the capability to complete my thesis.

This project, the size of a doctoral thesis cannot be completed alone and it is a great pleasure for me to thank many people who helped me in the pursuit of this doctoral work.

I would like to express my sincere gratitude to my Guide **Dr. Kiranmayee P.,** Associate Professor & HoD, Department of Cell biology and Molecular Genetics for her constructive criticism, and feedback throughout my research work. This wouldn't have been possible for me if it wasn't for her guidance and encouragement. I couldn't have asked for a better mentor for my Ph.D. research.

I extend my heartfelt thanks to **Dr. Sharath Balakrishna** his constant support and words of encouragement which has always kept my morale up.

My sincere thanks to **Dr. Venkateshwarlu Raavi.,** Assistant Professor, Dept. of CBMG for his support and encouragement.

I owe my gratitude to **Dr. Prabhakar Kamarthy,** Professor, Department of General Medicine, for his co-operation and support.

I sincerely thank the technicians from CDLS who have helped in collecting the samples from the patients and given me their valuable time.

My Special thanks to **Ms. Claire Dias, Mrs. Moutusi Saha, Mrs. Devinder Kaur and Mr. Amrik Kamboj** for their constant support throughout my journey of Ph.D.

I would also like to sincerely thank **Mrs. Priyadarshini, Mr. Shreenath, Mrs. Nagamani and Mrs. Bhagyalakshmi**. **N,** from the Department of Cell Biology and Molecular Genetics, for always being so co-operative and helpful in all possible ways.

I would like to thank **Mr. and Mrs. Venkatesh** for their valuable support in the need of the hour.

Last but not least I am immensely grateful to my parents **Mrs. Ranjana Shukla** and **Mr. Omprakash Shukla**, without whose support this wouldn't have been possible. Their encouragement and belief in me has kept me going.

A special thanks to my sister **Ms. Preeti Shukla** for always encouraging me and making me smile on my difficult days.

I dedicate my thesis to my grandfather **Late Mr. Lakhpati Shukla**, without him nothing would've been possible.

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ABBREVIATIONS

ADPN	Adiponutrin
AGPAT	Acyglycerol-3-phosphate acyltransferases
ALD	Alcoholic Liver Disease
ALKP	Alkaline Phosphatase
ALT	Alanine transaminase
AST	Aspartate aminotransferase
ApoB	Apolipoprotein B
BMI	Body mass index
CHC	Chronic Hepatitis C
CHREBP	Carbohydrate- Responsive Element Binding Protein
CVD	Cardiovascular disease
DAGs	Diacylglycerols
DMEM	Dulbecco's Modified Eagle Medium
DNL	De- novo lipogenesis
ELISA	Enzyme-linked immunosorbent assay
FATPs	Fatty acid transport proteins
FBS	Fetal Bovine serum
GGT	Gamma-glutamyl Transferase
GWAS	Genome Wide Association Studies
HCC	Hepatocellular carcinoma

HCV	Hepatitis C Virus
HepG2	Human hepatocellular cell lines
HSL	Hormone Sensitive Lipase
IL- β	Interleukin- 1β
Iκκβ	Inhibitor of nuclear factor kappaβ
LLTP	Large Lipid Transfer Protein
MGL	Monoacylglycerol Lipase
MTTP	Microsomal triglyceride transfer protein
NAFLD	Non-alcoholic Fatty Liver Disease
NASH	Non-alcoholic Steatohepatitis
NASH CRN	NASH clinical research network
NCCS	National Centre for Cell Science
NF-κβ	Nuclear factor-kappaβ
NIDDK	National Institute of Diabetes and Digestive and Kidney Disease
PA	Phosphatidic acid
PDI	Protein disulfide isomerase
PNPLA3	Patatin-like phospholipase domain containing-3
PPAR-γ	Peroxisome Proliferator-Activated Receptor gamma
ROS	Reactive oxygen species
SNPs	Single Nucleotide Polymorphisms
SOCS3	Suppressor of cytokine signalling 3
SREBP-1c	Sterol Regulatory Binding Protein-1
T2DM	Type 2 Diabetes Mellitus

TE	Transient Elastography
TG	Triglycerides
TNF-α	Tumor necrosis factor – alpha
VLDL	Very Low Density Lipoprotein

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ABSTRACT

Abstract

Non-alcoholic Fatty Liver Disease (NAFLD) is one the chronic liver diseases, usually linked to obesity, diabetes mellitus and characteristics of metabolic syndrome. NAFLD is defined as unusual accumulation of fats in absence of alcohol intake by an individual. The two major causes of fat accumulation in liver are; decreased fat degradation and decreased fat export. The fat accumulation is in the form of triglycerides. Hydrolysis of triglycerides is carried out by lipase enzymes and is exported by transfer protein. Adiponutrin (ADPN) plays a role in triglyceride breakdown and Microsomal triglyceride protein (MTP) plays a role in fat export. Therefore, the aim of the current study was to study ADPN and MTP in NAFLD patients.

The present study is an observational study. The serum sample of 60 NAFLD subjects was collected, and was divided based on the hepatic steatosis and NASH cases. These were subdivided into cases with and without comorbidities. The serum levels of adiponutrin (ADPN) and microsomal triglyceride protein (MTP) were estimated in study groups. An in-vitro study was carried out using Human hepatocellular cell lines (HepG2) under different combinations of glucose, fructose and sucrose to study their effect of ADPN and MTP levels.

The serum levels of ADPN and MTP were observed to be lower in NASH subjects as compared to hepatic steatosis cases. On further sub group analysis, it was observed that cases with comorbidities had lower serum levels of ADPN and MTP as compared to cases without comorbidities. The relationship of study molecules with triglycerides and serum transaminases (ALT and AST) was also assessed. A negative correlation was observed between study molecules and triglycerides as well as VLDL.

The serum transaminases showed a positive correlation with ADPN and weak negative correlation with MTP. The findings of in vitro study showed increased lipid accumulation and increased triglycerides levels after 7 days of treatment with glucose, fructose, sucrose and their combinations. The levels of ADPN and MTP were increased in in-vitro condition after 7 days of treatment.

In conclusion, the serum levels of ADPN and MTP decreases as NAFLD progresses. Therefore, ADPN and MTP can be considered as complementary markers that will help differentiate the stages of NAFLD and assess disease progression. Further the in-vitro study findings suggest that ADPN and MTP levels are affected by nutritional status.

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CHAPTER I
INTRODUCTION

Non-alcoholic Fatty Liver Disease (NAFLD) is one of the chronic liver diseases characterized by excess fat accumulation in absence of alcohol consumption [Kawano Y et al., 2013]. In India, the prevalence rate ranges from 9 to 32%, with 25% of the global population being affected [Kalra S et al., 2013]. Prevalence of NAFLD is increasing worldwide every year due to current trends in dietary and sedentary lifestyle [Fan JG et al., 2017]. Although a human liver can transport and store fats from the bloodstream and peripheral tissues, it cannot retain large amount of fat, and hence excessive fat accumulation is recognized as pathological condition.

The hallmark of NAFLD is accumulation of triglyceride (TG) in cytoplasm of hepatocytes that exceeds 5% of liver weight [Fabbrini E et al., 2015]. Usually majority of people will have simple steatosis that is only the accumulation of fat without any inflammation and hepatocellular injury and will never progress to advanced stages of liver disease. But few of the patients may progress to Non-alcoholic steatohepatitis (NASH) which includes inflammation and hepatocellular injury and further progress to cirrhosis followed by hepatocellular carcinoma (HCC) [Lambrecht J et al., 2021]. Advancing stages increases the risk of liver – related mortality and is also a leading indication of liver transplantation.

Multiple factors are found to be responsible for fat accumulation which include metabolic disorders like obesity, type 2 diabetes mellitus (T2DM), cardiovascular disease (CVD), hyperlipidemia, hypertension, genetic predisposition and the lifestyle of an individual. Obesity and Diabetes are primary risk factors of NAFLD [Martínez - Montoro JI et al., 2021].

Obesity is an independent risk factor for NAFLD [Li L et al., 2016]. According to meta-analysis obese NAFLD patients have higher transaminase levels, higher degree of hepatic steatosis, higher chance of developing hypertension, diabetes mellitus, hypertriglyceridemia, liver inflammation and liver fibrosis than non-obese NAFLD patients [Lu FB et al., 2018]. In obese patients, the increasing fat accumulation leads to adipocyte death and cytokine release, which adds to activation and chemotaxis of inflammatory cells [Du Plessis J et al., 2015]. This may further result in progression of NAFLD.

Prevalence of NAFLD with T2DM is 2-fold higher than in general population. The overall prevalence of NAFLD in T2DM patients is 55.2%. It accelerates the progression of liver disease in NAFLD [Younossi ZM et al., 2019]. Moreover, NAFLD in diabetic patients increases the risk of cardiovascular diseases and other diabetic complications [Targher G et al., 2007]. Insulin resistance causes increase in fatty acid release from adipose tissue [Kim DS et al., 2017]. In diabetic patients, increased hepatic free fatty acid influx directly causes lipotoxicity, which in turn causes inflammation and liver fibrosis [Tomita K et al., 2014, Marra F et al., 2018]. Oxidative stress induced by oxidation and metabolism of excessive free fatty acids in the liver results in additional damage to liver [Paradies G et al., 2014]. This eventually contributes in progression of NAFLD.

Prehypertension and increased hypertension are associated with NAFLD and its development. A recent study suggests that the presence of NAFLD is significantly associated with 1.55 fold increased risk of hypertension, whereas hypertension is significantly associated with 1.63 fold increased risk of NAFLD [Li G et al., 2022].

There are indications that the inflammation associated with NAFLD may promote the sympathetic nervous system activation and thus, induce hypertension [Carnagarin R et al., 2019].

NAFLD and CVD are both indicators of end-organ damage of the metabolic syndrome. An individual with initial stages of NAFLD is at risk of CVD. CVD's are one of the leading cause of deaths in NAFLD patients. The pathophysiology behind the association between NAFLD and CVD is not yet completely known. Although insulin resistance is thought to be a contributing factor, other pathways including oxidative stress, inflammation, and gut microbiota, may also play a role in pathophysiology of the link between NAFLD and CVD [Chiriac S et al., 2021].

NAFLD is a multifactorial disease and has a complex mechanism [Yu Y et al., 2019]. There are two major causes of excess fat accumulation in the liver; first is the decreased fat degradation and second is the decreased fat export. As mentioned above, fat accumulation is usually in the form of triglycerides (TGs) [Dongiovanni P et al., 2015]. Hydrolysis of TGs in hepatocytes is carried out by lipase enzyme. Lipase converts TGs into free fatty acid and glycerol.

Adiponutrin (ADPN) is a triglyceride lipase which mediates hydrolysis of TGs in hepatocytes [Pingitore P et al., 2019]. ADPN is encoded by Patatin- like phospholipase domain-containing protein 3 (PNPLA3) gene. PNPLA3 was first identified in potato tubers and therefore it is known as patatin-like phospholipase domain containing-3 gene. PNPLA3 belongs to PNPLA family which consists of nine members, among all these nine members, PNPLA3 shows highest homology with PNPLA2 [Kienesberger PC et al., 2009]. PNPLA2 also known as adipose triglyceride

lipase and plays a critical role in triglyceride metabolism by mediating a rate-limiting step in triglyceride hydrolysis. Location of human adiponutrin is on the long arm (q) of chromosome number 22 at 13.31 position and has molecular weight of 52.8kDa and encodes for 481 amino acids [Baulande S et al., 2001].

Several studies have demonstrated that ADPN gene variant is involved in hepatocellular lipid droplets remodeling and VLDL secretion as a major determinant of inter individual and ethnicity-related differences in hepatic fat content. The variant in this gene encodes for isoleucine to methionine substitution at 148 (I148M) position, which leads to impaired function of ADPN. The impaired function of ADPN leads to imbalance in TGs metabolism resulting in steatosis.

The stored fats in the liver is exported in the form of Very Low-Density Lipoprotein (VLDL) to peripheral tissues [Alves-Bezerra M et al., 2017]. VLDL is formed by incorporation of triglycerides into apolipoprotein B by Microsomal triglyceride protein (MTP) [Raabe M et al., 1999]. MTP is localized in endoplasmic reticulum of hepatocytes and enterocytes [Wetterau JR et al., 1991]. It was identified as major cellular protein capable of transferring neutral lipids like triglyceride and cholesterol. A human MTP gene is approximately 55kb in length and is located on long arm (q) of chromosome number 4. MTP is a heterodimer and consists of two subunits, P subunit (~58kDa) and M subunit (~97kDa). The P subunit is also known as protein disulfide isomerase (PDI). PDI by itself lacks lipid transfer activity. A non-covalent association of M subunit with PDI generates a fully functional lipid transfer complex that is MTP. The role of P subunit in biosynthesis of MTP is more likely

related to structural stabilization and solubilization of the complex [Hussain MM et al., 2012].

Lower hepatic expression of MTP plays a crucial role in development of NAFLD. The most common and widely investigated polymorphism in MTP gene is -493G > T (rs1800591), which contributes to increased risk of NAFLD [Li L et al., 2014]. MTP -493 G >T polymorphism may result in decrease in its protein and aberrant alterations of MTP synthesis and secretion, this influences the capacity for lipid export and induce dysregulation of hepatic lipid metabolism [Zheng W et al., 2014], thus contributing to development of NAFLD.

CHAPTER II
RATIONALE

NAFLD comprises of simple steatosis, NASH, and cirrhosis, leading to hepatocellular carcinoma. Diagnosis and prognosis of NAFLD is based on blood tests like liver function tests, various non-invasive medical imaging techniques like ultrasound, magnetic resonance imaging, and computerized tomography and scoring systems. However, the serum levels of liver enzymes are elevated in all liver disorders and are not specific to NAFLD. It was also found that 57% NAFLD cases had serum levels of enzymes within the reference range. Hence, serum transaminase levels alone could never be used as a screening test for NAFLD. The non-invasive medical imaging techniques available can only diagnose NAFLD but cannot differentiate the stages of NAFLD. Liver biopsy is the only gold standard for differentiating the stages of NAFLD. However, it is not possible to get a liver biopsy done on every patient because it involves chances of bleeding, bacterial infections, and accidental injury to a nearby organ and also due to high incidence of NAFLD. Simple steatosis is a benign stage that is characterized by fat accumulation but without inflammation of hepatocytes and is reversible. NASH is an advanced stage that involves inflammation of hepatocytes and scarring of tissue. This is an irreversible stage and the patients with NASH have a higher risk for liver-related morbidity and mortality than patients with simple steatosis. Moreover, as simple steatosis and NASH are asymptomatic, many patients are only diagnosed at advanced stages. So, it is very important to stratify the NAFLD patients and identify those who need more attention and regular follow-up.

This demands for non-or minimally invasive biomarkers to differentiate stages of NAFLD. Thus, this study was designed to analyze the levels of ADPN and MTP in

simple steatosis and NASH among NAFLD subjects due to their pivotal role in lipid metabolism. Since food habits contribute to the pathogenesis of NAFLD, the effect of nutritional status on ADPN and MTP under in vitro conditions was also evaluated.

CHAPTER III
AIM AND OBJECTIVES

Aim

To determine the role of Adiponutrin and Microsomal Triglyceride Protein in Non-alcoholic Fatty Liver Disease progression

Objectives

1. To compare the serum levels of ADPN and MTP between simple steatosis and NASH patients to assess their role in disease progression
2. To compare ADPN and MTP levels between simple steatosis and NASH patients with and without comorbidities
3. To correlate ADPN and MTP levels with Triglycerides, VLDL and serum transaminases (ALT and AST)
4. To study the effect of nutritional status on ADPN and MTP in an *in vitro* condition

CHAPTER IV
REVIEW OF LITERATURE

BACKGROUND:

Liver regulates lipid homeostasis through complex systems of biochemical, cellular and signaling mechanisms. Liver consists of different types of cells like Kupffer cells, endothelial cells, stellate cells and hepatocytes. Hepatocytes are the cells which control the metabolic functions like triglyceride metabolism. A human liver can store fats from circulation and can also transport to the peripheral tissues. Generally, liver cannot store much fats therefore, excess fat accumulation is considered as pathological condition. This pathological condition is termed as Non-alcoholic fatty liver disease (NAFLD). NAFLD is defined as fat build up in more than 5% of hepatocytes in absence of alcohol consumption by an individual. NAFLD is affecting over a quarter of global population and has emerged as highest prevalent type of chronic liver disease [Younossi ZM et al., 2016].

The likelihood of developing NAFLD is influenced by numerous risk factors.

1. **Metabolic disorders**

Metabolic disorders are considered as major health challenge, and is closely related with NAFLD. Metabolic disorders like diabetes, obesity, hypertriglyceridemia, hypertension, CVD contribute to progression of NAFLD [Gaggini M et al., 2013]. The pathophysiology of metabolic disorders and NAFLD is interconnected and therefore, metabolic disorders are one of the major risk factor contributing in NAFLD progression [Dongiovanni P et al., 2021]. As they are interlinked it becomes difficult to say sometimes whether NAFLD causes metabolic disorders or vice versa. A growing body of research indicates that NAFLD is not only an independent risk factor

for CVD and T2DM, but alps a substitute marker for metabolic syndrome [Armstrong MJ et al., 2014]. About one-third of the NAFLD patients meet the criteria for the metabolic syndrome, and approximately 90% of patients have more than one component of the syndrome [Marchesini G et al., 2003].

Intrahepatic fat accumulation in the form of triglycerides is the key feature of NAFLD and insulin resistance is thought to play a significant role in this process by promoting the transfer of free fatty acids from visceral fat storage to or peripheral lipolysis into the liver [Bugianesi E et al., 2005]. Numerous studies show that the metabolic disorders like obesity, insulin resistance, diabetes mellitus, dyslipidemia, and hypertension are significantly linked to NAFLD [Bedogni G et al., 2005, Marchesini G et al., 2003, Leite NC et al., 2009].

Sedentary lifestyle and consumption of high-fat diet

Lack of exercise or physical activity and intake of high calorie or high sugar diet leads to fat accumulation which eventually contributes to hepatic steatosis [Stevanović J et al., 2020]. Sedentary behavior in general has been linked to increased risk of metabolic syndrome, obesity, T2DM [Dunstan DW et al., 2007], and NAFLD [Kaminsky L, 2006]. Sedentary behavior has been recognized as an independent risk factor for NAFLD in prospective cohort studies [Ryu S et al., 2015], thus increase in sedentary lifestyle plays a potential role in development and progression of the disease.

Unhealthy lifestyle changes have been connected to global urbanization and modernization in the 20^{th} and 21^{st} centuries. As a result, during the past three decades,

both the mean worldwide body mass index (BMI) and the prevalence of obesity have dramatically grown. As mentioned above, obesity is one of the pathophysiological cause of NAFLD [NCD Risk Factor Collaboration, 2016]. Numerous animal studies have shown that a high-fat diet rapidly induces hepatic steatosis [McCuskey RS et al., 2004, Samuel VT et al., 2004, Kim SP et al., 2003]. A crossover design with 10 obese women and two consecutive isocaloric diets with either 16% or 56% of energy coming from a fat were placed in randomized order, and the liver fat was then measured by proton spectroscopy to directly test the relationship between total dietary fat and hepatic fat content in humans. On the low- fat diet, liver fat dropped by 20%, while it increased by 35% on high-fat diet. Change in fasting serum insulin concentrations was observed along with increase in liver fat [Westerbacka J et al., 2005].

Genetic predisposition

Genetics plays a crucial role across the spectrum of NAFLD pathogenesis. Genetic research on NAFLD includes heritability studies [Younossi Z et al., 2018, Eslam M et al., 2016], familial aggregation studies [Abdelmalek MF et al., 2006, Willner IR et al., 2001], candidate gene studies [Daly AK et al., 2011], and genome wide association studies (GWAS) [Chalasani N et al., 2018, Romeo S et al., 2008, Speliotes EK et al., 2011]. GWAS is now the standard method for examining the relationships between various phenotypes (diseases) and the millions of single nucleotide polymorphisms (SNPs) that make up the human genome. Over recent years, GWAS have dramatically improved our understanding of the genetic factors related to NAFLD susceptibility, development, and consequences [Eslam M et al., 2018].

There are two major causes of excess fat accumulation in the liver; first is the decreased fat degradation and second is the decreased fat export. The fat degradation is carried out lipases and the fat export is carried out by transfer protein. Adiponutrin (ADPN) is one of the widely studied protein which has lipase activity against triglycerides in liver. The triglycerides in the form of very low density lipoprotein (VLDL) are exported by microsomal triglyceride protein (MTP) from liver to peripheral tissues.

ADIPONUTRIN

Adiponutrin (ADPN) is encoded by Patatin like phospholipase domain containing protein-3 (PNPLA3) gene. It has multiple names in the literature like calcium-independent phospholipase A2 epsilon, chromosome 22 open reading frame 20 (C22orf20) PNPLA3 [Dong XC, 2019]. PNPLA3 is the most extensively researched gene in NAFLD. According to gene expression studies, the human ADPN gene is expressed in wide range of tissues, however liver has the highest levels of expression, followed by skin, adipose tissue, kidney, brain, and spleen [Huang Y et al., 2010, Pirazzi C et al., 2014]. Location of human adiponutrin is on the long arm (q) of chromosome number 22 at 13.31 position and has molecular weight of 52.8kDa and encodes for 481 amino acids [Balaunde S et al., 2001].

