ASSOCIATION OF SUBCLINICAL INFLAMMATION AND INSULIN RESISTANCE WITH NON ALCOHOLIC FATTY LIVER DISEASE IN PREDIABETIC SUBJECTS

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DEDICATION

TO MY BELOVED PARENTS

CERTIFICATE

This thesis titled "ASSOCIATION OF SUBCLINICAL INFLAMMATION AND INSULIN RESISTANCE WITH NONALCOHOLIC FATTY LIVER DISEASE IN PREDIABETIC SUBJECTS" is submitted in partial fulfillment of the requirement for the degree of MPhil (Biochemistry & Molecular Biology) Thesis under University of Dhaka. This work had been carried out in the Department of Biochemistry & Cell Biology, Bangladesh University of Health Sciences (BUHS) and Biomedical Research Group, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic disorders (BIRDEM) during the period of January 2013 to October 2013. To the best of my knowledge no part of the work has been submitted for another degree or qualification in any other institutes.

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DECLARATION

I hereby declare that this thesis entitled as **'ASSOCIATION OF SUBCLINICAL INFLAMMATION AND INSULIN RESISTANCE WITH NONALCOHOLIC FATTY LIVER DISEASE IN PREDIABETIC SUBJECTS'** is based on work carried out by me and no part of it has been presented previously to any academic institute or university for any higher degree.

The research work was carried out in the Department of Biochemistry & Cell Biology, Bangladesh University of Health Sciences (BUHS) and Biomedical Research Group, BIRDEM, Dhaka under the guidance of Dr Md Khalilur Rahman, Professor, Department of Biochemistry & Molecular Biology, University of Dhaka, and Dr Liaquat Ali, *Vice-Chancellor* & Professor, Department of Biochemistry & Cell Biology; Bangladesh University of Health Sciences (BUHS), Dhaka.

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LIST OF ABBREVIATIONS

µIU	Micro international unit
µl	Microliter
µmol	Micromole
1hPG	1-hour postprandial glucose
ADA	American Diabetes Association
AKB	Albumin
ALP	Alkaline phosphatase
ANA	Antinuclear antibody
BADAS	Bangladesh Diabetic Association
BIRDEM	Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders
BMI	Body mass index
BMRG	Biomedical Research Group
BUHS	Bangladesh University of Health Sciences
CHD	Coronary heart disease
CI	Confidence interval
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
ELISA	Enzyme linked immunesorbent assay
ESR	Erythrocyte sedimentation rate
FFA	Free fatty acid
FFM	Free fat mass
FFQ	Food frequency question
FLI	Fatty liver index
FSG	Fasting serum glucose
G/I	Glucose/insulin ratio
GGT	Gama glutamate transaminase
H_2O_2	Hydrogen peroxide
HbA_{1C}	Glycated hemoglobin
HCC	Hepatocellular carcinoma
HDL-c	High density lipoprotein cholesterol
HGP	Hepatic glucose production

HOMA%B	Homeostasis model assessment b cell function
HOMA%S	Homeostasis model assessment insulin sensitivity
HOMA-IR	Homeostasis model assessment insulin resistance
HPLC	High performance liquid chromatography
Hs-CRP	High sensitivity C-reactive protein
IDF	International diabetes federation
IFG	Impaired fasting glucose
IGR	Impaired glucose regulation
IGT	Impaired glucose tolerance
i-IFG	Isolated - impaired fasting glucose
i-IGT	Isolated-impaired glucose tolerance
IL-6	Interleukin-6
ISI	Insulin sensitivity index
LDL-c	Low density lipoprotein- cholesterol
mg/l	Milligram/liter
ml	Milliliter
mmol	Millimole
MS	Metabolic Syndrome
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa beta
NGT	Normal Glucose Tolerance
NHANES	National Health and Nutrition Examination Survey
nmol	Nanomole
OGTT	Oral glucose tolerance test
OPD	Out Patient Department
PPAR	Peroxisome proliferator-activated receptor
PPSG	Postprandial serum glucose
ROS	Reactive oxygen species
SBP	Systolic blood pressure
SD	Standard deviation
SGOT	Serum glutamate-oxaloacetate transaminase
SGPT	Serum glutamic pyruvic transaminase

SPSS	Statistical Package for Social Sciences
T Chol	Total cholesterol
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TG	Triglyceride
TNF-α	Tumor necrosis factor-alpha
TP	Total Protein
USA	United States of America
USG	Ultrasonogram
VLDL-c	Very low density lipoprotein cholesterol
WHO	World Health Organization
WHR	Waist to hip ratio
sICAM-1	Soluble intercellular adhesion molecule type 1

LIST OF SYMBOLS

Symbol	**Name**
%	Percentage
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal
$^{\circ}_{C}$	Degree centigrade
r	Spearman's correlation coefficient
ß	Regression coefficient
α	Alpha
κ	Kappa

ABSTRACT

Background: Prediabetes, inflammation and type 2 diabetes mellitus (T2DM) are believed to be associated with a worse metabolic profile in patients with nonalcoholic fatty liver disease (NAFLD). The pathophysiology involved in the development and progression of NAFLD is associated with insulin resistance and inflammation. Nevertheless, the association of subclinical inflammation and insulin resistance with NAFLD has not been studied among the Bangladeshi prediabetic subjects. ***Objectives****:* The aim of the study was to explore the proportion of prediabetic subjects with NAFLD and to investigate whether this association is mediated by suclinical chronic inflammation and insulin resistance. ***Design and Methods:*** Under a cross-sectional analytical design a total of 110 (Mean±SD age, 45±9 years) consecutive subjects were recruited. NAFLD was diagnosed on the basis of ultrasound assessment of the liver and were divided into a NAFLD group (n=48) and a non NAFLD group (n=62). All individuals underwent anthropometric and medical examinations. Among laboratory investigations, insulin secretory function (HOMA% B) and insulin sensitivity (HOMA% S) was calculated from fasting blood glucose and fasting serum insulin (pmol/l) values by Homeostasis Model Assessment (HOMA) using HOMA-CIGMA software. Fatty Liver Index, a recently identified correlate of NAFLD, was also estimated. Serum glucose was measured by glucose-oxidase mehod; lipid profile & liver enzymes were measured by enzyme colorimetric method, hs-CRP & insulin by enzyme immunoassay. ***Results:*** Forty four percent (n=48, 58.3 % of the men, 41.7 % of the women) of the study subjects had NAFLD consisting 29 (60%) IGT, 13 (27 %) IFG & 6 (13 %) IFG-IGT and 56% (n=62, 56.5 % of the men, 43.5 % of the women) of the study subjects had non NAFLD consisting 36 (58%) IGT, 21 (34%) IFG & 4 (8%) IFG-IGT. The prevalence of NAFLD was 44% among the prediabetes. Study subjects were age & BMI matched. WHR, percent body fat (%BF) and blood pressure (SBP & DBP) were significantly higher in NAFLD group compared to non NAFLD group (0.95±0.48 *vs.* 0.93±0.04, p=0.048), (32±8 *vs.* 28±6, p=0.021) and [134±34 *vs.* 112±15 & 93±26 *vs*. 76±17, (mmHg) p<0.001] respectively. Among the glycemic status, HbA_{1c} was significantly higher in NAFLD group compared to non NAFLD group [5.8±0.4 *vs.* 5.3±0.5, (%) p<0.001]. Among lipidemic profile, serum cholesterol & triglycerides were significantly higher in NAFLD group compared to non NAFLD group [198±44 *vs.* 182±38 (mg/dl), p=0.05) & (201±136 *vs.* 153±81 (mg/dl), p=0.04] whereas; HDL-c was significantly

lower [34±74 *vs.* 38±7 (mg/dl), p=0.007] in NAFLD group compared to non NAFLD group. Among liver enzymes, SGPT, SGOT & SGGT were significantly higher in NAFLD group compared to non NAFLD group [37±19 *vs.* 29±12 (IU/L), p=0.021], [36±21 *vs.* 26±8 (IU/L), p<0.001] and [34±12 *vs.* 24±11 (IU/L), p<0.001]. Among insulinemic status, postprandial insulin and HOMA-IR were significantly higher in NAFLD group compared to non NAFLD group [52 (11-170) *vs.* 35 (3-147) (μIU/ml), p=0.008] and [2.5 (0.9-6.9) *vs.* 1.9 (0.6-4.5), p=0.002] whereas; HOMA%S and HOMA%B were significantly lower in NAFLD group compared to non NAFLD group [43 (15-80) *vs.* 57 (22-164), p=0.002] and [110 (9-198) *vs.* 127 (52-198), p=0.001]. Among inflammatory status, serum hs-CRP and ESR were significantly higher in NAFLD group compared to non NAFLD group [3.7 (0.1-14.9) *vs.* 1.7 (0.2-13.2) (mg/l), p<0.001] and [25 (7-55) *vs.* 17 (5-55) (mm/hr), p=0.026]. On Spearman's correlation analysis fasting insulin, HOMA%B, & HOMA-IR showed significant positive correlation with BMI (r=0.573, p<0.001 & r=0.431, p= 0.003; r=0.544, p<0.001) & WC (r=0.353, p=0.024; r= 0.349, p=0.022 & r= 0.450, p=0.002 respectively) in NAFLD group. Whereas HOMA%B showed significant negative correlation with FBS (r=-0.367, p=0.018) in NAFLD subjects. Whereas hs-CRP showed significant positive correlation with BMI (r=0.459, p=0.003) and WC (r=0.339, p=0.035) while, it showed significant negative correlation with WHR (r=-0.334, p=0.038) in NAFLD group. Using binary logistic regression analysis, it was found that hs-CRP is a significant determinant of NAFLD [hs-CRP OR (95% CI): 1.2 (1.03-1.55), p=0.025] after adjustment of major confounders (age, BMI and sex). Moreover, in a different model HOMA-IR, HOMA%B and HOMA%S were also found to be significant determinants of NAFLD [HOMA-IR OR (95% CI): 2.44 (1.213-4.913), p=0.012], HOMA%B OR (95% CI): 0.95 (0.93-0.98), p=0.001] and [HOMA%S OR (95% CI): 0.92 (0.88-0.97), p=0.002] after adjustment of major confounders (age, BMI and sex). ***Conclusions:*** From the present data it may concluded that a high proportion (more than one-third) of the prediabetic subjects have NAFLD and the distribution of the disorder is almost similar in various subgroups of prediabetes. Subclinical chronic inflammation and insulin resistance seem to be independent mediators of the association between NAFLD and prediabetes. The data also indicate that the inflammatory condition and insulin resistance are associated with each other and those in turn are affected by central obesity and dyslipidemia in prediabetic subjects.

INTRODUCTION

An epidemic of diabetes threatens the health of large numbers of individual is developed and developing countries alike. Most of the recent growth in the prevalence of diabetes can be attributed to increases in T2DM, which now accounts for ~95% of all cases[1]. Patients with impaired glucose tolerance (IGT) and/or impaired fasting glucose (IFG) are now considered as being prediabetic, which indicates their relatively high risk for developing DM, moreover, it is associated with insulin resistance, subclinical inflammation, and cardiovascular diseases (CVDs)[2].

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of disorders characterized by predominantly macro vesicular hepatic steatosis that occur in individuals even in the absence of consumption of alcohol in amounts considered harmful to the liver. NAFLD is being increasingly recognized as a major cause of liver related morbidity and mortality. NAFLD represents a spectrum of clinical–pathological features ranging from simple steatosis, which is characterised by fatty infiltration only, to non-alcoholic steatohepatitis (NASH), which is characterised by inflammation and hepatocellular injury with or without fibrosis and cirrhosis. Most with NAFLD have an increase in liver fat content alone, which is apparently benign; others develop NASH that can progress to cirrhosis[3].

In developed countries, NAFLD is observed in 20-30% of the general population and in 75% of type 2 diabetic patients; necro-inflammatory activity and fibrosis coexist in the 2-3% of cases (nonalcoholic steatohepatitis, NASH) and may evolve in cirrhosis and liver failure in 20-25% of affected subjects[4]. Whereas, in developing countries the prevalence of NAFLD in diabetes is estimated at 34-74% and, in diabetes with obesity at virtually 100%. Currently, NAFLD is considered one of the leading causes of cryptogenetic cirrhosis[5]. Recently, an increased risk of cardiovascular disease in patients with NAFLD has been also suggested[6], based on the strong association between NAFLD and MS[7].

The pathogenesis of NAFLD is not completely understood. Among the factors thought to be involved are free fatty acid accumulation in the liver, hyperinsulinemia, inflammatory cytokines (such as tumor necrosis factor-alpha), mitochondrial damage, and free radicals that cause significant oxidative stress[8]. A mechanism by which hepatic steatosis causes insulin resistance involves an inhibition of insulin signalling at the level of the insulin receptor[9]. The central

mechanism by which insulin resistance causes hepatic steatosis appears to be via its effect on peripheral free fatty acid levels[10]. A net change in the amount of lipid in the liver will occur if there is a change in the balance between the liver's uptake or synthesis of fatty acids and the liver's disposal of fatty acids by oxidation or export[11]. Insulin resistance impairs the suppression of lipolysis, and this leads to an increased release of free fatty acids from adipose tissue so that more are delivered to and taken up by the liver[12]. This excess amount of free fatty acids can overload the hepatic mitochondrial beta oxidation system, the major pathway of fatty acid oxidation in the liver, leading to the accumulation of fatty acids in the liver[13]. However, overload of the hepatic mitochondrial beta oxidation system appears to be important. The resulting increase in oxidative processes promotes oxidative stress by leading to the generation of reactive oxygen species (ROS), free electrons and hydrogen peroxide (H_2O_2), which can directly damage mitochondrial DNA and impair mitochondrial function. ROS can also induce hepatic injury through lipid peroxidation within the hepatocytes and stimulation of cytokine synthesis, causing activation of inflammatory pathways[14].

Studies in nondiabetic subjects, in individuals with impaired glucose tolerance or impaired fasting glucose, and in patients with type 2 diabetes, as well as in the general population, have shown that markers of inflammation and proinflammatory cytokines, such as high sensitivity C-reactive protein, sialic acid, tumor necrosis factor-α (TNF-α), and interleukin-6 (IL-6), are positively correlated with measures of insulin resistance[15].

High sensitivity C-reactive protein (hs-CRP), a sensitive marker of systemic inflammation, has been shown to be increased in patients with type 2 diabetes mellitus. In addition, hs-CRP levels are elevated in individuals with features of the metabolic syndrome and with cardiovascular disease. Serum hs-CRP levels are elevated in patients with impaired glucose tolerance (IGT) or diabetes. A few prospective studies have shown that increased hs-CRP levels are an independent risk factor for future diabetes[15]. Although these findings indicate that hs-CRP levels in peripheral blood are closely associated with glucose levels, it remains unclear whether a relationship exists between hs-CRP levels and insulin resistance levels in the pre-diabetic range with NAFLD.

RATIONALE

Non-alcoholic fatty liver disease (NAFLD) is commonly associated with obesity, type 2 diabetes, dyslipidaemia, and insulin resistance. The prevalence of NAFLD in the general population has been reported as 15–30% of type 2 diabetic patients, 70–75% may have some form of NAFLD[3]. The pathogenesis of non alcoholic fatty liver disease has been closely related to insulin resistance; this disorder frequently co-exists with impaired glucose tolerance (IGT) or type 2 diabetes mellitus[12]. Subclinical inflammation is thought to be a major pathophysiologic mechanism associated with insulin resistance and this has been claimed to underlie many disorder of the metabolic syndrome family including T2DM. There is paucity of data regarding subclinical chronic inflammation and NAFLD in prediabetes. Serum levels of hs-CRP are usually elevated in obesity, dyslipidaemia and hyperglycaemia, all features of the metabolic syndrome. The relationship between hs-CRP and NAFLD is not well established[8]. Screening patients with impaired glucose regulation or type 2 diabetes mellitus for NAFLD could help the earlier diagnosis and treatment of these conditions preventing their possible complications, such as cardiovascular diseases. The association of subclinical inflammatory biomarker and insulin resistance status with NAFLD in T2DM subjects has been well studied, but that in prediabetic subjects no such prospective studies have been performed, nor have there been studies of the associations of these inflammatory markers along with insulin resistance with prediabetic subjects. In this regard, the present study has been undertaken to explore the association of hs-CRP and insulin resistance with non alcoholic fatty liver disease in patients with prediabetes.

HYPOTHESIS

A high proportion of prediabetic patients develop Non Alcoholic Fatty Liver Disease (NAFLD) and it, in turn is associated with subclinical chronic inflammation and insulin resistance.

OBJECTIVES

General Objective

The general objective of the study was to explore the association of subclinical inflammation and insulin resistance with nonalcoholic fatty liver disease in prediabetic subjects.

Specific Objectives

The specific objectives of the study were to:

- Explore the proportion of NAFLD among the prediabetic subjects;
- Determine the proportion of NAFLD in isolated IFG, isolated IGT and combined IFG-IGT groups;
- Measure serum insulin and hs-CRP level in the study groups;
- Investigate the association of hs-CRP with NAFLD in prediabetic subjects;
- Evaluate the association of insulin resistance with NAFLD and to investigate whether this association is mediated by hs-CRP in these subjects.

REVIEW OF THE LITERATURE

2.1. Concept of nonalcoholic fatty liver disease (NAFLD)

NAFLD is a clinical pathological condition representing a spectrum of histological findings from hepatic steatosis or fat accumulation in hepatocytes without inflammation, to hepatic steatosis with a necroinflammatory component that may or may not have fibrosis, or NASH. Fatty liver disease was defined as more than 5% of the hepatocytes containing fat or more than 5% of the liver weight due to fat. The term non alcoholic steatohepatitis (NASH) was developed by Ludwig in 1979 to describe an 'alcoholic–like liver disease that develops in people who do not drink alcohol. The pathological spectrum of NAFLD ranges from simple steatosis to steatohepatitis and cirrhosis. The risk factors for the development of NASH were identified as metabolic syndrome, obesity, type II diabetes mellitus (T2DM), and dyslipidemia. By the late 1990s, NASH was conceptualized as part of metabolic syndrome[16-20]. NAFLD is becoming a major public health problem due to the rising prevalence of obesity and T2DM worldwide[4,21]. NAFLD encompasses a spectrum of liver disorders characterized by macrovesicular hepatic fat accumulation alone (simple steatosis), or accompanied by signs of hepatocyte injury, mixed inflammatory cell infiltrate, and variable hepatic fibrosis in pericellular distribution (non-alcoholic steatohepatitis, NASH); NASH may lead to cirrhosis and hepatocellular carcinoma (HCC). Nonalcoholic fatty liver disease (NAFLD) is a clinical spectrum of liver abnormalities associated with obesity, a common liver disease, and also the most common cause of liver disease[22] that includes steatosis (increased liver fat without inflammation) and nonalcoholic steatohepatitis (NASH, increased liver fat with inflammation). NASH may lead to fibrosis, cirrhosis, and ultimately liver failure if it is not treated[22,23].

2.2. Prevalence of NAFLD: Globally, the incidence of NAFLD remains unknown because no prospective studies have been conducted. Further, the point prevalence of NAFLD at each of its different stages (simple steatosis, NASH, cirrhosis) remains poorly defined throughout the world[22-24,27]. The reported point prevalence of NAFLD varies widely, mainly based on the information available in a given population and the diagnostic criteria used[22-24]. With the increased prevalence of obesity, NAFLD is increasingly seen in males [24,25]. NASH may progress to cirrhosis in up to 20 percent of patients[23,26]. NASH is now recognized to be a leading cause of cryptogenic cirrhosis[26].

The prevalence depends upon the population (i.e., referral community, ethnic group) and the definition (e.g., level of aminotransferase elevation and/or ultrasonographic findings). The prevalence of NAFLD in the general population has been reported as 15–30%[29,30] of type 2 diabetic patients, 70–75% may have some form of NAFLD[31]. Recent data suggest that the presence of NAFLD in type 2 diabetes may also be linked to increased coronary artery disease (CAD) risk, independent of the risk conferred by components of the metabolic syndrome[32,33]. A high prevalence of NAFLD has been reported especially among patients with diabetes, estimated at 34–74 %[34]. However, the prevalence of NAFLD among nondiabetic individuals is also considerable, and insulin resistance is also common in these conditions[27]. In developed countries, NAFLD is observed in 20-30% of the general population[36,37] and in 75% of type 2 diabetic patients[19,38], necro-inflammatory activity and fibrosis coexist in the 2-3% of cases (nonalcoholic steatohepatitis, NASH) and may evolve in cirrhosis and liver failure in 20-25% of affected subjects[40-42].

