The Role of *Anopheles* Heme Peroxidases in Mosquito Immunity against Blood-Borne Antigens and Divergent Expression of anopheline Lineage-Specific Duplicated Genes

THESIS

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PARIK KAKANI

Under the Supervision of
Prof. Sanjeev Kumar
and
Co-supervision of
Dr. Pankaj Kumar Sharma

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CERTIFICATE

This is to certify that the thesis entitled **"The Role of *Anopheles* Heme Peroxidases in Mosquito Immunity against Blood-Borne Antigens and Divergent Expression of anopheline Lineage-Specific Duplicated Genes"** submitted by **Parik Kakani** ID No **2012PHXF0431P** for award of Ph.D. degree of the institute embodies original work done by her under my supervision.

Signature (Supervisor)

Name **SANJEEV KUMAR, Ph. D.**

Designation **Associate Professor, Department of Biological Sciences, BITS, Pilani**

Present address **Professor and Head, Department of Biotechnology**

Ch. Bansi Lal University (CBLU), Bhiwani, Haryana

Date:

Signature (Co-supervisor)

Name **PANKAJ KUMAR SHARMA, Ph. D.**

Designation **Assistant Professor, Department of Biological Sciences, BITS, Pilani**

Date:

ABSTRACT

Malaria, a vector-borne disease, affects approximately 3.2 billion people worldwide each year. The transmission of this disease is initiated by an infectious bite of female *Anopheles* mosquito which carries the parasite of genus *Plasmodium*. Malaria parasite requires human host to complete its asexual life cycle and female *Anopheles* to complete its sexual life cycle. A number of *Anopheles* species transmit human malaria parasite and in India, *Anopheles stephensi* is one of the major urban malaria vector.

The mosquito feeds on plant nectar. However, female mosquito requires blood meal for the development of eggs. During the blood meal, female *Anopheles* accidentally encounters the malaria parasite, *Plasmodium*. Several body compartments of mosquito are involved in the *Plasmodium* development and midgut is the first compartment that the parasite encounters in the mosquito. *Plasmodium* interacts with several molecules of mosquito midgut during its development. Molecules that positively regulate the development of *Plasmodium* are called as agonists. Also, *Plasmodium* undergoes massive losses during its development in the mosquito midgut and faces a sizeable bottleneck, suggested that midgut epithelial cells mount a potent immune response against it. Mosquito molecules that negatively regulate the *Plasmodium* development are known as antagonists. Currently, *Plasmodium* developmental stages of midgut are targeted to control malaria transmission. One such method is the development of vaccines against malaria in mosquito and known as Transmission Blocking Vaccines. Midgut-specific molecules can be used as vaccine candidates to control malaria transmission. Hence, targeting midgut molecules which are crucial for successful development of *Plasmodium* inside the mosquito host will prevent malaria transmission. Recently, in *Anopheles gambiae*, heme peroxidases such as HPX15, HPX2 and Duox are reported in modulating the midgut immunity. Functional studies of these heme peroxidases in *A. stephensi* may provide an opportunity to target these molecules as vaccine candidates in developing transmission blocking strategies.

In this thesis work, we carried out the molecular characterization of heme peroxidase gene HPX2 from Indian malaria vector *A. stephensi* and showed that it plays a crucial role in maintaining bacterial homeostasis and in limiting *Plasmodium* development inside the mosquito midgut. The AsHPX2 is a secreted protein of 692 amino acids. The orthologs of AsHPX2 are only present in mosquitoes. The expression

of this gene is reduced in blood fed midguts. RNAi based silencing of AsHPX2 gene in sugar fed midguts showed increased bacterial load. So, this gene is involved in maintaining the midgut bacterial homeostasis in the sugar fed mosquitoes. Silencing of AsHPX2 gene increased *Plasmodium* oocysts number and exhibited the anti-plasmodial property in the midguts in a way similar to its ortholog AgHPX2 in *A. gambiae*. In *A. gambiae* it is reported that HPX2 enhanced the clearance of *Plasmodium* ookinetes during midgut invasion. Thus, AsHPX2, a mosquito-specific gene may be targeted to design such strategy that can arrest *Plasmodium* development inside the mosquito.