Adiponutrin belongs to PNPLA family. PNPLA family comprises of nine members (PNPLA1-PNPLA9). The patatin-like phospholipase domain unites the members of PNPLA family [Wilson PA et al., 2006]. These 9 members are found in different human tissues and are involved in a wide range of cellular functions. The physiological function of some members is still unknown. PNPLA2 is highly

expressed in adipose tissues, and regulates the concentrations of stored lipids. PNPLA8 is a cardiac phospholipase, and upholds mitochondrial integrity. Whereas, PNPLA6 has been linked to the Golgi apparatus and endoplasmic reticulum in neurons, and is been suggested to be involved in maintaining axon integrity [Bamji-Mirza M et al., 2011].

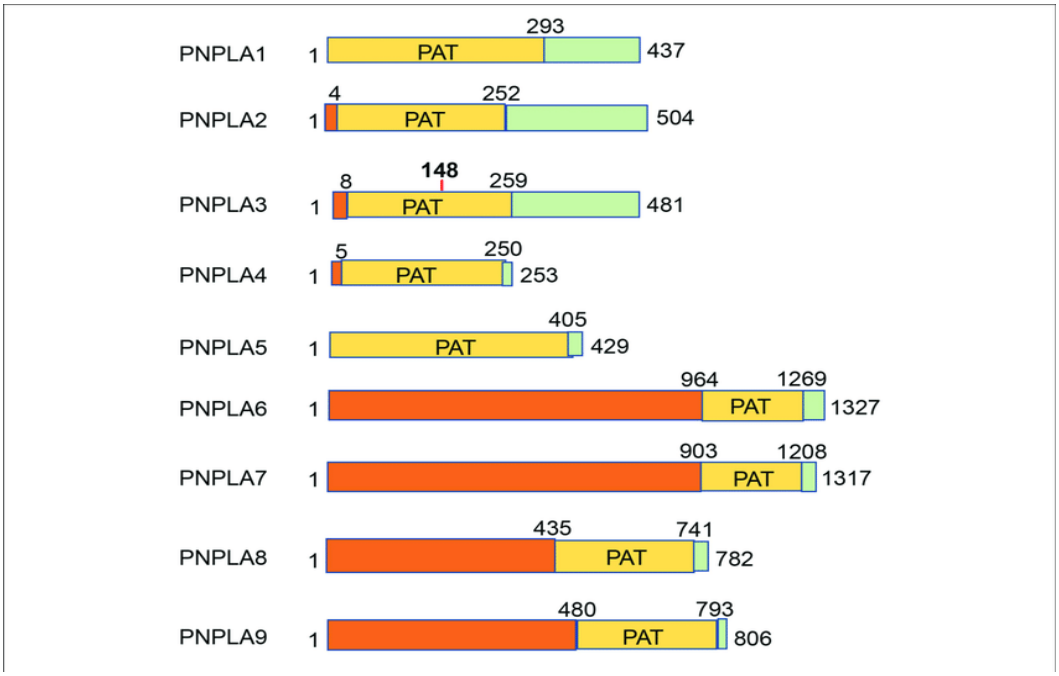


Fig. 1 Schematic representation of PNPLA family. The yellow color denotes the patatin phospholipase domain and the number on right denote the protein length. PNPLA: Patatin- like phospholipase domain containing enzymes

In vitro, ADPN gene exhibits triglyceride hydrolase as well as transacylase activity [Jenkins CM et al., 2004]. So it promotes either catabolism or anabolism of triglycerides. With respect to triglycerides containing mono and polyunsaturated fatty acids, it was observed that PNPLA3 gene has strong enzymatic activity against both of them [Huang Y et al., 2011, Li JZ et al., 2012]. Studies reveal that ADPN gene

expression decreases while fasting and increases after eating, specifying that the amount of protein released is controlled as necessary to aid in the processing and storing dietary fats. This gene is known to be highly affected by nutritional status [Huang Y et al., 2010].

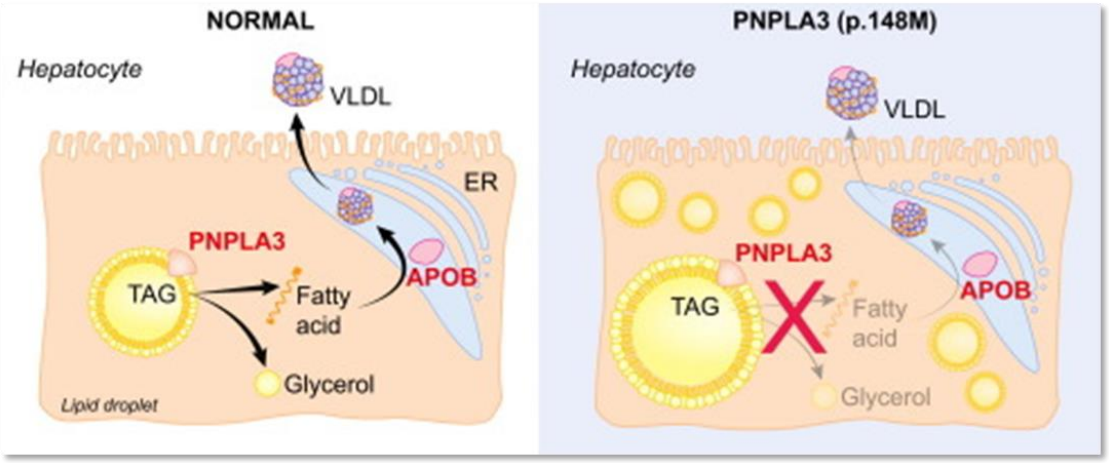


Fig. 2 Function of ADPN (PNPLA3) in Normal and Variant condition

According to a 2008 genome wide association study (GWAS), variation in PNPLA3 have been linked to NAFLD. Variation in PNPLA3 gene changes the amino acid isoleucine to methionine at 148 position, which is represented by I148M, and this variation is strongly associated with accumulation of fat content in hepatocytes due to loss of function [Romeo S et al., 2008]. Several studies have confirmed that I148M has association with hepatic fat, in adults as well as in developmental age [Yuan X et al., 2008, Johansson LE et al., 2008, Kotronen A et al., 2009, Kotronen A et al., 2009, Kollerits B et al., 2009, Sookoian S et al., 2009, Kantartzis K et al., 2009, Romeo S et al., 2010, Rotman Y et al., 2010, Speliotes EK et al., 2010, Valenti L et al., 2010, Sookoian S et al., 2011, Romeo S et al., 2010, Santoro N et al., 2010, Valenti L et al., 2010, Viitasalo A et al., 2015].

Structure of Adiponutrin

Consensus serine lipase motif is present in the conserved structural motif of the patatin-like domain, which is composed of a sheet sandwiched between two helices and differs from classical lipases by using a catalytic dyad rather than a catalytic triad to impact hydrolysis [Bruschi FV et al., 2017].

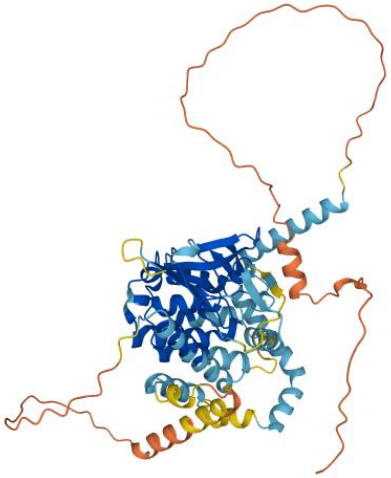


Fig. 3 Crystallized structure of Adiponutrin

ADPN in various disease conditions–

ADPN or ADPN gene is expressed in liver cells, therefore, is studied and associated only with the liver disorders. I148M is the most widely studied variant of ADPN gene, and is been associated with the liver disorders like Chronic Hepatitis C (CHC), Alcoholic liver disease (ALD), and NAFLD.

CHC is one of major causes of end-stage liver disease hepatocellular carcinoma [Fernández-Rodríguez A et al., 2013]. According to reports, steatosis has negative impact on the course of CHC, as it is linked to be more aggressive histological

characteristics, a quicker advancement of fibrosis, and a poorer response to treatment. Recent research has demonstrated that I148M variant affects the development of fibrosis in CHC patients. For the first time, Valenti et al., (2011) [Valenti L et al., 2011] studied two distinct groups of Italian patients with CHC and found that the ADPN gene variant influences the development of steatosis independent of age, sex, BMI, alcohol intake, and viral genotype. In the same study, ADPN gene promoted fibrosis, and risk of HCC in CHC patients. Additional clinical research showed that CHC patients with ADPN gene variation had a greater chance of developing advanced fibrosis [Rüeger S et al., 2015, De Nicola S et al., 2014]. However, the relationship between ADPN gene variant and the severity of CHC infection is still debatable and perhaps not direct, given that later investigations found no connection between ADPN gene variant and the viral genotype [Clark PJ et al., 2012, Moritou Y et al., 2014].

In developing countries, chronic viral hepatitis B and C is the leading cause of HCC, while alcohol intake accounts for about 45% of cases in North America and Europe [Bosch FX et al., 2004]. The study by Valenti et al., revealed the first conclusive link between hepatic cancer and ADPN gene variant, which was supported by subsequent research. A meta-analysis of European patients with cirrhosis strongly demonstrated a correlation between the I148M variant and HCC, with a stronger association with ALD than CHC related cirrhosis [Trépo E et al., 2014], indicating that this genetic variant exerts a profound influence on process triggering carcinogenesis beyond the pathological background. Numerous studies strongly showed the strong association between the I148M variant and the risk of HCC in relation to NAFLD [Liu YL et al., 2014, Burza MA, et al., 2012, Krawczyk M et al.,

2015]. Some of the studies evaluating association between ADPN gene I148M and liver damage are summarized in Table. 1

Table. 1 Studies evaluating the association between ADPN gene I148M and liver damage

Study	Number of Participants	Population	Findings of the study
Romeo et al. [Romeo S et al., 2008]	2111	American-European and African origin, Hispanics	I148M was associated with increase in liver fat in all participants, and increased levels of ALT, AST in Hispanics.
Valenti et al. [Valenti L et al., 2010]	432/321	Italy, UK	I148M NAFLD patients were associated with severity of steatosis, fibrosis and NASH.
Hotta et al. [Hotta K et al., 2010]	253/578	Japanese NAFLD/ Controls	I148M was associated with levels of AST, ALT, ferritin, and histological fibrosis stage. I148M was susceptible to NAFLD.

Zain et al. [Zain SM et al., 2012]	144/198	Chinese, Indian, Malay	G allele was positively correlated with susceptibility to fibrosis, NASH, and NASH severity.
Liu et al. [Liu YL et al., 2014]	100	European Caucasians with NAFLD-related HCC	I148M associated with greater risk of progressive NASH, fibrosis, and HCC.
Sookian and Pirola [Sookoian S et al., 2011]	16 studies	Meta-analysis	G allele had strong influence on liver fat accumulation and susceptibility of disease progression.
Krawczyk et al. [Krawczyk M et al., 2015]	5100	Meta-analysis	The ADPN gene variant is associated with increased risk of developing HCC in NAFLD patients

Hepatic fat accumulation results either due to increased fat synthesis or decreased fat export [Postic C et al., 2008]. Hence, steatosis may also result from alterations in the release of lipoproteins. Apolipoprotein B (ApoB)100 is a mediator of liver secretion, when triglyceride is available, apoB is lapidated and a VLDL particle is

produced, this mechanism is mediated by Microsomal Triglyceride Transfer Protein (MTTP/MTP.)

MICROSOMAL TRIGLYCERIDE TRANSFER PROTEIN

Triglycerides are exported from liver in the form of VLDL particles. As mentioned above, VLDL are formed by incorporation of triglyceride into apolipoprotein B (apoB) by (MTTP/MTP) [Adams LA et al., 2005]. Alterations of MTP/apoB synthesis have been proposed as one of the potential mechanisms in pathogenesis of NAFLD leading to decreased capacity for lipid export [Charlton M et al., 2002, Namikawa C et al., 2004]. MTP is mainly synthesized in liver and small intestine and, is an endoplasmic reticulum resident protein [Wetterau JR et al., 1991]. MTP and apoB may share a common evolution of origin because the amino acid sequence of MTP is homologous to N-terminal 20% of apoB [Mann CJ et al., 1999, Van der Horst DJ et al., 2009].

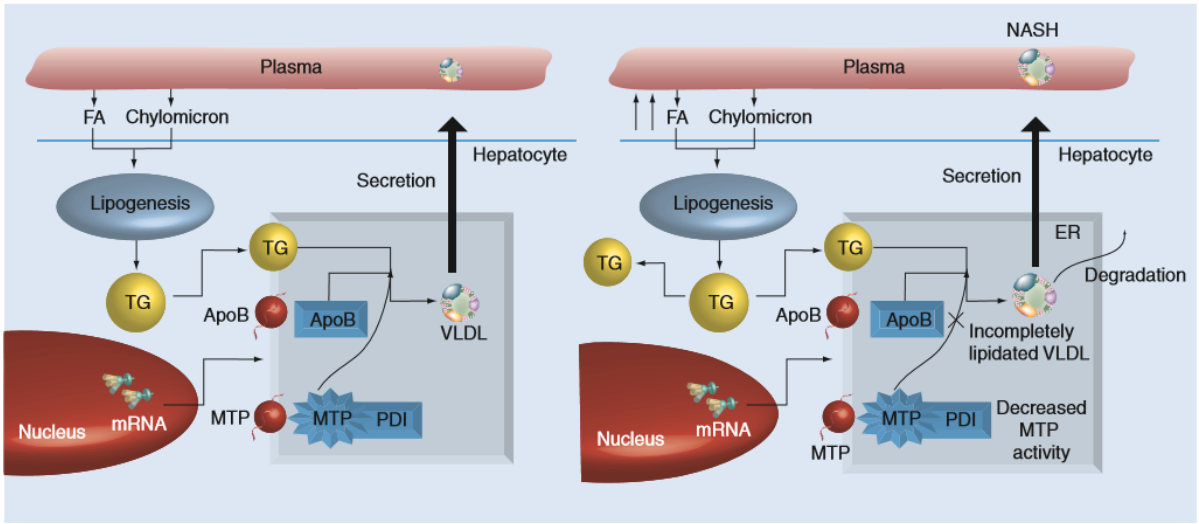


Fig. 4 Physiological role of MTP in Normal and NAFLD (NASH) conditions

In vitro, MTP was found to be capable of transferring triglycerides between vesicles and therefore, proposed as lipid transfer protein during apoB maturation [Atzel A et al., 1993]. An interaction between MTP and NAFLD is been identified with the help of genetic studies. A polymorphism at promoter region (-493G/T) of MTTP is associated in NASH with type 2 Diabetes Mellitus [Bernard S et al., 2000]. The G allele is associated with decreased MTP transcription and is prone to increased triglyceride content in hepatocytes.

Structure of MTP

In 1984, Wettarau and Ziversmith identified a protein from bovine liver microsomes that sped up the transfer of phospholipids, triglycerides, and cholesteryl esters between biological membranes. This protein was precursor of MTP [Wetterau JR et al., 1984]. MTP belongs to the large lipid transfer protein (LLTP) superfamily [Shelness GS et al., 2005]. Members of LLTP superfamily have β-barrel and α-helical domains in their amino-terminal sections, but their carboxyl-terminal lipid binding domains differ to reflect the various amount and type of lipid they are able to bind [Shoulders CC et al., 2005]. While the other members of this protein family may have evolved to move lipids outside of cells rather than inside of cells, MTP may be the first lipid transfer protein that evolved to move proteins [Hussain MM et al., 2012, Sellers JA et al., 2005].

MTP is a heterodimeric complex comprised of two sub-units. The first is the particular MTP subunit, which is 894 amino acids long (57kDa) and expressed mostly in hepatocytes and enterocytes in humans. The ubiquitously expressed multifunctional

protein disulfide isomerase (PDI) makes up the second subunit (55kDa) [Wetterau JR et al., 1990]. In the early stages of protein folding, PDI, a member of the thioredexin family, catalyses the oxidation and isomerization of disulfide bonds [Ellgaard L et al., 2005]. The function of MTP is independent of PDI's isomerase activity [LAMBERG A et al., 1996]. PDI does not have any lipid transfer activity per se, but it does appear to be crucial for preserving the complex's activity and solubility. The MTP component aggregates into insoluble compounds in absence of PDI [Wetterau JR et al., 1991].

There are three structural regions in MTP;

- An amino terminal β-barrel domain (amino acids 22-297),
- Central α-helical region (amino acids 298-603), and
- A carboxyl-terminal domain (amino acids 604-894) (Fig. 3) [Hussain MM et al., 2003, Hussain MM et al., 2012].

According to the evidence, apoB interacts with both the amino-terminal and central sections, but the central region is where the PDI binding occurs, while the carboxyl domain is where lipid binding and transfer takes place.

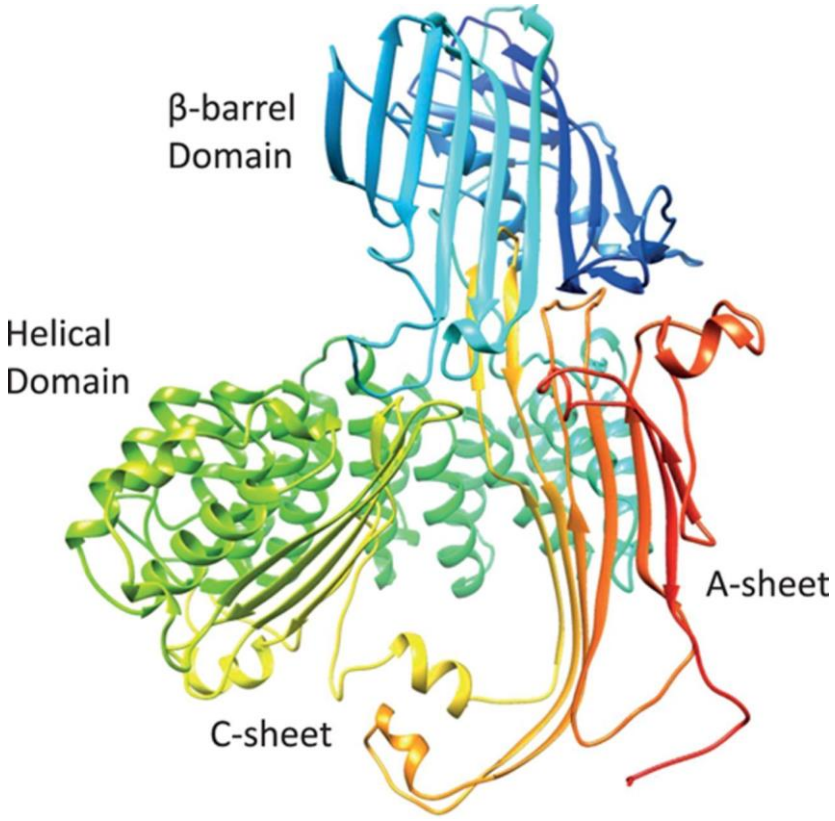


Fig. 5 Crystallized structure of MTP

MTP in various disease conditions –

Hepatitis C Virus (HCV) is the leading cause of chronic viral hepatitis. Patients who develop this condition have higher risk of cirrhosis and HCC. HCV circulates as lipo-viral particles that are rich in triglycerides and also contain ApoB, HCV RNA, and core proteins [Negro F, 2010, Mirandola S et al., 2010]. Studies investigating whether lipoprotein synthesis or MTP contribute to the spread of the HCV virus were sparked by the presence of apoB-lipoproteins in the body. It has now been established by numerous studies that viral production requires MTP activity and lipoprotein assembly [Huang H et al., 2007, Gastaminza P et al., 2008, Domitrovich AM et al.,

2005]. Low MTP activity has been reported in T2DM patients with polymorphism at promoter region of MTP gene [Phillips C et al., 2004].

Abetalipoproteinemia is a rare genetic disorder of lipoprotein metabolism affecting <1 per million people. Inability to produce chylomicrons or VLDL ad lack of MTP expression or activity are linked to a number of clinical symptoms that have an impact on several organ systems. The condition serves as a well-known example of the outcomes of total MTP inhibition in humans [Hooper AJ et al., 2015].

HISTOLOGY (STAGES) AND PATHOGENESIS OF NAFLD–

The fat accumulation is usually in the form of triglycerides and this is the dominant characteristic of NAFLD. As mentioned above, NAFLD is a broad spectrum of simple steatosis to HCC. Simple steatosis is characterized by hepatocellular damage in absence of inflammation, and this stage is usually considered as benign. NASH is characterized by hepatocellular damage with inflammation. NASH if left untreated it progresses to fibrosis, which is scarring of tissue. This further results in cirrhosis, followed by HCC.