Asia-Pacific region, the prevalence of non-alcoholic fatty liver disease (NAFLD) has been increasing over the past two decades. The risk factors are similar to those in other ethnic populations; but it is important to adopt the regional (ethnic-specific) anthropometric criteria to define overweight, obesity (including central obesity) and metabolic syndrome. To be noted, even using strict ethnic-specific criteria, a high percentage (15-21%) of Asia-Pacific NAFLD subjects in some series have been found to be non-obese, i.e. to have a normal body mass index (BMI) (17.5-22.4 kg/m^2) or to be overweight (BMI 22.5-24.9 kg/m^2)[24].

Asian population are highly predisposed to develop insulin resistance, the metabolic syndrome, T2DM and CHD; more than white Caucasians[43,44]. They have abnormal body composition consisting of high body fat and abdominal adiposity that may partially explain the high prevalence of NAFLD[43].

2.3. Pathogenesis of NAFLD: The pathogenesis of NAFLD is not completely understood. It is characterized by accumulation of triglycerides within the hepatocytes. Lipids can enter the liver either through diet, FFA, or lipogenesis. Once inside the liver, lipids can be esterified with glycerol into TG, while lipogenesis involves the conversion of precursors like glucose to FFA, where they are esterified with glycerol to produce TG. Liver TG can be broken down to FFA and exported via mitochondrial oxidation, or packaged into VLDL for export to various other tissues. In a normal liver, this TG flux is in balance so the net amount of TG stored in the liver is very

low, however in states of nutrient excess or dysfunction of one or more of these pathways, TG accumulation can occur leading to metabolic impairments[50]. Insulin resistance is thought to play an important role in the triglyceride accumulation. Excess intracellular fatty acids, oxidant stress, ATP depletion, and mitochondrial dysfunction all contribute to hepatocyte injury and inflammation followed by fibrosis. Not surprisingly, the most common laboratory abnormality in patients with NAFLD is mild to moderate elevation of serum aminotransferases. As in the histological study of diabetic patients with abnormal LFTs by Salmela *et al* (1984) elevated level of transaminase in NAFLD does not predict severity of liver histology[45]. Among the factors thought to be involved are free fatty acid accumulation in the liver, hyperinsulinemia, inflammatory cytokines (such as tumor necrosis factor-alpha), mitochondrial damage, and free radicals that cause significant oxidative stress[46,47]. NAFLD is associated with insulin resistance-related diseases, such as obesity, metabolic syndrome, atherosclerosis, and type 2 diabetes[48].

The pathogenesis of NAFLD in overweight and obese individuals is not exactly known. It appears to be related to insulin resistance. There are important clinical associations between NAFLD and elements of the metabolic syndrome, including insulin resistance, dyslipidemia, and hypertension, independent of the degree of obesity. Recent study results have shown that the presence of NAFLD indicated aggravating insulin resistance and derangement of gluconeogenetic pathways even in nondiabetic patients[49]. Other reports have proposed that NAFLD is more closely associated with insulin resistance rather than metabolic syndrome itself and precedes the manifestation of other metabolic derangements[50-51]. Although the current definition of nondiabetic status is dichotomous, that is, prediabetes (impaired fasting glucose and impaired glucose tolerance) and normoglycemia, several studies suggest that a risk gradient exists even within the normoglycemic range. Metabolic derangements were reported to worsen continuously with increasing glucose levels, even though the level of fasting glucose was within the normal range. Also, recent studies have reported a linear increase in the risks of diabetes and cardiovascular disease with increasing glucose levels within the normoglycemic range. Therefore, individuals with NAFLD should be carefully evaluated for each of these comorbidities and have counseling at national level about nutrition, physical activity, and tobacco use to help prevention of the development of cardiovascular disease and type 2 diabetes mellitus along with chronic liver disease.

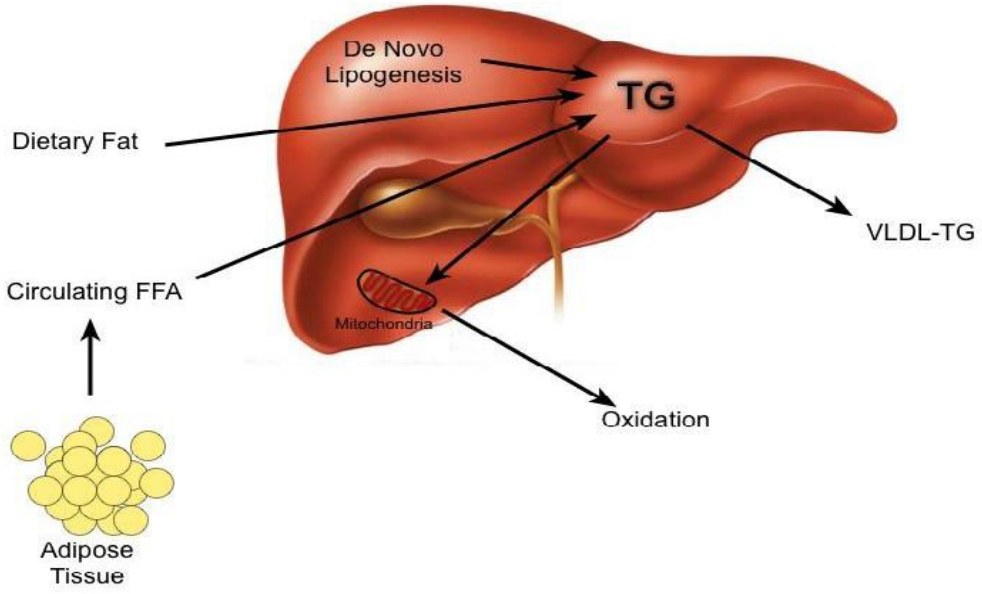

Figure 2.1: Input and output pathways of lipid to and from the liver

2.4. Natural history of NAFLD: Natural history of NAFLD is quite variable. There are some inherent draw backs in studying natural history of NAFLD[52]. Firstly there is no definitive laboratory test for diagnosis. Various studies published have different definitions. Published series using serial biopsies for histological progression have limitation of short follow up and selection bias. While cohort studies examining clinical outcomes have remarkably varied definition of NAFLD and clinical outcome may be shadowed by other diseases. Due to these conflicting results many workers considered NAFLD as a medical curiosity[51-56]. While others consider it as major cause of chronic liver disease with impending epidemic[57-61] over the last 5 years there is addition of valuable information on NAFLD. NAFLD is considered as a liver manifestation of a generalized fat storage disorder (metabolic syndrome) with an increased risk of cardiovascular events, extra hepatic malignancies as compared to liver related mortality[57-61].

There has been much interest with regards to the actual natural history of NAFLD. Current literature lack good longitudinal studies some include non-standard definitions and diagnostic methods for NAFLD and often lack controls. The long-term clinical outcome of NAFLD is still controversial; although it has been described that prognosis varies with the degree of histologic injury. Despite the limitations with sampling variability, liver biopsy remains the gold standard in NAFLD studies. Histology at time of diagnosis has been found to be the best predictor of disease progression. Benign steatosis without inflammation has a low likelihood of progression, whereas the presence of inflammation predicts progression to advanced fibrosis. Even in patients

with fibrosis without inflammation, the risk of progression to advanced fibrosis is less. Patients with any inflammation in the setting of steatosis, have 2.5 times the likelihood of developing advanced fibrosis. About 7% of NASH patients with compensated cirrhosis will develop HCC within 10 years, while 50% will require a transplant or die from liver related causes. Recently, some authors have described HCC in the non-cirrhotic fatty liver. How this might impact on disease management or surveillance is not yet known. NASH patients have a risk of increased overall mortality (compared to the general population) and increased liver related mortality (compared to patients with benign steatosis alone). Some studies have demonstrated an increased risk of cardiovascular mortality as compared to the general population. Type 2 diabetic patients with NAFLD also have been described to have higher cardiovascular morbidity than type 2 diabetics without NAFLD[62].

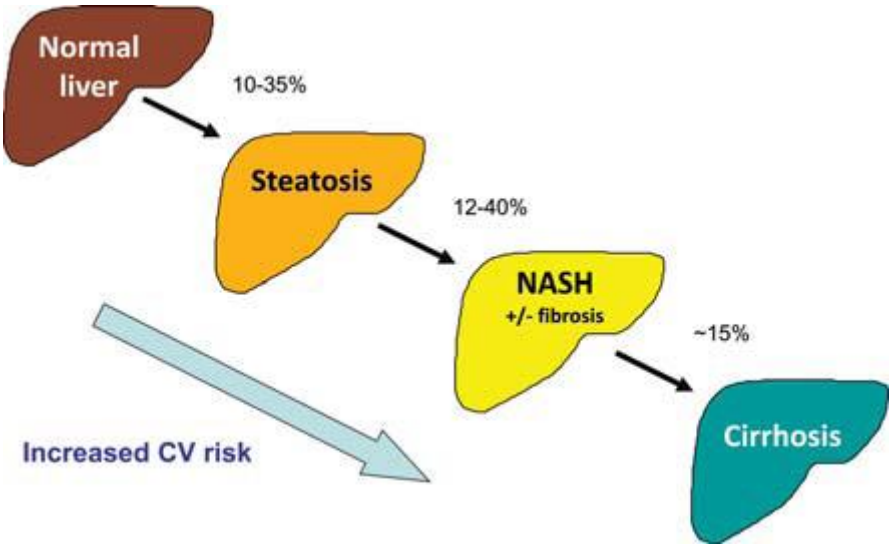

Figure 2.2: Variable progression of non-alcoholic fatty liver disease (usually over several years), with different grades of severity in each stage of simple steatosis and non-alcoholic steatohepatitis

2.5. Diagnosis of NAFLD: NAFLD is a clinical diagnosis based on the presence of transaminitis and fatty liver changes on ultrasound. The exclusion of other liver diseases, specifically alcohol-related liver disease, is a requisite criterion for diagnosis of primary NAFLD. However, NAFLD can co-exist with other liver diseases such as chronic hepatitis C and hepatitis B. The proposed criteria for the diagnosis of NASH include (i) a histologic picture of steatohepatitis; (ii) convincing evidence of minimal or no alcohol consumption; and (iii) absence of serological evidence of viral hepatitis[63].

Although histological examination remains the gold standard for the diagnosis of NASH, pathological definition is often not possible in community-based research studies and clinical practice settings[64-66]. Alternatively, operational definitions of NAFLD have been proposed in which biochemical criteria and hepatic imaging (ultrasonography, computed tomography, and magnetic resonance imaging) are used[68-71]. Among them, ultrasonographic definition of steatosis has most frequently been used in epidemiological research studies[67-69] and this approach has been endorsed by Asia-Pacific regional guidelines[70]. Diagnosis of fatty liver by ultrasonography is defined by the presence of at least two of three abnormal findings: diffusely increased echogenicity ('bright') liver – with liver echogenicity greater than kidney or spleen, vascular blurring, and deep attenuation of ultrasound signal[67]. In addition, other liver diseases should be excluded[67-70]. Imaging may confirm the presence of fatty liver, indicated by increased echogenicity. However, the severity of liver involvement does not correlate with radiographic features, clinical features, or the degree of elevation of liver transaminases. Asian population as a race is a risk factor for diabetes mellitus and obesity because increase in incidence of NASH in this ethnic group.

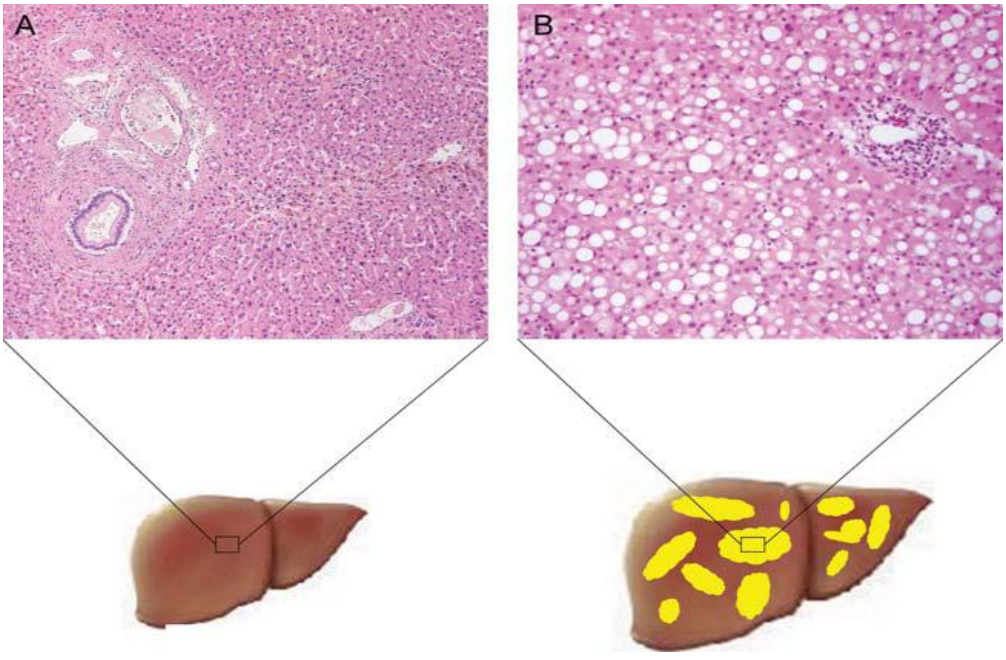

Figure 2.3: (A) Histological section of normal liver tissue compared with (B) simple steatosis, showing fat accumulation in hepatocytes

2.5.1. Laboratory abnormalities in NAFLD: Most patients with NAFLD have abnormal aminotransferases with elevated ALT and AST. The degree of transaminitis is often mild and is usually within 1–4 times the upper limit of normal, with ALT higher than AST. However, degree of ALT elevation does not correlate with histological severity of steatosis or fibrosis. A large proportion of NAFLD patients have normal liver enzymes, and a fraction of these patients may have significant NASH-related fibrosis despite normal ALT levels. Alkaline phosphatase (ALP) levels may also be mildly raised in NAFLD, up to twice the upper limit of normal. Similarly gamma glutamyltransferase (GGT) levels may be raised, although there is little data on the frequency and significance of GGT elevation in NAFLD. Bilirubin, albumin and prothrombin time are usually not affected in fatty liver disease until cirrhosis and liver failure develop. In patients without prior known type 2 diabetes mellitus, the presence of glucose intolerance and insulin resistance should be evaluated with fasting blood glucose, insulin levels and HbA_{1c}. Thirty to 50% of patients with NASH are likely to have either diabetes or glucose intolerance. Fasting lipid profiles shows the presence of coexisting hypertriglyceridemia and/or elevated low-density lipoprotein (LDL) levels in 20–80% of NAFLD patients. Elevated serum auto-antibodies are elevated in 10–25% of patients with NAFLD. Low titre (<1:160) antinuclear antibody (ANA) positivity has been documented in up to 33% of NAFLD patients[71].

2.5.2. Liver enzyme & development of diabetes

The liver, a major site of insulin clearance, plays an important role in maintaining normal glucose concentrations during fasting and postprandially[72]. Recently, several cohort studies have shown that serum GGT[72,73-77], ALT[78-80] and AST[81] levels are predictors of diabetes. In one of these reports, a study on Pima Indians[79] found that high serum ALT levels were a significant risk factor for diabetes, although no clear association between serum GGT and diabetes was seen. On the other hand, serum GGT levels, but not AST levels, have been identified as an independet predictor of incident diabetes in British men selected from lists of general practitioners[73]. Moreover, the Mexico City Diabetes Study found that serum AST is an independent risk factor for future diabetes in multivariable adjustment, whereas no association was observed between serum GGT or ALT and the development of diabetes[81]. Furthermore, it also remains unknown whether liver enzyme markers are stronger predictors of future diabetes than well-known risk factors for diabetes, such as adiposity, insulin resistance, and inflammation.

2.5.3. Clinical manifestations of NAFLD

Fatty liver is the accumulation of fat (i.e., macrovesicular steatosis) within the hepatic parenchyma. NAFLD, the presence of fat infiltration in the liver in the absence of excessive alcohol consumption and other causes of liver disease, is the most common cause of fatty liver, with a prevalence as high as 30% in many populations[82]. NAFLD may lead to fibrosis[83], cirrhosis[84], liver cancer[85,86], liver failure requiring liver transplant[87], and mortality[88], and it is associated with type 2 diabetes, metabolic syndrome, and other cardiovascular risk factors[89]. Although NAFLD represents a major public health challenge, its natural history and determinants are incompletely understood because of limitations in diagnostic technologies and because this condition is often asymptomatic until very late, severe complications occur. In addition, because of the risk of progression to more advanced stages, early noninvasive detection of fatty liver disease is clinically important.

NAFLD is a silent disease more than half of the patients are diagnosed accidentally without any symptoms. Majority of the patients have normal physical examination. 25-50% may have clinically identifiable hepatomegaly. Mild to modest ALT, AST elevation is found in 25-50% of patients, remaining patients have normal liver enzymes[91-99].

Fatty Liver can be defined on ultrasonography characteristics as given below in day to day clinical practice[100,101]

Ultrasonographic criteria:

- Presence of 2 of the following 3 with or without elevated ALT
- A) Bright hepatic echo texture as compared to kidney and spleen;
- B) Blurring of hepatic veins;
- C) Loss of deep echo- discontinuous diaphragm;

Magnetic resonance spectroscopy and liver histology may be more accurate than ultrasound but their utility in daily clinical practice remains unclear.

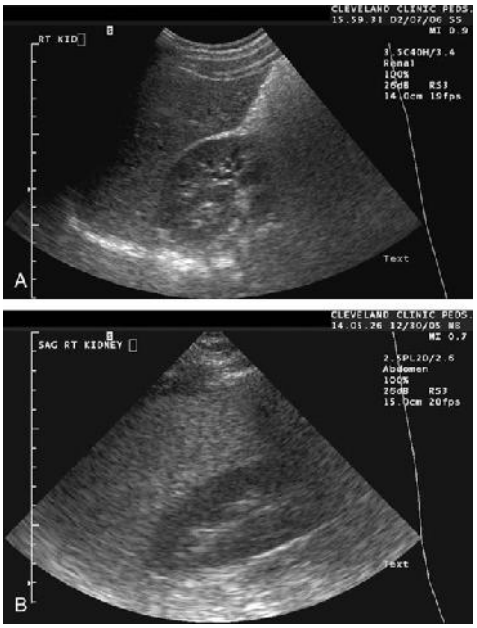

Figure 2.4: Sonographic features in fatty liver. A, Normal liver: same echogenicity as the kidney. B, Fatty liver: increased echogenicity compared with the kidney

2.6. Management of NAFLD

All patients diagnosed to have NAFLD after evaluation should be treated for abnormalities if present e.g. diabetes, and dyslipidemia and glucose intolerance. In case of doubt of severity of liver disease, patients should undergo liver biopsy. In the absence of diabetes and dyslipidemia but presence of NASH on histology, with abnormal glucose tolerance tests or presence of insulin resistance should be considered for treatment with insulin sensitizers. Treatment modalities are still evolving and no drug has been proved useful in the treatment of NAFLD in the absence of predisposing conditions. Currently life style modifications including dietary restrictions and exercise should be recommended as cornerstone of the therapy. The general recommendations for the diet are individualized to achieve energy deficit of 500 to 1000 kcal per day depending on the patients BMI, reduced saturated fat and total fat less than 30% of the total energy intake, reduced refined sugars and increase soluble fiber intake. Physical activities recommended 60 minutes per day at least 3 days a week and progressively increase the exercise to five times a week. Pharmacological and surgical methods of weight loss should be used in morbidly obese patients or moderately obese patients with significant risk factors[102-104].