Studies exploring the mosquito's innate immune defense mechanism against *Plasmodium* and detailing the importance of the midgut microbiota in vector competence may contribute towards the development of effective control strategies. In our study, we characterized Dual Oxidase (Duox) gene in Indian malaria vector and showed that it plays a crucial role in bacterial homeostasis and also in *Plasmodium* development in the mosquito midgut. Duox in *A. stephensi* is a transmembrane protein with N-terminal cytoplasmic heme peroxidase domain and a non-cytoplasmic NADPH oxidase domain at C-terminal. In addition, it also has a calcium binding domain and seven transmembrane domains. The AsDuox ortholog, AgDuox in *A. gambiae* performs tyrosine crosslinking of a mucin layer in the midgut. This mucin layer acts as a physical barrier and protects midgut commensal bacteria and *Plasmodium* against midgut immunity. Silencing of AsDuox in either sugar fed or blood fed midguts revealed increased endogenous bacterial growth. Thus, we assumed that AsDUOX gene plays a dual role, on one hand, it protects the bacteria from midgut immunity by creating low immunity zone through midgut barrier formation, and on the other hand, it controls their over-growth due to its anti-bacterial nature. The expression of this gene is induced in exogenous bacteria supplemented blood fed midguts and has a strong negative correlation with the growth of bacteria in these midguts. This indicates that Duox is one of the major molecules of midgut immunity.

AsDuox gene also plays important role in *Plasmodium* development and silencing of this gene suppressed *Plasmodium* oocysts number through activation of TEP1 (Thioester-containing proteins) molecules. Previously, in *A. gambiae,* it has been reported that Duox gene supports the development of *Plasmodium* and silencing of AgDuox reduced the number of *Plasmodium* oocysts. These findings explored that agonist role of Duox is conserved in both *A. stephensi* and *A. gambiae*. Thus, Duox gene is an important molecule of innate immunity against pathogens in *Anopheles*. Hence, this molecule might serve as a universal target to manipulate mosquito immunity

and midgut bacterial population that can be used to arrest *Plasmodium* development inside the vector host.

Heme peroxidases belong to multi-gene family in which gene duplication event is very common. So, we were interested in studying the gene duplication event in heme peroxidase family of *Anopheles*. We found that previously reported heme peroxidase HPX15, a transmission blocking vaccine candidate, in *A. gambiae* and *A. stephensi* has its tandemly duplicated paralog named HPX14. The duplicated genes are are flanked by the presence of boundary elements and might act as an independent domain of gene expression. We found that duplicated genes are under the purifying selection and hence, might maintain two distinct functional copies. We found that both the genes are functional and the mRNA levels of AsHPX15 gene are higher than AsHPX14 gene in the midguts. However, the spatial and temporal expression of AsHPX14 gene might be suppressed by CTCF (CCCTC-binding factor), an insulator protein. To reveal the function of AsHPX14 gene, we silenced the AsHPX15 gene in the midguts. We found that expression of AsHPX14 gene is induced in AsHPX15 silenced bacteria supplemented blood fed midguts but not in AsHPX15 silenced blood fed midguts against respective controls. The high bacterial load in absence of barrier formation (silencing of AsHPX15) cause some signal to displace CTCF protein and induce the expression of AsHPX14 gene. This data suggested that HPX14 gene may have a role in immunity against bacteria but not in physiology. Hence, we conclude that there is no redundancy in the function of duplicated gene. This strengthen the potentiality of AsHPX15 as a vaccine candidate to block *Plasmodium* transmission. This study suggests the potential functional roles of CTCF in the mosquito. This can be used to improve mosquito transgenesis. This can also provide a new model for the study of CTCF function in a species with medical significance. CTCF can be explored in managing and regulating genome-wide chromatin architecture and gene expression.

This thesis work contributes to a better understanding of the mosquito immune system to the malaria parasite, mosquito midgut microbiota interactions, mechanisms that maintain midgut homeostasis and chromatin organization of two duplicated heme peroxidases involved in the immunity. It would be of great interest to study the exact molecular mechanism that controls the expression of these heme peroxidases in the midgut. In future, functional study of these heme peroxidases in different anophelines may explore their use for widespread control of malaria.