The mechanism resulting in NAFLD is unclear. Several mechanisms have been proposed, but insulin resistance seems to be pivotal in the pathogenesis of both NAFLD and type 2 diabetes [Shulman GI., 2000, Tarantino G et al., 2013]. The pathological progression of NAFLD follows tentatively a “three-hit” process [Jou J et al., 2008] namely steatosis, lipotoxicity and inflammation. Steatosis results from the interplay between diet, gut microbiota [Jiang W et al., 2015, Kirpich IA et al., 2015], genetic factors [Romeo S et al., 2008], and de novo lipogenesis via upregulation of

lipogenic transcription factors like sterol regulatory binding protein-1 c (SREBP1c), carbohydrate- responsive element binding protein (CHREBP), and peroxisome proliferator-activated receptor gamma (PPAR-γ) [Anderson N et al., 2008]. Primarily, fatty acid (FA) is stored in the adipose tissue as triglyceride. However, in obese subjects, fatty acids seem to be misrouted from their primary storage site to ectopic sites like skeletal and hepatic tissues for re-esterification into diacylglycerols (DAGs), perhaps through increased adipocyte lipolysis. The uptake of fatty acid by these organs probably is facilitated by fatty acid transport proteins (FATPs) and FAT/CD36 (fatty acid translocase) which have been shown to be elevated in obese subjects and NAFLD patients [Greco D et al., 2008, Fabbrini E et al., 2009].

Steatosis leads to increased signaling of the transcription factor NF-κβ (nuclear factor-kappaβ), through the upstream activation of Iκκβ (inhibitor of nuclear factor kappaβ [NF- κβ]). The activation of NF- κβ induces the production of pro-inflammatory mediators like TNF-α (tumor necrosis factor – alpha), IL- 6 (Interleukin-6) and IL- β (Interleukin- 1β). These cytokines contribute to the recruitment and activation of kupffer cells (resident hepatic macrophages) [Anderson N et al., 2008] to mediate inflammation in NASH [Ramadori, G et al., 200, Joshi-Barve S et al., 2007]. Additionally, TNF-α and IL- 6 have been reported to play a role in hepatic insulin resistance through the up-regulation of SOCS3 (Suppressor of cytokine signaling 3) [Persico M et al., 2007, Torisu T et al., 2007].

The excess fat in the liver causes lipotoxicity and leads to organelle failure mainly mitochondria dysfunction and endoplasmic reticulum stress [Browning JD et al., 2004, Bell M et al., 2008]. Dysfunctional mitochondria have an elevated capacity

to oxidize fatty acid resulting in production of ROS (Reactive oxygen species) and causing oxidative stress due to an imbalance between the production of ROS and protective oxidants. Oxidative stress in NAFLD patients [Sanyal AJ et al., 2001, Tiniakos DG et al., 2010] is regarded as third result that eventually leads to hepatocyte death. The pathogenesis of NAFLD seem to be a vicious cycle of steatosis, lipotoxicity and inflammation resulting in intricate alterations in the histopathological and biochemical features of the liver.

Mechanism of Triglyceride metabolism:

Triglyceride metabolism:

The assembly of Triglyceride molecules constitutes the principal means by which the liver stores and exports fatty acids. Under normal conditions, the liver stores little triglyceride, but exports considerable amounts in the form of VLDL particles that deliver Fatty acids to muscle and fat tissue, depending on nutritional status. Prior to NAFLD development, it was thought that the excess triglycerides stores contributed to lipotoxicity [Day CP et al., 1998]. However, emerging theories contend that increased triglycerides storage and release of very-low density lipoproteins protect against fatty acid mediated hepatotoxicity.

Triglyceride synthesis:

In most mammalian cell types, the G3P pathway is the principal route for the synthesis of triglyceride, contributing over 90% of total Triglyceride synthesis [Coleman RA et al., 2000, Declercq PE et al., 1984]. The first and rate limiting step of this pathway is the esterification of long-chain acyl-CoA to G3P, which is catalyzed

by mitochondrial and microsomal G3P acyltransferase (GPAT) enzymes. Lysophosphatidic acid (LPA) molecules produced in this reaction are then acylated to form phosphatidic acid (PA) by the acyglycerol-3-phosphate acyltransferases (AGPAT) present in the endoplasmic reticulum membrane. PA can be converted into cytidine diphosphate diacylglycerol (CDP-DG), which is a substrate for the synthesis of certain glycerolphospholipids and cardiolipins [Heacock AM et al., 1997, Shindou H et al., 2009] or can be dephosphorylated by phosphatidate phosphohydrolase (PAP, synonym Lipin) to form DG, which serve as precursor molecules for the synthesis of triglyceride, as well as phosphatidylcholine (PC) and phosphatidylethanolamine (PE) [Coleman RA et al., 2000, Dowhan W, 1997]. DG acyltransferase (DGAT) catalyzes the acylation of DG, constituting the final step of triglyceride synthesis [Coleman RA et al., 2004]. Newly synthesized triglyceride molecules are then directed from endoplasmic reticulum lipid bilayer to form cytosolic LDs [Goh VJ et al., 2013, Wilfling F, et al., 2014].

The second step is triglyceride synthesis is mediated by AGPAT enzymes. Although 10 AGPAT enzymes have been identified on the basis of sequence of homology, the enzymatic activity for LPA acylation has been confirmed only for few [Takeuchi K et al., 2009]. Certain isoforms initially described as AGPAT were later reclassified into different acyltransferases groups. For example, AGPAT6 & AGPAT8 are currently designated as GPAT4 & GPAT3 respectively [Cao J et al., 2006, Chen YQ et al., 2008, Nagle CA et al., 2008]. AGPAT1 & AGPAT2 are localized in the endoplasmic reticulum [Eberhardt C et al., 1999, Gale SE et al., 2006, Kume K et al., 1997, West J et al., 1997] and are highly expressed in the liver [West J

et al., 1997, Eberhardt C et al., 1997, STAMPS AC et al., 1997, Takeuchi K et al., 2009] but the contribution of these enzymes in hepatic metabolism is not known.

VLDL assembly and secretion:

Triglyceride rich VLDL particles represent the mechanism by which fatty acids are exported from the liver and delivered to muscle for oxidation and adipose tissue for storage, respectively. VLDL assembly is a 2-step process that begins in the endoplasmic reticulum lumen.

In the 1st step Microsomal triglyceride transfer protein acts to incorporate a small amount of triglyceride in apoB100 as it is being translated by ribosomes and translocated across the endoplasmic reticulum membrane [Gordon DA et al., 1995]. In the 2nd step, additional triglyceride is packaged into the nascent apoB100-containing particles as they are traverse from the endoplasmic reticulum to the Golgi apparatus to form VLDL particles [Cohen DE et al., 2013].

The link between increased VLDL secretion and metabolic diseases, such as insulin resistance and diabetes, is well established [Cohen DE et al., 2013, Subramanian S et al., 2012, Zoltowska M et al., 2004]. The development of insulin resistance leads to hepatic triglyceride accumulation owing to both enhanced fatty acids uptake into the liver and increased DNL. The greater availability of triglyceride together with MTP promote the overproduction of VLDL particles and greater plasma triglyceride concentrations [Cohen DE et al., 2013]. In non-diabetic obese NAFLD cases, VLDL-TG secretion rates are elevated up to 2-times in comparison to normal subjects [Green CJ et al., 2014].

Reductions in VLDL secretions can also lead to hepatic steatosis. This occurs in the setting of genetic defects in apoB100 and microsomal triglyceride protein, which leads to hypobetalipoproteinemia and abetalipoproteinemia, respectively [Welty FK, 2014].

Lipolysis:

The lipid droplets associated adipose triglyceride lipase, which is also known as PNPLA2 is the rate limiting step in triglyceride lipolysis in adipocytes. The resulting Di-glycerides molecules are then hydrolyzed by the hormone sensitive lipase (HSL) to release mono-glycerides. In the final step, the monoacylglycerol lipase (MGL) cleaves mono-glyceride into glycerol and fatty acids [Lafontan M et al., 2009]. Studies on gain and loss of function have revealed that hepatic ATGL is required for the lipolysis of triglyceride stored in lipid droplets, controls substrate availability for fatty acid oxidation, and modulates the progression of hepatic steatosis [Ong KT et al., 2011, Reid BN et al., 2008, Wu JW et al., 2011]. Less is known concerning MGL and HSL activities in hepatocytes, although HSL expression is down-regulated in livers of NAFLD patients [Kohjima M et al., 2007].

Diagnosis and classification of NAFLD -

Since, the currently used routine procedures cannot tell the difference between simple steatosis and steatohepatitis, diagnosing NAFLD is difficult. Simple steatosis and steatohepatitis can be distinguished by liver biopsy, which is regarded as the gold standard for defining NAFLD. However, because of the increased risk of bleeding and complications, it is not advised for regular use.

Numerous non-invasive diagnostic technologies have been described in past ten years. A precise diagnosis is necessary for NAFLD to be classified. Some of the classification systems available include scoring systems by Matteoni [Matteoni CA et al., 1999], Brunt [Brunt EM et al.,1999], NASH CRN (Clinical Research Network) system [Kleiner DE et al., 2005], and the SAF (steatosis, activity and fibrosis) system [Bedossa P et al., 2012].

Thus, the various NAFLD classification systems can produce disparate outcomes, adding variation to scientific research. Matteoni and colleagues carried out one of the ground breaking studies with the most participants and the longest follow-up for stratifying NAFLD patients [Matteoni CA et al., 1999]. The Matteoni's system was based on fat accumulation, inflammation, ballooning degeneration, Mallory hyaline and fibrosis. NAFLD patients were put into 4 groups; Type I (Simple fatty liver), Type II (steatohepatitis), Type III (steatonecrosis) and Type IV (steatonecrosis plus either Mallory hyaline/ fibrosis). Type I was relatively benign whereas the necrotic forms were considered aggressive. The aggressive forms have higher risk of cirrhosis and liver-related death. Though this system helps to identify patients at risk of cirrhosis and liver-related death, it does not take into account NAFLD in children.

The system developed by Brunt [Brunt EM et al., 1999, Brunt EM et al., 2004] is semi-quantitative and evaluates the unique lesions in NASH. It unifies steatosis and steatohepatitis into a 'grade' and fibrosis into 'stage' [Angulo P, 2002]. Steatosis is graded on a scale of 1 to 3 depending on the percentage of hepatocytes affected (< 33% = 1, 33 – 66% = 2, > 66% = 3). Steatohepatitis was similarly graded on a scale of 1 to 3 (1 = Mild, 2 = Moderate, 3 = Severe) but on basis pf the severity and extent of

steatosis, ballooning, lobular inflammation and portal inflammation. Fibrosis on the other hand was staged on a scale of 1 to 4. Brunt's system does not cover the entire spectrum of NAFLD as defined by Matteoni's system.

Additionally, it was not designed to evaluate NAFLD in children [144]. In 2005, the pathology committee of the NASH clinical research network (NASH CRN) of the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) came up with a scoring system and NAFLD activity score (NAS) for use in clinical trial [Kleiner DE et al., 2005]. The scoring system was intended to address the full spectrum of lesions of NAFLD. The histological features considered were grouped into five categories, each with a scoring scale. These features which were independently associated with NASH, included steatosis (0-3), lobular inflammation (0-3), fibrosis (0-4), and miscellaneous features like Mallory's hyaline and glycogenated nuclei. The NAS is the unweighted sum of steatosis, lobular inflammation, and hepatocellular ballooning scores. NAS of ≥5 was found to correlate with the diagnosis of NASH and biopsies with score of less than 3 were classifies as "not NASH". Notwithstanding, not all the biopsies with ≥5 NAS meet the diagnostic criteria of definite NASH and should be used carefully in establishing the presence or absence of NASH [Brunt EM et al., 2011]. In a number of experimental work involving humans and rodents, a NAS score of at least 4 was considered as NASH [Canet MJ et al.,2014, Ferslew BC et al., 2015].

Later on, SAF (steatosis, activity, fibrosis) system was proposed. SAF system considered steatosis, lobular inflammation, and ballooning in defining fatty liver and NASH. The activity is defined as the sum of the grades of lobular inflammation and

ballooning and ranges from 0-4. The presence of NAFLD is defined by steatosis in the presence of any degree of activity. This implies that the definition of either NAFL or NASH requires the presence of steatosis (1-3) and varying degree of activity [Bedossa P et al., 2012, Kleiner DE et al., 2016]. These scores were used to predict the advance form of NASH and to analyze whether patient needs the biopsy or not. The scoring system would also make use of parameters like BMI, levels of liver enzymes (AST/ALT ratio), Age of the patient, level of triglycerides etc.

The other common method for diagnosing NAFLD is the scanning or imaging tests. Steatosis is usually detected incidentally by imaging test like computed tomography (CT) and ultrasound. Notably, these methods are not sensitive enough, can only identify fat when 20-30% percent of the live parenchyma is affected, and cannot precisely measure the amount of hepatic fat present. Modern techniques like transient elastography (TE)-based controlled attenuation parameter (CAP), MR-proton density fat fraction (MR-PDFF), and magnetic resonance (MR) imaging-based spectroscopy are more sensitive and enable reliable assessment of hepatic steatosis [Younossi ZM et al., 2018].

However, each of these imaging techniques have advantages and disadvantages that must be taken into account before use. Ultrasound is used to assess steatosis, but has poor sensitivity, negative predictive value, is unable to detect mild steatosis, and is nit quantitative. The MR elastography is used to assess fibrosis, but has limited availability and high cost. Looking at TE, which is used to assess fibrosis, the accuracy is reduced in obese cases or high BMI [Cleveland E et al., 2018].

Reliable noninvasive techniques to detect NASH are limited. Measuring the serum biomarkers is one of the commonly used method for primary screening or diagnosis. Liver enzymes or serum aminotransferases, are usually used in clinical practice as a surrogate for inflammation or liver related disorders, but has poor predictive value for NASH. Patients with hepatic steatosis also have serum levels of the enzymes within the normal range [Chalasani N et al., 2018, ASL-EASD-EASO –guidelines, 2016]. Hence, these are not the reliable methods to specifically detect the NAFLD or its progression. However, noninvasive or less invasive detection of NAFLD and its stages remains key diagnostic challenge, and requires further research.

TREATMENT FOR NAFLD

Currently, there are no effective and specific therapies available for NAFLD apart from changes in lifestyle that lead to improved physical fitness and weight loss are advised. The backbone of any treatment strategy should be modest weight loss of about 7-10% and exercise that improves liver histology, insulin resistance, and quality of life [Promrat K et al., 2010, Dixon JB et al., 2004]. While total calorie intake is significant, there is growing evidence that suggests fructose consumption plays a special role in NAFLD patients, with fructose intake being linked to higher levels of fibrosis [Abdelmalek MF et al., 2010]. According to recent research, vigorous exercise is more closely related to histological improvement than moderate exercise [Kistler KD et al., 2011]. Unfortunately, many patients find it difficult to implement these adjustments, and among those who do have trouble maintaining it over time [Centis E et al., 2010]. It is therefore advised that a behavioral approach, such as

cognitive behavioral therapy, or at least the engagement of a dietician can be a part of this treatment.

The Pharmacological therapies include Insulin sensitizers, Incretin-based therapies, Lipid lowering agents, cytoprotective and antioxidant agents, Anti-tumor necrosis factor-alpha agents etc. are used. Surgeries like bariatric surgery is used as therapeutic option for morbidly obese patients [Beaton MD, 2012].

CHAPTER V
MATERIAL AND METHODS

STUDY DESIGN

The present study was an observational study carried out in the Department of Cell Biology and Molecular Genetics, Sri Devaraj Urs Academy of Higher Education and Research, Kolar, Karnataka. The subjects were enrolled from the Department of General Medicine of R.L.J. Hospital and Research Centre, the teaching hospital of Sri Devaraj Urs Medical College, a constituent college of Sri Devaraj Urs Academy of Higher Education and Research, Tamaka, Kolar, Karnataka. The study was conducted from November 2019 to November 2021. The study was carried out by following the guidelines of the Declaration of Helsinki and the study was approved by the Institutional Ethics Committee (SDUMC/KLR/IEC/41/2019-20). The written consent form was obtained from all the study participants, prior to the recruitment.

Selection of Study Participants

The study included 60 NAFLD subjects in the age group of 25-65 years who were divided into simple steatosis and NASH. Simple steatosis included 10 subjects and NASH included 50 subjects.

ATP III guidelines -National cholesterol education program [ATP III guidelines, 2001] and ultrasonography were followed to differentiate the stages. Cases with high serum levels triglycerides (>150mg/dL) along with impression of hepatomegaly with fatty infiltration were considered as NASH. In addition, cases with hs-CRP within the normalrange (<10mg/L) were considered as simple steatosis and hs-CRP >10mg/L were considered as NASH in this study.

Further, subjects of each stage were sub-divided into 2 groups, where patients with comorbidities like obesity, hyperlipidemia, hypertension, T2DM and CVD were categorized as group 1 and those without comorbidities as group 2. Simple steatosis with comorbidities had 5 subjects and without comorbidities 5 subjects, whereas NASH had 25 cases with comorbidities and 25 without comorbidities.

Subjects with other chronic liver diseases like hepatitis, Chronic Obstructive Pulmonary Disease, alcohol consumption, smoking history were excluded from the study.

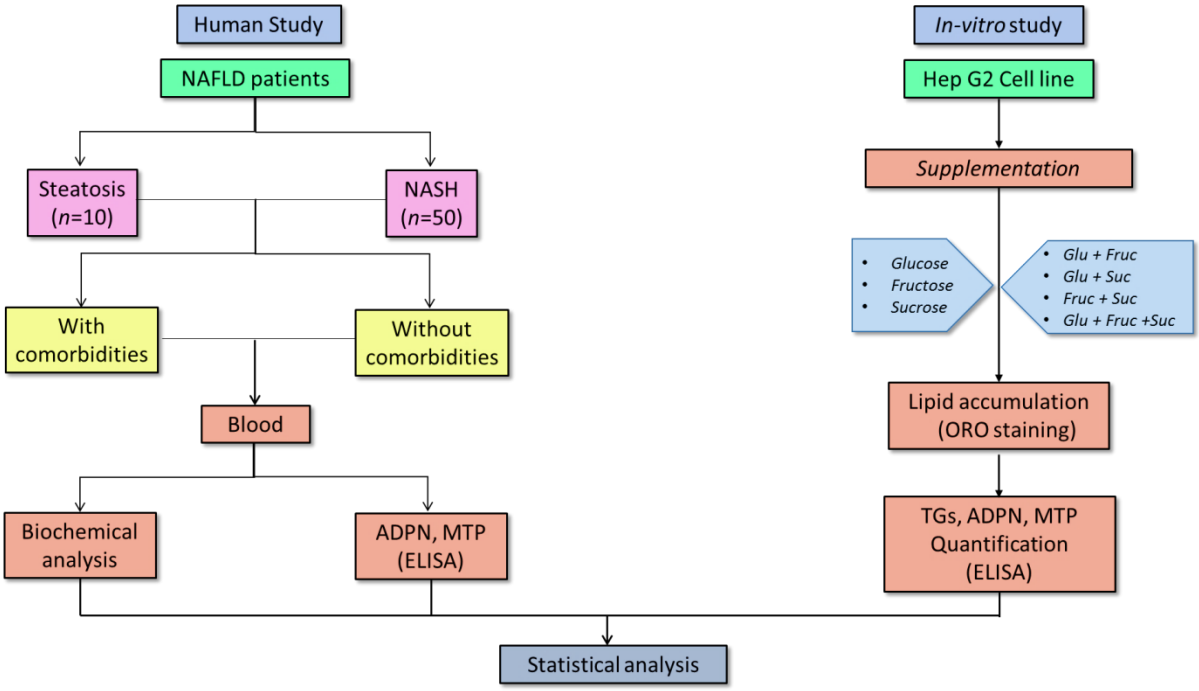


Fig.6 Flow chart for study design

Sample Collection

Venous blood sample (5ml) was collected from all the subjects. 1ml of blood was collected in tubes containing sodium fluoride for FBS estimation and 4ml of blood in no-anticoagulant tube for investigations like liver function tests, lipid profile,

and hs-CRP. The biochemical parameters were analyzed by standard methods using Vitros 5.1 FS clinical chemistry analyzer (Ortho Clinical Diagnostics).

Table. 2 Measured Biochemical parameters

Sr.no	Parameters	Equipment	Reference range	Test method
1.	Highly sensitive- C reactive protein (hs-CRP) (mg/L)	Vitros 5,1	0.5-10	Immunoturbidimetry
2.	Fasting Blood Sugar (FBS) (mg/dL)		70-110	Glucose oxidase-peroxidase
3.	Aspartate aminotransferase (AST) (U/L)		8-48	Oxaloacetate Decarboxylase, Pyruvate Oxidase/Peroxidase
4.	Alanine transaminase (ALT) (U/L)		7-55	Multipoint enzymatic by using LDH

5.	Alkaline Phosphatase (ALKP) (U/L)		42-128	P-Nitrophenyl phosphate as substrate
6.	Gamma-glutamyl Transferase (GGT) (U/L)		< 48	L-gamma-glutamyl-p-nitroanilide as substrate
7.	Albumin (g/dL)		3.5-5.2	Bromocresol green
8.	Serum cholesterol (mg/dL)		< 200	Cholesterol oxidase peroxidase method
9.	Triglycerides (mg/dL)		< 150	Lipase glycerol kinase peroxidase method
10.	HDL (mg/dL)		> 60	Direct precipitation method
11.	VLDL (mg/dL)		< 30	Friedwald's formula

Estimation of ADPN and MTP

For estimation of the study molecules, serum was separated within 2 hours of sample collection by centrifugation at 3000rpm. The serum was then aliquoted and stored at -70˚C until further analysis. Prior to the analysis, the samples were thawed at room temperature, vortexed, and centrifuged. ADPN and MTP serum levels were estimated using commercially available Enzyme-linked immunosorbent assay (ELISA) kits (Cloud Clone Corp., USA, # SEH868Hu, and # SEC641Hu respectively).