2.7. Inflammation

Inflammatory processes are now recognized to play a central role in the pathogenesis of atherosclerosis and its complications[105]. High sensitivity C-reactive protein (hs-CRP) is one of the most sensitive markers of systemic inflammation and synthesized by the liver in response to cytokines[106]. Chronic, low-level inflammation is an important factor in the initiation and progression of atherosclerosis[107-109]. Recent studies have shown that hs-CRP is strongly associated with various components of the metabolic syndrome, and especially with measures of obesity[110,111,113-115]. A growing body of evidence implicates adipose tissue in general and visceral adiposity in particular, as key regulators of inflammation, coagulation, and fibrinolysis. Proinflammatory cytokines such as tumour necrosis factor-alpha (TNF- α) and interleukin-6 (IL-6) are produced in adipose tissue[114,116] and are considered to be an important source of basal production of IL-6, the chief stimulator of the production of hs-CRP in the liver. Although hyperglycaemic milieu can potentially promote production of inflammatory mediators, the relation between glycaemic status and markers of subclinical inflammation is controversial[111,112,114] and few studies have directly examined the association between fasting plasma glucose and plasma concentrations of hs-CRP.

2.7.1. Studies evaluating the association of NAFLD with inflammation

NAFLD plays a central role in the pathway which connects the metabolic syndrome, obesity and inflammation. The possible mechanistic pathways include increased oxidative stress, subclinical inflammation, an adipocytokine profile, endothelial dysfunction and lipid abnormalities. Recent evidence suggests that the severity of the liver histology in the NAFLD patients is closely associated with the markers of early atherosclerosis and the components of the metabolic syndrome. Abdominal obesity, type 2 diabetes, insulin resistance, hypertension and dyslipidaemia–the typical components of the metabolic syndrome are the co-existing pathological conditions which are frequently associated with NAFLD and their co-existence within the same individual increases the likelihood of having more advanced forms of NAFLD[119-121]. Park *et al* (2005) conducted a study on NAFLD and showed that the hs-CRP, TNF-α and IL-6 concentrations were higher in the obese than in the non-obese individuals. Adipose tissue is an important source of cytokines, and adiposity contributes to the proinflammatory milieu[15]. Lipid accumulation occurs primarily in hepatocytes, while inflammation occurs as a result of cytokine release from kupffer cells and adipose tissue. Inflammation leads to hepatic stellate cell activation and the induction of fibrosis. TNF-α has been considered to be a key player in the progression from simple fatty liver to NASH. TNF-α is produced by macrophages in the adipose tissue and it is increased in obesity. Free fatty acids can induce the expression of TNF-α in hepatocytes through the activation of NF-κB, thereby linking the increased influx of free fatty acids which are seen in hepatic steatosis, to the progression of inflammation. In adipocytes, TNF-α down regulates the adiponectin production. hs-CRP, synthesized in hepatocytes, is an acute-phase reactant that increases nonspecifically in bacterial infections, immuno-inflammatory diseases and malignant disorders. Obesity, particularly abdominal adiposity, is characterised by low-grade systemic inflammation. In prospective studies, high hs-CRP levels have been shown to predict the metabolic syndrome[123,124], T2DM[125] and coronary heart disease (CHD)[126]. Increased hs- CRP levels have been shown to correlate with generalised and abdominal adiposity in Asian population[127,128]. Interestingly, systemic sub-clinical inflammation could be contributed by hepatic inflammation as well as from visceral adipose tissue. Recent data also show that hs-CRP is a biomarker for NAFLD in some ethnic groups (Japanese), while no association has been shown by others (Europeans)[129].

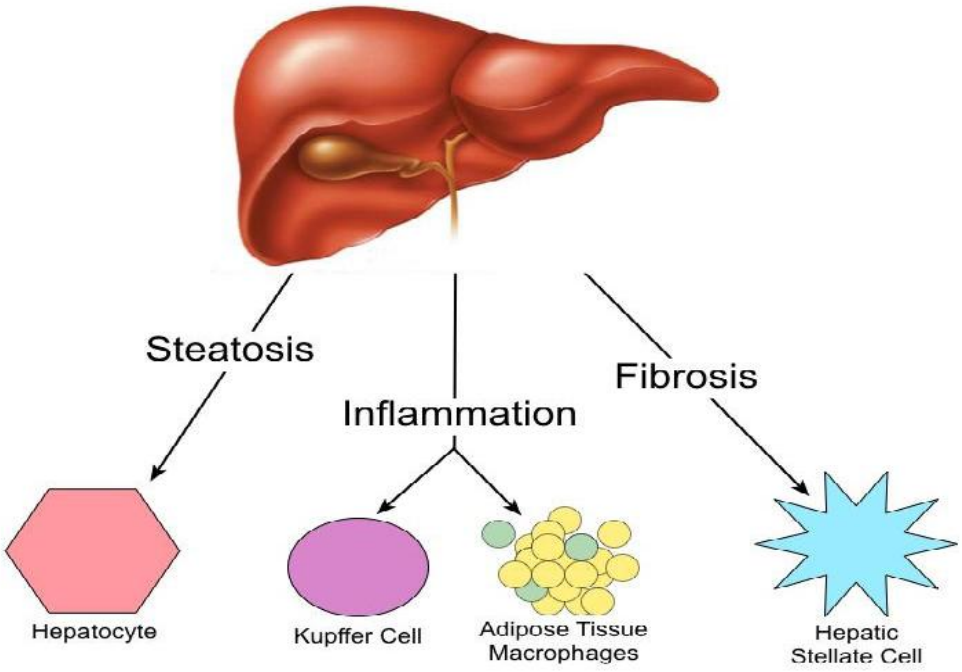

Figure 2.5: Liver cell types involved in the progression of NAFLD

2.8. Prediabetes

Pre-diabetes is a condition in which blood glucose levels are higher than normal but not high enough for a diagnosis of diabetes. This condition is sometimes called impaired fasting glucose (IFG) or impaired glucose tolerance (IGT). IFG and IGT indicate a metabolic stage intermediate between normal glucose homeostasis and diabetes. Historically the term IGT was first introduced by the National Diabetes Data Group in 1979 and later, the same term was adopted by World Health Organization (WHO). In 1997, the ADA published report mentioned IFG as a new category, which was also adopted in 1999 WHO report. Patients with IFG and/or IGT are now referred to as having 'prediabetes' by ADA or 'impaired glucose regulation' (IGR) by WHO. These categories are a part of the natural history of diabetes and not a type of diabetes. They are not interchangeable and represent different abnormalities of glucose regulation, one in fasting state and one postprandial (WHO, 1999). According to fasting and post load glucose concentration, at present patients with IGR or prediabetes may be stratified into three subcategories -1) Isolated IGT; 2) Isolated IFG; and 3) Combination of the two 'IFG-IGT' (ADA, 2005).

2.8.1. Impaired fasting glucose (IFG)

The WHO criterion for impaired fasting glucose differs from the ADA criteria because the normal range of glucose is defined differently. WHO keep the upper limit of normal at under 6.1 mmol/l (110 mg/dL), whereas the ADA lowered the upper limit of normal to a fasting glucose under 5.6 (100 mg/dL). WHO and ADA criteria for diagnosing as IFG is as follows:

- WHO criteria: fasting plasma glucose level from 6.1 mmol/l (110 mg/dL) to 6.9 mmol/l (125 mg/dL) (WHO, 2006)[134]
- ADA criteria: fasting plasma glucose level from 5.6 mmol/l (100 mg/dL) to 6.9 mmol/l (125 mg/dL). (ADA, 2005)[133]

Epidemiological studies suggest that subjects with impaired fasting glucose (IFG) have lower insulin sensitivity and higher insulin secretion[135]. IFG is associated with insulin resistance and increased risk of cardiovascular pathology, although of lesser risk than impaired glucose tolerance (IGT). IFG sometimes progresses to type 2 diabetes mellitus. There is a 50% risk over 10 years of progressing to overt diabetes. IFG is also a risk factor for mortality [136].

2.8.2. Impaired glucose tolerance (IGT)

IGT is defined as fasting plasma glucose <6.1 mmol/l and 2h plasma glucose between 7.8 and 11.0 mmol/l (ADA, 2005). In this stage blood glucose values are higher than the defined normal levels but not high enough to meet the diagnostic criteria for diabetes. IGT is associated with insulin resistance and increased risk of cardiovascular pathology. IGT may precede type 2 diabetes mellitus by many years. It is also a risk factor for mortality[137]. An earlier prospective study in Pima Indians, in which insulin action and insulin secretion were estimated from fasting and post challenge plasma insulin concentrations during an OGTT, suggested that insulin resistance might play a predominant role in the development of IGT, whereas insulin secretory dysfunction might be the major factor determining whether individuals with IGT progress to diabetes[138] Studies in other populations, however, found that a low early-phase insulin secretion predicted the transition from NGT to IGT[138] and that insulin resistance predicted the progression from IGT to diabetes[139,140].

2.8.3. Combined IFG-IGT

Studies involving Bangladeshi as well as other population demonstrated some subjects have feature of both IFG and IGT, they are termed as combined IFG-IGT. They have fasting plasma

glucose 6.1- 6.9 mmol/l and 2h plasma glucose 7.8 - 11.0 mmol/l (ADA, 2005). In one particular study in Denmark the progression of IFG-IGT to diabetes found to be 28% per year[141]. IFG and IGT are asymptomatic and predict future diabetes or cardiovascular diseases[142]. The main features of IFG/IGT are: i) a stage in the natural history of disordered glucose metabolism, ii) can lead to any type of diabetes, iii) increased risk of progression to diabetes, iv) increased risk of cardiovascular diseases, v) little or no risk of micro vascular diseases, and vi) some patient may revert to normoglycemic[143,144].

2.8.4. Prevalence of prediabetes

The prevalence of IFG and IGT increases with age[145]. The prevalence of IFG tends to plateau in middle age whereas the prevalence of IGT rises into old age[144]. IGT is more prevalent than IFG, less than or equal to 50% of people with IFG has IGT and 20-30% with IGT also has IFG. The rising prevalence of IGT is assumed to increase from 8.2 to 9.0% worldwide and 7.1 to 7.8% in Bangladesh from 2003 to 2005 in adults (20-79 yrs age groups). The prevalence of IFG found to be similar in men and women, but IGT is more frequent in women. From DECODA (Diabetes Epidemiology: Collaborative analysis of Diagnostic criteria in Asia) study; it was found that IGT was more prevalent than IFG in all Asian populations studied for all age-groups[145]. A recent study in rural Bangladeshi population, the prevalence of IFG, IGT and newly detected T2DM were found 1.3%, 2% and 7% respectively. IFG, IGT and combined IFG-IGT were more prevalent in females than males[147]. The rising prevalence rate of IGT may be mainly due to diabetogenic lifestyle factors that lead to obesity and increasing life expectancy. Interestingly, there is a tendency for the prevalence rates of IGT to decline as those of diabetes rise, perhaps suggesting that areas with a high ratio of IGT: diabetes are at an earlier stage of the diabetes epidemic and thus may be a particular target for preventive strategies.

2.8.5. Pathogenesis of prediabetes

Progression to overt diabetes from a pre-diabetic state occurs gradually over a period of many years and is characterized by worsening insulin resistance and insulin secretory dysfunction and gradual increases in fasting and prandial plasma glucose concentrations[148,149]. Even though IFG and IGT represent intermediate stages of glucose intolerance, epidemiological studies demonstrated that they are likely to be distinct conditions with different pathophysiological etiologies[150]. Individuals with isolated IFG manifest mainly of hepatic insulin resistance, but

have relatively normal skeletal muscle insulin sensitivity. In contrast, those with isolated IGT are characterized by more severe muscle insulin resistance and less severe hepatic insulin resistance. Differences in insulin secretory abnormalities are also apparent between subjects with isolated IFG and isolated IGT. Whereas those with isolated IFG have defects in first-phase or early insulin secretion, individuals with isolated IGT have more severe defects in second-phase or late insulin secretion. As might be expected, individuals with combined IFG-IGT manifest both hepatic and muscle insulin resistance as well as impairments in both first and second phase insulin secretion. Among subjects with pre-diabetes, those with combined IFG-IGT most closely resemble subjects with type 2 diabetes[148,150]. Similar findings were seen in many studies showing IFG associated with more β cell failure[148] and IGT with predominant insulin resistance and features of insulin resistance. Some studies found that both IFG and IGT have similar impairment of insulin action[148,151]. But other studies claimed opposite ideas that subjects with IFG had more insulin resistance and features of insulin resistance and those with IGT more defective insulin secretion in early and late phase[135].

2.8.6. Insulin resistance

Insulin resistance refers to the impairment of the physiological action of insulin i.e. blunting of insulin's action at circulating concentrations that are normally effective. Insulin resistance in fat cells results in hydrolysis of stored triglyceride, which elevates free fatty acids in the blood plasma. Insulin resistance in muscle reduces glucose uptake, whereas insulin resistance in liver reduces glucose storage, with both effects serving to elevate blood glucose. High plasma levels of insulin and glucose due to insulin resistance often lead to metabolic syndrome and T2DM. It was established that insulin resistance is an early feature in the natural history of type 2 diabetes. Defects in the following three main steps are involved in the generation of insulin resistance- a) insulin binding to the cell membrane receptor b) insulin receptor phosphorylation and c) intracellular insulin signaling.

The primary insulin responsive tissues include liver, muscle, and fat. In the liver, insulin controls hepatic glucose production, thereby preventing unnecessary elevations in fasting plasma glucose levels, and similarly, after a meal when glucose is absorbed from the gastrointestinal tract, insulin inhibits endogenous (hepatic) glucose production[152]. In fat cells, whereas glucose uptake is under the control of insulin via the GLUT4 mobilization to the plasma membrane[153,154], the major effect of insulin is to inhibit lipolysis[155]. Finally, muscle is the major organ responsible for

insulin-induced glucose uptake and accounts for 80% of whole-body glucose disposal after the meal by facilitated glucose transport, again via GLUT4[154]. All of the above events are initiated by the interaction of insulin with the cell-surface insulin receptors followed by a varied number of postreceptor signaling cascades, eventuating in the appropriate biological responses. One of the major common phenomena seen in obesity, the metabolic syndrome, and type 2 diabetes is insulin resistance[156], with insulin unable to regulate hepatic glucose production, lipolysis in fat cells, and whole-body (muscle) glucose disposal.

2.8.7. Insulin resistance and the development of NAFLD

Several studies have highlighted that insulin resistance is a characteristic feature of NAFLD[157-160], even when subjects are not obese[161]. However, NAFLD per se cannot be considered a cause for insulin resistance but rather a consequence as shown by studies in subjects genetically predisposed to NAFLD. On the other hand NAFLD is highly prevalent among patients with type 2 diabetes (up to 70%) that show increased hepatic triglyceride accumulation independently of BMI[157]. Insulin resistant subjects with NAFLD show reduced insulin sensitivity not only at the level of the muscle but also at the level of the liver and adipose tissue[157,161,162]. In insulin-resistant conditions, the adipose tissue becomes resistant to the antilipolytic effect of insulin and the release of fatty acids is increased[163]. Insulin resistance is accompanied by increased insulin levels that, in the presence of increased lipolysis and/or increased fat intake, promote hepatic triglyceride synthesis[157]. Adipose tissue insulin resistance is quantified using the index Adipo-IR (FFA × INS)[157,164] that reflects the inability of insulin to suppress peripheral lipolysis. In subjects with NAFLD, even if not obese, FFA concentrations and Adipo-IR are increased compared to control subjects[161,165], despite an increase in both hepatic and systemic lipid oxidation[161] and in VLDL-TG secretion[166,167]. Adipo-IR is also a marker of hepatic liver injury[162].

Under postprandial conditions, an important source of FFA is due to the increased spillover from chylomicrons[168]. The increased spillover reflects the inefficiency in dietary fat storage and results in excess FFA. FFA are taken up by organs saturating their oxidative capacity[161] and accumulated as ectopic fat, mainly as intramyocellular and hepatic lipids but also as cardiac and pancreatic fat. It has been hypothesized that ectopic fat could be a defense mechanism against lipotoxicity[169,170] and that subjects with NAFLD develop NASH and cirrhosis only in consequence of a second hit due to increased inflammation and reactive oxygen species[171].

2.8.8 Prediabetes and inflammation

Pre-diabetes identifies subjects with impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) at high risk for type 2 diabetes; moreover, it is associated with insulin resistance, subclinical inflammation, and cardiovascular diseases (CVDs)[172-175]. According to recent ADA guidelines[177], prediabetes is an earlier stage in the hyperglycaemic continuum that is associated with increased future risk of developing diabetes and CVD[178,179]. However, studies examining the association between hs-CRP and prediabetes among participants without diabetes are limited[176,180,181]. Few studies have shown an association between hs-CRP and prediabetes among specific population groups, including older black and white participants in the USA, middle-aged Japanese[181] and clinical patients in China[180]. The prevalence of diabetes is increasing in epidemic proportions among Asians[182,177] and it has also been shown that Asians have lower levels of hs-CRP than Western populations[183,184].

2.9. Studies evaluating the association of NAFLD with hs-C-reactive protein and insulin resistance

Insulin resistance is associated with an increase of free fatty acids (FFAs) flux that contributes to increased TG production that, in turn, stimulate assembly and secretion of VLDL in hepatocytes. Fat accumulation in the liver is associated with oxidative stress and lipid peroxidation. Furthermore NAFLD subjects have increased secretion of inflammatory markers, plasma glucose and a decrease in HDL concentration. The consequence of this physiological dysfunction is increased risk for the development of diabetes and atherosclerosis and increased risk for coronary artery disease. hs-CRP (an acute phase protein) has been thought to be synthesized in the liver, with a plasma half-life of 18 hours. However, the extrahepatic expression of hs-CRP has been detected in macrophages and smooth-muscle cells from atherosclerotic plaques[185]. It plays an important role in the inflammatory process and is recognised as a useful biochemical marker of inflammation. Increasing epidemiological evidence supports the notion that low-grade inflammation, as reflected by elevated levels of hs-CRP, is associated with glucose intolerance[186] and various vascular diseases including atherosclerosis, stroke, ischaemic heart disease, and peripheral vascular disease. There is evidence for the presence of hs-CRP in human adipose tissue and growing evidence that adipose tissue can induce chronic low-grade inflammation by producing proinflammatory cytokines such as interleukin-6[187]. A certain degree of inflammatory process in subjects with NAFLD could be suspected, but data examining the direct association

between hs-CRP and is sparse. This study aimed to evaluate whether higher low-grade systemic inflammation as measured by serum hs-CRP level and insulin resistance are associated with NAFLD using data from Bangladeshi prediabetes.

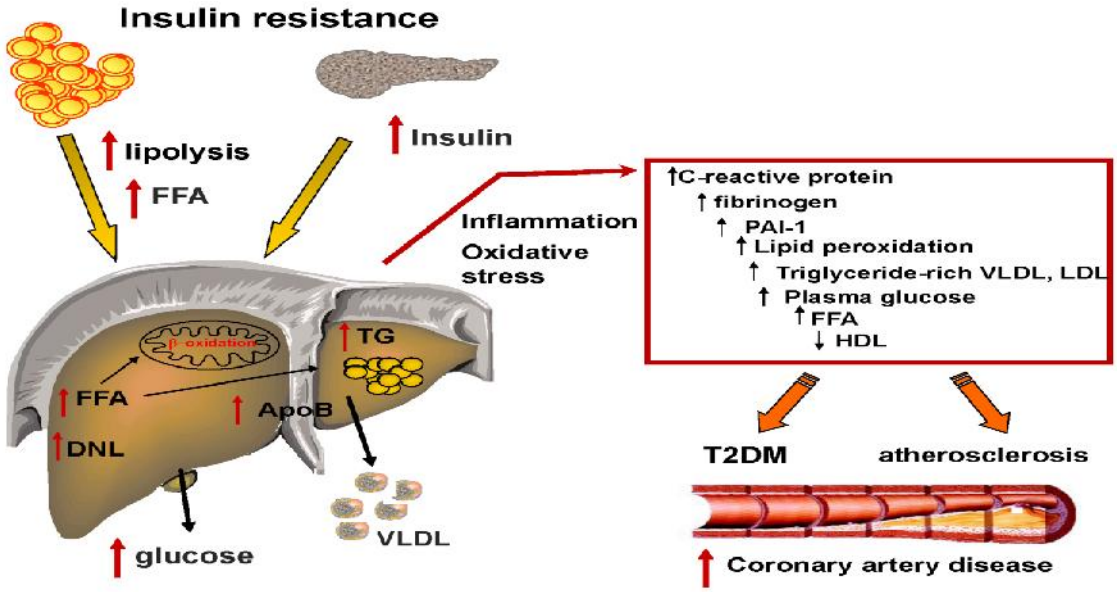

Figure 2.6: Link between insulin resistance and inflammation

SUBJECTS AND METHODS

3.1. Study design

3.1.1. Study type

This was an observational analytic study with a cross-sectional design.