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ACC	American College of Cardiology
ADA	American Diabetic Association
AGES study	Age, Gene/Environment Susceptibility study
AHA	American Heart Association
Apo	Apolipoprotein
AQPap	Aquaporin adipose
Arbiter 6-HALTS	Arterial Biology for the Investigation of the Treatment Effects of Reducing Cholesterol 6-HDL and LDL Treatment Strategies in Atherosclerosis
ATP-III	Adult Treatment Panel
AUC	Area under the curve
BMI	Body mass index
BP	Blood pressure
CAD	Coronary artery disease
CE	Cholesteryl ester
CETP	Cholesteryl ester transfer protein
CHD	Coronary heart disease
ChREBP	Carbohydrate-responsive element-binding protein
CI	Confidence interval
CMR	Cardiometabolic risk
CRP	C-reactive proteins
CT	Computed tomography
CVD	Cardiovascular disease
DBP	Diastolic blood pressure
DNP	Dinitrophenol
ELISA	Enzyme linked immunosorbent assay
FFA	Free fatty acid

Fox-1	Forkhead Box 01
HbA1C	Glycosylated haemoglobin
HDL-C	High density lipoprotein cholesterol
HMG-CoA	3-hydroxy-3-methyl-glutaryl-CoA reductase
HOMA	Homeostatic model assessment
HTGL	Hepatic triglyceride lipase
HU	Hounsfield Unit
IDF	International Diabetes Federation
IDL	Intermediate density lipoprotein
IHF	Indian Heart Foundation
IL-6	Interleukin-6
INSPIRE ME IAA	The International Study of Prediction of Intra-abdominal Adiposity and Its Relationship with Cardiometabolic Risk/Intra-abdominal Adiposity
IRS	Insulin receptor substrate
LDL-C	Low density lipoprotein cholesterol
LMCs	Low and middle income countries
LpL	Lipoprotein lipase
MANCOVA	Multivariate analysis of covariance
MR	Magnetic resonance
MRI	Magnetic resonance imaging
MRS	Magnetic resonance spectroscopy
MS	Metabolic syndrome
MTP	Microsomal transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCEP	National Cholesterol Education Program
PAI-1	Plasminogen activator inhibitor-1

PI3	Phosphoinositol-3
PkC	Protein kinase C
PROCAM	The Prospective Cardiovascular Munster study
ROC	Receiver operating curve
SBP	Systolic blood pressure
Se	Sensitivity
SERPINS	Serine protease inhibitors
Sp	Specificity
SREBP	Sterol regulatory element-binding proteins
TC	Total cholesterol
T2DM	Type 2 diabetes mellitus
TG	Triglycerides
TNF-α	Tumour necrosis factor- alpha
tPA	Tissue type plasminogen activators
uPA	Urokinase type plasminogen activators
US	Ultrasonography
VAD	Visceral adiposity dysfunction
VAHIT	Veterans Affair HDL Intervention Study
VAI	Visceral adiposity index
VLDL	Very low density lipoprotein
WHO	World Health Organization
+LHR	Positive likelihood ratio
-LHR	Negative likelihood ratio
PPV	Positive predictive value
NPV	Negative predictive value

CHAPTER 1 INTRODUCTION

1.1 Introduction

In Indian context, cardiovascular disease (CVD) and diabetes are increasingly becoming an epidemic. Out of more than 10.5 million deaths reported annually, 18% (males 20%, females 17%) deaths were due to CVD, corresponding to 1.8 million deaths annually. In adults aged 25-69 years, more than 25% of deaths, most of them premature, were due to CVD. Further, it is reported that risk of acute myocardial infarction (MI) due to known diabetes was 33%, implying that diabetes is a major factor that contributes to these deaths (Gupta, 2010). Despite availability of standard treatment, the 'residual risk' of vascular events such as atherosclerosis in patients with diabetes still persists. Although statin use reduces the levels of LDL-C (Low Density Lipoprotein-Cholesterol), there is only a moderate reduction in total mortality (Fruchart et al., 2008; Fruchart et al., 2013). The cause and effect relationship between diabetes and CVD is controversial. Both are caused by similar abnormal lifestyles and lead to similar end-stage disease in form of widespread blockages of large and small vessels (Gupta, 2010). American Diabetes Association (ADA) and American Heart Association (AHA) introduced the term 'cardiometabolic risk' (CMR) to designate the risks of diabetes and CVD. CMR signifies the overall risk of developing type 2 diabetes mellitus (T2DM) and/or CVD including MI and stroke, which is due to a cluster of modifiable risk factors. In addition to well known classical risk fa...rs (such as smoking, raised LDL-C, hypertension, elevated blood glucose), modifiable risk factors include the emerging risk factors closely related to abdominal obesity, such as insulin resistance, reduced high density lipoprotein cholesterol (HDL-C), high triglycerides (TG) and inflammatory markers.