The protocol for quantification of Serum ADPN levels is as follows:

1. Samples, standards and reagents were prepared as per the manufacturer's guidelines.
2. 1000µl of sample or standard were added to the respective wells of the plate.
3. The plate was Incubated for an hour at 37˚C.
4. The sample and standard were aspirated from the wells and 100µl of Detection Reagent A was added to each well. The plate was incubated for an hour at 37˚C.
5. Reagent was aspirated and wells were washed using washing buffer.
6. Detection Reagent B was added to each well and the plate was incubated for 30 minutes at 37˚C.
7. After incubation period, reagent was aspirated and washed.
8. 90µl of substrate solution was added to each well, followed by incubation for 10-20 minutes at 37˚C.

9. 50µl of stop solution was added to each well and absorbance was recorded at 450nm.

The protocol for quantification of Serum MTP levels is as follows:

1. Samples, standards and reagents were prepared as per the manufacturer's guidelines.
2. 1000µl of sample or standard were added to the respective wells of the plate.
3. The plate was Incubated for an hour at 37˚C.
4. The sample and standard were aspirated from the wells and 100µl of Detection Reagent A was added to each well. The plate was incubated for an hour at 37˚C.
5. Reagent was aspirated and wells were washed using washing buffer.
6. Detection Reagent B was added to each well and the plate was incubated for 30 minutes at 37˚C.
7. After incubation period, reagent was aspirated and washed.
8. 90µl of substrate solution was added to each well, followed by incubation for 10-20 minutes at 37˚C.
9. 50µl of stop solution was added to each well and absorbance was recorded at 450nm.

In-vitro culture:

Cell line maintenance

Human hepatocellular Cell lines (HepG2) were procured from National Centre for Cell Science (NCCS), Pune. Cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) low glucose media supplemented with 10% Fetal Bovine serum (FBS) and 1% antibiotic solution and was kept at 37ºC in 5% CO2 atmosphere [Freshney RI, 1986]. Sub-culturing was done for every 2 days after achieving 80% confluence.

Cell culture treatment

A 12-well plate seeded with 1000µl of cell suspension at an optimum density of 20,000 cells per well. Cells were allowed to grow for 24 hours to reach 80% confluence and were then treated supplements of glucose, fructose, sucrose, and their combinations at a concentration of 25mM [Bruschi FV et al., 2020]. Plates were incubated for 4 days and 7 days at 37°C in a 5% CO2 atmosphere.

Table no. 3 The supplements and their combinations used in cell culture experiment

Sr. No.	Supplements and combination
1	Glucose
2	Fructose
3	Sucrose
4	Glucose + Fructose
5	Glucose + Sucrose
6	Fructose + Sucrose
7	Glucose + Fructose + Sucrose

Primary Screening

Primary screening was done to confirm the lipid accumulation by Oil Red O staining.

Secondary Screening

The secondary screening was done for triglyceride quantification (TRUEchemie TRIGLYCERIDES TEST KIT) followed by quantification of ADPN and MTP using commercially available kits.

The protocol for triglyceride quantification is as follows:

1. After the cell culture treatment, 1000µl of supernatant from all culture conditions was transferred to 1.5ml tubes and 100µl of triglyceride reagent was added. Tubes were incubated at 37°C for 10minutes.
2. Post incubation, absorbance was recorded at 505nm.

Statistical Analysis

The data obtained were statistically analyzed using SPSS V20 (International Business Machine Corporation, Armonk, New York) software and Graph Pad Prism 9.1. The Shapiro-Wilk test was used to check the normal distribution of data and the data were found to be normally distributed. Unpaired student t-test was used to compare the means between two groups. The relationship between the variables was analyzed using Pearson's correlation. p-value < 0.005 was considered statistically significant and < 0.001 as highly significant.

CHAPTER VI
RESULTS

I. Demographic and Biochemical parameters of the study participants

The demographic characteristics of study subjects are represented in Table no. 4. Significantly increased hs-CRP levels were observed in NASH patients compared to simple steatosis patients indicating inflammation in the NASH patients. The serum levels of liver enzymes like AST, and ALT were within the reference range in simple steatosis and NASH subjects (Reference range; AST: 8-48 U/L and ALT: 7-55 U/L). Serum levels of AST and ALKP showed a statistically significant difference when compared between simple steatosis and NASH patients. No significant difference was observed in lipid profile between simple steatosis and NASH patients.

Table no.4 Demographic and Biochemical parameters of simple steatosis and NASH subjects

Parameter	Simple steatosis (*n* = 10)	NASH (*n* = 50)	*p* Value
Age (years)	46.5 ± 10.18	48.85 ± 10.43	1.02
Gender(F/M)	50%/50%	64%/ 36%	0.41
BMI (kg/m^2)	27.56 ± 7.82	27.69 ± 7.17	0.65
hs-CRP (mg/L)	7.136 ± 2.24	32.08 ± 36.36	**< 0.0001****
FBS (mg/dL)	184.5 ± 105.02	169.67 ± 80.86	0.24
LFT			
ALT (U/L)	50.4 ± 35.38	43.71 ± 38.41	0.85
AST (U/L)	40.7 ± 19.18	49.21 ± 45.45	**0.008***
ALKP (U/L)	125.2 ± 85.42	98.49 ± 50.13	**0.02***
Albumin (g/dL)	3.42 ± 0.66	3.38 ± 0.79	0.59
GGT (U/L)	26.3 ± 10.39	26.38 ± 10.19	0.85
Lipid profile			
Serum cholesterol (mg/dL)	128.5 ± 58.62	158.67 ± 57.71	0.86
Triglyceride (mg/dL)	193.2 ± 80.30	217.71 ± 97.16	0.07
HDL (mg/dL)	24.6 ± 6.619	27.30 ± 9.24	0.28
LDL (mg/dL)	64.2 ± 29.32	66.56 ± 42.46	0.23
VLDL (mg/dL)	38.64 ± 16.06	43.54 ± 19.43	0.07

Values are expressed as Mean ± SD. * $p < 0.05$ statistically significant ** $p < 0.001$ statistically highly significant.

II. Serum levels of ADPN and MTP in Simple steatosis and NASH patients.

a. Serum levels of ADPN in Simple steatosis and NASH patients.

When serum levels of ADPN were measured and compared between Simple steatosis and NASH subjects, the mean serum levels of ADPN were significantly decreased in NASH subjects (2.50 ± 1.77) as compared to subjects with simple steatosis (3.44 ± 1.90) ($p < 0.001$) (Fig.7).

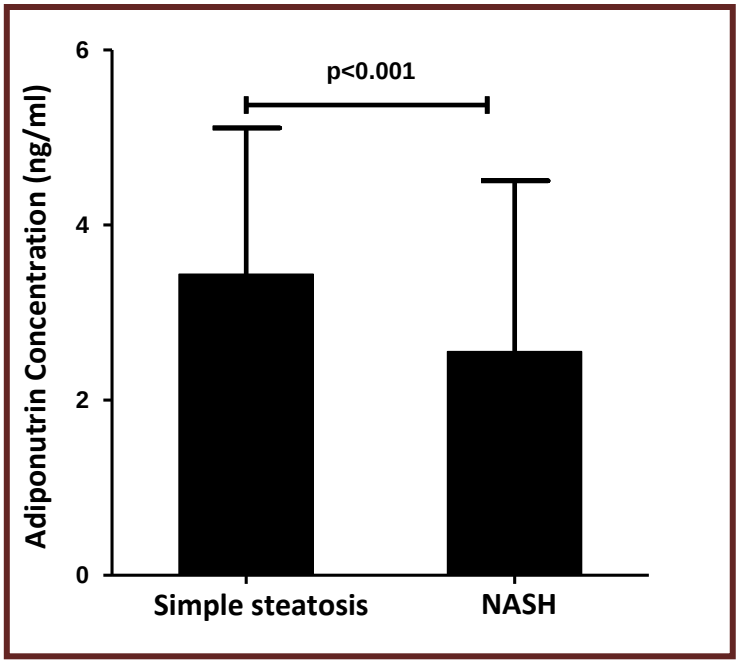


Fig. 7 Serum levels of Adiponutrin between Simple steatosis and NASH of NAFLD patients

b. Serum levels of MTP in Simple steatosis and NASH patients

When mean serum levels of MTP were compared, NASH subjects showed decreased levels of MTP (1.93 ± 0.61) as compared to simple steatosis subjects (1.48 ± 1.02) (Fig.8).

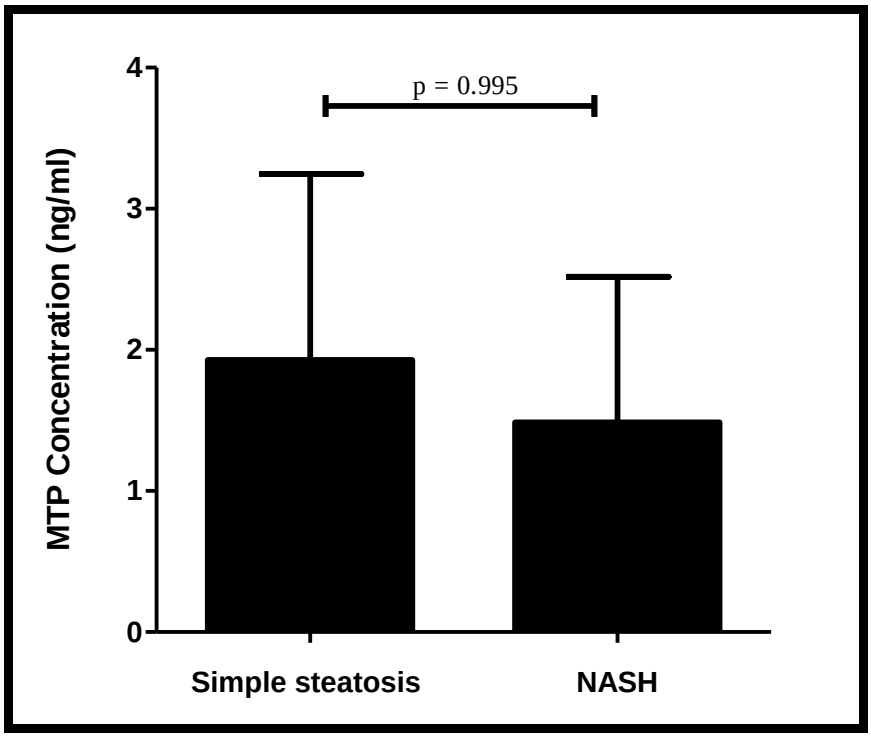


Fig. 8 Serum levels of MTP between Simple steatosis and NASH in NAFLD patients

III. Serum levels of ADPN and MTP in simple steatosis and NASH patients with and without comorbidities

The demographic characteristics of cases with comorbidities (Group 1) and cases without comorbidities (Group 2) were represented in Table no.5. Mean serum levels of hs-CRP, FBS, systolic BP, ALT, AST, ALKP, GGT, TG, and VLDL was significantly higher in cases with comorbidities as compared to cases without comorbidities ($p < 0.001$).

Table no. 5 Demographic and Biochemical parameters of cases with comorbidities (Group 1) and cases without comorbidities (Group 2)

Parameter	NAFLD Cases with comorbidities (Group 1) (*n* = 30)	NAFLD Cases without comorbidities (Group 2) (*n* = 30)	*p* Value
Age (years)	50.63 ± 10.32	46.42 ± 9.80	0.77
Gender(F/M)	60%/40%	53.33%/46.67%	0.32
BMI (kg/m^2)	34.81 ± 1.74	20.75 ± 0.98	**0.0017***
hs-CRP (mg/L)	47.14 ± 40.86	9.91 ± 2.48	< **0.0001****
FBS (mg/dL)	238.90 ± 70.38	107.03 ± 21.79	< **0.0001****
Systolic BP (mmHg)	131.09 ± 4.35	111.61 ± 6.30	**0.041***
Diastolic BP (mmHg)	82.81 ± 3.16	77.30 ± 2.74	0.42
LFT			
ALT (U/L)	68.22 ± 41.49	21.96 ± 10.56	< **0.0001****
AST (U/L)	70.97 ± 53.75	25.55 ± 6.55	< **0.0001****
ALKP (U/L)	123.5 ± 63.61	82.33 ± 21.72	< **0.0001****
Albumin (g/dL)	3.39 ± 0.82	3.36 ± 0.74	0.57
GGT (U/L)	35.06 ± 3.08	17.94 ± 6.83	< **0.0001****
Lipid profile			
Serum cholesterol (mg/dL)	190.55 ± 37.93	117.31 ± 47.39	0.22
Triglyceride (mg/dL)	283.94 ± 87.54	146.06 ± 20.93	< **0.0001****
HDL (mg/dL)	27.72 ± 8.86	26 ± 9.31	0.78
LDL (mg/dL)	64.53 ± 35.71	68.94 ± 45.001	0.20
VLDL (mg/dL)	56.79 ± 17.51	29.21 ± 4.19	< **0.0001****

Values are expressed as Mean ± SD. * $p < 0.05$ statistically significant ** $p < 0.001$ statistically highly significant.

On comparison of ADPN serum levels between cases with and without comorbidities, cases with comorbidities had significantly decreased levels (0.98 ± 0.55) as compared to cases without comorbidities (3.84 ± 1.75). This decrease was highly significant among NASH subjects ($p < 0.001$) (Fig. 10).

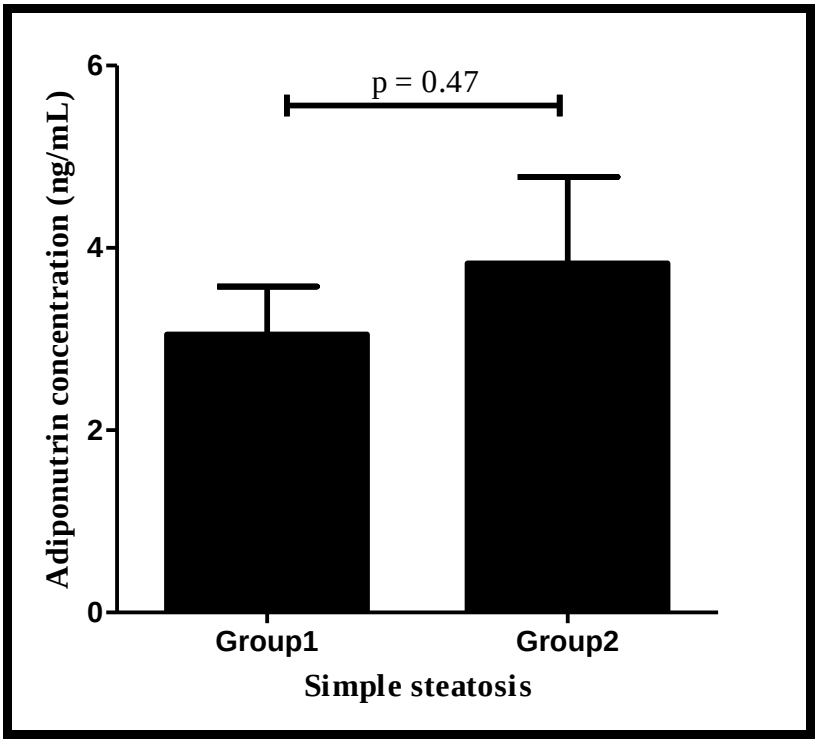


Fig. 9 Serum levels of ADPN between cases with comorbidities (Group 1) and cases without comorbidities (Group 2) in simple steatosis subjects

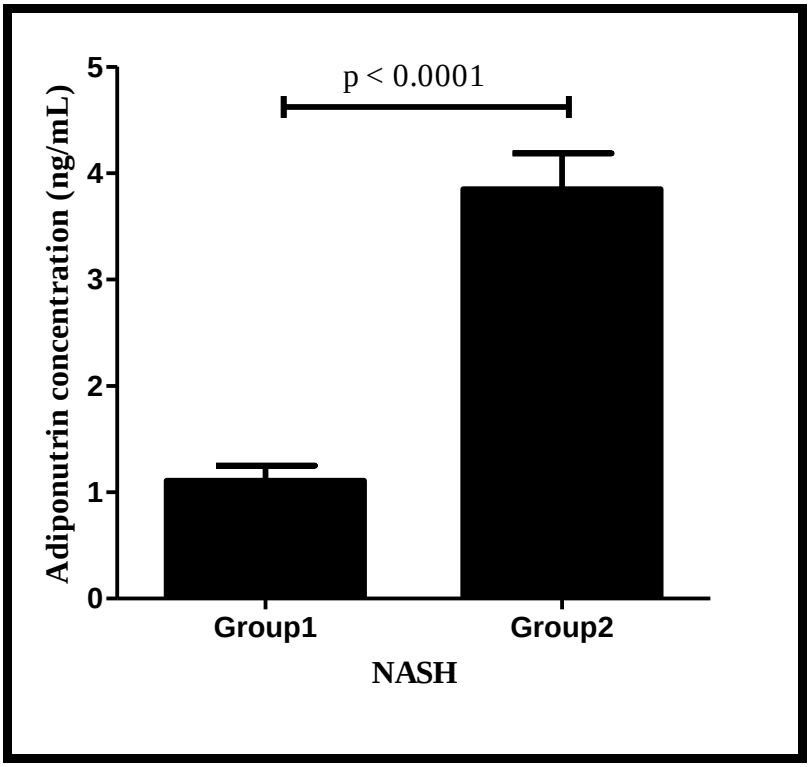


Fig. 10 Serum levels of ADPN between cases with comorbidities (Group 1) and cases without comorbidities (Group 2) in NASH subjects

Mean MTP levels were significantly lower in NAFLD cases with comorbidities (0.79 ± 0.39) as compared to cases without comorbidities (3.06 ± 0.61) in simple steatosis patients (p = 0.006) (Fig.11), but non-significant in NASH patients (1.39 ± 1.02) (1.57 ± 1.01) (p = 0.532) (Fig.12).

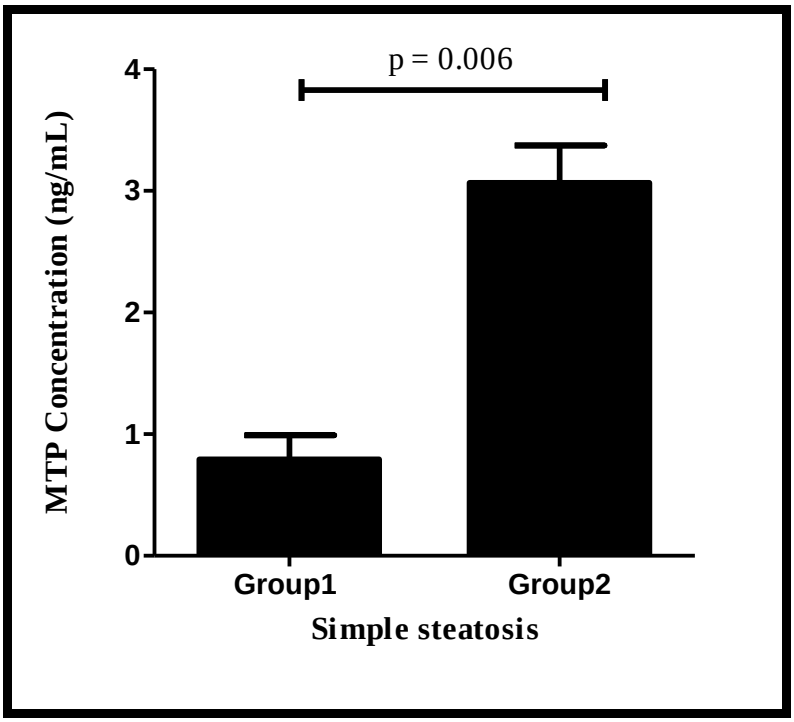


Fig. 11 Serum levels of MTP between cases with comorbidities (Group 1) and cases without comorbidities (Group 2) in simple steatosis subjects

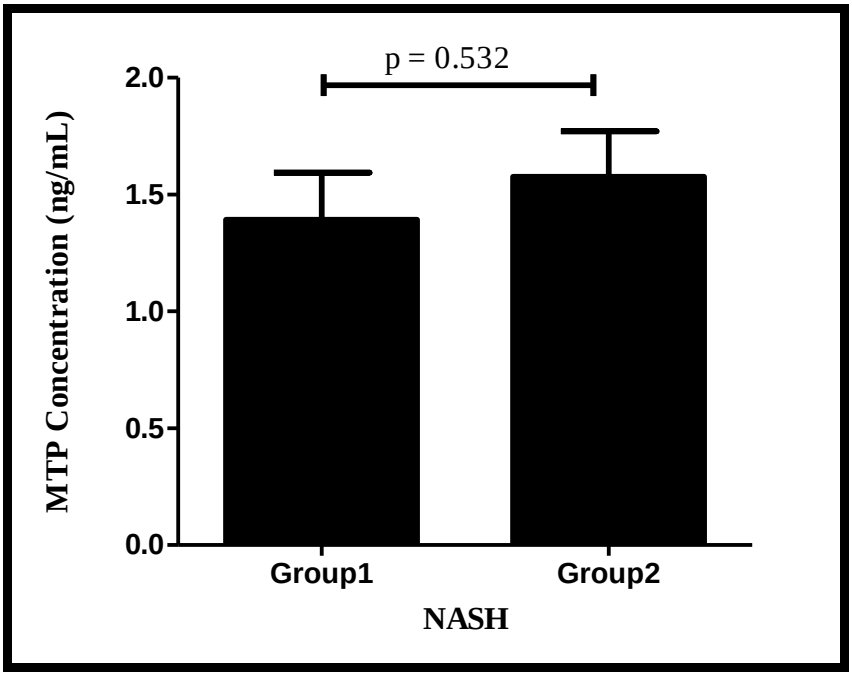


Fig. 12 Serum levels of MTP between cases with comorbidities (Group 1) and cases without comorbidities (Group 2) in NASH subjects

IV. Correlation analysis of ADPN and MTP levels with triglycerides, VLDL, and serum transaminase (ALT, and AST)

The correlation analysis between ADPN v/s TGs and ADPN v/s VLDL in NAFLD subjects showed a significant positive correlation (r = 0.241, p = 0.05). Further correlation in simple steatosis subjects showed a negative correlation whereas, a significant positive correlation was observed in NASH subjects (r = 0.308, p = 0.025).