3.1.2. Place of study

The study was conducted in the Department of Biochemistry & Cell Biology of Biomedical Research Group (BMRG), Research Division, Bangladesh Institute of Research and Rehabilitation in Diabetes, Endocrine and Metabolic Disorders (BIRDEM) and Bangladesh University of Health Sciences (BUHS), Dhaka, Bangladesh.

3.1.3. Duration of study

This study was conducted during the period of January 2013 - October 2013.

3.1.4. Sample size calculation

Minimum number of samples for study was calculated as 90 considering the regression analyses as statistical tools using the following formula (Appendix III):

N≥50+8m (N=Sample size; m=number of predictors)

N≥90

3.1.5. Study subjects

A total number of 150 (one hundred and fifty) subjects were purposively recruited in the study irrespective of race, religion and socioeconomic status. Diabetes and prediabetes were diagnosed following WHO criteria (WHO, 2006). Of the total, upper abdomen ultrasonogram had done on 110 (one hundred and ten) subjects of which 62 subjects were non NAFLD and 48 were NAFLD.

Table 3.1: WHO Diabetes criteria (2006)[134]

Condition	Fasting glucose mmol/l	2 hour glucose mmol/l
Normal	<6.1	<7.8
Impaired fasting glycaemia (IFG)	≥ 6.1− ≤6.9	<7.8
Impaired glucose tolerance (IGT)	<7.0	≥7.8− ≤11.0
Diabetes mellitus	≥7.0	≥11.1

The test was performed using a glucose load containing the equivalent of 75g anhydrous glucose dissolved in water. Informed written consent was taken from all participants. Subjects were collected from OPD of BIRDEM/BUHS by standard criteria. Physical examination for anthropometric measurements including BMI, WHR, of each subject

was done by appropriate technique. Serum was processed and stored by standard methods. An ultrasound examination was performed to identify NAFLD. All tests was performed with a single probe by one experienced radiologist, thus minimizing inter- and intra observer variability. This method has a sensitivity and specificity of 89% and 93%, respectively[188].

3.2. Recruitment of the subjects

Subjects were collected from the Out-Patient Department (OPD) of BIRDEM/BUHS who came for checking their glycemic status. After taking brief history, preliminary selection was done, and the purpose of the study was explained in details to each subject and their verbal consent was taken. They were advised to take unrestricted carbohydrate diet, to do normal physical activities and to avoid drugs that significantly interfere with blood glucose level (like glucocorticoids, oral contraceptives containing levonorgestrel or high-dose estrogen, phenytoin, high-dose thiazide diuretics, etc.) for 3 days. They were also advised to abandon the program if they became sick. Then they were requested to report to BMRG-BIRDEM/BUHS after 3 days at morning between 8.00-9.00 am following an overnight (8-14 hours) fasting. When the subjects reported, informed written consent was taken. A predesigned case record form (Appendix II) was used to record relevant clinical, medical, demographic, socio-economic data such as age, sex, educational status and occupational status from the consenting subjects. Physical examination for anthropometric measurements of each subject was done on the very first day of the visit.

3.3. Selection criteria

Respondents were included in the study if fulfill the following inclusion and/ or exclusion criteria:

3.3.1. Inclusion criteria

Equal number (n=50) of non diabetic, healthy, age, sex, and BMI matched prediabetic subjects was selected without family history of diabetes. Study subjects also have to be without pregnancy and any systemic illness.

3.3.2. Exclusion criteria

Subjects suffering from any systemic illness like acute severe septic conditions, acute and chronic cardiac disease, hepatic, renal, acute and chronic respiratory failure, stroke and type 1 diabetes. No recent change (≥10%) in body weight; No current medication and pregnant subjects was excluded from the study.

3.4. Ethical implications

Ethical aspect and procedure for maintaining confidentiality

1. Ethical clearance for the study was taken from the concerned departments from where we will collect out study subjects.
2. The entire study subject was thoroughly appraised about the nature, purpose and implications of the study as well as entire spectrum of benefits and risks of the study.
3. Subjects were assured about their confidentiality and freedom to withdraw them from the study any time.
4. Written consent of all the study subjects was taken free of duress and without exploiting any weakness of the subject.
5. There is minimum physical, psychological, social and legal risk during collection of blood, Blood pressure measurement & physical examinations and these was done after taking informed consent and proper safety method.
6. For safeguarding confidentiality and protecting anonymity each of the patients was given a special ID number which was followed in each and every step of the procedure.
7. A signed informed consent was taken from the patient after explaining her about the nature, objective, procedure, risks and benefits and implications of the study.
8. No drugs-experimental new drug or placebo was used here.

Ethical approval for this research protocol was obtained from the Ethical Review Committee of Bangladesh Diabetic Association (BADAS). *Ref no: BADAS-ERC/13/00106*

3.5. Anthropometric measurements

3.5.1. Height (m)

Standing height was measured using appropriate scales (Detect-Medic, Detect scales INC, USA) without shoes. The patient was positioned fully erect, with the head in the Frankfurt plane (with the line connecting the outer canthus of the eyes and the external auditory meatus perpendicular to the long axis of the trunk); the back of the head, thoracic spine, buttocks, and heels touched the vertical axis of the anthrop meter and the heels were together. Height was recorded to the nearest 5 mm.

3.5.2. Weight (kg)

The balance was placed on a hard flat surface and checked for zero balance before measurement. The subjects were in the center of the platform wearing light cloths without shoes. Weight was recorded to the nearest 0.5 Kg.

3.5.3. Calculation of BMI (Kg/m^2)

Body mass index (BMI) of the subjects was calculated using following formula:

BMI = Weight (kg)/ Height (m^2)

3.5.4. Waist circumference (cm)

Waist circumference was measured to the nearest 0.5 cm with a soft non-elastic measuring tape. The tape was snug, but not so tight as to cause skin indentation or pinching. The waist circumference was taken to the nearest standing horizontal circumference between the lower border of the 12th rib and the highest point of the iliac crest on the mid-axillary line at the end of normal expiration.

3.5.5. Hip circumference (cm)

Hip circumference was measured on the maximum circumference over the buttocks using soft non-elastic measuring tape and reading was taken to the nearest 0.5 cm. Participants were asked to breath normally, the reading were taken after gentle exhaling. The measuring tape was held firmly, ensuring its horizontal position. The tape was loose enough to allow the observer to place one figure between the tape and subject's body.

3.5.6. Calculation of WHR

Waist to hip ratio (WHR) of the study subjects was calculated as the ratio of waist circumference divided by hip circumference.

3.5.7. Body fat mass (%)

Body fat mass was measured by Omron Body Fat Monitor. Height in cm, weight in kg, age in yrs and sex of patients were set to the monitor. Then the patient held the monitor by both hands with upper limbs horizontal in standing position. The machine was then put on and body fat mass (%) was recorded from the monitor.

3.5.8. Measurement of blood pressure

Blood pressure was measured using Barometric Sphygmomanometer. Standard protocol was followed to record blood pressure data. Blood pressure was measured in sitting position, with calf at the level of the heart. After 10 minutes of rest a second reading was taken and average was recorded. Recorded Korotkoff sound I (the first sound) and V (the

disappearance of sound) denoted the systolic blood pressure (SBP) and diastolic blood pressure (DBP), respectively (according to WHO-IHS).

3.6. Laboratory methods

3.6.1. Sample collection and storage

After overnight fasting (8-14 hours) blood was collected between 8.00-9.00 am. Venous blood (~6 ml) was obtained by venipuncture following standard procedure. Subjects were then allowed to drink glucose (35 g in 300 ml of water). They were requested not to take any food and be rested for two hours. After 2 hours of glucose intake the second blood sample (3.00 ml venous blood) was taken. Fasting and postprandial blood samples were taken into plain tube (~6 cc), allowed to clot for 30 minutes and serum was separated by centrifugation for 10 min at 3000 rpm and then the serum was collected at least 600 µl in each four aliquot. Blood samples were maintained at 4^0c until separation and serum was frozen at -30^0c within an hour of sample collection. One aliquot was used for measuring OGTT, lipid profile, liver enzymes, creatinine, second aliquot for Insulin / C-peptide and the third aliquot for inflammatory marker (hs-CRP) measurement respectively. The remaining aliquot was frozen at -30^0 C for further measurement. Serum was not allowed to be thawed until the assay is performed.

3.6.2. Calculation of B-cell function and insulin resistance

Insulin secretory function (as assessed by insulin secretion HOMA% B) and insulin resistance (as assessed by insulin sensitivity HOMA% S) was calculated from fasting blood glucose and fasting serum insulin (pmol/l) values by Homeostasis Model Assessment (HOMA) using HOMA-CIGMA software.

3.6.3. Body fat analysis

Total body fat was determined by bioimpedometry.

3.6.4. Fatty liver index and insulin sensitivity index calculation

Fatty liver index (FLI) and insulin sensitivity index ($ISI_{Matsuda}$) was calculated by previous reported formula[242,245].

3.6.5. Analytical methods

- Serum glucose was measured by glucose-oxidase method (Randox, UK).
- HbA_1c was measured by HPLC method.

- Serum lipid profile (Total cholesterol, TG, and HDL-c) and clinical enzymes like liver enzymes (SGOT, SGPT, GGT and ALP) was measured by enzymatic-colorimetric method (Randox, UK).
- Serum insulin & hs-CRP were determined by an ELISA method (DRG-International, Germany)
- Insulin secretory function (HOMA%B) and insulin sensitivity (HOMA%S) was calculated by Homeostasis Model Assessment (HOMA) using HOMA software and
- NAFLD was diagnosed by ultrasound.

3.7. Data analysis

We grouped the prediabetes subjects (IFG, IGT & combined IFG-IGT) into alcoholic and non alcoholic fatty liver disease of which 59% (n=65) subjects was IGT, 31% (n=34) was IFG and 10% (n=11) was IFG-IGT. NAFLD was defined as any degree of fatty liver in the absence of alcohol intake. NAFLD, if present, was classified based on standard ultrasonographic criteria as:

Grade 1 (mild steatosis): slightly increased liver echogenicity with normal vessels and absent posterior attenuation.

Grade 2 (moderate steatosis): moderately increased liver echogenicity with partial dimming of vessels and early posterior attenuation.

Grade 3 (severe steatosis): diffusely increased liver echogenicity with absence of visible vessels and heavy posterior attenuation.

Data were expressed as mean±SD, median (range) and/ or number where appropriate. Comparison between two groups was done using Students unpaired‘t’ test for normally distributed variables and Mann-Whitney U for skewed data. Correlation analyses between variables for skewed data were examined by the Spearman’s correlation test. The variables of baseline patient characteristics with different distributions between the non NAFLD and NAFLD groups were entered in a binary logistic regression model to test for independent associations. P values lower than 0.05 were considered statistically significant. Statistical analyses were performed using Statistical Package for Social Science (SPSS) for Windows version 15.0 (SPSS Inc., Chicago, ILL).

RESULTS

4. A. Frequency distribution

4. A. I. Frequency distribution of NAFLD and non NAFLD among the total study subjects

Among the total study subjects (n=110), 56% (n=62) subjects was non NAFLD, 44% (n=48) was NAFLD.

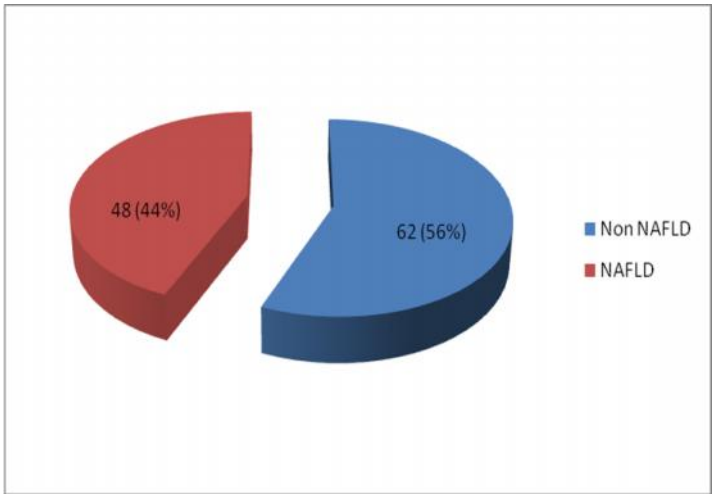

Figure 4.1: Distribution of NAFLD and non NAFLD among the total study subjects

4. A. II. Frequency distribution of NAFLD and non NAFLD among the studied IGT subjects

Among the IGT subjects (n=65), 55% (n=36) subjects was non NAFLD, 45% (n=29) was NAFLD.

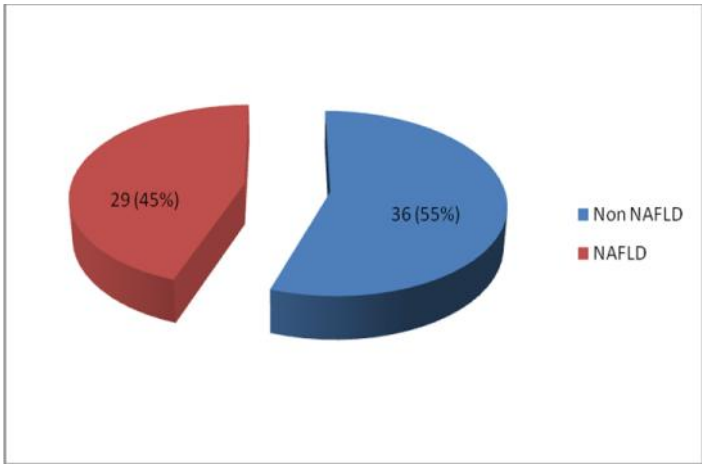

Figure 4.2: Distribution of NAFLD and non NAFLD among the IGT subjects

4. A. III. Frequency distribution of NAFLD and non NAFLD among the studied IFG subjects

Among the IFG subjects (n=34), 62% (n=21) subjects was non NAFLD, 38% (n=13) was NAFLD.

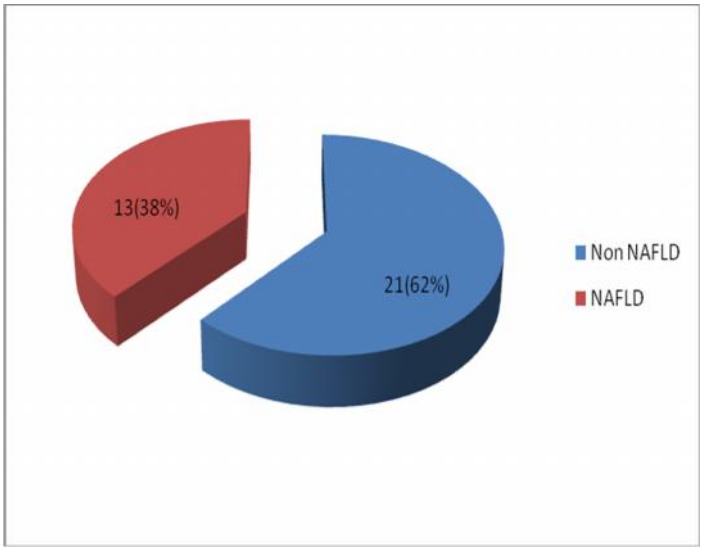

Figure 4.3: Distribution of NAFLD and non NAFLD among the IFG subjects

4. A. IV. Frequency distribution of different grades of fatty liver among the total study subjects

Among the total study subjects (n=110), 61% (n=55) subjects was normal, 40% (n=36) was Grade-I, 5% (n=6) was Grade-II and 3% (n=3) was Grade-III fatty liver after liver ultrasonography.

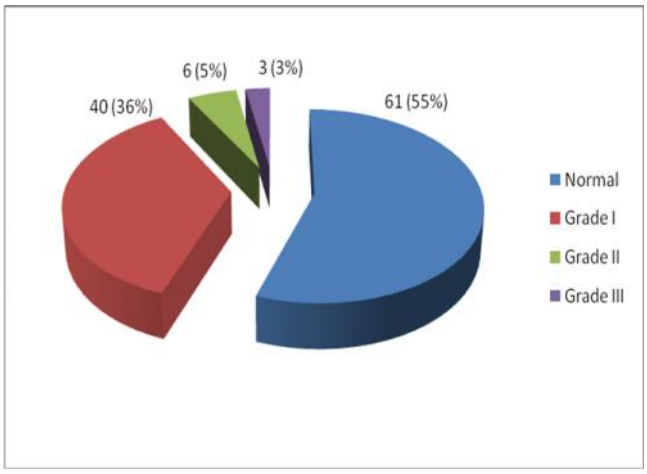

Figure 4.4: Distribution of different grades of fatty liver among the total study subjects

4.1. Sociodemographic, anthropometric and clinical characteristics of the study subjects

4.1.1. Gender

Of the total 110 subjects 28 (54.3 %) were male and 20 (41.7 %) were female had NAFLD while 35 (56.5 %) were male and 27 (43.5 %) were female had normal liver ultrasonography (Table 4.1).

4.1.2. Age (year)

Mean (±SD) age in the NAFLD subjects were 45±8 and in non NAFLD were 46±9. Mean age did not show statistically significant difference between the two groups (Table 4.1).

4.1.3. Body mass index (BMI, kg/m^2)

Mean (±SD) BMI in the NAFLD subjects were 25±3 and in non NAFLD were 24±4. Mean BMI did not show statistically significant difference between the two groups (Table 4.1).

4.1.4. Waist & Hip circumferences

Mean (±SD) Waist & Hip circumferences (WC & HC) in the NAFLD subjects were 93±7 *vs.* 88±8 and 98±7 *vs.* 95±8 respectively. Mean WC in the NAFLD group was significantly higher compare to the non NAFLD (p=0.011) (Table 4.1).

4.1.5. Waist to hip ratio (WHR)

Mean (±SD) WHR in the NAFLD subjects were 0.95±0.48 and in non NAFLD were 0.93±0.04. Mean WHR in the NAFLD group was significantly higher compare to the non NAFLD (p=0.048) (Table 4.1).

4.1.6 Percent body fat (%BF)

Mean (±SD) %BF in the NAFLD subjects were 32±8 and in non NAFLD were 28±6. Mean %BF in the NAFLD group was significantly higher compare to the non NAFLD (p=0.021) (Table 4.1).

4.1.7. Free fat mass (FFM)

Mean (±SD) FFM in the NAFLD subjects were 21±6 and in non NAFLD were 21±12. Mean FFM did not show statistically significant difference between the two groups (Table 4.1).

4.1.8. Systolic blood pressure (SBP, mmHg)

Mean (±SD) SBP in the NAFLD subjects were 134±34 and in non NAFLD were 112±15. Mean SBP in the NAFLD group was significantly higher compare to the non NAFLD ($p<0.001$) (Table 4.1).

4.1.9. Diastolic blood pressure (DBP, mmHg)

Mean (±SD) DBP in the NAFLD subjects were 93±26 and in non NAFLD were 76±17. Mean DBP in the NAFLD group was significantly higher compare to the non NAFLD ($p<0.001$) (Table 4.1).

4.1.10. Fasting & postprandial serum glucose (mmol/l)

Mean (±SD) Fasting & Postprandial serum glucose in the NAFLD and non NAFLD subjects were 5.80±0.43 *vs.* 5.95±0.50 and 4.30±1.56 *vs.* 7.56±1.51 respectively. Mean Postprandial serum glucose in the NAFLD group was significantly higher compare to the non NAFLD ($p=0.025$) (Table 4.1).