Atherogenic dyslipidemia is a clinical condition characterized by elevated levels of serum TG levels and LDL particles with low levels of HDL-C. It is often observed in patients with obesity, insulin resistance and T2DM; hence also referred as either diabetic dyslipidemia or dyslipidemia of metabolic syndrome (MS) and is considered as an important CVD risk factor. It may even be a factor responsible for coronary artery

disease (CAD) occurring before other major complications such as diabetic retinopathy or nephropathy (Manjunath et al., 2013).

Obesity is often associated with the development of insulin resistance and has been linked to pathogenesis of T2DM and CVD. The link between obesity and atherogenic dyslipidemia, both in absence and presence of insulin resistance, is not fully understood (Bamba and Rader, 2007). Nonetheless, adverse metabolic sequels are not uniformly observed in obese individuals. It is reported that about 30% of obese men and women are metabolically healthy, that is, do not have hypertension, dyslipidemia, or disturbances in glucose metabolism (Snel et al., 2012).

In general, it is accepted that variance in CVD risk factors can be better explained by obesity represented by increased abdominal and liver fat as compared to obesity represented by body mass index (BMI) (Speliotes et al., 2010; Smith et al., 2013). Adipose tissue serves to conserve energy by storing TGs as reserve fuel. Subcutaneous adipocytes account for around 80% of the total body fat, followed by visceral adipose tissue (around 10%) and non-adipose tissues or ectopic sites (approximately10%) such as liver, skeletal muscles etc. (Roden, 2006; Ghosh, 2014). Ectopic sites have no capacity to store excess fat without causing harm (Snel et al., 2012), which strengthens the fact that distribution of fat considerably accounts for metabolic heterogeneity of obesity (Marinou et al., 2014). Liver, one of the major sites of ectopic fat storage, plays a pivotal role in maintaining energy homeostasis during fasting-fed transitions & in buffering carbohydrate load by suppression of hepatic glucose production and promoting liver glycogen deposition (Savage et al., 2007). Liver fat can be derived from: (i) adipose tissue lipolysis leading to increased free fatty acid (FFA) release (ii) dietary chylomicrons (iii) *de novo* lipogenesis in liver. In general, dietary chylomicrons are the major source of liver fat but in patients with increased visceral adiposity, adipose tissue lipolysis contributes most to increased liver fat (Marra and Lotersztajn, 2013).

Several studies using Computed Tomography (CT) & Magnetic Resonance Imaging (MRI) have reported direct and independent correlation between visceral and liver fat

content while subcutaneous adipose tissue has not been related to liver fat content. This may be due to unique anatomical location of visceral fat providing FFAs & adipokines to the liver through portal blood flow (Gastaldelli and Basta, 2010; Fabbrini et al., 2009). However, if visceral fat was a major contributor to metabolic risk, visceral adipose tissue should be the major source of systemic FFA flux to the liver via portal vein, which is not the case. Visceral fat contributes to only 15% of the total systemic FFA whereas the majority of FFA are contributed by non-splanchnic adipose tissue. This observation raises the doubt over considering visceral adipose tissue being major contributor of metabolic abnormalities (Patel and Abate, 2013). High liver fat causes hepatic insulin resistance leading to fasting hyperglycemia, loss of post-prandial suppression of gluconeogensis and dyslipidemia. Increased lipid availability leads to overproduction of TG rich very low density lipoprotein (VLDL) particles. A high liver fat content can, by itself, largely explain the hyperinsulinemic, hyperglycemic, hypertriglyceridemic, and elevated apolipoprotein B (ApoB) dysmetabolic state independent of contribution from visceral adipose tissue. Thus, studying the causes and metabolic consequences of hepatic fat has become a major field of research for scientists investigating the pathogenesis of T2DM (Stefan et al., 2011). In addition, body fat distribution has been reported to be associated with altered postprandial lipid metabolism or postprandial lipemia (Nabeno-Kaeriyama et al., 2010). Postprandial lipemia refers to a transient increase in blood lipids, particularly TG, which occurs after fatty meal. Zilversmit first proposed that postprandial hypertriglyceridemia to be the most common risk of atherogenesis (Nakano et al., 2011). Studies have shown that postprandial TG is an independent predictor of coronary heart disease (CHD) even when adjusted for fasting TG or HDL-C (Nabeno-Kaeriyama et al., 2010). Approximately, 80% of TG increased in postprandial plasma was TG derived from remnant lipoprotein (Enkhmaa et al., 2010). Patients with T2DM tend to have higher total TG levels after eating a meal compared with people with normal glucose tolerance (Van Dieren et al., 2011).