Moreover, the correlation analysis between MTP v/s triglycerides and MTP v/s VLDL in NAFLD subjects showed a negative correlation (r = -0.177, p = 0.16). Further correlation on simple steatosis subjects showed a significant negative correlation (r = -0.665, p = 0.036) whereas, NASH subjects also showed a negative correlation but the difference was insignificant (r = -0.080, p = 0.563) (Table no.6).

Table no. 6 Correlation of ADPN, and MTP with Triglycerides and VLDL serum levels in NAFLD patients

		NAFLD stages (S1 + S2)		Simple steatosis (Group1 + Group2)		NASH (Group1 + Group2)	
		r	***p***	***r***	***p***	***r***	***p***
Triglycerides	**ADPN**	0.241	**0.05***	-0.131	0.718	0.308	**0.025***
	MTP	-0.177	0.16	-0.665	**0.036***	-0.080	0.563
VLDL	**ADPN**	0.241	**0.05***	-0.131	0.718	0.308	**0.025***
	MTP	-0.177	0.16	-0.665	**0.036***	-0.080	0.563

Pearson's correlation was used. * $p < 0.05$ statistically significant. ** $p < 0.001$ statistically highly significant.

Liver enzymes like AST and ALT showed a significant positive correlation with the serum levels of ADPN, whereas MTP showed a weak negative correlation (Table no.7).

Table no. 7 Correlation between ADPN and MTP levels with Serum transaminases

	ADPN		MTP	
	r	***p***	***r***	***p***
AST	0.398	**0.001****	-0.039	0.758
ALT	0.349	**0.005***	-0.029	0.821

Pearson's correlation was used. * $p < 0.05$ Statistically significant. ** $p < 0.001$ Statistically highly significant.

V. To study the effect of nutritional status on ADPN and MTP in *in vitro* setup

The nutritional supplements used for *in vitro* study is indicated in Table no.3. Oil Red (ORO) staining was done for primary screening and confirmation of lipid accumulation. Based on differentiation and lipid droplet formation observed under microscope, cells were subjected to primary screening. The screening was done after 4 and 7 days of treatment, where a significant increase in lipid accumulation was observed in cells treated for 7 days when compared to 4 days. This suggests that as the duration of supplementation increases the lipid accumulation also increases. Further, it was found that supplementation with sucrose increased the lipid accumulation (Fig.15).

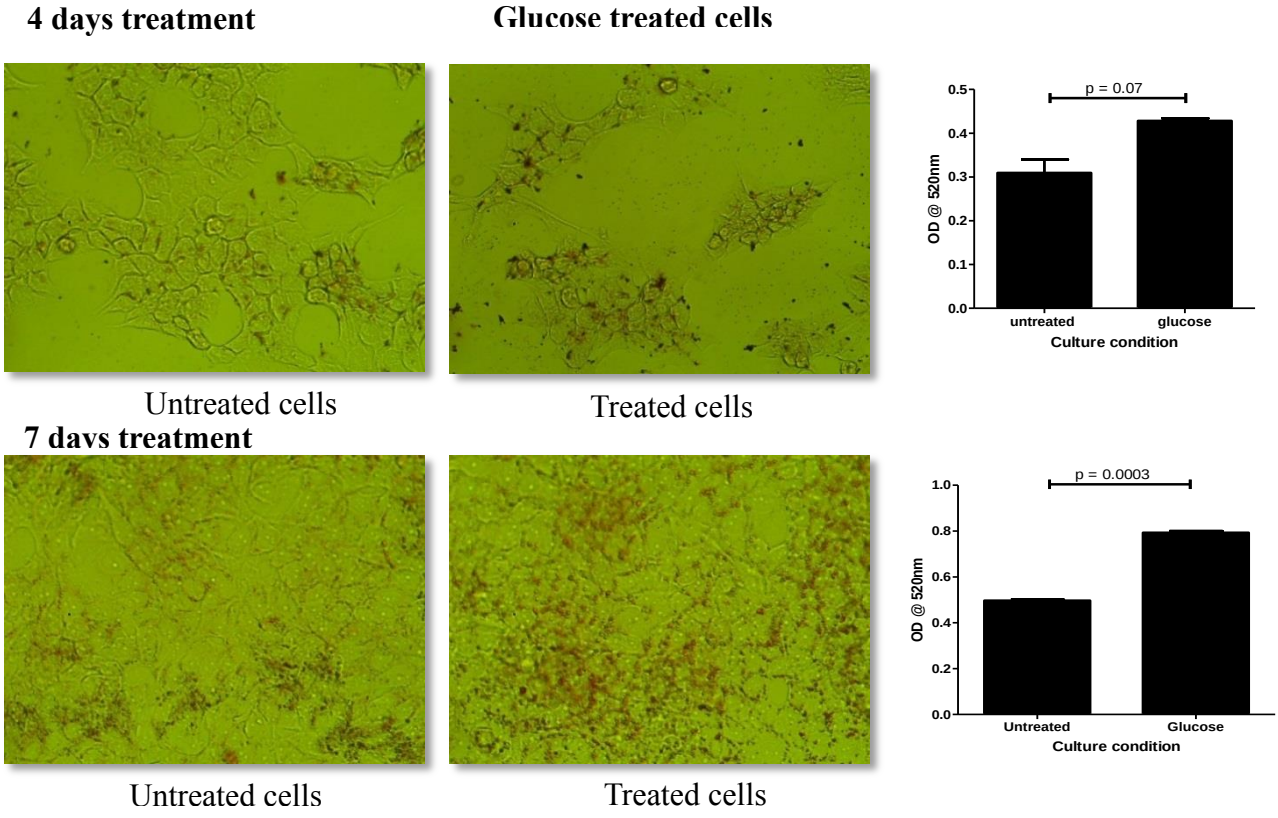


Fig. 13. Lipid accumulation in HepG2 cells after 4 and 7 days of glucose supplementation

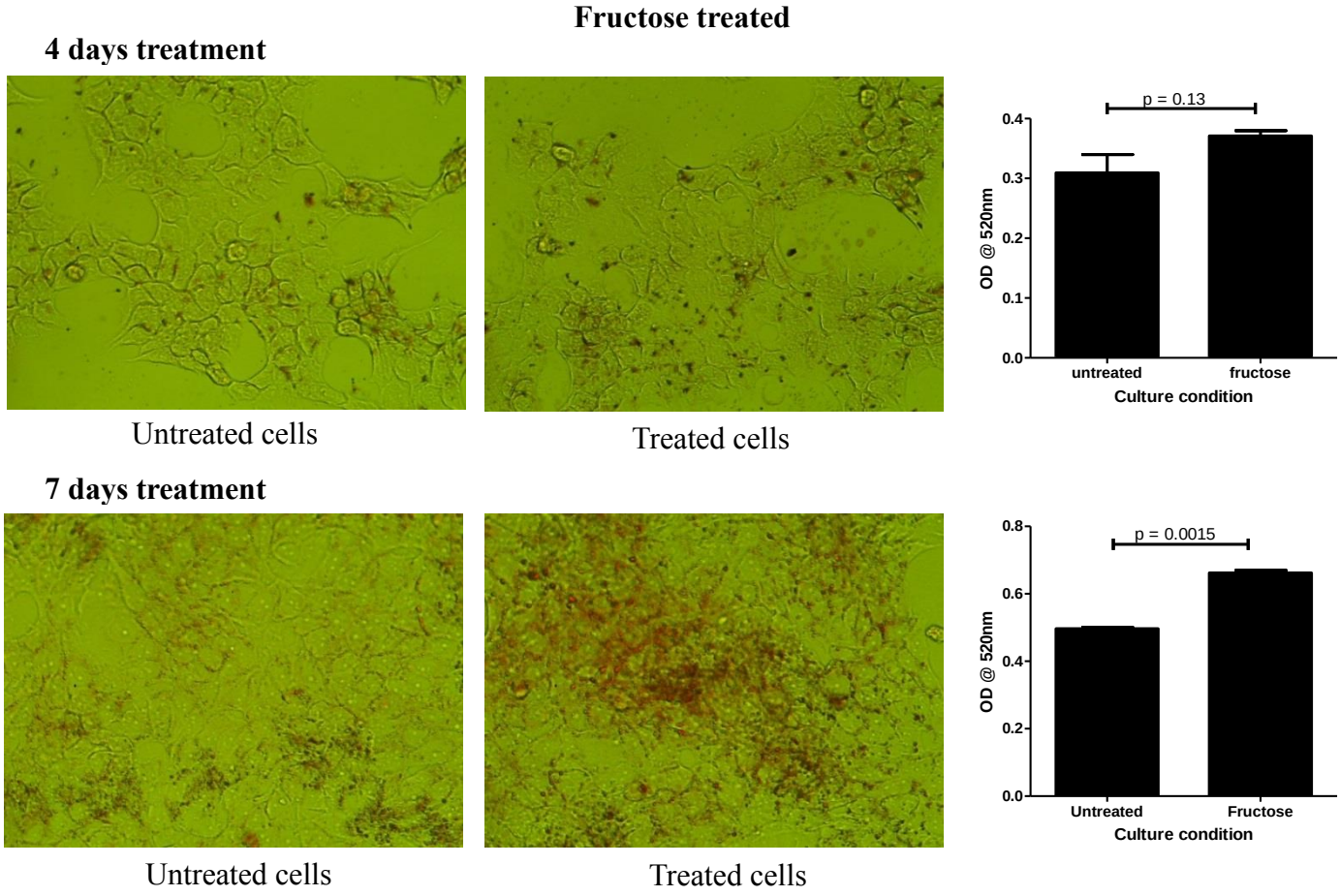


Fig.14 Lipid accumulation in HepG2 cells after 4 and 7 days of fructose supplementation

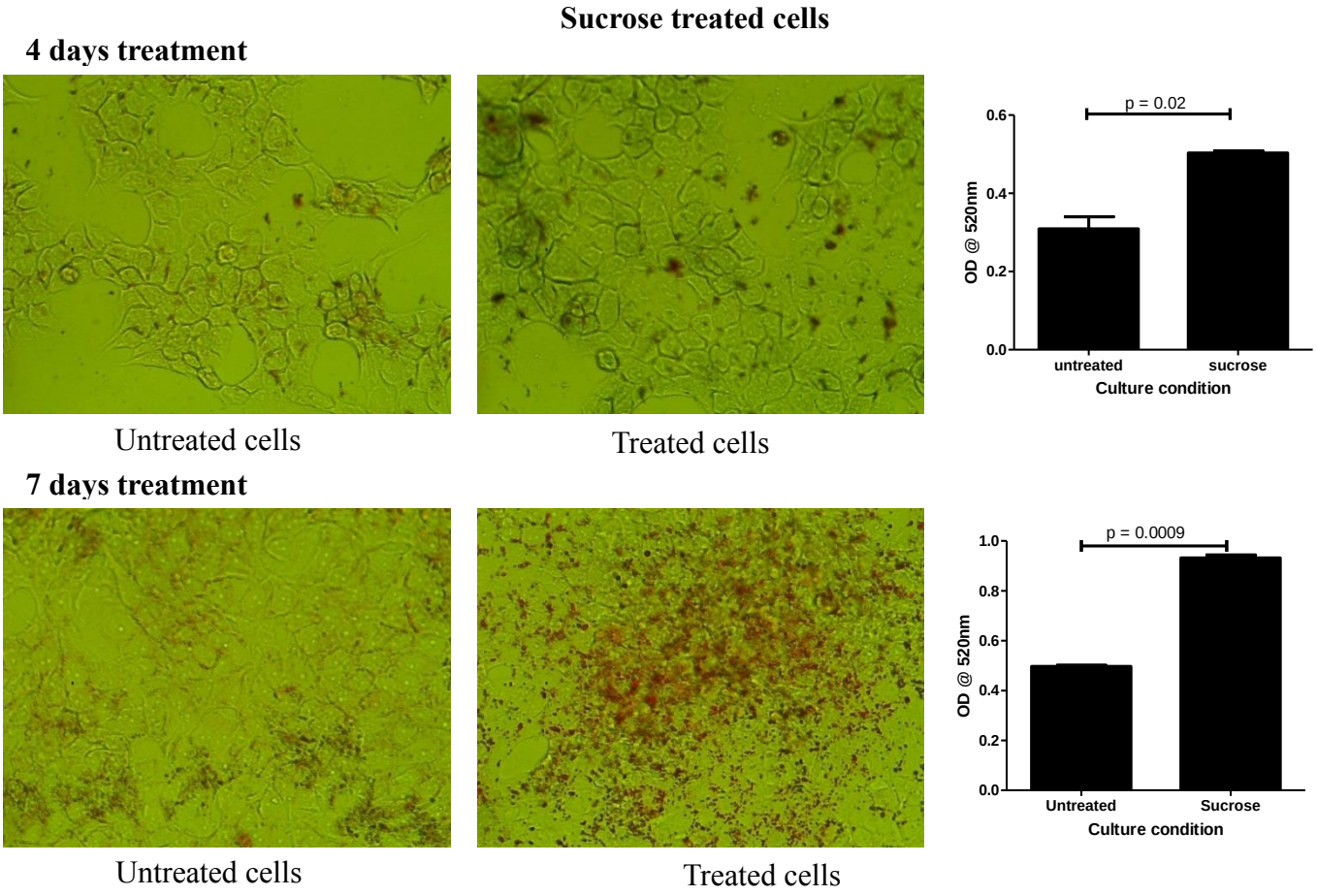


Fig. 15 Lipid accumulation in HepG2 cells after 4 and 7 days of sucrose supplementation

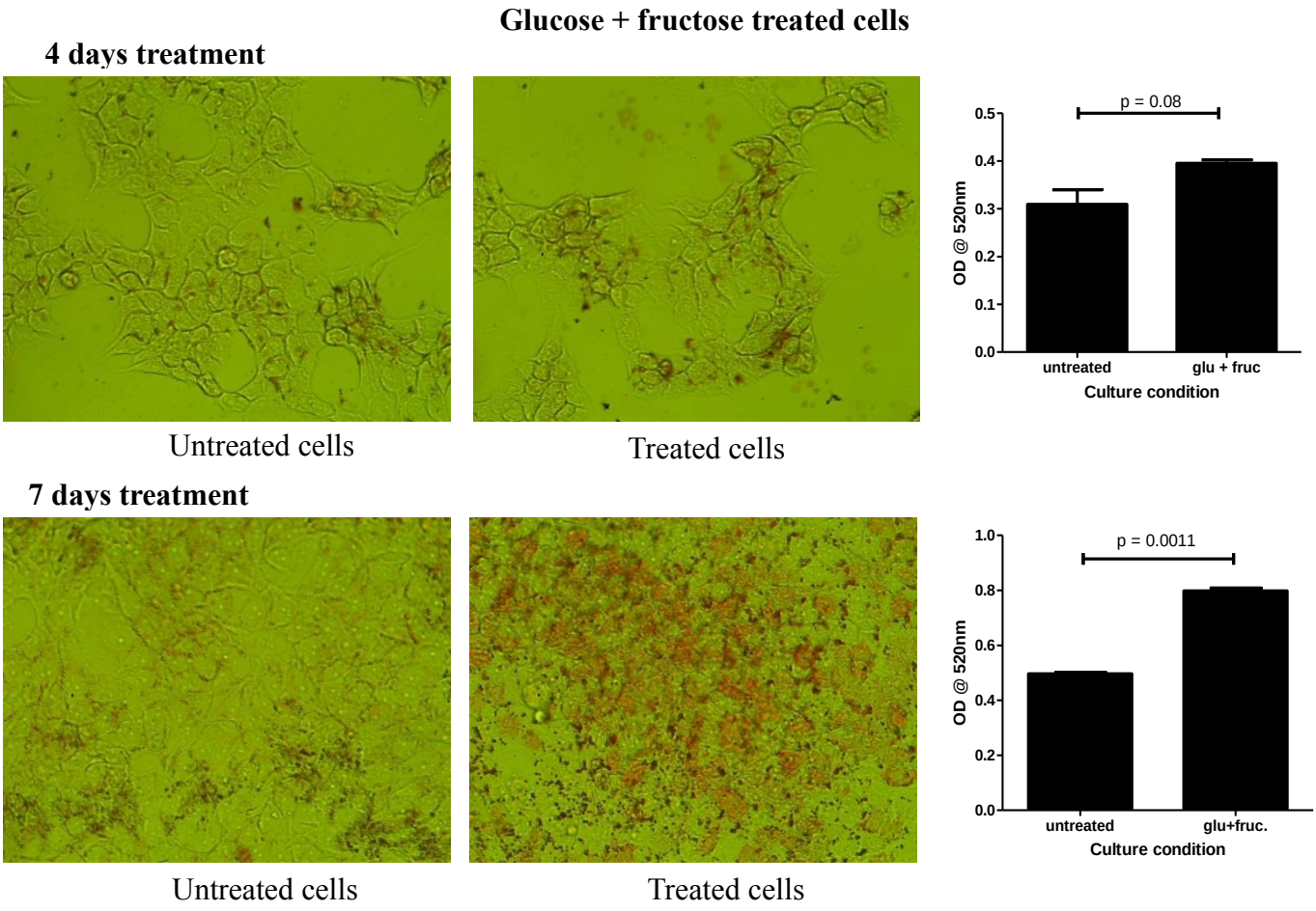


Fig. 16 Lipid accumulation in HepG2 cells after 4 and 7 days of glucose and fructose supplementation

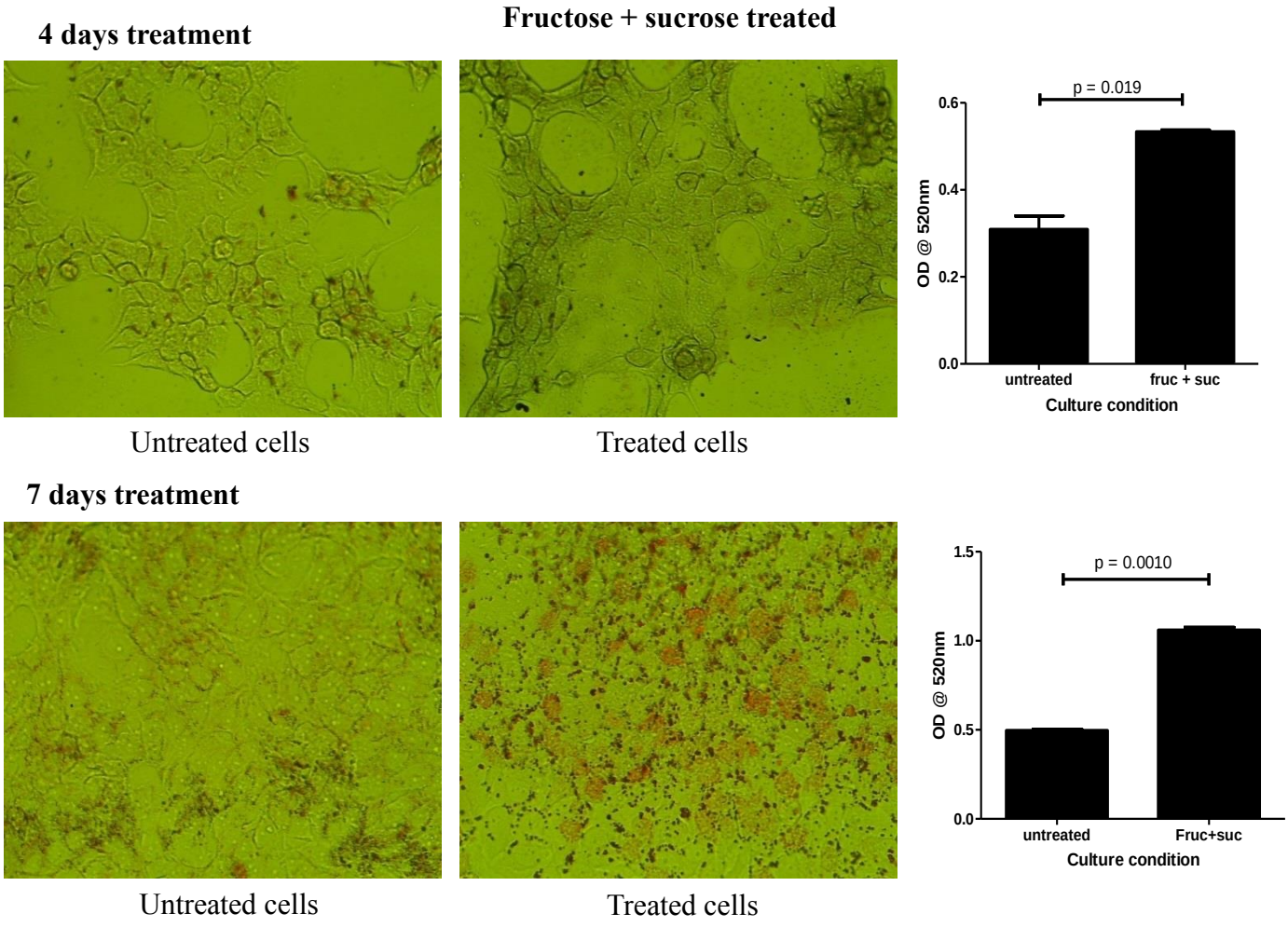


Fig. 17 Lipid accumulation in HepG2 cells after 4 and 7 days of fructose and sucrose supplementation