4.1.11. HbA_{1C} (%)

Mean (±SD) HbA_{1C} in NAFLD subjects were 5.88±0.41 and in non NAFLD subjects were 5.39±0.55. Mean HbA_{1C} in the NAFLD group was significantly higher compare to the non NAFLD ($p=0.005$) (Table 4.1).

4.1.12 Triglyceride (TG, mg/dl)

Mean (±SD) TG in NAFLD subjects were 201±36 and in non NAFLD subjects were 153±81. Mean TG in the NAFLD group was significantly higher compare to the non NAFLD ($p=0.042$) (Table 4.1).

4.1.13. Total cholesterol (mg/dl)

Mean (±SD) total cholesterol in NAFLD subjects were 198±44 and in non NAFLD subjects were 182±38. Mean total cholesterol in the NAFLD group was significantly higher compare to the non NAFLD ($p=0.050$) (Table 4.1).

4.1.14. High density lipoprotein cholesterol (HDL-c, mg/dl)

Mean (±SD) HDL-c in NAFLD subjects were 34±7 and in non NAFLD subjects were 38±7. Mean HDL-c in the NAFLD group was significantly higher compare to the non NAFLD ($p=0.007$) (Table 4.1).

4.1.15. Low density lipoprotein cholesterol (LDL-c, mg/dl*)*

Mean (±SD) serum LDL-c in NAFLD subjects was 191±44and in non NAFLD subjects was 190±36, which did not show statistically significant difference between the two groups (Table 4.1).

4.1.16. Serum Glutamate-pyruvate transaminase (SGPT) (U/L)

Mean (±SD) SGPT in NAFLD subjects were 37±19 and in non NAFLD subjects were 29±12. Mean SGPT in the NAFLD group was significantly higher compare to the non NAFLD (p=0.021) (Table 4.1).

4.1.17. S γ-Glutamyl transaminase (SGGT) (U/L)

Mean (±SD) SGGT in NAFLD subjects were 34±12 and in non NAFLD subjects were 24±11. Mean SGGT in the NAFLD group was significantly higher compare to the non NAFLD (p<0.001) (Table 4.1).

4.1.18. S Alkaline phosphatase (ALP) (U/L)

Mean (±SD) serum ALP in NAFLD subjects were 105±24 and in non NAFLD subjects were 104±28, which did not show statistically significant difference between the two groups (Table 4.1).

4.1.19. S Glutamate-oxaloacetate transaminase (SGOT) (U/L)

Mean (±SD) SGOT in NAFLD subjects were 36±21 and in non NAFLD subjects were 26±8. Mean SGOT in the NAFLD group was significantly higher compare to the non NAFLD (p=0.005) (Table 4.1).

4.1.20. S Total protein (g/l)

Mean (±SD) serum TP in NAFLD subjects were 5.96±1.81 and in non NAFLD subjects were 6.35±1.45, which did not show statistically significant difference between the two groups (Table 4.1).

4.1.21 S Albumin (mg/dl)

Mean (±SD) serum ALB in NAFLD subjects were 48±4 and in non NAFLD subjects were 47±4, which did not show statistically significant difference between the two groups (Table 4.1).

4.1.22. Fatty Liver Index

Median (range) FLI in NAFLD subjects were 32 (2-204) and in non NAFLD subjects were 16 (2-518). Median (range) FLI in the NAFLD group was significantly higher compare to the non NAFLD (p=0.005) (Table 4.1).

Table 4.1: Demographic anthropometric & clinical characteristics among the total study subjects (n=110)

Variables (Mean±SD)	**Group I NAFLD patients** (n=48)	**Group II Non NAFLD patients** (n=62)	*t/p values*
Sex n (%)			
Male	25 (56.8)	32 (59.3)	-
Female	19 (43.2)	22 (40.7)	-
Age (yrs)	45±8	46±9	-0.361/0.722
BMI (kg/m^2)	25±3	24±4	-1.175/0.241
Waist circumference (cm)	93±7	88±8	-2.594/**0.011**
Hip circumference (cm)	98±7	95±8	-1.542/0.125
WHR	0.95±0.48	0.93±0.04	-1.952/**0.048**
% Body fat	32±8	28±6	-2.281/**0.021**
Fat free mass (kg)	21±6	21±12	0.134/0.890
SBP (mm Hg)	134±34	112±15	-4.000/**<0.001**
DBP (mm Hg)	93±26	76±17	-3.645/**<0.001**
Fasting blood sugar (mmol/l)	5.80±0.43	5.95±0.50	1.612/0.104
Postprandial blood sugar (mmol/l)	4.30±1.56	7.56±1.51	-2.354/**0.025**
HbA_{1C} (%)	5.88±0.41	5.39±0.55	-4.974/**0.005**
Serum Cholesterol (mg/dl)	198±44	182±38	1.902/**0.050**
Serum Triglycerides (mg/dl)	201±36	153±81	-2.025/**0.042**
HDL-c (mg/dl)	34±7	38±7	2.752/**0.007**
LDL-c (mg/dl)	191±44	190±36	-0.184/0.852
SGPT (U/L)	37±19	29±12	-2.351/**0.021**
SGGT (U/L)	34±12	24±11	-3.784/**<0.001**
S ALP (U/L)	105±24	104±28	-0.268/0.789
SGOT (U/L)	36±21	26±8	-2.901/**0.005**
S Total protein (g/l)	5.96±1.81	6.35±1.45	1.180/0.241
S Albumin (mg/dl)	48±4	47±4	-0.466/0.643
FLI	32 (2-204)	16 (2-518)	776/**0.005**

Results were expressed as Number (%), Mean±SD & Median (range); n=number of subjects; BMI, body mass index; WHR, waist to hip ratio; %BF, percent body fat; FFM, free fat mass; SBP, systolic blood pressure; DBP, diastolic blood pressure; postprandial serum glucose (serum glucose 2 hours after 75g glucose load); TG, triglyceride; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; SGPT, Serum Glutamate-pyruvate transaminase; SGGT , Serum Gama-Glutamyl transaminase; S ALP, serum alkaline phosphatase; SGOT, Serum glutamate-oxaloacetate transaminase; FLI, Fatty Liver Index; NAFLD, non alcoholic fatty liver disease.

4.2. Inflammatory and insulinemic status of the study subjects

4.2.1. Erythrocyte sedimentation rate (mm/hr)

Median (range) ESR in NAFLD subjects were 25 (7-55) and in non NAFLD subjects were 17 (5-55). Median (range) ESR in the NAFLD group was significantly higher compare to the non NAFLD (p=0.026) (Table 4.2).

4.2.2. High sensitivity C-reactive protein (mg/l)

Median (range) hs-CRP in NAFLD subjects were 3.7 (0.1-14.9) and in non NAFLD subjects were 1.7 (0.2-13.2). Median (range) hs-CRP in the NAFLD group was significantly higher compare to the non NAFLD (p<0.001) (Table 4.2).

4.2.3. Fasting insulin (µIU/ml)

Median (range) serum insulin in NAFLD subjects were 16 (6-57) and in non NAFLD subjects were 14 (4-44). Median (range) serum insulin did not show significant difference between the two groups (Table 4.2).

4.2.4. Postprandial insulin (µIU/ml)

Median (range) Postprandial insulin in NAFLD subjects were 52 (11-170) and in non NAFLD subjects were 35 (3-147). Median (range) Postprandial insulin in the NAFLD group was significantly higher compare to the non NAFLD (p=0.008) (Table 4.2).

4.2.5. HOMA%S

Median (range) HOMA%S in NAFLD subjects were 43 (15-80) and in non NAFLD subjects were 57 (22-164). Median (range) HOMA%S in the NAFLD group was significantly lower compare to the non NAFLD (p=0.002) (Table 4.2).

4.2.6. HOMA%B

Median (range) HOMA%B in NAFLD subjects were 110 (9-198) and in non NAFLD subjects were 127 (52-198). Median (range) HOMA%B in the NAFLD group was significantly lower compare to the non NAFLD (p=0.001) (Table 4.2).

4.2.7. HOMA-IR

Median (range) HOMA-IR in NAFLD subjects were 2.5 (0.9-6.9) and in non NAFLD subjects were 1.9 (0.6-4.5). Median (range) HOMA-IR in the NAFLD group was significantly higher compare to the non NAFLD (p=0.002) (Table 4.2).

4.2.8. Insulin sensitivity index (ISI $_{Matsuda}$)

Median (range) $ISI_{Matsuda}$ in NAFLD subjects were 2 (0.09-2976) and in non NAFLD subjects were 4 (0.23-634). Median (range) $ISI_{Matsuda}$ in the NAFLD group was significantly lower compare to the non NAFLD (p=0.006) (Table 4.2).

4.2.9. Glucose/insulin ratio (G/I)

Median (range) G/I in NAFLD subjects were 0.3 (0.1-3.4) and in non NAFLD subjects were 0.4 (0.2-1.3). Median (range) G/I did not show significant difference between the two groups (Table 4.2).

Table 4.2: Inflammatory & insulinemic parameters among the total study subjects (n=110)

Variables (Mean±SD)	**Group I NAFLD patients**	**Group II Non NAFLD patients**	***U/p values***
ESR (mm/hr)	25 (7-55)	17 (5-55)	803/**0.026**
hs-CRP (mg/l)	3.7 (0.1-14.9)	1.7 (0.2-13.2)	442/**<0.001**
Fasting Insulin (μIU/ml)	16 (6-57)	14 (4-44)	1104/0.551
Postprandial Insulin (μIU/ml)	52 (11-170)	35 (3-147)	785/**0.008**
HOMA%S	43 (15-80)	57 (22-164)	722/**0.002**
HOMA%B	110 (9-198)	127 (52-198)	632/**0.001**
HOMA-IR	2.5 (0.9-6.9)	1.9 (0.6-4.5)	715/**0.002**
ISI $_{Matsuda}$	2 (0.09-2976)	4 (0.23-634)	767/**0.006**
G/I ratio	0.3 (0.1-3.4)	0.4 (0.2-1.3)	1064/0.462

Results were expressed as Median (range). n=number of subjects; HOMA%B, B cell function assessed by homeostasis model assessment; HOMA%S, insulin sensitivity assessed by homeostasis model assessment; HOMA-IR , insulin resistance assessed by homeostasis model assessment; ISI $_{Matsuda}$, Insulin sensitivity index proposed by Matsuda and DeFronzo; G/I, Glucose/Insulin ratio; NAFLD; non alcoholic fatty liver disease.

4.3. Spearman's correlation of insulinemic status with some significant variables

Bivariate Spearman's correlation analyses were performed for fasting insulin, HOMA%S, HOMA%B and HOMA-IR with clinical, anthropometrical and other biochemical variables in the non NAFLD and NAFLD group. Fasting insulin, HOMA%B, & HOMA-IR showed significant positive correlation with BMI & waist circumference (r=0.573, p<0.001 & r=0.431, p= 0.003; r=0.544, p<0.001 & r=0.353, p=0.024; r= 0.349, p=0.022 & r= 0.450, p=0.002 respectively) in NAFLD group. Whereas HOMA%S showed significant negative correlation with BMI & waist circumference (r=--0.490, p=0.001 & r=-0.357, p= 0.019 respectively) in NAFLD group.

On the other hand fasting insulin & HOMA-IR showed significant positive correlation with waist circumference (r=0.292, p=0.032 & r=0.380, p=0.005 respectively) in non NAFLD group. While HOMA%S showed significant negative correlation with waist circumference (r=-0.320, p=0.019) in non NAFLD group. HOMA%B showed significant negative correlation with FBS (r=-0.367, p=0.018) in NAFLD subjects. Fasting insulin & HOMA-IR showed significant positive correlation with FBS (r=0.290, p=0.033 & r=0.315, p=0.022 respectively) in non NAFLD group. HOMA-IR showed significant positive correlation with WHR (r=0.268, p=0.052) in non NAFLD group (Table 4.3).

Table 4.3: Spearman's correlation of insulinemic status with some significant variables in non NAFLD and NAFLD subjects (n=110)

Group *(r/p)*	**BMI**	**WC**	**WHR**	**FBS**	**2 h AG**	**HbA_{1C}**	**Chol**	**TG**	**HDL-c**	**LDL-c**	**hs-CRP**
Fasting Insulin (μIU/ml)											
Non NAFLD	0.183	0.292	0.045	0.290	-0.039	-0.055	0.068	0.105	-0.207	-0.016	0.182
	0.186	**0.032**	0.747	**0.033**	0.780	0.693	0.627	0.448	0.133	0.908	0.231
NAFLD	0.573	0.431	0.095	-0.113	0.113	-0.085	-0.056	0.061	-0.104	-0.147	0.117
	0.000	**0.003**	0.538	0.464	0.464	0.584	0.718	0.692	0.502	0.346	0.479
HOMA%B											
Non NAFLD	-0.052	0.071	0.095	-0.046	0.258	0.005	0.182	-0.067	-0.022	0.195	0.106
	0.714	0.612	0.498	0.742	0.062	0.969	0.192	0.632	0.877	0.166	0.493
NAFLD	0.544	0.353	-0.050	-0.367	0.236	-0.149	-0.086	0.027	0.186	-0.060	0.111
	0.000	**0.024**	0.757	**0.018**	0.138	0.352	0.592	0.869	0.244	0.714	0.519
HOMA%S											
Non NAFLD	-0.188	-0.320	-0.249	-0.136	0.150	0.102	-0.086	-0.207	0.073	0.025	-0.067
	0.178	**0.019**	0.072	0.331	0.284	0.467	0.540	0.138	0.601	0.858	0.665
NAFLD	-0.490	-0.357	-0.012	0.097	-0.150	0.119	0.060	0.005	0.096	0.125	-0.018
	0.001	**0.019**	0.937	0.535	0.337	0.446	0.703	0.972	0.541	0.432	0.913
HOMA-IR											
Non NAFLD	0.240	0.380	0.268	0.315	-0.069	0.025	0.104	0.235	-0.139	0.012	0.172
	0.084	**0.005**	**0.052**	**0.022**	0.625	0.858	0.461	0.091	0.321	0.935	0.264
NAFLD	0.349	0.450	0.172	-0.128	0.024	-0.243	0.066	0.128	0.041	-0.057	0.029
	0.022	**0.002**	0.271	0.412	0.877	0.116	0.675	0.414	0.792	0.719	0.864

Results were expressed as Pearson's correlation coefficient r and statistical significance p <0.05.

4.4. Spearman's correlation of hs-CRP with some significant variables

Bivariate Spearman's correlation analyses were performed for hs-CRP with clinical, anthropometric, biochemical variables and insulinemic profile in NAFLD and non NAFLD groups. hs-CRP showed significant positive correlation with BMI and WC (r=0.459, p=0.003 and r=0.339, p=0.035 respectively) while it showed significant negative correlation with WHR (r=-0.334, p=0.038) in NAFLD group. There was no significant correlation of hs-CRP with any other variables (Table 4.4).

Table 4.4: Spearman's correlation of hs-CRP with some significant variables in the total study subjects (n=110)

Group *(r/p)*	**BMI**	**WC**	**WHR**	**FBS**	**2 h AG**	**HbA_{1C}**	**Chol**	**TG**	**HDL-c**	**LDL-c**	**HOMA %S**	**HOMA %B**	**HOMA-IR**
						hs-CRP (mg/l)							
Non NAFLD	0.201	0.269	0.138	0.093	0.263	0.076	0.285	0.179	0.089	0.259	-0.002	0.219	0.199
	0.186	0.074	0.366	0.544	0.081	0.622	0.058	0.240	0.563	0.086	0.988	0.154	0.196
NAFLD	0.459	0.339	-0.334	-0.152	0.010	0.221	0.084	-0.185	0.229	0.216	-0.283	0.293	0.225
	0.003	**0.035**	**0.038**	0.356	0.952	0.176	0.611	0.259	0.161	0.193	0.085	0.082	0.175

Results were expressed as Pearson's correlation coefficient r and statistical significance p <0.05.

4.5. Binary logistic regression analysis taking NAFLD as dependant variable after adjusting the effects of major confounders

Binary logistic regression analysis was done using NAFLD as dependent variable and age, sex BMI as independent variables. hs-CRP showed significant positive association (ß=0.234, p=0.025) with NAFLD after adjusting the major confounding variables (age, sex & BMI) (Table 4.5).

Table 4.5: Binary logistic regression to evaluate the contribution of hs-CRP on NAFLD after adjusting the effects of major confounders

Variables	**Coefficient**	**S. E.**	**P-Value**	**Odds Ratio**	**95% C.I.**	
					Lower	**Upper**
Constant	*-3.342*	*2.166*	*0.123*	*0.035*	*	*
Age	0.029	0.028	0.301	1.030	0.974	1.088
BMI	0.060	0.059	0.303	1.062	0.947	1.192
Sex	-0.302	0.522	0.563	0.739	0.266	2.055
hs-CRP	0.234	0.104	**0.025**	1.264	1.030	1.551

Dependent variable: NAFLD; Adjusted R^2=0.110 the level of significance at p<0.05.

4.6. Binary logistic regression analysis taking NAFLD as dependant variable after adjusting the effects of major confounders

Binary logistic regression analysis was done using NAFLD as dependent variable and age, sex BMI and hs-CRP as independent variables. HOMA-IR was found to be significant independent determinant of NAFLD (ß=0.892, p=0.012) after adjusting the major confounding variables (age, sex, BMI and hs-CRP) (Table 4.6).

Table 4.6: Binary logistic regression to evaluate the contribution of insulin resistance on NAFLD after adjusting the effects of major confounders

Variables	Coefficient	S. E.	P-Value	Odds Ratio	95% C.I.	
					Lower	Upper
Constant	*-4.548*	*2.377*	*0.056*	*0.011*	*	*
Age	0.029	0.030	0.332	1.030	0.971	1.092
BMI	0.034	0.062	0.582	1.035	0.917	1.168
Sex	-0.270	0.553	0.625	0.763	0.258	2.257
hs-CRP	0.165	0.100	0.099	1.179	0.970	1.435
HOMA-IR	0.892	0.357	**0.012**	2.441	1.213	4.913

Dependent variable: NAFLD; Adjusted R^2=0.315; the level of significance at p<0.05.

4.7. Binary logistic regression analysis taking NAFLD as dependant variable after adjusting the effects of major confounders

Binary logistic regression analysis was done using NAFLD as dependent variable and age, sex BMI and hs-CRP as independent variables. HOMA%S and HOMA%B were found to be significant independent determinants of NAFLD (ß=-.076, p=0.002 and ß=-0.043, p=0.001 respectively) after adjusting the major confounding variables (age, sex, BMI and hs-CRP) (Table 4.7).

Table 4.7: Binary logistic regression to evaluate the contribution of insulin seceretory capacity and insulin sensitivity on NAFLD after adjusting the effects of major confounders

Variables	Coefficient	S. E.	P-Value	Odds Ratio	95% C.I.	
					Lower	Upper
Constant	*-4.548*	*2.377*	*0.056*	*0.011*	*	*
Age	0.040	0.037	0.278	1.041	0.968	1.119
BMI	0.106	0.070	0.130	1.112	0.969	1.277
Sex	-0.902	0.656	0.169	0.406	0.112	1.467
hs-CRP	0.144	0.103	0.162	1.154	0.944	1.412
HOMA%S	-0.076	0.025	**0.002**	0.926	0.883	0.972
HOMA%B	-0.043	0.013	**0.001**	0.958	0.933	0.984

Dependent variable: NAFLD; Adjusted R^2=0.364; the level of significance at p<0.05.

4.8. Relationship of hs-CRP with HOMA-IR among the total study subjects (n=110)

There was significant positive association of hs-CRP with insulin resistance (r=0.051, p=0.049) among the total study subjects (Fig: 4.5).