In clinics or laboratory settings postprandial lipid kinetics can be studied by oral fat load test. Typically, the oral fat load includes the administration of a test meal or drink with a high fat content (e.g. 1 g/fat per kilogram of body weight) after an overnight fast. The increase in postprandial TG levels depends on the amount of dietary fat in the test meal; a very low (5 g) or low (<15 g) dose of dietary fat generally does not increase postprandial TG; moderate doses (30–50 g) dose dependently increase postprandial TG, whereas very high doses (>80 g) result in an exaggerated postprandial TG response (Enkhmaa et al., 2010).

Further, alterations in plasma lipoprotein-lipid concentrations are known to increase the risk of CAD in both men and women. However, at all ages, the prevalence of CAD in women is lower than in men, and the gender difference in plasma lipoprotein-lipid levels as well as in the prevalence of T2DM are believed to be responsible, at least in part, for the higher CAD risk observed in men. An increased visceral fat area has been reported in men compared with women and this factor could also contribute to the gender difference in the CAD risk profile (Couillard et al., 1999).

CHAPTER 2 LITERATURE REVIEW

2.1 Concept of cardiometabolic risk

Preclinical and clinical studies in the field of cardiovascular medicine have enhanced understanding of non-modifiable and modifiable risk factors for CVD. In 1968, deaths from CHD were at peak and declined significantly in later years. Data suggests that, around 44% of the reduction was due to treatment of cardiovascular risk factors such as hypercholesterolemia, hypertension, smoking, and physical inactivity. This was indeed achieved because of the attention driven towards the unmet need of treating the disease and by addressing the risk factors both at the clinical level and through public health policies.

However, the current overconsumption of processed and energy-dense food products of poor nutritional value combined with sedentary lifestyle contributed to the emergence of new drivers of CVD risk: obesity and T2DM. This has partially counterweighted the progress being made towards improvement in cardiovascular outcomes (Chiha et al., 2012; Despres, 2012). World is undergoing rapid transition; urbanization and economic growth continues to catalyse sporadic changes in diet and life style that promote positive energy balance. Low-income and middle-income countries (LMCs) like those in Sub-Saharan Africa and South-East Asia, including India, currently encompasse the majority of patients with diabetes and CVD and are predicted to continue to do so in future decades due to inadequate medical attention (Kelly et al., 2008; Malik et al., 2012; Cappuccio, 2014).

Dyslipidemia associated with T2DM is characterized by hypertriglyceridemia, raised small and dense LDL particles, which are more atherogenic than the more buoyant forms and low HDL-C. Compared to non-diabetics, patients with T2DM are more predisposed to CV mortality and morbidity due to a complex combination of various risk factors. Many of these risk factors could have a common history for both diabetes and CVD. And thus, it has been hypothesized, that both disorders (T2DM and CVD) come independently from a “common soil” (Martin-Timon et al., 2014).

The prevalence of diabetes has increased substantially over last few decades. According to World Health Organization (WHO) report, from 171 million cases in year

2000, the number has dramatically increased to 366 million in 2011; half of these are undiagnosed. Further, the total number of people with diabetes mellitus is projected to be 552 million by the year 2030. Around 80% of these affected people live in LMCs (Martin-Timon et al., 2014; Ramachandran et al., 2014).

In 1988, Reaven described the insulin resistance syndrome or syndrome X, now called the metabolic syndrome. It was originally defined by the presence of hyperinsulinemia, varying degrees of glucose tolerance, hypertriglyceridemia and low plasma HDL-C concentration (Reaven et al., 1988). Remarkably, obesity or waist circumference was not part of the original definition till 2001. For the purpose of unifying the definition and optimizing clinical utility, Adult Treatment Panel (ATP) III of the National Cholesterol Education Program (NCEP) came out with working definition for the MS to be characterized by central obesity, hypertriglyceridemia, low plasma HDL-C, hypertension and dysglycemia, i.e. impaired fasting glucose (NCEP ATP III, 2001).