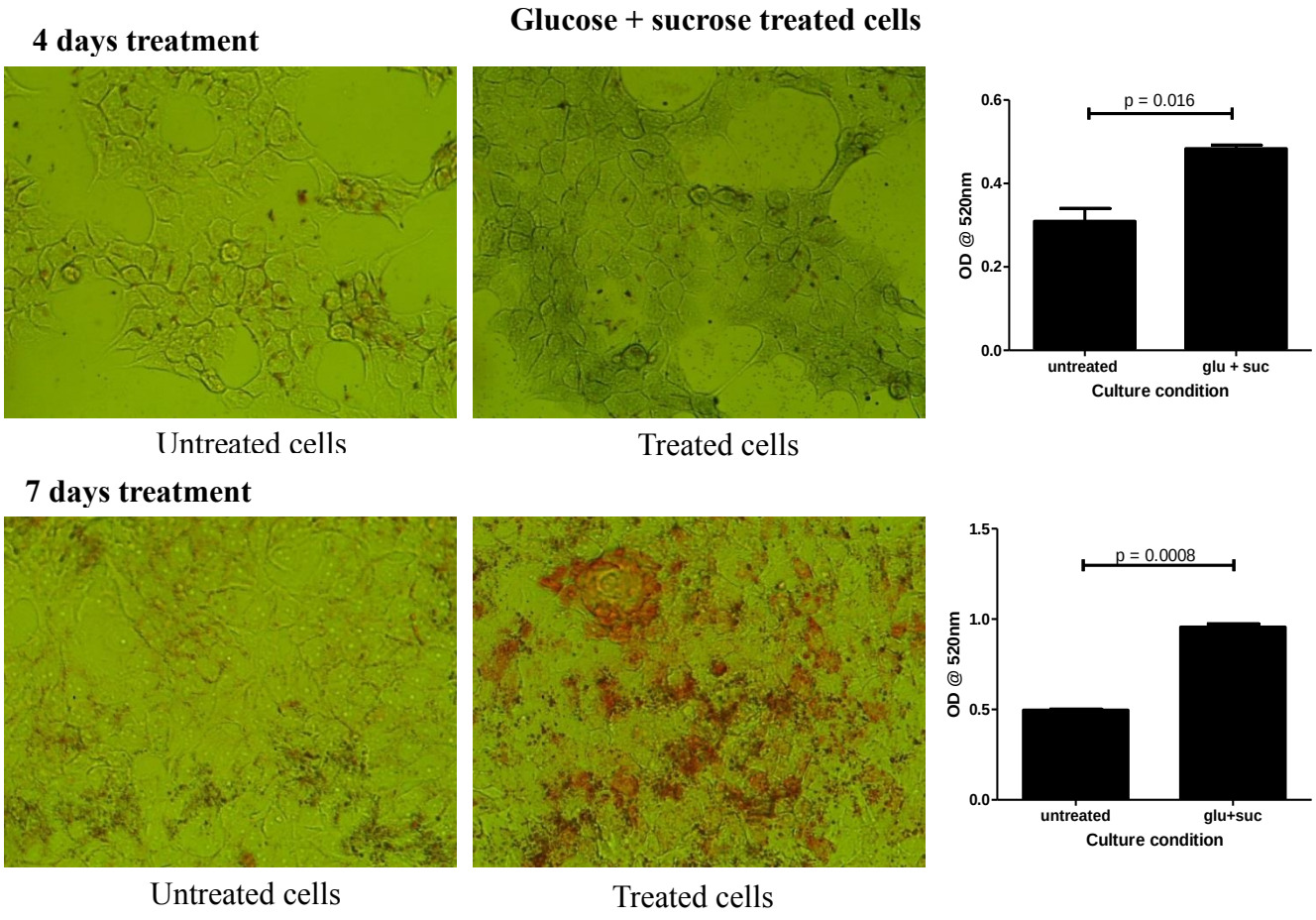


Fig. 18 Lipid accumulation in HepG2 cells after 4 and 7 days of glucose and sucrose supplementation

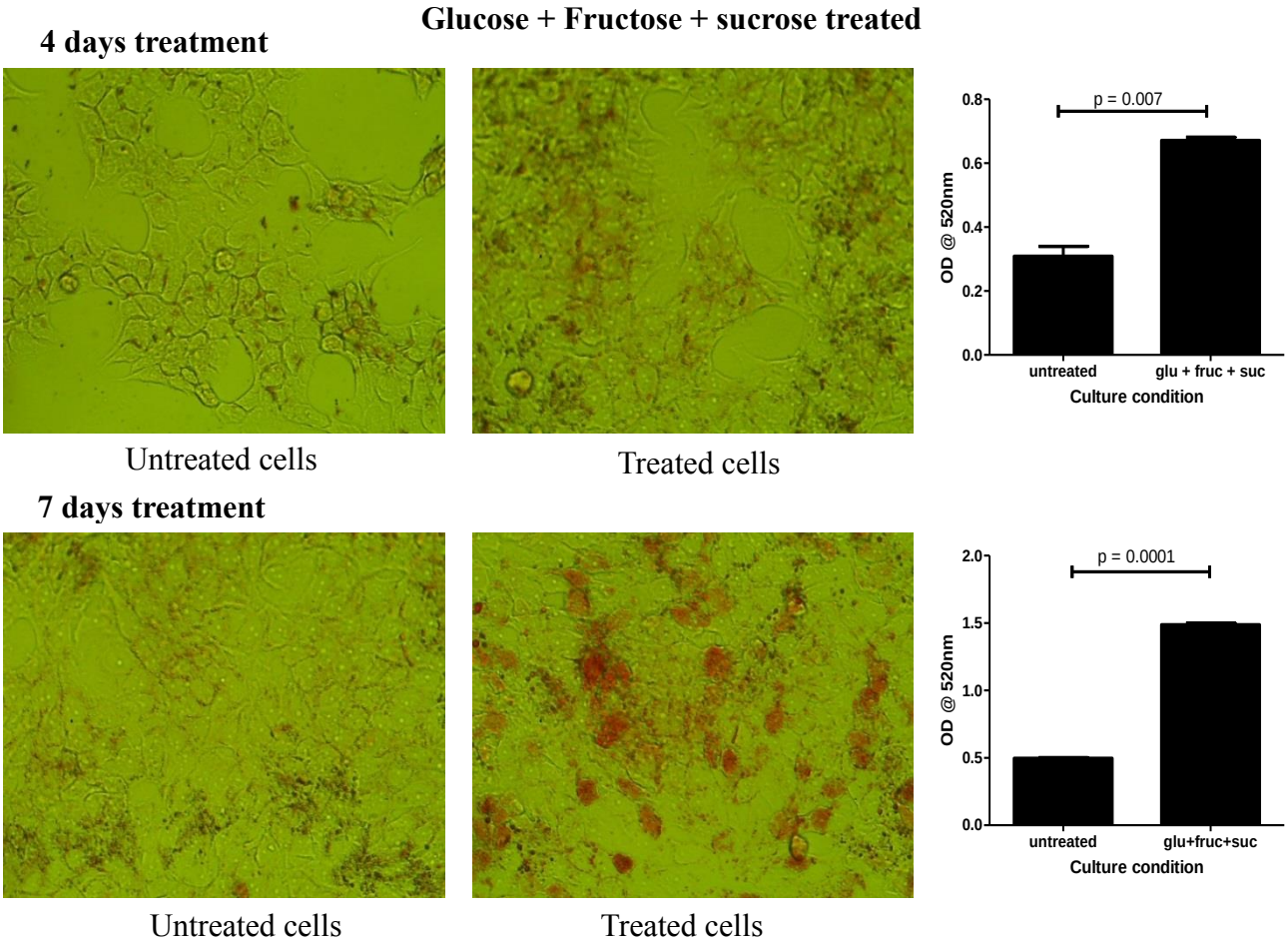


Fig. 19 Lipid accumulation in HepG2 cells after 4 and 7 days of glucose, fructose and sucrose supplementation

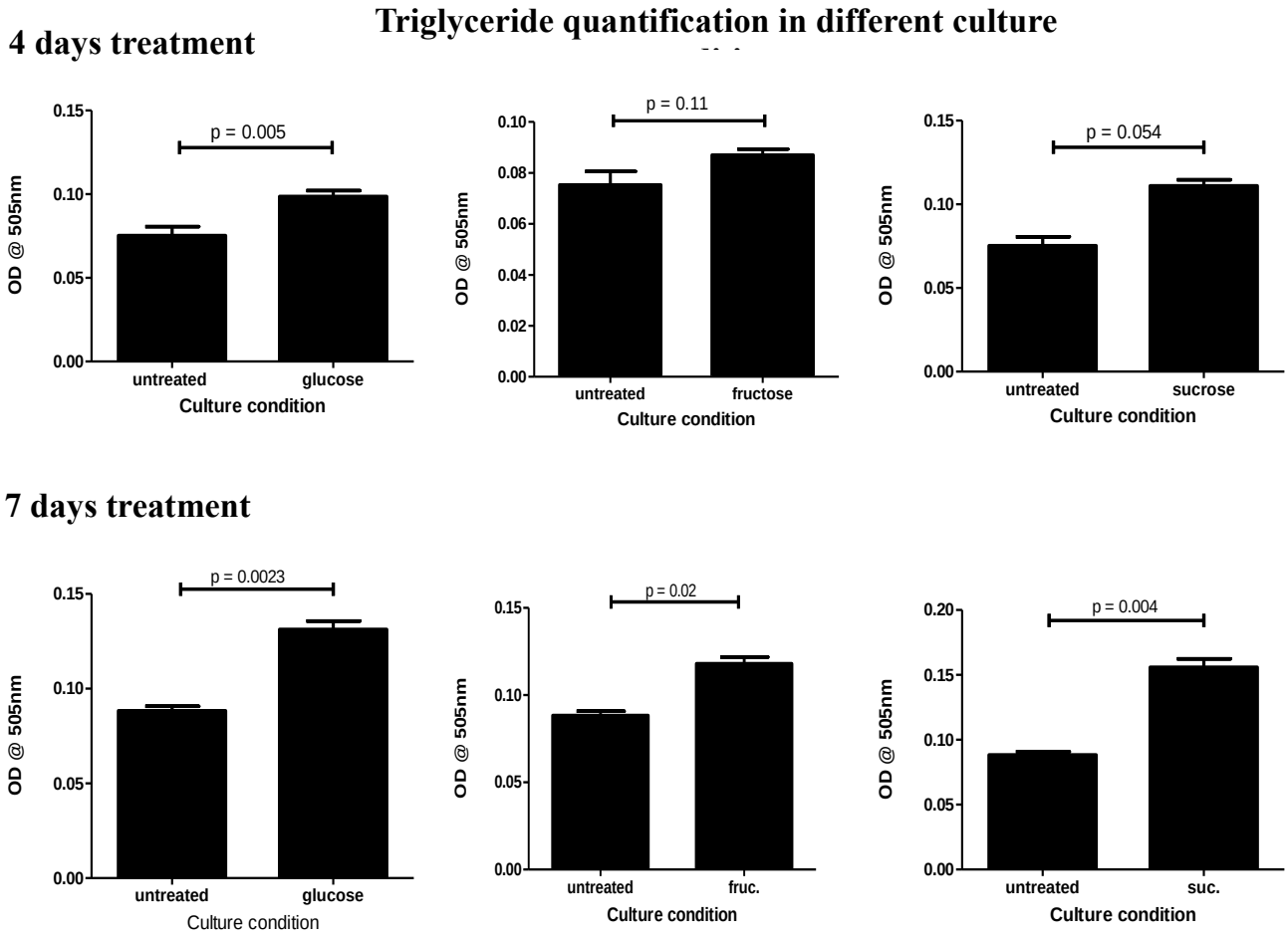
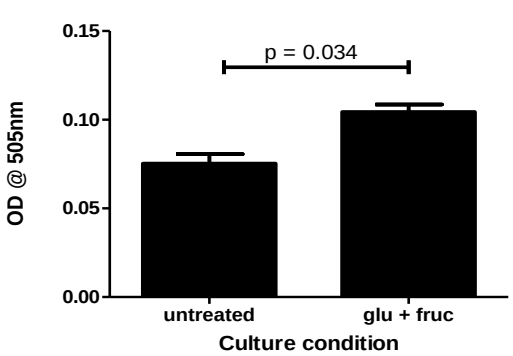
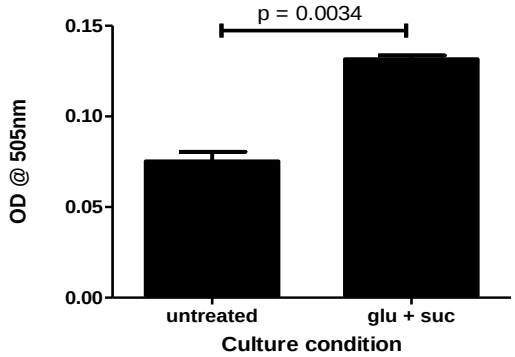


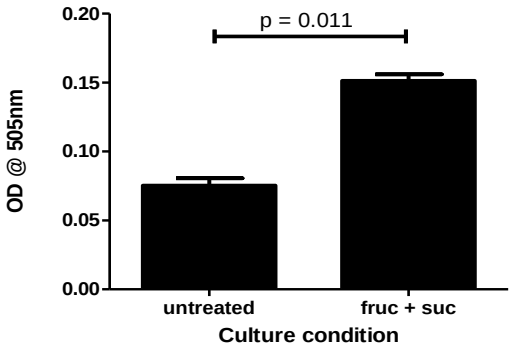
Fig. 20 Triglyceride quantification after 4 and 7 days of supplementation with glucose, fructose, and sucrose

Effect of combination treatment on Triglyceride

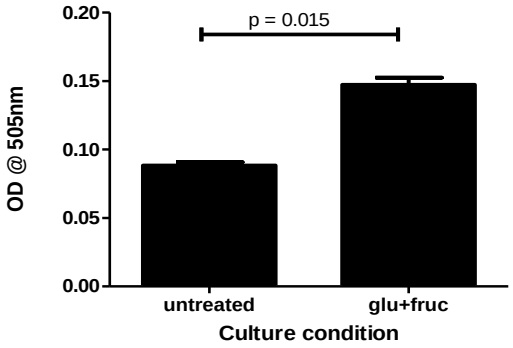
4 days treatment

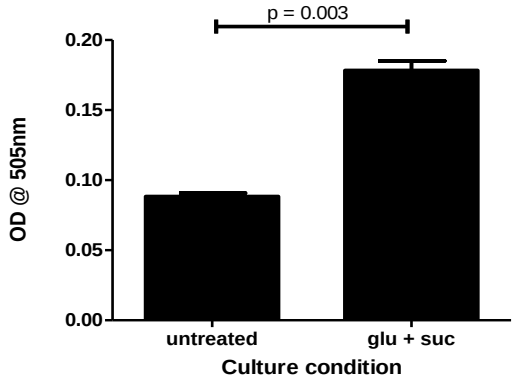


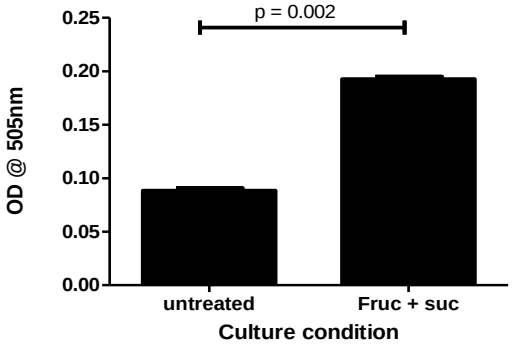




7 days treatment







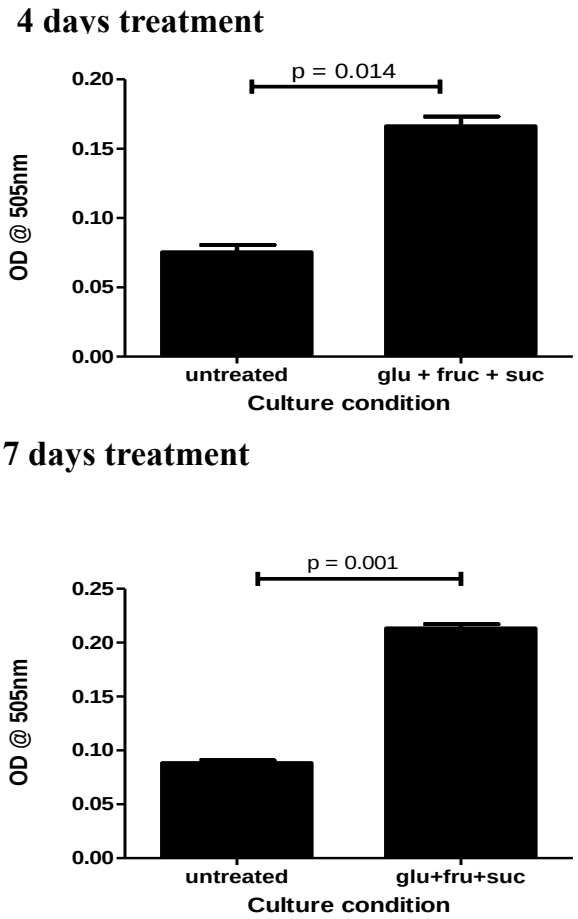
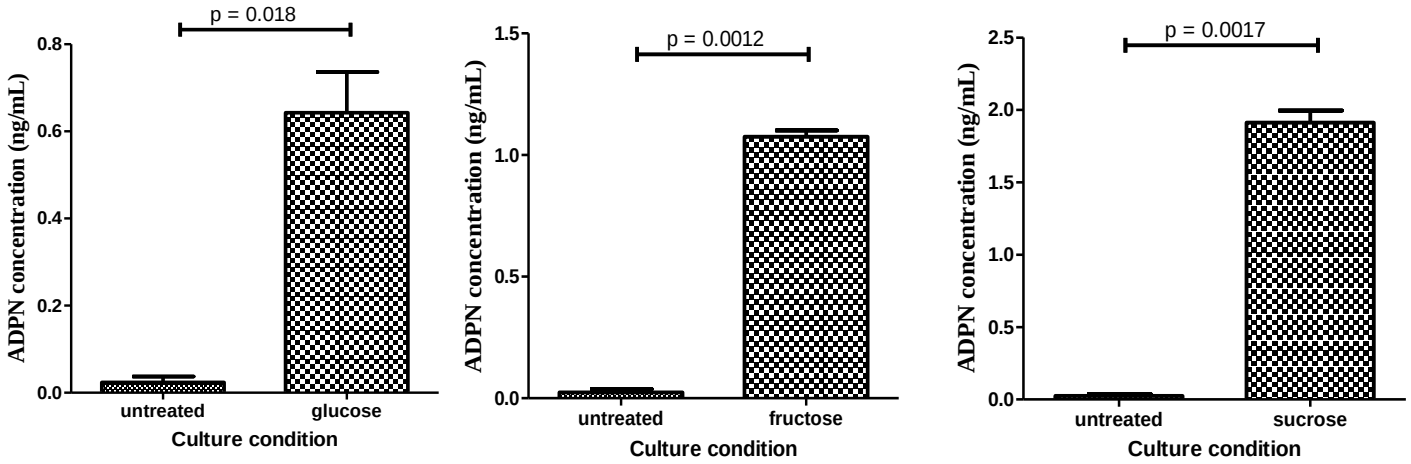


Fig. 21 Triglyceride quantification after 4 and 7 days of supplementation with different combinations of glucose, fructose, and sucrose

Triglyceride quantification was done for secondary screening. The concentration of triglyceride was significantly increased in cells treated with glucose, fructose, sucrose and their combinations as compared to untreated group. An important observation of this study was, significantly higher concentration of triglyceride in cells treated with fructose, sucrose and their combination as compared to glucose supplementation. It was also found that as the duration of supplementation increased, triglyceride accumulation also increased (Fig.20 and 21). This suggests that excess consumption of sugars like glucose, fructose and sucrose contribute to lipid accumulation leading to NAFLD progression.