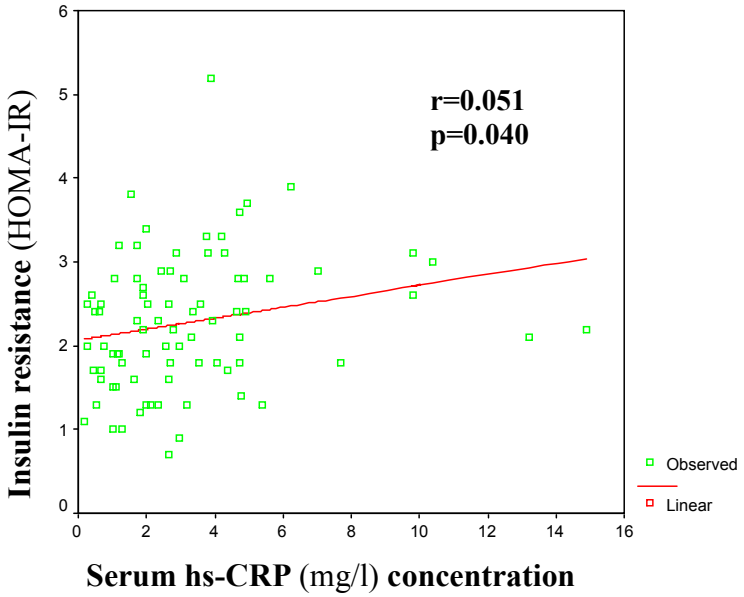

Figure 4.5: Relationship of hs-CRP with HOMA-IR among the total study subjects

DISCUSSION

NAFLD describes a spectrum of various conditions. This spectrum is mainly characterized by histological findings of macrovesicular hepatic steatosis in individuals consuming little or no alcohol. NAFLD possesses many components of the metabolic syndrome such as obesity, DM, hypertriglyceridemia but may also occur in patients with insulin resistance without obesity. Recently, ultrasonographic examination has been proposed as an alternative non-invasive, cheap and reliable technique to evaluate intra-abdominal fat thickness and liver steatosis. In this ultrasonographic study, we demonstrated the relationships between subclinical inflammation and insulin resistance among prediabetic subjects. In this study, 44 % of the prediabetic subjects were affected by NAFLD which is comparable with the prevalence found in other studies[188-191].

This is the first cross-sectional study in this research area in which comprehensive analysis of High sensitivity C-reactive protein (hs-CRP), anthropometric and metabolic covariates has been researched among Bangladeshi prediabetes with NAFLD. In this study, the NAFLD was associated with hs-CRP and insulin resistance (IR) in prediabetic subjects having NAFLD. hs-CRP has a short life of around 18 h, and the elevation of serum hs-CRP usually reflects its synthesis in response to a pathological process. Thus, hs-CRP has been considered as a useful nonspecific biochemical marker of chronic inflammation[192]. Several case and control studies have supported an association between elevated serum level of hs-CRP and the presence of NAFLD. In contrast, some studies failed to show an association of hs-CRP with the histological severity of NAFLD. The current study has also shown higher hs-CRP levels in prediabetes with NAFLD as compared to those without NAFLD. The findings of present study are in line with previous data on cross-sectional association between the hs-CRP and NAFLD in Japanese and Korean Asians[193]. In the cross-sectional study by Park *et al* (2004), elevated hs-CRP level was associated with NAFLD in apparently healthy nonobese Korean men.

While the pathophysiology of NAFLD remains incompletely understood, accumulation of triglycerides in hepatocytes in presence of oxidative stress, lipid peroxidation, pro-inflammatory cytokines (e.g. TNF-α, IL-6) appears to be important[195]. When hepatocytes get damaged, liver-specific macrophages (‘Kupffer Cells’) get activated and secrete more TNF-α and IL-6 into the blood. TNF-α and IL-6 are considered to induce hepatic production of the acute phase protein hs-CRP[196]. Elevation of serum hs-CRP usually reflects its synthesis in response to a pathological

process. In vivo release of interleukin-6 (IL-6), linked closely to hs-CRP pathway, but not tumor necrosis factor-a (TNF-α), which is related to insulin resistance, has been reported in human subcutaneous adipose tissue (SAT). We speculate that relatively larger SAT mass (truncal and peripheral SAT) is likely to generate relatively higher amounts of hs-CRP and preferentially drive this pathway rather than the insulin resistance pathway, although both appear to be interlinked. These findings agree with those of previous studies[197-198].

The study of Koruk *et al* (2003) reported that increased level of hs-CRP could be helpful in the diagnostic work-up of patients with fatty liver disease. Moreover, Yoneda *et al* (2007) added that hs-CRP could be a clinical feature that not only distinguishes NASH from simple nonprogressive steatosis. However, these findings disagree with those of Wieckowska *et al* (2007) they found no significant difference in levels of hs-CRP among NAFLD patients.

Insulin resistance and diabetes mellitus (DM) are known to contribute to the progression of non-alcoholic fatty liver disease (NAFLD). However, the relationship between glucose metabolism and NAFLD is not well known. Present study showed a higher level of fasting and postprandial insulin which is in line with earlier studies reported by Kimura *et al* (2011) where they investigated whether secretion patterns of glucose and insulin could influence the histological severity in NAFLD patients without prior known T2DM. Manchanayake *et al* (2001) found NAFLD patients have postprandial hyperinsulinemia, and OGTT reveals a high frequency of previously unsuspected IGT or T2DM. Hyogo *et al* (2012) suggests that increased early phase of insulin secretion may contribute to nonalcoholic fatty liver disease activity score in patients with normal glucose tolerance.

The presence of NAFLD among prediabetes or T2DM is associated with significant hepatic IR compared with subjects without a fatty liver. Current evidence suggests that insulin resistance plays a central role in the pathogenesis of NAFLD in T2DM[189]. In a recent study, Peterson *et al* (2005) showed that prevalence of insulin resistance is higher in Asian population and was associated with increased hepatic triglyceride content and plasma IL-6 concentration[205]. Several studies have suggested the association of NAFLD with IR and hs-CRP in healthy control and T2DM. However, only limited data show elevation of serum IR and hs-CRP level in prediabetes and their association with NAFLD[206,207].

In the present study, NAFLD was independently associated with insulin resistance and inflammation in prediabetic subjects. Previous studies have suggested that NAFLD is closely associated with insulin resistance. Furthermore, the current data provide evidence that insulin resistance might be an independent risk factor for NAFLD in prediabetes that lack confounding factors such as a history of diabetes, hypertension, fasting hyperglycemia, or high blood pressure[208].

We found that insulin resistance assessed by HOMA-IR in the NAFLD group increased as the blood glucose level increased compared to that of the non NAFLD group. This suggests that an increase in blood glucose level has a different meaning on insulin resistance by NAFLD status in nondiabetic subjects, assuming that progression of glucose tolerance status seems to be associated with the insulin resistance in subjects with NAFLD more closely than in non NAFLD individuals.

The present study showed a higher value of WC and WHR which is in line with the earlier studies[209,210]. Hypertension and especially systolic hypertension is also an independent predictor of NAFLD by Dixon *et al* (2001). We found a higher mean SBP & DBP in NAFLD compared to non NAFLD which is in agreement with several studies and showed a higher BP in NAFLD patients[209,210]. Another major finding of this study is that the risk of NAFLD development increased with increasing HbA_{1c} level in nondiabetic subjects. Current study subjects showed a higher HbA_{1c} level compared to control which is supporting earlier study[212] that showed a higher level of serum HbA_{1c} was independently associated with NAFLD.

Particular emphasis in the diagnosis of NAFLD is paid to disorders in lipid metabolism. The results of our study also confirmed this association. Patients with NAFLD had significantly higher concentrations of TC, TG and lower HDL-c. These observations are fully consistent with previous observations of Targher *et al* (2007). Higher levels of triglycerides were more observed in patients with NAFLD, which may possibly reflect a greater accumulation of fatty acid into the liver, higher IR and a greater tendency to develop into NASH[214].

We also calculated the fatty liver index (FLI), a simple and accurate predictor of hepatic steatosis in the general population[215]. The KORA-F4-Study 2011 found that the FLI is a useful approximation in a population-based study that cannot collect biopsy data due to ethical concerns[216]. The FLI has also been used in the French D.E.S.I.R Study in association with

incident diabetes[217]. They concluded that the FLI is predictive of diabetes in men and women independently of traditional risk factors and suggested that the index should be used by hepatologists to better identify patients at high risk of diabetes. Our study showed a higher FLI among the NAFLD patients, other published reports showed the similar results[218].

Our study was an attempt to explore the association between increased hs-CRP and insulin resistance with NAFLD and this relationship raises the possibility that inflammatory processes that accompany NAFLD contribute to the systemic inflammation observed in subjects with prediabetes. There is no consensus regarding the mechanism for the association between metabolic disorders and chronic subclinical inflammation and several possible explanations have been suggested. A remarkable finding of the current study is the strong independent association of serum hs-CRP and insulin resistance with presumed NAFLD. The association between hs-CRP and HOMA-IR also remained consistent by binary logistic regression analysis even after adjusting for age, gender and BMI, which supports the fact that fatty liver correlates with inflammation and insulin resistance among prediabetic subjects.

CONCLUSIONS

From the present data it may concluded that a high proportion (more than one-third) of the prediabetic subjects have NAFLD and the distribution of the disorder is almost similar in various subgroups of prediabetes. Subclinical chronic inflammation and insulin resistance seem to be independent mediators of the association between NAFLD and prediabetes. The data also indicate that the inflammatory condition and insulin resistance are associated with each other and those in turn are affected by central obesity and dyslipidemia in prediabetic subjects.

LIMITATIONS

- The diagnosis of NAFLD was based on ultrasonography and was not confirmed by liver biopsy;
- Correlation between the different stages of NAFLD (by histologic picture) and the levels of serum inflammatory markers could not be done;
- To date hs-CRP has yielded the most promising results, but its use should be validated in a larger cohort of patients so studies in larger prediabetes cohorts having NAFLD are needed to re-formulate the association of serum inflammatory markers and insulin resistance with NAFLD;
- It is an analytical study with a cross-sectional design; thus, no causal association between hs-CRP and other interacting molecules with NAFLD could be explored.

RECOMMENDATIONS

- The study needs to be completed involving adequate number of patients to provide optimum statistical power;
- Prospective studies with appropriate design should be undertaken to investigate the casual association between insulin resistances and hs-CRP with NAFLD in prediabetes;
- Other techniques for assessing insulin resistance (QUICKI and Clamp) should also be employed in such studies;
- The association of various inflammatory markers should be explored in greater details among Bangladeshi prediabetes with NAFLD.

BIOCHEMICAL ANALYSES

The following biochemical parameters were analyzed for the study.

8.1. ESTIMATION OF GLUCOSE

Glucose was estimated by enzymatic colorimetric (GOD-PAP) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.1.1. Principle

Glucose is determined after enzymatic oxidation in the presence of glucose oxidase. The hydrogen peroxide formed reacts with phenol and 4-aminophenazone under catalysis of peroxidase to form a red violet quinoneimine dye as indicator (Trinder, 1969).

$$\text{Glucose} + H_2O \xrightarrow{\text{Glucose Oxidase}} \text{Gluconic acid} + H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone} + \text{phenol} \xrightarrow{\text{Peroxidase}} \text{Quinoneimine} + 4H_2O$$

8.1.2. Reagents

Contents	Initial concentration of solution
Phosphate Buffer	0.1 mol/L, pH 7.0
Phenol	11 mol/L
4-aminophenazone	0.77 mmol/L
Glucose oxidase	≥1.5 kU/L
Peroxidase	≥1.5 kU/L
Glucose Standard	5.55 mmol/L (100 mg/dL)
Uranyl Acetate	0.16%

8.1.3. Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

8.1.4. Procedure

The method determines glucose without deproteinization. The instrument was calibrated before estimation. Serum and reagent were taken in specific cup. They were arranged serially into the Auto analyzer. The Auto analyzer was programmed for the estimation of glucose and allowed to run with following procedure:

5 µl sample and 500 µl reagent were mixed and incubated at 37° C for 10 minutes. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

8.1.5. Calculation of the result

Optical densities or absorbances were fed into a computer and calculation was done using the software program. Values for the unknown samples were calculated by extrapolating the absorbance for the standard using following formula.

$$\text{Glucose concentration (mmol/L)} = \frac{A_{Sample}}{A_{Standard}} .55$$

8.2. ESTIMATION OF HbA_{1C} (GLYCATED HEMOGLOBIN)

8.2.1. Principle

The VARIANT II Hemoglobin A_{1c} Program utilizes the principles of ion exchange high performance liquid chromatography (HPLC). The samples are automatically mixed and diluted on the VARIANT II Sampling Station (VSS) and injected into the analytical cartridge. The VARIANT II Chromatographic Station (VCS) dual pumps deliver a programmed buffer gradient, of increasing ionic strength, to the cartridge where the hemoglobins are separated based on their ionic interactions with the cartridge material. The separated hemoglobins then pass through the flow cell of the filter photometer, where changes in the absorbance at 415nm are measured. An additional filter at 690nm corrects the background absorbance. The VARIANT II Clinical Data Management (CDM) software performs reduction of the raw data collected from each analysis. Two-level calibration is used for adjustment of the calculated HbA1c values. A sample report and a chromatogram are generated by CDM for each sample. The A1c peak is shaded. This area is calculated using an exponentially modified gaussian (EMG) algorithm that excludes the labile A_{1c} and carbamylated peak area from the Hb A_{1c} peak area.

8.2.2. Specimen preparation

Whole blood were used as specimen

1. Allowed sample tubes to reach room temperature (15-30^0c) before performing the assay. No sample preparation was required. Mixed the tube prior to loading was not necessary. The sample tube were loaded into the *VARIANT II TURBO* sample racks and placed on the Sampling Station conveyor belt. Used special rack insert for 12, 8 and 14 mm diameter tubes. Removed all inserts for 16 mm diameter tubes. Tubes with a height of 75 mm were acceptable for use.

2. If the sample was in an abnormal size/type tube, or if the height of the sample in the tube appeared to be less than 25 mm, then the sample must be prediluted. Before pipetting, thoroughly mixed the sample by gently inverting the tube. To prediluted, pipet 1.5 mL of Wash/Diluent Solution into a labeled 1.5 mL vial, followed by 5 µL of the whole blood sample. Caped the sample vial and mixed thoroughly. Used a microvial adapter for prediluted samples.

8.2.3. Reagent preparation

All reagents and Buffer solutions were prepared followed the procedure described in the *VARIANT II TURBO* operation manual.

8.2.4. Elution Buffer and Wash/Diluent Solution

1. Allowed the Elution Buffers and Wash/Diluent Solution to reach room temperature (15-30^{0}c) before performing the assay. Mixed each bottle by gently inverting prior to installation.
2. The Elution Buffers and Wash/Diluent Solution Will be stable Until the expiration date when stored unopened at 15-30^{0}c. After opening the bottles, Elution Buffer A and Wash/Diluent Solution were Stable for 4 weeks, and Elution Buffer B was stable for 8 weeks, when stored at 15-30^{0}c.
3. With a new recorder pack installed one bottle of each reagent and followed the procedure for *Installing a New Recorder pack Lot*.
4. The Wash/Diluent Solution was interchangeable between Recorder Pack Lots.

8.2.5. Whole Blood Primer

A fresh aliquot of Whole Blood Primer when installing a new analytical cartridge and/or guard cartridge were used.

1. The Whole Blood Primer will be stabled until the expiration date when stored unopened at 2-8^{0}c.
2. Prepared the Whole Blood Primer by adding 1 mL of deionized water to the vial.
3. Allowed to stand for 10-15 minutes at 15-30^{0}c.
4. Swirled gently to dissolve and ensure complete mixing.
5. Written the reconstitution date on the label. The reconstituted Whole Blood Primer was stable for 1 day when stored at 2-8^{0}c.
6. The Whole Blood Primer was interchangeable between lots.

8.2.6. Hemoglobin A_{1c} Calibrators

Reconstituted and stored HbA_{1c} Calibrators as directed in the *Calibrator/Diluent Set Inserted*

Controls

- Reconstituted and stored the controls according to the manufacturer's package inserted.
- Bio-Rad Lyphocheck Diabetes Controls must be diluted 1:300 prior to analysis. Pipetted 1.5 mL of Wash/Diluent Solution into a labeled 1.5 mL vial, followed by 5 µL of the reconstituted control. Caped each control vial and mixed thoroughly.
- Bio-Rad Liquichek Diabetes Controls must be diluted 1:200 prior to analysis. Pipetted 1.0 mL Wash/Diluent Solution into a labeled 1.5 mL vial, followed by 5 µL of the control. Caped each control vial and mixed thoroughly.

8.2.7. Cartridge Set

The Cartridge stored at 2-8^{0}c. The analytical cartridge was stable for 4 weeks when installed on the instrument. The guard cartridge was stable for 2 weeks when installed in the instrument.

8.2.8. Procedure

1.Sample was prepared as described in Specimen Preparation.

2.The reagents and prepared patient specimens was placed into the VARIANT as indicated bellow.

Well	Reagent
STAT Well	Hemoglobin Primer
1	HbA_{1c} Calibrator
2	HbA_{1c} Calibrator
3	HbA_{1c} Calibrator
4	Normal Control
5	Abnormal Control
6 to N	Patient Hemolysates
N+1	Normal Control
N+2	Abnormal Control

3. Reagent was stored at 2-8 C when not in use.
4. The run was initialized as VARIANT Operation Manual.
5. After analysis of the calibrator , the calibration response factor for hemoglobin A_{1c} was automatically calculated. The calibration response factor was used in the calculation of area percentage of hemoglobin A_{1c} for all subsequent analysis in the run.
6. At the completion of each run , the system automatically initiated a five minutes Wash cycle. At the completion of the wash cycle, the system entered the idle mode.

Expected Value Range

8.2.9. Hemoglobin A_{1c} Ranges

The following HbA_{1c} ranges may be used for interpretation of results, however , factors such as duration of diabetes, adherence to therapy and the age of the patients should be considered in assesing the degree of blood glucose control. These values are for non pregnant individuals. Action Suggested depends on individuals patients circumstances. Such action may include enhanced diabetes self-management education, co-management with diabetes team, referral to an endocrinologist, change in pharmacological therapy, initiation or increased self-monitoring of blood glucose or more frequent contact with the patient.

Hemoglobin A_{1c} (%)	Degree of Glucose Control
8	Action Suggested
<7	Goal
< 6	Non diabetic level

8.3. ESTIMATION OF TOTAL CHOLESTEROL

Total cholesterol was measured by enzymatic endpoint method (Cholesterol Oxidase/Peroxidase) method in the Hitachi 704 Automatic Analyzer, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.3.1. Principle

The cholesterol is determined after enzymatic hydrolysis and oxidation. The indicator quinoneimine is formed from hydrogen peroxide and 4-aminoantiphyrine in the presence of phenol and peroxidase (Richmond, 1973).

8.3.2. Reagents

Contents	Initial concentration of solution
Phosphate Buffer	0.1 mol/L, pH 7.0
Phenol	11 mol/L
4-aminophenazone	0.77 mmol/L
Glucose oxidase	≥1.5 kU/L
Peroxidase	≥1.5 kU/L
Glucose Standard	5.55 mmol/L (100 mg/dL)
Uranyl Acetate	0.16%

8.3.3. Materials

- Micro-centrifuge tube
- Micropipettes and pipettes
- Disposable tips
- Automatic Analyzer (Boehringer Mannheim, 704; HITACHI)

8.3.4. Procedure

Serum and reagents were taken in specific cup or cell. They were arranged serially. Then ID number for each test was entered in the Auto analyzer. 5 µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the Auto lab. The reaction occurred in reaction cell or cup. The absorbance of the sample and the standard against the reagent blank were measured at 500 nm within 60 minutes.

8.3.5. Calculation of the result

Concentration of cholesterol in the sample was calculated by using software program with the following formula.

Cholesterol concentration (mg/dL) = $\frac{A_{Sample}}{A_{Standard}}$ oncentration of standard.