NCEP-ATP III committee members recognized the importance of insulin resistance as a core metabolic abnormality associated with a constellation of atherogenic and diabetogenic risk factors/markers, but they also emphasized that the most prevalent form of insulin resistance in clinical practice was abdominal obesity. In patients likely to have excess abdominal fat, the committee recommended that attention be paid to five parameters (waist circumference, TG, HDL-C, fasting glycaemia, blood pressure) in order to identify individuals with MS, prioritizing waist circumference over BMI when estimating the amount of abdominal fat. This recommendation to measure waist girth in clinical practice was a giant conceptual leap forward (Grundy, 2005; Despres et al., 2006; Vidal and Jimenez, 2016).

The criteria of MS are interrelated, but the pathophysiology of their relation is not yet fully understood. The long-standing debate about how to define this syndrome led to the appearance of two distinct schools of thought: the insulin resistance-based and the ectopic fat deposition-based hypothesis. Both suggested mechanisms remain equivocal and debated.

ADA and AHA introduced the term 'cardiometabolic risk' (CMR) to designate the risks of diabetes and CVD. CMR signifies the overall risk of developing T2DM and/or CVD including MI and stroke, which is due to a cluster of modifiable risk factors. In addition to well known classical risk factors (such as smoking, raised LDL-C, hypertension, elevated blood glucose), modifiable risk factors include the emerging risk factors closely related to abdominal obesity, such as insulin resistance, reduced HDL-C, high TG and inflammatory markers. CMR is based on the concept of the risk continuum. Abdominal obesity has been linked strongly to multiple CMR factors such as atherogenic dyslipidemia (hypertriglyceridaemia and low HDL-C), elevated blood glucose, insulin resistance and inflammation, which are major drivers of CVD and T2DM (Figure 1) (Vasudevan and Ballantyne, 2005; Gelfand and Cannon, 2006; Neeland et al., 2012).

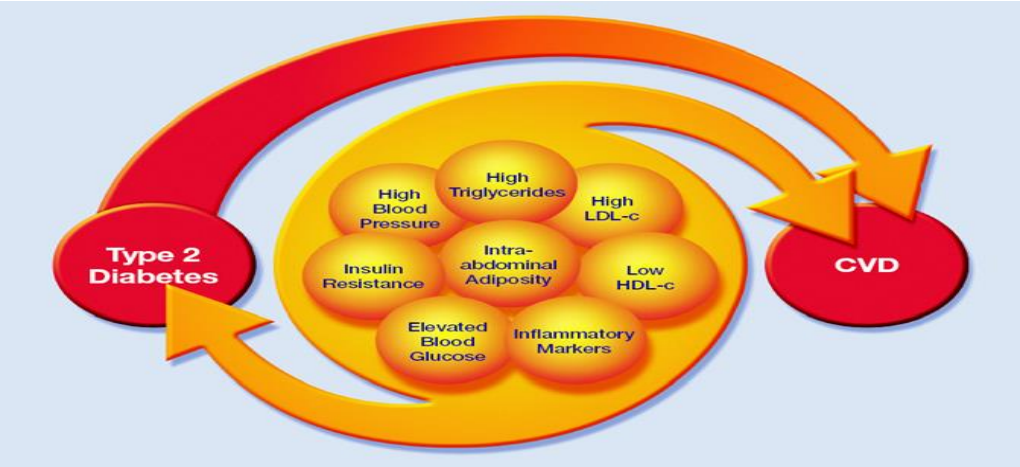


Figure 1: Concept of global cardiometabolic risk

(Gelfand and Cannon, 2006)

2.2 Pathogenesis of cardiometabolic risk

CMR is multifactorial in its origin and its pathogenesis is underlined by two tightly intertwined conditions: obesity and insulin resistance. These conditions can be attributed to be the "common soil" for CMR (Grundy et al., 2005; Enas et al., 2007).

While it still remains debatable as to which, obesity or insulin resistance, plays a pivotal role in the pathogenesis of CMR, some researchers have deciphered obesity as the most essential factor in the pathogenesis of cardiometabolic anomaly. Patients

with obesity or insulin resistance show specific lipid abnormalities, i.e. hypertriglyceridemia, low HDL-C and raised non HDL-C with either normal or elevated LDL-C levels. Such dyslipidemic profile in turn promotes atherosclerosis and further contribute to the residual CVD risk observed among these patients even after attaining LDL-C reduction to treatment goals with statin therapy and optimum treatment of other comorbidities (Boden et al., 2011). T2DM patients are often diagnosed with such lipid abnormality but the prevalence varies with population and with varying degree of abdominal adiposity (Taskinen, 2005; Nazare et al., 2012; Warraich et al., 2015).