ADPN quantification in different culture conditions after 7 days
p = 0.018
ADPN concentration (ng/mL)
0.0
0.2
0.4
0.6
0.8
untreated
glucose
Culture condition
p = 0.0012
ADPN concentration (ng/mL)
0.0
0.5
1.0
1.5
untreated
fructose
Culture condition
p = 0.0017
ADPN concentration (ng/mL)
0.0
0.5
1.0
1.5
2.0
2.5
untreated
sucrose
Culture condition

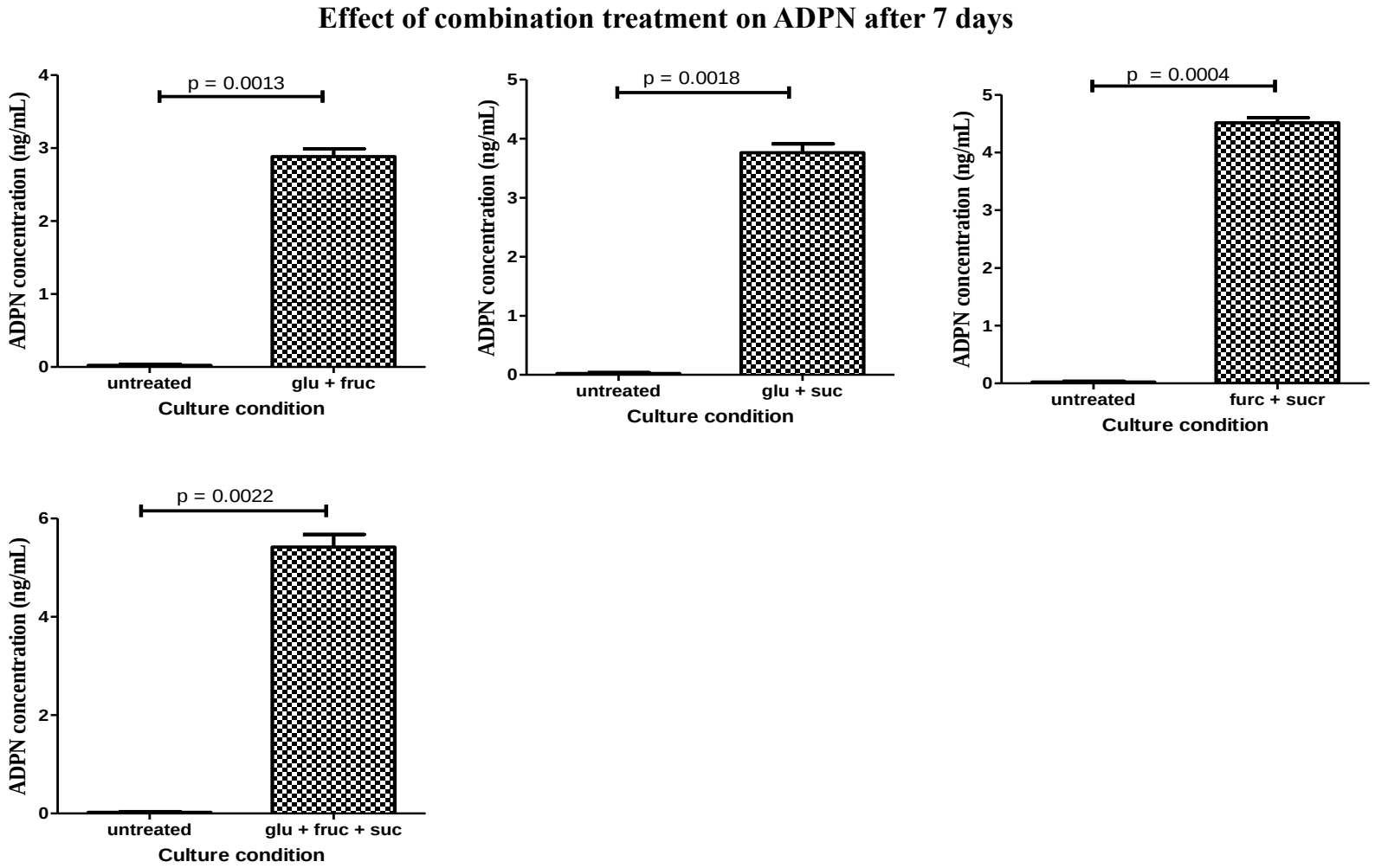
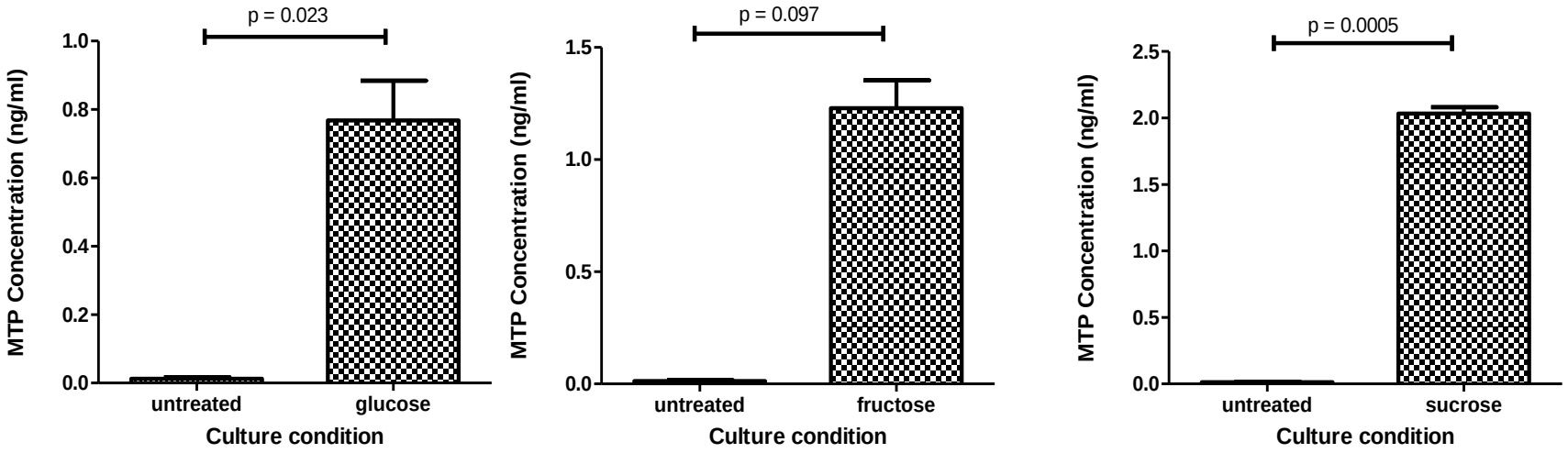


Fig. 22 ADPN quantification after 7 days of supplementation of glucose, fructose, sucrose and their combinations

MTP quantification in different culture conditions after 7 days



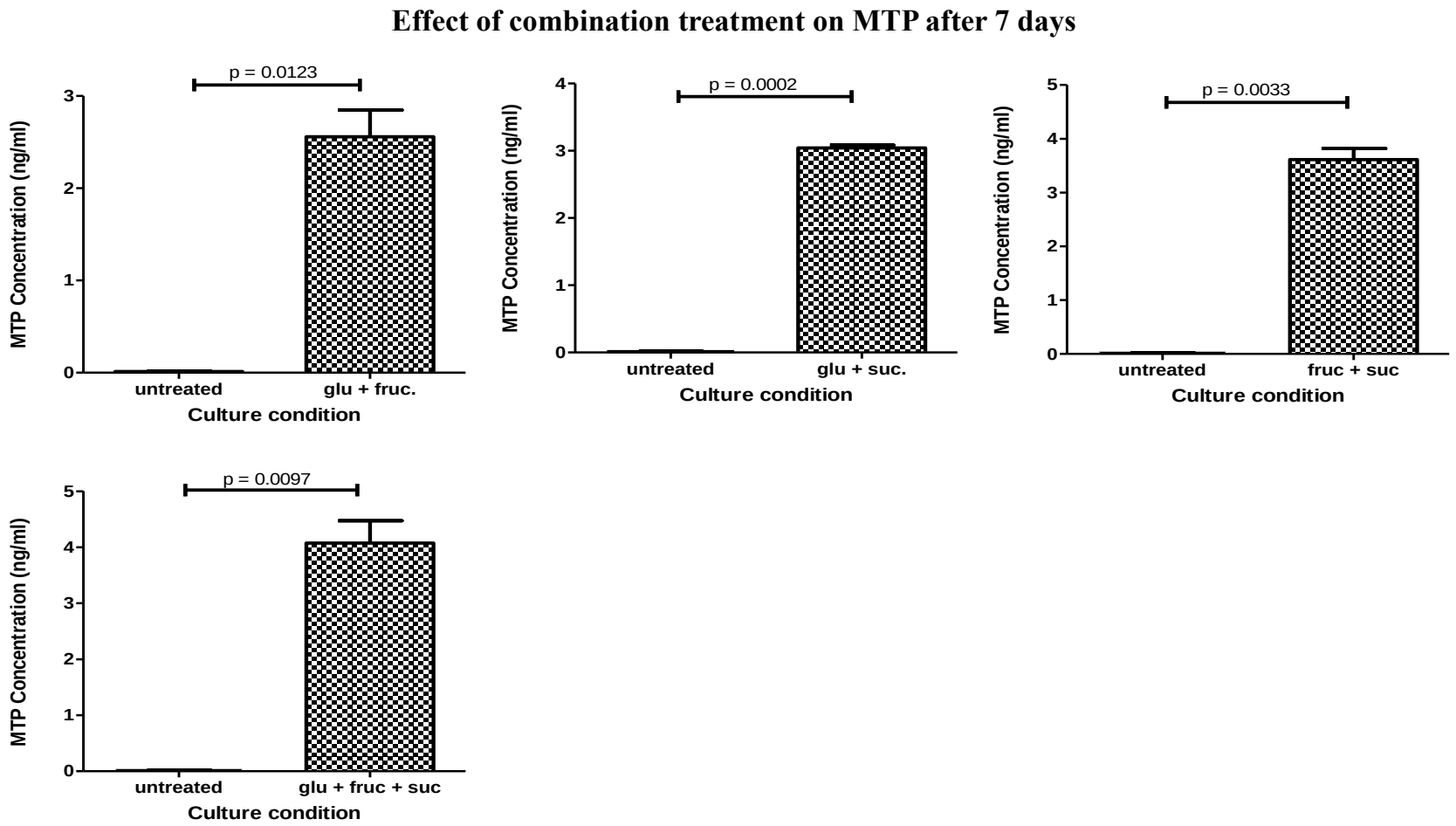


Fig. 23 MTP quantification after 7 days supplementation of glucose, fructose, sucrose and their combinations

The results indicated significantly increased levels of ADPN in cells treated with glucose, fructose, sucrose and their combination as compared to untreated group (Fig.22). Similarly, MTP levels were also increased cells treated with glucose, fructose, sucrose and their combinations as compared to untreated group (Fig.23).

CHAPTER VII
DISCUSSION

NAFLD is considered as a complex disease trait with relevant interactions between genetics and environment [Eslam M et al., 2018]. The sedentary lifestyle and intake of high fat or high sugar diet is one the most common contributor to hepatic steatosis [Vos MB et al., 2013]. This lifestyle if prolongs results in progression of NAFLD. Also, hepatic steatosis is asymptomatic, therefore is usually diagnosed accidentally [Vachliotis I et al., 2021]. The further stages of NAFLD are irreversible and as there is no specific treatment available for NAFLD except healthy lifestyle and regular exercise, there is a need to diagnose and treat NAFLD in initial stages [Chalasani N et al., 2018]. Improvement in diets and exercise are the first-line therapy for NAFLD [Younossi ZM et al., 2021].

The pathogenesis of NAFLD is not completely understood. Different hypotheses have been formulated, leading initially to the "two-hit hypothesis". According to "two-hit", a sedentary lifestyle causes hepatic fat buildup. High fat diets, obesity, and insulin resistance are first hit, while liver sensitization to additional stressors is the second strike. Inflammatory cascades and fibrogenesis are triggered by the "second-hit" [Peverill W et al., 2014]. A more realistic explanation of NAFLD pathogenesis is provided by "multiple-hit" hypothesis which takes into account several insults acting collectively on genetically susceptible patients to cause NAFLD [Buzzetti E et al., 2016]. The multiple hit includes insulin resistance, hormones secreted from adipose tissues, gut microbiome, dietary components, and genetic and epigenetic elements [Buzzetti E et al., 2016, Ballestri S et al., 2016, Borrelli A et al., 2018, Gonzalez-Jaramillo V et al., 2019]. The most relevant genetic association of NAFLD was seen in PNPLA3, which encodes ADPN [Kotronen A et al., 2009].

To the best of our knowledge this is first study to quantify the levels of ADPN and MTP in simple steatosis and NASH subjects of NAFLD patients. The aim of this study was to measure the levels of ADPN and MTP in serum sample of NAFLD subjects and in in-vitro conditions, to analyze whether these molecules can be helpful in differentiating the stages of NAFLD and assess disease progression**.** In order to achieve this aim, the first objective designed was to compare the serum levels of ADPN and MTP in simple steatosis and NASH patients to assess their role in disease progression.

1. **To compare the serum levels of ADPN and MTP in simple steatosis and NASH patients to assess their role in disease progression**

   The result indicates decreased degradation of triglycerides in NASH patients as compared to simple steatosis resulting in increased hepatic TG content. The findings of this study suggest protective role of ADPN against NAFLD progression. Till date, no biochemical study has been done to assess serum levels of ADPN. Thus, the findings of this study can be substantiated by reported genetic studies. Genome-wide association studies (GWAS) have established the involvement of PNPLA3 single nucleotide polymorphism (SNP) in the development and progression of NAFLD, especially the I148M variant (rs738409 C/G) [Anstee QM et al.,2020]. Romeo' et al., [Romeo S et al., 2008] were the first to report SNP in the ADPN gene which encodes for the I148M variant. This variant was strongly associated with increased liver fat content. I148M variant is been associated with the degree of liver injury and all the histopathological

characteristics of NAFLD which includes NASH, fibrosis, cirrhosis, and development of hepatocellular carcinoma [Dongiovanni P et al., 2013].

As the name suggests, MTP is involved in the transfer of triglycerides from hepatocytes to peripheral tissue. Decreased serum levels of MTP observed in the advanced stage of NAFLD in this study indicates decreased triglyceride export and thus resulting in its accumulation. An interaction between MTP and NAFLD has been identified with the help of genetic studies. A polymorphism at the promoter region (-493G/T) of MTP gene is associated in NASH patients with type 2 Diabetes Mellitus. The G allele is associated with decreased MTP transcription and is prone to increased triglyceride content in hepatocytes. Transversion of base guanine to thymine at 493 positions in the promoter region of MTP is found to be associated with decreased transcription level of MTP and failure in triglyceride secretion from hepatocytes which increases the susceptibility to NAFLD [Al-Qarni R et al., 2020]. Several studies have investigated gene expression-based staging of NAFLD to understand its progression and identify the effective treatment [Li L et al., 2014].

2. **To associate ADPN and MTP levels of Simple steatosis and NASH patients with and without comorbidities**

Comorbidities such as Diabetes mellitus, hypertension, and obesity have been increasingly associated with NAFLD development and progression [Mavrogiannaki AN et al., 2013]. Therefore, it was considered worthwhile to assess whether co-existence of the comorbidities in NAFLD subjects would have any impact on the levels of ADPN and MTP.

NAFLD subjects with comorbidities have an increased risk of liver-related morbidity and mortality [Bang KB et al., 2015]. The results of this study shows that ADPN and MTP level decreases in cases with comorbidities than in cases without comorbidities hence, supporting the role of comorbidities in NAFLD progression.

3. **To correlate ADPN and MTP levels with Triglycerides, VLDL and serum transaminases (ALT and AST)**

   As unusual accumulation of triglyceride is one of the main histological characteristic of NAFLD, therefore relation between triglyceride and ADPN, MTP was assessed. Liver transaminases like ALT and AST play vital role in liver metabolism. Mild elevated serum transaminases are primary abnormality seen in NAFLD patients. Though triglycerides and serum transaminases are evaluated in NAFLD, but they are not specific to NAFLD. Some NAFLD cases have serum transaminases within the reference range. Also, there are cases who are not obese but have NAFLD which are also termed as lean NAFLD.

4. **To study the effect of nutritional status on ADPN and MTP in and *in vitro* setup**

   Intake of high fat or high sugar diet and sedentary lifestyle are one of the risk factors of NAFLD. Consumption of sugars like glucose, fructose and sucrose in higher concentration can contribute in NAFLD pathogenesis. Therefore, in the present study, effect of these sugars on ADPN and MTP was studied using HepG2 cell lines in an in-vitro setup. The results showed significant increase in lipid

accumulation after treatment of glucose, sucrose, fructose and their combinations for 7 days.

Fructose consumption is a key player in development of NAFLD [Jeypal S et al., 2017]. Studies have demonstrated that fructose has a major role in inducing fatty liver [Yilmaz Y, 2012]. Fructose increases hepatic de novo lipogenesis in a dose dependent manner and de novo lipogenesis has been shown to be abnormally upregulated in NAFLD patients. A significant consumption of fructose contributes to hepatic steatosis. Simple steatosis can be reversed through dietary changes and physical exercise. But excessive consumption of fructose promotes various processes like inflammation and cellular stress. These processes are responsible for irreversibility and progression of NAFLD. In a study looking at dietary history, fructose consumption was found to be nearly 2-3 fold greater in NAFLD patients than controls [Ouyang X, et al., 2008].

Sucrose in combination with high fat diet has also been demonstrated to induce NASH [Sun S et al., 2021]. High sucrose diet also promotes NAFLD via metabolites like short chain fatty acids [Wang CW, 2016].

Dietary contributors are required to be investigated because these are readily modifiable and may be the key differentiator in why some progress.

It is also well known that excess nutritional intake results in lipid accumulation. The excess lipids are stored in lipid droplets of the cell. Accumulation of a high concentration of lipid droplets is toxic for the cell. This accumulation causes cell stress resulting in release of proteins from the cell. Thus,

the observation of in vitro study adds to the existing knowledge of nutritional status in the progression of NAFLD.

SUMMARY & CONCLUSION

The present study was designed to measure the serum levels of ADPN and MTP in simple steatosis and NASH subjects among NAFLD patients due to their pivotal role in lipid metabolism. The serum levels of ADPN and MTP were significantly decreased in NASH as compared to hepatic steatosis patients. This indicates that as the NAFLD progresses the serum levels of ADPN and MTP decreases. The in-vitro study was performed to check the effect of nutritional status on ADPN and MTP. The cells treated with glucose, fructose, sucrose and their combinations showed a significant increase lipid accumulation and triglyceride levels after 7 days of supplementation. The study demonstrated that ADPN and MTP levels were affected by nutritional status. Therefore, ADPN and MTP can be considered as complementary markers to differentiate the stages of NAFLD and assess disease progression. Hence, ADPN and MTP can be considered as complementary markers that will help differentiate the stages of NAFLD and assess disease progression.

NEW KNOWLEDGE GENERATED

New knowledge generated

The new knowledge generated in through study was:

1. Study introduces Adiponutrin and MTTP for differentiating steatosis from steatohepatitis in NAFLD stages with and without comorbidities.
2. This is the first study to quantify the protein levels of Adiponutrin and MTTP in human serum sample of NAFLD subjects.
3. Study shows that inverse relationship between Adiponutrin protein levels and NAFLD disease progression.
4. Study shows the effect of simple sugars like Glucose, fructose and sucrose on Adiponutrin and MTTP in in-vitro setup.

LIMITATIONS

Limitations

There are few limitations to the present study. The small sample size, the larger sample size would give better insights. Cirrhosis and HCC stage samples should have also been considered for the analysis. In in-vitro condition, prolonged treatment is required to get the parallel outcome like human serum sample. Furthermore, genetic analysis for ADPN and MTP could provide better explanation for reduced levels of ADPN and MTTP and confirmation of the results.

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PRESENTATIONS AND PUBLICATIONS

Presentations:

1. Shukla Mayuri, Kunder Mamatha, Balakrishna Sharath, Kamarthy Prabhakar. Serum levels of Adiponutrin in stages of Non-alcoholic Fatty liver Disease. Poster presented at ABGCON 2021, National virtual conference and workshop; 2021 Aug 27 to Aug 28; Sri Balaji Medical College and Hospital, Chennai.
2. Shukla Mayuri, Kunder Mamatha, Balakrishna Sharath, Kamarthy Prabhakar. Quantification of MTP in NAFLD patients. Oral presentation at International Webinar on Diabetes and Health Care; 2022 March 29 and 29.

Publications:

1. Shukla M, Kunder M, Kamarathy P, Balakrishna S. Quantification of Microsomal triglyceride transfer protein in Non-alcoholic fatty liver disease patients: A Cross-sectional Study.Journal of Clinical Diagnostic and Research.2022 Sept 01;16(9):BC01-BC04.
2. Shukla M, Kunder M, Kamarthy P, Balakrishna S. Serum levels of adiponutrin in differentiating non-alcoholic steatohepatitis from simple steatosis in non-alcoholic fatty liver disease patients. Romanian Journal of Diabetes Nutrition and Metabolic Diseases. 2022 Jun 23;29(2):261-7.