8.4. ESTIMATION OF TRIGLYCERIDE (TG)

Serum triglyceride was measured by enzymatic colorimetric (GPO-PAP) method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.4.1. Principle

Sample triglycerides incubated with a lipoprotein lipase liberate glycerol and fatty acids. Glycerol is converted to glycerol-3-phosphate by glycerol kinase and ATP.Glycerol-3-phosphate oxidase (GPO) oxidizes glycerol-3-phosphate into dihydroxy acetone phosphate and H_2O_2. In the presence of peroxidase, hydrogen peroxide oxidizes the chromogen-4-aminoantipyrine and 4-chlorophenol to a violet colored complex. The quinone formed is proportional to the amount of

triglycerides present in the sample. The principle is based on the following reaction system (Fossati and Prencipe, 1982).

$$\text{Triglyceride} \xrightarrow{Lipase} \text{glycerol + Fatty acids}$$

$$\text{Glycerol + ATP} \xrightarrow[Kinase]{Glycerol} \text{glycerol-3-phosphate + ADP}$$

$$\text{Glycerol-3-phosphate + } O_2 \xrightarrow[Oxidase]{Glycerol\ Phosphate} \text{dihydroxy acetone phosphate + } H_2O_2$$

$$2H_2O_2 + \text{4-aminophenazone + 4- chlorophenol} \xrightarrow{Peroxidase} \text{Quinoneimin+HCl+ } 4H_2O$$

8.4.2. Reagents

Content	Concentrations in the Test
Buffer	
Pipes Buffer	40 mmol/L, pH 7.6
4-choloro-phenol	5.5 mmol/L
Magnesium-ions	17.5 mmol/L
8.4.3. Enzyme Reagent	
4-aminophenazone	
ATP	1.0 mmol/L
Lipases	>150 U/ml
Glycerol-3-phosphate oxidase	1.5 U/ml
Standard	2.29 mmol/L (200 mg/dL)

8.4.4. Materials

- Micropipettes and pipettes
- Disposable tips
- Auto analyzer (Boehringer Mannheim, 704; HITACHI)

8.4.5. Procedure

Serum and reagents were taken in specific cup. They were arranged serially. Then ID number for test was entered in the analyzer. Five (5) µl sample and 500 µl reagent were mixed and incubated at 37^0C for 5 minutes within the cell. Reading was taken at 500 nm.

8.4.6. Calculation of result

Triglyceride concentration was calculated by following formula:

$$\text{Triglyceride concentration (mg/dL)} = \frac{A_{Sample}}{A_{Standard}} \times \text{Concentration of standard.}$$

8.5. ESTIMATION OF HIGH DENSITY LIPOPROTEIN (HDL) CHOLESTEROL

High density lipoprotein cholesterol (HDL-C) was measured by Differential Precipitation, Enzymatic colorimetric test & Endpoint method using reagent of Linear Chemicals, Spain.

8.5.1. Principle

HDL (High Density Lipoprotein) is separated from chylomicrons, VLDL (very low density lipoprotein) and LDL (Low density lipoprotein) by precipitating reagent (phosphotungstic acid-magnesium chloride). After centrifugation, the cholesterol content of HDL fraction, which remains in the supernatant, was determined by the enzymatic colorimetric method using CHOD-PAP (Friedwald *et al*., 1972).

8.5.2. Materials and reagents

1. Precipitant Buffer
2. Lipid Controls
3. Randox aqueous Cholesterol Standard: 200 mg/dL
4. Reagent solution for cholesterol CHOP-PAP assay.
5. Pipettes (5 µl –50 µl, 100 µl-1000 µl) and Pipette Tips.
6. Multi-Channel Pipettes and Pipette Tips: 50-300 µl
7. Buffer and Reagent Reservoirs
8. Vortex Mixture
9. Deionized Water
10. Microtiter Plate Reader capable of reading absorbency at 450 nm 590 nm
11. Orbital Microtiter Plate Shaker
12. Absorbant Paper

8.5.3. Reagents composition

Phosphotungstic Acid: 0.55 mmol/L

Magnesium Chloride: 25 mmol/L

8.5.4. Standard Preparation

Dilute Randox aqueous cholesterol standard (200 mg/dL) with deionized water by volume of 0, 20, 40, 50, and 100 µl. The final volume was 200 µl.

8.5.5. Assay Procedure

1. 100 ml serum sample was taken in microcentrifuge tube.
2. 250 ml HDL-C precipitant was added.
3. Mixed well and allowed to sit for 10 minutes
4. The mix components were vortexed and centrifuged for 15 minutes at 4000 rpm.
5. 30 µl of each Standard was transferred in first six wells.
6. 30 µl of clear supernatant was transferred into the other wells.

7. 250 µl of cholesterol reagent was then added into all the 96 wells quickly using multi-channel pipettes.
8. Incubated for 5 minutes at 37^0C on orbital microtiter plate shaker.
9. Absorbance was read at 490 nm.

8.5.6. Calculation of the result

The HDL-C value of each sample was obtained as follows:

The net absorbance value for each level, obtained by subtracting the value for the HDLC concentration (mg/dL) from the value of individual. The smooth linear curve was drawn and the results of unknown samples were calculated using logistic function.

8.6. ESTIMATION OF LOW DENSITY LIPOPROTEIN (LDL) CHOLESTEROL

The LDL-Cholesterol level in serum was calculated by using by Friedewald formula (Friedwald *et al.,* 1972).

Formula

$$\text{LDL cholesterol} = \{\text{Total cholesterol} - (\text{HDL cholesterol} + \frac{1}{5} x \quad \text{Triglyceride})\}$$

8.7. ESTIMATION OF SERUM GLUTAMATE-PYRUVATE TRANSAMINASE (SGPT)

Serum glutamate-pyruvate transaminase (GPT) or alanine aminotransferase (ALT) was estimated by UV method using ALT (GPT) opt. kit (RANDOX) (IFCC, 1980).

8.7.1. Principle

$$\alpha\text{- oxogluterate + L – alanine} \xrightarrow{\text{SGPT}} \text{L-glutamate + pyruvate}$$

$$\text{Pyruvate + NADH + H}^+ \xrightarrow{\text{LDH}} \text{L-lactate + NAD}^+$$

8.7.2. Reagents

Contents	Concentration in the test
1. Buffer/Substrate	
Tris biffer	100 mmol/L, pH 7.5
L-alanine	0.6 mol/L
2. Enzyme/Coenzyme/α-oxoglutarate	
α-oxoglutarate	15 mmol/L
LD	≥1.2 U/ml
NADH	0.18 mmol/L

8.7.3. Preparation of Solutions

1. Buffer/Substrate: Buffer/Substrate supplied in the kit was used as it is.
2. Enzyme/Coenzyme/α-oxoglutarate: One vial of Enzyme/Coenzyme/α-oxoglutarate2 was reconstituted with the appropriate volume of Buffer/Substrate 1:

2 ml	for the	20 x	2 ml	kit (AL 1200)
10 ml	for the	20 x	10 ml	kit (AL 1205)
20 ml	for the	5 x	20 ml	kit (AL 1268)

Cat No AL 2360 5 x 100 ml

One vial of Enzyme/Coenzyme/α-oxoglutarate 2 was reconstituted with a portion of Buffer/Substrate 1 and then the entire content was transferred to bottle 1 rinsing bottle 2 several times.

8.7.4. Procedure

Wavelength:	340 nm (Hg 334 nm or Hg 365 nm)	
Cuvette:	1 cm light path	
Temperature:	25/30/37^{o}C	
Measurement:	against air	
Pipetted into cuvette:	Macro	Micro
Serum	0.2 ml	0.1 ml
Enzyme/Coenzyme/α-oxoglu-tarate 2	2.0 ml	1.0 ml

Mixed and initial absorbance was read after 1 minute. Again after 1, 2 and 3 minutes the absorbance was read. The absorbance change per minute was noted and if the value is between

0.11 and 0.16 at 340 nm/Hg 340 nm

0.06 and 0.08 at Hg 365 nm

Only then the values for the first 2 minutes were used for the calculation.

8.7.5. Calculation

To calculate the ALT activity the following formulae was used:

U/L	=	1746	x	ΔA 340 nm/min
U/L	=	1780	x	ΔA Hg 334 nm/min
U/L	=	3235	x	ΔA Hg 365 nm/min

Normal values in serum

	25^{o}C	**30^{o}C**	**37^{o}C**
Men	Up to 22 U/L	Up to 29 U/L	Up to 40 U/L
Women	Up to 17 U/L	Up to 22 U/L	Up to 31 U/L

Linearity

If the absorbance change per minute exceeds

0.16 at 340 nm/Hg 334 nm

0.08 at Hg 365 nm

0.1 ml of sample was diluted with 0.9 ml pf 0.9% NaCl solution and reassessed. The result was multiplied by 10.

8.8. ESTIMATION OF SERUM GLUTAMATE-OXALOACETATE TRANSAMINASE (SGOT)

Serum GOT was measured by enzyme kinetic method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.8.1. Principle

α-oxoglutarato reacts with L-aspartate in the presence of AST to form L-glutamate plus oxaloacetate. The iddicator reaction utilizes the oxaloacetate for a kinetic determination of NADH consumption.

$$\alpha\text{-oxoglutarate} + \text{L-aspartate} \xrightarrow[\text{MDH}]{\text{AST}} \text{L-glutamate} + \text{oxaloacetate}$$

$$\text{oxaloacetate} + \text{NADH} + H^{+} \xrightarrow[\text{MDH}]{\text{LDH}} \text{L-malate} + NAD^{+}$$

8.8.2. Procedure:

Aspirate fresh dd H_2O and perform a new Gain Calibration in flow mode. Select AST in the Run Test screen and carry out a water bank as instructed.

Pipette into a test tube:

Sample	0.1ml
Reagent	1.0ml

Mix and aspirate into the Rx Monza.

8.8.3. Calculation

To calculate the AST activity uses the following formulae.

U/I=1746 x ΔA 340 nm/min

U/I=1780 x ΔA Hg 334 nm/min

U/I=3235 x ΔA Hg365 nm/min

8.8.4. Normal value in Serum

Temperature	25ºC	30ºC	37ºC
Mane	Up to 18 U/l	Up to 25 U/l	Up to 37 U/l
Women	Up to 15 U/l	Up to 21 U/l	Up to 31 U/l

8.9. ESTIMATION OF SERUM GAMA-GLUTAMYL TRANSAMINASE (SGGT)

Serum GGT was measured by enzyme kinetic method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.9.1. Colorimetric Method

The substrate L-γ-glutamyl-3-carboxy-4-nitroanilide, in the presence of glycylglycine is converted by γ-GT in the sample to 5-amino-2-nitrobenzoate which can be measured at 405nm.

8.9.2. Principle

L-γ-glutamyl-3-carboxy-4-nitroanilide+ glycylglycine

↓ γ-GT

L-γ-glutamylglycylglycine+5-amino-2-nitrobenzoate

8.9.3. Sample collection and preparation

Serum: use only non-haemolysed serum

Plasma: use only EDTA plasma that is free from hemolysis. Other anticogulants interfere with this test. γ-GT in serum and plasma is stable for 7 days at +2ºC to +8ºC or for 3month at -20ºC.

Test Method

Wavelength	Hg 405nm (400-420nm)
Cuvette	1 cm light path
Temperature	25ºC,30ºC,37ºC
Measurement	Against air

Pipette into cuvette

Sample	0.10ml
Reagent (25ºC,30ºC,37ºC)	1.00ml

Mix read initial absorbance and start timer simultaneously. Read again after 1,2 and 3 min.

8.9.4. Calculation

To calculate the GGT activity uses the following formulae.

U/L=1158 x ΔA 405 nm/min

8.9.5. Normal value in serum values

Temperature	25ºC	30ºC	37ºC
Mane	6-28 U/l	8-38 U/l	11-50 U/l
Women	4-18 U/l	5-25 U/l	7-32 U/l

8.10. ESTIMATION OF SERUM ALKALINE PHOPHATASE (SALP)

Serum ALP was measured by enzyme kinetic method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.10.1. Colorimetric Method

This is an optimized standard method according to the recommendations of the Deutsche Gesellschaft für Klinische Chemie.

8.10.2. Principle

p-nitrophenylphosphate + H_2O ⟶ ALP ⟶ Phosphate + p-nitrophenol

Sample

Serum or heparinized plasma. Samples are stable for 5 days when stored at +2ºC to +8º C.

8.10.3. Procedure

Wavelength	Hg 405nm
Cuvette	1 cm light path
Temperature	25ºC,30ºC,37º C
Measurement	Against air

Method

Pipette into cuvette	Macro	Semi-micro	Micro
Sample	0.05ml	0.02ml	0.01ml
Reagent(25ºC,30ºC,37º,C)	3.00ml	-	0.50ml

Mix read initial absorbance and start timer simultaneously. Read again after 1,2 and 3 min.

8.10.4. Calculation

To calculate the ALP activity uses the following formulae.

U/I=3300xΔA 405 nm/min MACRO

U/I=2760 xΔA 405 nm/min SEMI-MICRO

U/I=2760 xΔA 405 nm/min MICRO

8.10.5. Normal Value In Serum

Temperature	25ºC	30ºC	37ºC
Men/Women	60-170U/l	73-207U/l	98-279U/l

References:

1. Rec. GSCC (DGKC);J. Cli.Chem.cli.Biochem.1972;10:182.

2. Englehardt A., et al Aerztl Labor 1970; 16:42.

8.11. DETERMINATION OF ALBUMIN

Serum ALB was measured by enzyme colorimetric method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.11.1. Principle

The measurement of serum albumin is based on its quantitative binding to the indicator 3,3',5,5'-tetrabromom cresol sulphonephthalein (bromocresol green, BCG). The albuminBCGcomplex absorbs maximally at 578 nm, the absorbance being directly proportional to the concentration of albumin in the sample.

8.11.2. Sample

Serum, heparinized plasma or EDTA-plasma. Normal procedure for collecting and storing serum may be used for samples to be analysed by this method. Serum is stable for 3 days at +2 to +8C or 6 month at -20C.

8.11.3. Procedure:

Using fresh dd H_2O perform a new Gain Calibration in cuvette mode. Select ALB in the Run Test screen and carry out a water bank as instructed.

Pipette into a cuvette

	Reagent Blank SO	Standard SI	Sample
dd H_2O	10µl		
Standard		10µl	
Sample			10µl
Reagent	3000µl	3000µl	3000µl

Mix, incubate for 20min at 20ºC,-25ºC or 10min at 37º, C insert into the Monza flowcell holder and press Read within 60 mins.

8.11.4. Manual Calculation

The albumin concentration in the sample may be calculated from the following formula.

$$\text{Albumin Concentration (g/l or g/dl)} = \frac{A_{sample}}{A_{standard}} \text{ concentration of standard.}$$

ALB Normal value in serum

Adults	38-44 g/l (3.8-4.4 g/dl)
Neonates	38-42 g/l (3.8-4.2 g/dl)

8.12. DETERMINATION OF TOTAL PROTEIN

Serum TP was measured by enzyme colorimetric method in the Automatic Analyzer, Hitachi 704, Hitachi Ltd., Tokyo, Japan using reagents of RANDOX Laboratories Ltd., UK.

8.12.1. Principle

Cupric ions, in an alkaline medium, interact with protein peptide bonds resulting in the formation of a colored complex.

8.12.2. Sample

Serum, heparinized plasma or EDTA-plasma

8.12.3. Procedure:

Using fresh Distill H_2O performs a new Gain Calibration in cuvette mode. Select the Total Protein Program in the Run Test screen and carry out a water bank as instructed.

8.12.4. Pipette into cuvette

	Reagent Blank SO	Standard SI	Sample	Sample blank
Distilled H_2O	0.02ml			
Standard (CAL)		0.02ml		
Serum			0.02ml	0.02ml
R1	1.0ml	1.0ml	1.0ml	
R2				1.0ml

Mix, incubate for 30 min at +20ºC to +25º C before reading as instructed on screen.

8.12.5. Manual calculation

1. When measurements are taken at Hg546 nm, total protein concentration may be calculated as follows.

Tot. Prot. Conc. (g/l) =190 x A_{sample}

Tot. Prot. Conc. (g/dl) =190 x A_{sample}

2. When using a standard:

$$\text{Tot. Prot. Conc.} = \frac{A_{Sample}}{A_{Standard}} \text{ x concentration of standard.}$$

Normal Values Serum:

	g/dl	g/l
Adults	6.4-8.3	64-83

8.8. DETERMINATION OF ERYTHROCYTE SEDIMENTATION RATE

8.8.1. Lab objective

The student will able to perform, within 2 mm/hr accuracy compared with the instructor's value, three erythrocyte sedimentation rates using the Westergren method.

8.8.2. Principle

The erythrocyte sedimentation rate (ESR), also called the sed rate, measures the settling of erythrocytes in diluted human plasma over a specified time period. This numeric value is determined (in millimeters) by measuring the distance from the bottom of the surface meniscus to the top of erythrocyte sedimentation in a vertical column containing diluted whole blood that has remained perpendicular to its base for 60 minutes. Various factors affect the ESR, such as RBC size and shape, plasma fibrinogen, and globulin levels, as well as mechanical and technical factors.

The ESR is directly proportional to the RBC mass and inversely proportional to plasma viscosity. In normal whole blood, RBCs do not form rouleaux; the RBC mass is small and therefore the ESR is decreased (cells settle out slowly). In abnormal conditions when RBCs can form rouleaux, the RBC mass is greater, thus increasing the ESR (cells settle out faster).

The Westergren method is preferred by NCCLS standards because of its simplicity and greater distance of sedimentation measured in the longer Westergren tube. The straight tube is 30 cm long, 2.5 mm in internal diameter, and calibrated in millimeters from 0-200. Approximately 1 mL of blood is required. The method it replaces is called the Wintrobe method.

8.8.3. Specimen

Fresh anticoagulated blood collected in EDTA. Blood should be at room temperature and should be no more than 2 hours old. If anticoagulated blood is refrigerated, the test must be set up within 6 hours. Hemolyzed specimens cannot be used.

8.8.4. Reagents, supplies, and equipment

1. Westergren tubes
2. Westergren rack
3. Disposable pipettes
4. 0.5 ml sodium chloride in puncture ready vials
5. Leveling plate for holding the Westergren rack
6. Timer

8.8.5. Quality control

Commercial controls are available for this procedure. They will not be used for this exercise.

8.8.6. Procedure

1. Collect whole blood anticoagulated with EDTA.
2. Label the puncture ready vial with the patient's name.
3. Remove cap from the puncture ready vial and add well mixed blood up to the line (see illustration).
4. Replace cap and invert 8 times making sure the blood and saline mix well.
5. Carefully insert the Westergren tube into plunge able vial cap of blood/diluent mixture twisting as you push the tube down.
6. Place the tube in the Westergren rack to a vertical position and leave undisturbed for exactly 1 hour.
7. Set timer for 1 hour.
8. After 1 hour has passed, read the distance in millimeters from the bottom of the plasma meniscus to the top of the sedimented erythrocytes. Do not include the buffy coat in this measurement. (The buffy coat is the layer of white cells and platelets at the interface of red cells

and plasma. It is usually negligible, but may be noticeable in cases of leukocytosis or thrombocytosis.)

8.8.7. Reporting results

Normal values

Adult male 0-15 mm/hr

Adult female 0-20 mm/hr

8.14. ESTIMATION OF FASTING SERUM INSULIN

8.14.1. Principle

The DRG Insulin ELISA Kit is a solid phase enzyme-linked immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the Insulin molecule. An aliquot of patient sample containing endogenous Insulin is incubated in the coated well with enzyme conjugate, which is an anti-Insulin antibody conjugated with Biotin. After incubation the unbound conjugate is washed off. During the second incubation step Streptavidin Peroxidase Enzyme Complex binds to the biotin-anti-Insulin antibody. The amount of bound HRP complex is proportional to the concentration of Insulin in the sample. Having added the substrate solution, the intensity of colour developed is proportional to the concentration of Insulin in the patient sample.

8.14.2. Reagents

1. Microtiterwells, 12 x 8 (break apart) strips, 96 wells; Wells coated with anti-Insulin antibody (monoclonal).
2. Zero Standard, 1 vial, 3 mL, ready to use 0 µIU/mL Contains non-mercury preservative.
3. Standard (Standard 1-5), 5 vials, 1 mL, ready to use; Concentrations: 6.25 - 12.5 – 25 - 50 and 100 µIU/mL, Conversion: µIU/mL x 0.0433 = ng/mL x 23.09 = µIU/mL The standards are calibrated against international WHO approved Reference material NIBSC 66/304.; Contain non-mercury preservative.
4. Enzyme Conjugate, 1 vial, 5 mL, ready to use, mouse monoclonal anti-Insulin conjugated to biotin; Contains non-mercury preservative.
5. Enzyme Complex, 1 vial, 7 mL, ready to use, Streptavidin-HRP Complex Contains non-mercury preservative.
6. Substrate Solution, 1 vial, 14 mL, ready to use, Tetramethylbenzidine (TMB).