2.2.1 Adiposity, Insulin Resistance and Atherogenic Dyslipidemia

'Atherogenic lipid triad' is recognized as the common culprit for predisposing CV risk. It refers to (a) raised TG values (> 150 mg/dl); (b) low HDL-C (<50 mg/dl in females and < 40 mg/dl in males) values; (c) increased LDL particles which are smaller and denser than normal. This lipid phenotype has also been defined as atherogenic dyslipidemia (Rashid et al., 2015).

Interestingly, raised LDL-C level is not a distinct feature of T2DM or obesity (Chaudhary et al, 2012; Grundy, 2015). The extent to which TG directly promotes disease or represents a biomarker of risk has been debated for decades. The largest and most comprehensive meta-analysis included 29 prospective studies and 262,525 participants, proving a strong and highly significant association between TG and coronary risk. Adjustment for HDL-C attenuated the magnitude but did not abolish the significant association between TG and coronary risk (Sarwar et al., 2007).

Distinguishing feature of insulin sensitive phenotype includes a normal body weight without abdominal or visceral obesity, being moderately active, and consuming a diet low in saturated fats. Alternatively, insulin-resistant individuals need not be clinically obese; but they nevertheless commonly have an abnormal fat distribution. Regardless of the relative contributions of visceral fat and abdominal subcutaneous fat to insulin resistance, a pattern of abdominal obesity correlates more strongly with the insulin resistance than lower body obesity (Kaur, 2014). By definition, insulin resistance is a pathophysiological condition in which a normal insulin concentration does not

adequately produce a normal insulin response in the peripheral target tissues such as adipose, muscle, and liver (van der Valk et al., 2014; Teixeira et al., 2015). Insulin resistance appears to play an important role in the development of atherogenic dyslipidaemia. It is associated with enhanced lipolysis brought about by variety of lipases, including lipotprotein lipase (LpL), hormone-sensitive lipase and endothelial lipase as well as reduced FFA uptake and esterification leading to an increased FFA level in the circulation which in-turn enhance FFA flux into non-adipose tissues, including the liver and muscle (Klop et al., 2013; Patel & Abate, 2013; Tchernof and Despres, 2013; Kaur, 2014; van der Valk et al., 2014). Raised FFA pool causes accumulation of TG in liver and also competes with glucose for its uptake in skeletal muscle (Johnson and Olefsky, 2013; Crist et al., 2015). Increase in liver fat also leads to enhanced production of VLDL cholesterol, further leading to raised FFA and TG flux into skeletal muscles and other tissues, further inducing insulin resistance and increasing lipolysis in adipose tissue (Grundy, 2006; Saponaro et al., 2015). Thus, a vicious cycle is instituted as it results in hepatic steatosis which, in turn, exacerbates hepatic and peripheral insulin resistance (Mahajan et al., 2015).

Insulin resistance and hypertriglyceridemia is associated with formation of small and dense LDL particles (Gerber et al., 2013; Stahlman et al., 2013). In hypertriglyceridaemic states, large TG-rich VLDL molecules accumulate (Figure 2). When VLDL is lipolysed by LpL, a population of LDL particles with changed apoB conformation is produced. These particles fail to bind efficiently to LDL receptors and have a prolonged residence time in the circulation. Cholesteryl ester transfer protein (CETP) is secreted by the adipose tissue and its activity and mass is increased in obese patients. CETP is an important determinant of lipoprotein composition because it facilitates transfer of cholesteryl esters (CE) from CE-rich lipoproteins to TG-rich lipoproteins in exchange for TG (Hovingh et al., 2015). This causes enrichment of HDL and LDL with TG, making them preferred substrate for hepatic triglyceride lipases (HTGL) (Klop et al., 2013). HTGL hydrolyses the TG and phospholipid present in TG-rich LDL resulting in formation of small dense LDL (Lopez-Rios et al., 2011). Thus, it seems

that the presence of large triglyceride-rich VLDL particle is a prerequisite for small dense LDL formation. However, small dense LDL is also observed in patients with T2DM and insulin resistance with close to normal TG levels. This might be explained by increased HTGL activity commonly seen in patients with T2DM (Taskinen and Boren, 2015). Small dense LDL is associated with higher vascular risk due to reduced LDL receptor mediated clearance, increased retention in the arterial wall and increased susceptibility to oxidation (Lamarche, 1998).