ANNEXURES

SRI DEVARAJ URS ACADEMY OF HIGHER EDUCATION & RESEARCH

SDUAHER

SRI DEVARAJ URS MEDICAL COLLEGE

Tamaka, Kolar

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2. **Dr. Sujatha.M.P, (Member Secretary),** Assoc. Prof. of Anesthesia, SDUMC,
3. Dr. C.S.Babu Rajendra Prasad, Prof. of Pathology, SDUMC
4. Dr. Srinivasa Reddy.P, Prof. & HoD of Forensic Medicine, SDUMC
5. Dr. Prasad.K.C, Professor of ENT, SDUMC
6. Dr. Sumathi.M.E Prof. & HoD of Biochemistry, SDUMC.
7. Dr. Bhuvana.K, Prof. & HoD of Pharmacology, SDUMC
8. Dr. H.Mohan Kumar, Professor of Ophthalmology, SDUMC
9. Dr. Hariprasad, Assoc. Prof Department of Orthopedics, SDUMC
10. Dr. Pavan.K, Asst. Prof of Surgery, SDUMC
11. Dr. Talasila Sruthi, Assoc. Prof. of OBG, SDUMC
12. Dr. Mahendra.M , Asst. Prof. of Community Medicine, SDUMC
13. Dr. Mamata Kale, Asst. Professor of Microbiology, SDUMC

No. SDUMC/KLR/IEC/41/2019-20 **Date:06-06-2019**

PRIOR PERMISSION TO START OF STUDY

The Institutional Ethics Committee of Sri Devaraj Urs Medical College, Tamaka, Kolar has examined and unanimously approved the Ph.D study entitled **"Studies on adiponutrin and microsomal trigtyceride protein for differentiating stages of non- alcoholic fatty liver disease"** being investigated by **Ms. Shukla Mayuri Omprakash,** Dr. Mamatha Kunder[1], Dr.Sharath.B & Prabhakar.K[2] in the Departments of Cell Biology and Molecular Genetics, Biochemistry[1] & Medicine[2] at Sri Devaraj Urs Medical College, Tamaka, Kolar. **Permission is granted by the Ethics Committee to start the study.**

Sujatha M.P

Member Secretary

Member Secretary

Institutional Ethics Committee

Sri Devaraj Urs Medical College

Tamaka, Kolar.

Chairman

CHAIRMAN

Institutional Ethics Committee

Sri Devaraj Urs Medical College

Tamaka, Kolar

Note: The same Institutional Ethics Clearance No. can be used for Presentation & Publication. However Presentation to be preceded before Publication.

CASE HISTORY SHEET

Title of the study: Studies on Adiponutrin and Microsomal Triglyceride Protein in Non-alcoholic Fatty Liver Disease patients

Case No:	
Patient's Name	
IP/OP Number	
Age	
Gender	M ☐ F ☐
Occupation	
Contact number	
E-mail	
Address	
Past History	
Hypertension	
Diabetes	
Heart diseases	
Smoking	
Alcohol intake	
Physical Examination	

Height	
Weight	
BMI	
BP	
Biochemical Investigations	
Hs-CRP	
FBS	
AST	
ALT	
ALKP	
GGT	
Albumin	
Serum cholesterol	
Triglycerides	
HDL	
VLDL	

INFORMATION SHEET

Title of the study: Studies on Adiponutrin and Microsomal Triglyceride Protein in Non-alcoholic Fatty Liver Disease patients

Principal investigator: Ms. Shukla Mayuri Omprakash

Consent for the interview:

Non-alcoholic fatty liver is a most common cause of chronic liver disorder, which results due to accumulation of fats affecting the normal functions of liver. It further progresses to non-alcoholic steatohepatitis (NASH) followed by fibrosis and cirrhosis. Multiple factors like metabolic syndrome (MS), genetic and environmental factors are causes for NAFLD. In PNPLA3 gene, change in isoleucine amino acid by methionine at 148 protein position 148 leads to accumulation of triglycerides in hepatocytes. This is one of the most common variant found to be associated with NAFLD. Hepatic fat accumulation can be results of increased fat synthesis or decreased fat export. Triglycerides are exported from liver in the form of Very low density protein(VLDL). VLDL's are formed by incorporation of apolipoprotein B (apoB) and Microsomal Triglyceride Protein(MTP). Alterations in apoB and MTP is also one of the potential mechanism in pathogenesis of NAFLD. Genetic studies have proven interaction between NAFLD and MTP. Therefore, estimating levels of adiponutrin and microsomal triglyceride protein can help us in differentiating stages of NAFLD.

In this regard, I would like to ask you some questions regarding your present & past health conditions. You do not have to answer any question that you not want to answer & you may end this interview at any time you want to. I will take about half an

hour to ask the questions. I would like to take your consent to participate in this study & if you're willing to participate I will be drawing 5ml of blood from you to perform the tests.

Participation in this study does not involve any cost for you. The study is not only beneficial to you but to the community at large. The results gathered from this study will be beneficial in management of the diseases. All information collected from you will be strictly confidential & will not be disclosed to any outsider except if it is required by the law. This information collected will be used only for research. This information will not reveal your identity. This study has been approved by the local review board & has been started only after their formal approval.

There is no compulsion to participate in this study. You will be no way affected if you do not wish to participate in the study. You are required to sign only if you voluntarily agree to participate in this study. Further you are at a liberty to withdraw from the study at any time. Be assured that your withdrawal will not affect your treatment by the concerned physician in any way. This document will be stored in the safe locker in the Cell Biology and Molecular Genetics Department & a copy given to you for information.

For any further clarification you are free to contact me.

Name: Ms. Shukla Mayuri Omprakash

Mobile no.: 9561687132

ರೋಗಿಯ ಮಾಹಿತಿ ಹಾಳೆ

ಶೀರ್ಷಿಕೆ:- ಆಲ್ಕೊಹಾಲ್ಯುಕ್ತವಲ್ಲದ ಕೊಬ್ಬಿನ ಪಿತ್ತಜನಕಾಂಗದ ರೋಗಿಗಳಲ್ಲಿ ಅಡಿಪೋನ್ಯೂಟ್ರಿನ್ ಮತ್ತು ಮೈಕ್ರೋಸೋಮಲ್ ಟ್ರೈಗ್ಲಿಸರೈಡ್ ಪ್ರೋಟೀನ್‌ಗಳ ಮೇಲಿನ ಅಧ್ಯಯನಗಳು

ಪ್ರಿನ್ಸಿಪಲ್ ಇನ್ವೆಸ್ಟಿಗಾರ್ಟರ್:- ಮಿಸ್. ಶುಕ್ಲಾ ಮಯೂರಿ ಓಂಪ್ರಕಾಶ್

ಸಂದರ್ಶನಕ್ಕೆ ಅನುಗುಣವಾಗಿ:-

ಆಲ್ಕೋಹಾಲಿಕ್ ಅಲ್ಲದ ಕೊಬ್ಬಿನ ಯಕೃತ್ತು ರೋಗ (NAFLD) ದೀರ್ಘಕಾಲದ ಯಕೃತ್ತಿನ ಅಸ್ವಸ್ಥತೆಯ ಒಂದು ಸಾಮಾನ್ಯ ಕಾರಣವಾಗಿದೆ, ಇದು ಯಕೃತ್ತಿನ ಸಾಮಾನ್ಯ ಕ್ರಿಯೆಗಳ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವ ಕೊಬ್ಬುಗಳ ಸಂಗ್ರಹದಿಂದ ಉಂಟಾಗುತ್ತದೆ. ಇದು ಮತ್ತಷ್ಟು ಆಲ್ಕೊಹಾಲ್ಯುಕ್ತ ಸ್ಟಿಟೋಹೆಪಟೈಟಿಸ್ (NASH) ಗೆ ತುತ್ತಾಗುತ್ತದೆ ಮತ್ತು ನಂತರ ಫೈಬ್ರೋಸಿಸ್ ಮತ್ತು ಸಿರೋಸಿಸ್ಗೆ ಕಾರಣವಾಗುತ್ತದೆ. ಮೆಟಾಬಾಲಿಕ್ ಸಿಂಡ್ರೋಮ್ (ಎಂಎಸ್), ಜೆನೆಟಿಕ್ ಮತ್ತು ವಾತಾವರಣ ಅಂಶಗಳು ಕಾರಣಗಳಾಗಿವೆ. PNPLA3 ವಂಶವಾಹಿಗಳಲ್ಲಿ, 148 ಪ್ರೋಟೀನ್ ಸ್ಥಾನ 148 ರಲ್ಲಿ ಮೀಥಿಯೋನಿನ್ ನಿಂದ ಐಸೊಲುಸಿನ್ ಅಮೈನೊ ಆಸಿಡ್ನಲ್ಲಿ ಬದಲಾವಣೆ ಹೆಪಟೊಸೈಟ್ಗಳಲ್ಲಿ ಟ್ರೈಗ್ಲಿಸರೈಡ್ಗಳ ಸಂಗ್ರಹಕ್ಕೆ ಕಾರಣವಾಗುತ್ತದೆ. NAFLD ನೊಂದಿಗೆ ಸಂಯೋಜಿತವಾಗಿರುವ ಅತ್ಯಂತ ಸಾಮಾನ್ಯವಾದ ರೂಪಾಂತರಗಳಲ್ಲಿ ಇದು ಒಂದಾಗಿದೆ. ಹೆಪಾಟಿಕ್ ಕೊಬ್ಬು ಶೇಖರಣೆ ಹೆಚ್ಚಿದ ಕೊಬ್ಬು ಸಂಶ್ಲೇಷಣೆಯ ಫಲಿತಾಂಶಗಳು ಅಥವಾ ಕೊಬ್ಬಿನ ರಫ್ತು ಕಡಿಮೆಯಾಗುತ್ತದೆ. ಟ್ರೈಗ್ಲಿಸರೈಡ್ಗಳನ್ನು ಯಕೃತ್ತಿನಿಂದ ಕಡಿಮೆ ಸಾಂದ್ರತೆಯ ಪ್ರೋಟೀನ್ (VLDL) ರೂಪದಲ್ಲಿ ರಫ್ತು ಮಾಡಲಾಗುತ್ತದೆ. ಅಪೊಲಿಪೋಪ್ರೋಟೀನ್ B (apoB) ಮತ್ತು ಮೈಕ್ರೊಸೋಮಲ್ ಟ್ರೈಗ್ಲಿಸರೈಡ್ ಪ್ರೋಟೀನ್ (MTP) ಅನ್ನು ಸಂಯೋಜಿಸುವ ಮೂಲಕ VLDL

ಗಳು ರೂಪುಗೊಳ್ಳುತ್ತವೆ. NAFLD ನ ರೋಗಕಾರಕದಲ್ಲಿನ ಸಂಭವನೀಯ ಯಾಂತ್ರಿಕ ವ್ಯವಸ್ಥೆಯಲ್ಲಿ apoB ಮತ್ತು MTP ಯ ಪರಿವರ್ತನೆಗಳು ಕೂಡಾ ಒಂದಾಗಿದೆ. ತಳೀಯ ಅಧ್ಯಯನಗಳು NAFLD ಮತ್ತು MTP ಯ ನಡುವೆ ಪರಸ್ಪರ ಸಂವಹನ ನಡೆಸಿದವು. ಆದ್ದರಿಂದ, ಅಡಿಪೋನಟ್ರಿನ್ ಮತ್ತು ಮೈಕ್ರೊಸೋಮಲ್ ಟ್ರೈಗ್ಲಿಸರೈಡ್ ಪ್ರೋಟೀನ್ಗಳ ಅಂದಾಜು ಮಟ್ಟಗಳು NAFLD ಹಂತಗಳನ್ನು ವಿಭಜಿಸುವಲ್ಲಿ ನಮಗೆ ಸಹಾಯ ಮಾಡಬಹುದು.

ಈ ನಿಟ್ಟಿನಲ್ಲಿ, ನಿಮ್ಮ ಪ್ರಸ್ತುತ ಹಿಂದಿನ ಆರೋಗ್ಯ ಪರಿಸ್ಥಿತಿಗಳ ಬಗ್ಗೆ ಕೆಲವು ಪ್ರಶ್ನೆಗಳನ್ನು ನಾನು ಕೇಳಲು ಬಯಸುತ್ತೇನೆ. ನೀವು ಯಾವುದೇ ಉತ್ತರವನ್ನು ಉತ್ತರಿಸಬೇಕಾದ ಅಗತ್ಯವಿಲ್ಲದಿದ್ದಲ್ಲಿ ನೀವು ಈ ಸಂದರ್ಶನವನ್ನು ಕೊನೆಗೊಳಿಸಬಹುದು. ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನಾನು ಸುಮಾರು ಅರ್ಧ ಘಂಟೆ ತೆಗೆದುಕೊಳ್ಳುತ್ತೇನೆ. ನಾನು ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನಿಮ್ಮ ಒಪ್ಪಿಗೆಯನ್ನು ತೆಗೆದುಕೊಳ್ಳಲು ಬಯಸುತ್ತೇನೆ. ನಿಮ್ಮ ಪರೀಕ್ಷೆ ನಡೆಸಲು ನಾನು ನಿಮ್ಮ 5 ಮಿಲಿ ರಕ್ತವನ್ನು ಸೆಳೆಯುತ್ತಿದ್ದೇನೆ.

ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸುವಿಕೆಯು ನಿಮಗೆ ಯಾವುದೇ ವೆಚ್ಚವನ್ನು ಒಳಗೊಂಡಿರುವುದಿಲ್ಲ. ಅಧ್ಯಯನವು ನಿಮಗೆ ಅನುಕೂಲಕರವಲ್ಲ ಆದರೆ ಸಮುದಾಯಕ್ಕೆ ದೊಡ್ಡದಾಗಿದೆ. ಈ ಅಧ್ಯಯನದಿಂದ ಸಂಗ್ರಹಿಸಿದ ಫಲಿತಾಂಶಗಳು ರೋಗಗಳ ನಿರ್ವಹಣೆಗೆ ಅನುಕೂಲಕರವಾಗಿರುತ್ತದೆ. ಕಾನೂನಿನಿಂದ ಅಗತ್ಯವಿದ್ದರೆ ಹೊರತು ನಿಮ್ಮಿಂದ ಸಂಗ್ರಹಿಸಲಾದ ಎಲ್ಲಾ ಮಾಹಿತಿಯನ್ನು ಕಡ್ಡಾಯವಾಗಿ ಗೌಪ್ಯವಾಗಿ ಬಹಿರಂಗಪಡಿಸಲಾಗುವುದಿಲ್ಲ. ಸಂಗ್ರಹಿಸಿದ ಈ ಮಾಹಿತಿಯನ್ನು ಸಂಶೋಧನೆಗೆ ಮಾತ್ರ ಬಳಸಲಾಗುತ್ತದೆ. ಈ ಮಾಹಿತಿಯು ನಿಮ್ಮ ಗುರುತನ್ನು

ಬಹಿರಂಗಪಡಿಸುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನವು ಸ್ಥಳೀಯ ಔಪಚಾರಿಕ ಅನುಮೋದನೆಯ ನಂತರ ಮಾತ್ರ ಪ್ರಾರಂಭವಾಗಿದೆ.

ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಯಾವುದೇ ನಿರ್ಬಂಧವಿಲ್ಲ. ನೀವು ಅಧ್ಯಯನದಲ್ಲಿ ಭಾಗವಹಿಸಲು ಬಯಸದಿದ್ದರೆ ನಿಮಗೆ ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ. ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ನೀವು ಸ್ವಯಂಪ್ರೇರಣೆಯಿಂದ ಸಮ್ಮತಿಸಿದರೆ ಮಾತ್ರ ನೀವು ಸಹಿ ಮಾಡಬೇಕಾಗುತ್ತದೆ. ಮತ್ತಷ್ಟು ನೀವು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಅಧ್ಯಯನದಿಂದ ಹಿಂದೆಗೆದುಕೊಳ್ಳಲು ಸ್ವಾತಂತ್ರ್ಯದಲ್ಲಿದ್ದಾರೆ. ನಿಮ್ಮ ಹಿಂತೆಗೆದುಕೊಳ್ಳುವಿಕೆಯು, ಯಾವುದೇ ರೀತಿಯಲ್ಲಿ ಯಾವುದೇ ಚಿಕಿತ್ಸೆಯ ಮೇಲೆ ಪರಿಣಾಮ ಬೀರುವುದಿಲ್ಲ ಎಂದು ಖಚಿತಪಡಿಸಿಕೊಳ್ಳಿ. ಮಾಹಿತಿಗಾಗಿ ನಿಮಗೆ ನೀಡಿದ ನಕಲನ್ನು ಜೀವಕೋಶ ಜೀವಶಾಸ್ತ್ರ ಮತ್ತು ಅಣು ತಳಿಶಾಸ್ತ್ರ ವಿಭಾಗದಲ್ಲಿ ಸುರಕ್ಷಿತ ಲಾಕರ್ನಲ್ಲಿ ಈ ಡಾಕ್ಯುಮೆಂಟ್ ಸಂಗ್ರಹಿಸಲಾಗುವುದು

ಯಾವುದೇ ಹೆಚ್ಚಿನ ಸ್ಪಷ್ಟೀಕರಣಕ್ಕಾಗಿ ನೀವು ನನ್ನನ್ನು ಸಂಪರ್ಕಿಸಲು ಮುಕ್ತವಾಗಿರುತ್ತೀರಿ.

ಮಿಸ್ .ಶುಕ್ಲಾ ಮಯೂರಿ ಓಂಪ್ರಕಾಶ್

ಮೊಬೈಲ್ ಸಂಖ್ಯೆ. 9561687132

INFORMED CONSENT

I understand that I remain free to withdraw from this study at any time.

I have read or had read to me & understood the purpose of this study & the confidentiality of the information that will be collected & disclosed during the study.

I have had the opportunity to ask my questions regarding the various aspects of this study & my questions have been answered to my satisfaction.

I agree to participate in this study & authorize the collection & disclosure of my personal information as outlined in this consent form.

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Participant's name & signature/thumb impression Date

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Name and Signature of the witness Date

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Signature of the principal investigator Date

ಸಮ್ಮತಿಯ ಪ್ರಮಾಣಪತ್ರ

ಈ ಅಧ್ಯಯನದಿಂದ ನಾನು ಯಾವುದೇ ಸಮಯದಲ್ಲಿ ಹಿಂತೆಗೆದುಕೊಳ್ಳಲು ಮುಕ್ತನಾಗಿರುತ್ತೇನೆ ಎಂದು ನಾನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

ನಾನು ಓದಿದ್ದೇನೆ ಅಥವಾ ನನಗೆ ಓದಿದ್ದಾರೆ ಮತ್ತು ಈ ಅಧ್ಯಯನದ ಉದ್ದೇಶ ಮತ್ತು ಅಧ್ಯಯನದ ಸಮಯದಲ್ಲಿ ಸಂಗ್ರಹಿಸಿದ ಮತ್ತು ಬಹಿರಂಗಗೊಳ್ಳುವ ಮಾಹಿತಿಯ ಗೌಪ್ಯತೆಯನ್ನು ಅರ್ಥಮಾಡಿಕೊಂಡಿದ್ದೇನೆ.

ಈ ಅಧ್ಯಯನದ ವಿವಿಧ ಅಂಶಗಳ ಬಗ್ಗೆ ನಾನು ಪ್ರಶ್ನೆಗಳನ್ನು ಕೇಳಲು ನನಗೆ ಅವಕಾಶವಿತ್ತು ಮತ್ತು ನನ್ನ ಪ್ರಶ್ನೆಗಳಿಗೆ, ನನ್ನ ತೃಪ್ತಿಗೆ ಉತ್ತರ ನೀಡಲಾಗಿದೆ.

ನಾನು, ಈ ಅಧ್ಯಯನದಲ್ಲಿ ಪಾಲ್ಗೊಳ್ಳಲು ಒಪ್ಪುತ್ತೇನೆ ಮತ್ತು ಈ ಸಮ್ಮತಿಯ ರೂಪದಲ್ಲಿ ವಿವರಿಸಿರುವಂತೆ ನನ್ನ ವೈಯಕ್ತಿಕ ಮಾಹಿತಿಯ ಸಂಗ್ರಹಣೆ ಮತ್ತು ಬಹಿರಂಗಪಡಿಸುವಿಕೆಯನ್ನು ದೃಢೀಕರಿಸುತ್ತೇನೆ.

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ಭಾಗವಹಿಸುವವರ ಹೆಸರು ಮತ್ತು ಸಹಿ / ಹೆಬ್ಬೆರಳು ಗುರುತು ದಿನಾಂಕ

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ಸಾಕ್ಷಿಯ ಹೆಸರು ಮತ್ತು ಸಹಿ ದಿನಾಂಕ

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ಪ್ರಧಾನ ತನಿಖಾಧಿಕಾರಿಯ ಸಹಿ: ದಿನಾಂಕ

RECOMMENDATION

Since the underlying mechanism of NAFLD is complex with multiple interactions. Targeting ADPN and MTP and increasing its level so as to increase their activity can be helpful in treating NAFLD and preventing its progression.