7. Stop Solution, 1 vial, 14 mL, ready to use, contains 0.5 M H2SO4, Avoid contact with the stop solution. It may cause skin irritations and burns.
8. Wash Solution, 1 vial, 30 mL (40X concentrated).

8.14.3. Reagent Preparation

Wash Solution

Deionized water was added to the 40X concentrated Wash Solution. 30 mL of concentrated Wash Solution was diluted with 1170 mL deionized water to a final volume of 1200 mL. The diluted Wash Solution is stable for 2 weeks at room temperature.

8.14.4. Procedure

1. Microtiter wells were secured in the frame holder.
2. 25 µL of each Standard, control and samples were dispensed with new disposable tips into appropriate wells.
3. 25 µL Enzyme Conjugate was dispensed into each well. Thoroughly mixed for 10 seconds.
4. Incubated for 30 minutes at room temperature.
5. The contents of the wells were shaked out briskly. The wells were rinsed 3 times with diluted Wash Solution (400 µL per well). The wells were striked sharply on absorbent paper to remove residual droplets.
6. 50 µL of Enzyme Complex was added to each well.
7. Incubated for 30 minutes at room temperature.
8. The contents of the wells were shaked out briskly. The wells were rinsed 3 times with diluted Wash Solution (400 µL per well). The wells were striked sharply on absorbent paper to remove residual droplets.
9. 50 µL of Substrate Solution was added to each well.
10. Incubated for 15 minutes at room temperature.
11. The enzymatic reaction was stopped by adding 50 µL of Stop Solution to each well.
12. The absorbance (OD) of each well was determined at 450 ± 10 nm with a microtiter plate reader.

8.14.5. Calculation

Optical densities obtained were used to calculate values of unknown samples using software, Kinetic Calculation. Results were calculated by expanding standard curve.

8.15. DETERMINATION OF INSULIN SECRETORY CAPACITY AND INSULIN SENSITIVITY

Homeostasis Model Assessment (HOMA) is a simple widely used method which derives separate indices of B cell secretion (HOMA B) and insulin sensitivity (HOMA S) from the Serum glucose and insulin concentrations under basal conditions by using mathematical formula or software. Using HOMA, insulin sensitivity (HOMA-IR) is calculated as [fasting insulin (mu/l) × fasting glucose (mmol/l)/22.5]. The HOMA model has been incorporated in a simple MS-DOS-based computer program (HOMA-CIGMA software) that allows rapid determination of % B (B cell secretion) and % S (insulin sensitivity) from measured values. Although the simple equation gives a qualitatively useful approximation of the model prediction, most authors prefer the computer model. In this study HOMA-CIGMA software was used.

8.16. DETERMINATION OF HIGH SENSITIVITY C-REACTIVE PROTEIN

8.16.1. Principle of the hs-CRP ELISA

Microtiter strips coated with anti-hs-CRPantibody are incubated with diluted standard sera and donor samples. During this incubation step hs-CRP is bound specifically to the wells. After removal of the unbound serum proteins by a washing procedure, the antigen-antibody complex in each well is detected with specific peroxidase-conjugated antibodies. After removal of the unbound conjugate, the strips are incubated with a chromogen solution containing tetra methyl benzidine and hydrogen peroxide: a blue colour develops in proportion to the amount of immunocomplex bound to the wells of the strips. The enzymatic reaction is stopped by the addition of 0.5M H2SO4 and the absorbance values at 450 nm are determined. A standard curve is obtained by plotting the absorbance values versus the corresponding standard values. The concentration of hs-CRP in donor samples is determined by interpolation from the standard curve.

8.16.2. Reagents

1. **Coated Microtiter strips**

12 x 8-well strips coated with monoclonal antibodies to human hs-CRP.

2. **Standard Sera** - 5 vials, each containing 1/10 prediluted hs-CRP standard solutions (0.2 ml): 0 - 0.4 - 1 - 5 - 10 µg/ml. Calibrated against the NIBSC 1st International Standard, 85/506.

Contain 0.09 % NaN_3 and antimicrobial agents as preservatives.

3. **Conjugate** - 1 vial, containing peroxidase conjugated monoclonal anti-human hs-CRP antibodies (12 ml). Contains antimicrobial agents and an inert red dye.

4. **Specimen Dilution Buffer** - 1 vial, containing 40 ml dilution buffer 5x concentrated.

Contains 0.09 % NaN_3 and antimicrobial agents and an inert green dye.

5. **Washing Solution -** 1 vial, containing 50 ml 20 x concentrated phosphate buffered washing solution.

6. **Chromogen Solution -** 1 vial, containing 15 ml of a solution containing H_2O_2 and tetramethylbenzidin.

7. **Stopping Solution** -1 vial, containing 12 ml of 0.5M H_2SO_4

8.16.3. Materials required

1. Precision micropipettes and standard laboratory pipettes.
2. Clean standard laboratory volumetric glassware.
3. Clean glass tubes for the dilution of the samples.
4. A microtiterplate reader capable of measuring absorbencies at 450 nm

8.16.4. Reconstitution of the Reagents

Washing Solution: Dilute 50 ml of concentrated Washing Solution (5) to 1000 ml with distilled water. Reconstituted solution can be stored at least 1 month, store at 2 °C – 8 °C. At higher temperatures, the concentrated Washing solution (5) may appear cloudy, without affecting its performance. Upon dilution, the solution will be clear.

Sample Diluent: Dilute 40 ml of the concentrated Sample Diluent to 200 ml with distilled water. Reconstituted solution can be stored at least 3 months or as long as solution remains clear. Store at 2 °C – 8 °C.

8.16.5. Assay Procedure

1. The 10 x prediluted standard sera (2) are diluted 1:100 as follows: pipette 10 µl of each calibrator into separate glass dilution tubes. Add 990 µl of diluted Specimen Dilution Buffer (4) and mix carefully.

2. The donor samples are diluted 1:1000 in two consecutive steps: pipette 10 µl of each donor sample into separate glass dilution tubes and add 990 µl of diluted Specimen Dilution Buffer (4). Mix thoroughly. Add 450 µl of diluted Specimen Dilution Buffer to 50 µl of these 100 x prediluted samples. Mix thoroughly. Warning: do not store the diluted samples for more than 8 hours.

3. Pipette 100 µl of the diluted calibrators and samples into each of a pair of adjacent wells (1).

4. Incubate the covered microtiter strips for 30 ± 2 min at room temperature.

5. Wash the microtiter strips three times with Washing Solution. This can either be performed with a suitable microtiter plate washer or by briskly shaking out the contents of the strips and immersing them in washing solution. During the third step, the washing solution is left in the strips for 2-3 min. Change washing solution for each cycle. Finally empty the microtiter strips and remove excess fluid by blotting the inverted strips on adsorbent paper.

6. Add 100 µl of Conjugate Solution (3) and incubate the covered microtiter strips for 30 ± 2 min at room temperature.

7. Repeat the washing procedure as described in step 5.
8. Add 100 µl of Chromogen (6) Solution to each well.
9. Incubate for 10 ± 2 min at room temperature. Avoid light exposure during this step.
10. Add 50 µl of Stopping Solution (7) to each well.
11. Determine the absorbance of each well at 450 nm within 30 min following the addition of acid.

8.16.6. Results

The average absorbance value of each calibrator is plotted against the corresponding CRP-value and the best calibration curve (e.g. log/linear) is constructed. Use the average absorbance of each donor sample obtained in the hsCRP ELISA to determine the corresponding value by simple interpolation from the curve. Depending on the experience and/or availability of computer capability, other methods of data reduction may be used.

Minimal detectable concentration

The minimal detectable concentration is approximately 0.02 µg/ml

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Appendix I

সম্মতি পত্র

প্রকল্পের নামঃ বাংলাদেশের ডায়াবেটিসের পূর্বাস্থার রোগীদের মধ্যে যকৃতে চর্বি জমা হওয়ার সাথে ইনসুলিন প্রতিরোধক এবং প্রদাহ এর সম্পর্ক।

গবেষনাকারীর নামঃ ইসরাত আরা হোসেন, প্রভাষক, প্রানরসায়ন ও কোষবিদ্যা বিভাগ, বি ইউ এইচ এস

আমি এই মর্মে অবগত হয়েছি যে, প্রি-ডায়বেটিস রোগীদের সুফল বয়ে আনার জন্য এই গবেষনা কাজটি পরিচালিত হচ্ছে। আমি এই গবেষনা কাজটির উদ্দেশ্য ও ধরন সম্পর্কে পরিষ্কার ধারনা পেয়েছি। আমি নিশ্চিত হয়েছি যে, এই অংশগ্রহনের ফলে আমি কোন প্রকার শারীরিক এবং মানষিক ক্ষতির সম্মুখীন হবোন্া । উপরন্তু আমি আমার শারীরিক অবস্থা সম্পর্কে অবগত হয়ে উপকৃত হবো। এর পর ও যদি কোন প্রকার ক্ষতির সম্মুখীন হই তাহলে গবেষনাকারী ক্ষতিপূরন দিতে বাধ্য থাকবে । আমি এটাও নিশ্চিত যে, এই গবেষনা কাজে সংগৃহীত আমার পরিচয় এবং অন্যান্য তথ্যাবলী সম্পুর্ন গোপন রাখা হবে।

আমি বুঝতে পারছি যে, এই গবেষনা পদ্ধতি সংক্রান্ত যে কোন প্রশ্ন করার সুযোগ আমাকে দেওয়া হবে এবং পরবর্তিতে কোন প্রশ্ন থাকলে গবেষনাকারী সাথে যোগাযোগ করতে পারব।

আমি যে কোন সময় এই গবেষনা প্রকল্প থেকে আমার সম্মতি উঠিয়ে নিতে পারব এবং এর জন্য আমাকে কোন জবাবদিহি করতে হবে না ।

আমি সেচ্ছায় এবং সজ্ঞানে এই গবেষনা কাজে অংশ গ্রহন করতে রাজি আছি।

গবেষনায় অংশ গ্রহনের সুবিধাদি ঃ

এই গবেষনায় অংশ গ্রহন করলে আপনি ব্যক্তিগত ভাবে সরাসরি লাভবান হবেন না, তবে ভবিষ্যতে আরো উন্নত স্বাস্থ্য সম্পর্কে অবগত হতে পারবেন ।

বিকল্প ঃ

এই গবেষনায় অংশগ্রহণ করা কিংবা না করার ব্যাপারে আপনার সিদ্ধান্ত চূড়ান্ত বা অংশগ্রহণ করার পর যে কোন সময় আপনি নিজেকে গবেষনা থেকে সারিয়ে নিয়ে পারবেন ।

খরচ ঃ

এই গবেষনায় অংশ গ্রহণের জন্য আপনার প্রকৃত চিকিৎসা খরচের বাহিরে কোন খরচ নাই বা আপনাকে কোন টাকা পয়সা দেয়া হবে না ।

গোপনীয়তা ঃ

এই গবেষনার সময় আপনার যাবতীয় তথ্য অত্যন্ত যত্নের সাথে গোপন রাখা হবে । আপনার আইডি নম্বর সম্বলিত সব ধরনের কাগজপত্রে আপনার নাম ও ঠিকানা বসিয়ে গোপনীয়তার সাথে রাখ্া হবে এবং ব্যক্তিগত বিষয়াদি তথ্য বিশ্লেষন, প্রতিবেদন, তৈরীতে এবং প্রকাশনার কাজে ব্যবহার কাজে ব্যবহার হবে না এবং গবেষনার পরীক্ষক ব্যতীত কারো কাছে প্রকাশ করা হবে না ।

স্বেচ্ছামূলক অংশ গ্রহন ঃ

এই গবেষনায় আপনার অংশগ্রহন সম্পূর্ণ স্বেচ্ছামূলক । আপনি গবেষনায় অংশগ্রহনে অস্বীকৃতি জানাতে পারেন অথবা গবেষনা থেকে নিজেকে প্রত্যাহার করে নিতে পারেন । এই ফরমে স্বাক্ষর করলে আপনার আইনগত কোন অধিকার খর্ব হবে না ।

প্রশ্নাবলী ঃ

যদি আপনার কোন প্রশ্ন থাকে তবে দয়া করে জিজ্ঞেস করুন । আমরা তার উত্তর প্রদান করার যথাসাধ্য চেষ্টা করব। যদি ভবিষ্যতে আপনার অতিরিক্ত কোন প্রশ্ন থাকে তাহলে গবেষনারত ইসরাত আরা হোসেন, -এ যোগাযোগ করতে পারেন ।

সম্মতির স্বীকারোক্তিঃ

আমি গবেষনায় নিয়োজিত চিকিৎসক এর সাথে এই গবেষনা নিয়ে আলোচনায় সন্তুষ্টি প্রকাশ করছি । আমি এটা বুঝেছি যে গবেষনায় অংশগ্রহন স্বেচ্ছামূলক এবং আমি যে কোন সময় কোন বাধ্যবাধকতা ছাড়াই গবেষনা থেকে নিজেকে বিরত রাখতে পারি । আমি উপরোক্ত শর্তগুলো পড়েছি/আমার সম্মুখে পঠিত হয়েছে এবং স্বেচ্ছায় গবেষনায় অংশগ্রহন করতে সম্মতি জ্ঞাপন করছি।

অংশগ্রহণকারীর স্বাক্ষর / টিপসই (নিরক্ষর হলে)

.....

নাম ঃ গবেষণাকারীর স্বাক্ষর ঃ

পিতা / স্বামীর নাম ঃ

ঠিকানা ঃ

তারিখ ঃ

Appendix I*a*

INFOFRMED CONSENT FORM

Name of the title: Non alcoholic fatty liver disease in prediabetes and its association with insulin resistance and subclinical inflammation

Name of Principal Investigator: Israt Ara Hossain, Lecturer, Dept of Biochemistry & Cell Biology, BUHS, Dhaka.

I have clearly informed that this project work has been conducted for the welfare of prediabetes patients. I also get clear concept on the pattern and notion of the study. I confirmed that after enrolled myself in this assignment I did not face any threat both physically and mentally moreover, I will be benefited after getting the test results about my physical condition. In spite of this, if I faced any threat then the investigator will be liable to compensate that. I am also ascertained that the collected identity of patients and others information should all be kept in confidential.

I understand that I will be given any opportunities about the purpose of the study and in addition of these, if I seek any question then I can easily contact with the investigator.

I could withdrawal my consent at any time from this project and for this I won't face any query.

I agree to participate in this project work with willingly and sensibly.

.....

Signature of the participant/hand mark (if illiterate)

.....

Name: Signature of Investigator

Father/Husband's name:

Address:

Date:

Appendix II

Case Record Form

Sl No. FL- **Date:**

1. a. Name: _____

b. Father's / Husband's Name: _____

c. Mother's Name: _____

2. Age: _____years **3. Gender:** Male/ Female

4. Marital status: Married_____yrs/ Unmarried

5. Area: Rural/ Urban/ Semi urban

6. Address

Permanent:_____

_____

7. Phone: Off: _____ Cell: _____

Res: _____ Tnt: _____

8. Socioeconomic status

- Earning capacity (1. Earner 2. Dependent) _____
- No of earners in the family _____
- No of family members sharing the same kitchen_____
- Totally family monthly expenditure _____

9. Educational status: _____ yr/ yrs

10. Occupational status

Present occupation: _____ Duration: _____

History of past occupations: _____ Duration: _____

11. Religion: Muslim/Hindu/Christian/ Buddhist

12. Any history of past illness or any chronic diseases

a. Subject: IFG/ IGT/ IFG-IGT/ Normal

b. Patient history: Hypertension_____ Diabetes_____

Renal disease_____ Cardiac disease_____

Hepatic disease_____

GDM in previous pregnancy_____

No. of child: _____Before 28 weeks of gestation

After 28 weeks of gestation

c. Family history: Hypertension_____ Diabetes_____

Renal disease_____ Cardiac disease_____

Hepatic disease_____

13. Physical Activity Level (minutes per day)

At Work: _____At Home: _____During Leisure Time: _____m/d

14. History of addiction

a. Smoking: Yes/ No (if yes; duration _____ yrs; Average ____ sticks/ day)

Past history: Yes/ No (if yes; duration _____yrs; Average ____ sticks/ day)

b. Tobacco leaf chewing/ using powder (Gull): Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

c. Betel leaf chewing: Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

d. Tobacco Powder sniffing: Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

e. Drinking alcohol: Yes/ No (if yes; duration _____ yrs)

Past history: Yes/ No (if yes; duration _____ yrs)

15. History of medication

a. Present medications:

b. Past medications:

16. Physical examinations

A. Anthropometry

a. Height (cm): _____b. Weight (Kg): _____c. BMI (kg/ m^2): _____

d. Skinfold thickness (mm): _____ e. Triceps: _____ f. Subscapular: _____

g. Waist circumference (cm): _____ h. Hip circumference (cm): _____

i. Waist/ Hip ratio: _____ j. Percent body fat: _____

k. Free fat mass (FFM): _____ l. Change in body weight in last 3 months: _____

B. Blood pressure (BP) in mm Hg: SBP_____ DBP_____

17. Investigations

Sl No.	Biochemical parameters	Result	Result in SI unit	Reference Range
1	S Glucose (F)	mg/dl	mmol/L	3.6- 5.6
2	S Glucose 2 hrs after 75 gm glucose load	mg/dl	mmol/L	<7.8
3	S Cholesterol	mg/dl	mmol/L	150- 200
4	S HDL-C	mg/dl	mmol/L	M:>55;F:>65
5	S LDL-C	mg/dl	mmol/L	<150
6	S TG	mg/dl	mmol/L	50- 150
7	S Creatinine	mg/dl	mmol/L	0.67- 1.2
8	S GPT	U/L		Upto 40

Appendix III

Calculation of sample size:
N > 50+8m

(Here N=Sample Size; m= number of predictors (which was 8);

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17.4 How many subjects?

The ratio of number of cases to TVs has to be substantial otherwise the solution will be perfect and meaningless! If there are more IVs than cases, one can find a regression solution that completely predicts the DV for each case, but only as an artefact of the ratio of cases-to-IVs.

How many subjects does it require to do a regression analysis? The answer is that it depends on the desired power (l-(3). significance level (a), the number of predictors and the expected effect sizes.

A simple answer is N > 50 + 8m, where m is the number of IVs for testing the multiple correlation and N > 104 +m for testing individual predictors. For example, if you plan 5 predictors, you need 50 + 8(5) = 90 cases and 104 + 5 = 109 cases for testing individual predictors. If you arc interested in both the overall correlation and the individual IVs, calculate N both ways and choose the larger number of cases. These calculations are based on significance level of 5% (a=0.05) and 80% power (p = 0.20).

A higher cases-to-IV ratio is needed when the DV is skewed, or when a small effect size is anticipated or substantial measurement error is expected from unreliable variables. A more complex rule that takes into account size effects N > (8//~^{2}) + (m - 1), where/^{2} » 0.01, 0.15 and 0.35 for small, medium and large effects, respectively. More precisely estimated effect sizes can be calculated by/^{2} = R^2/(l - R^2), where R^2 is the coefficient of determination.

If step wise multiple regression is to be used, yet more cases are needed and a case-to-IV ratio of 40 to 1 is reasonable. If you cannot measure as rhany cases as you would like you can delete some IVs or create one IV that is a composite of several others and then use the new composite IV in the analysis in place of the original IVs.

It is also possible to have too many cases. As the number of cases becomes quite large, almost any

multiple correlation will depart significantly from zero, even one mat predicts negligible variance in theDV.

It is essential that you verify that the analysis included as many cases as you think it should have, because, by default, regression programs delete cases for which mere are missing values on any of the variables. This can lead to substantial loss of cases. If you have missing values you can choose to estimate them rather than delete the cases.