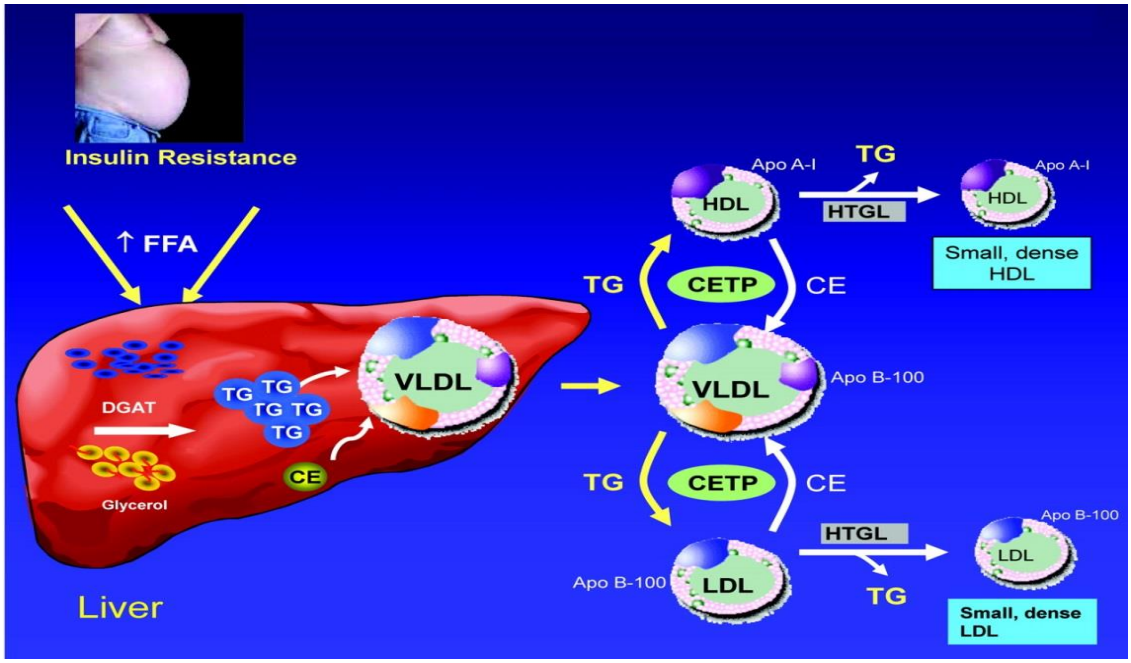


Figure 2: Metabolic consequences of insulin resistance and hypertryglyceridemia

ApoA-I - Apolipoprotein A-I; ApoB-100 - Apolipoprotein B-100; CE - Cholesteryl Ester; CETP – Cholesteryl Ester Transfer Protein; DGAT - Diacylglycerolacyltransferase; FFA - Free Fatty Acid; HDL - High-Density Lipoprotein; HTGL – Hepatic Triglyceride Lipase; LDL - Low-Density Lipoprotein; TG - Triglyceride; VLDL - Very Low-Density Lipoprotein. (Miller M et al., 2011)

Elevated VLDL levels also have its impact over the composition of HDL, again mediated by CETP and HTGL (Chapman et al., 2011). Apparently, decrease in circulating HDL levels in insulin resistant state has been linked to overproduction of TG-rich lipoprotein. Low HDL-C levels represent an independent risk factor for CVD (Athyros et al., 2004; Taskinen, 2005). In hypertriglyceridemia condition, CETP mediates an

exchange of CE and TG between TG-rich VLDL and HDL, making TG enriched HDL depleted of CE (Rashid et al., 2002). These small and dense HDL particles are prone to degradation through hydrolysis of their TG component by HTGL and further apolipoprotien A1 (ApoA1) in liver (Kakafika et al., 2006, Rashid et al., 2003). TG rich HDL lacks the ability to carry out reverse cholesterol transport effectively (Lewis et al., 2002). Alternatively, it is being hypothesized that insulin resistance driven change in FFA flux in the liver may reduce hepatic production of apoA1 and subsequent reduction in HDL assembly. Thus, both CETP and reduction in apoA1 are responsible for decrease in HDL-C mass and HDL particle size (HDL-3). It is proven that with increase in the number of CMR factors, the HDL phenotype shifts predominantly towards small HDL-3 while there is a reduction in large HDL-2 particles, resulting in a decreased HDL-2/HDL-3 ratio. In addition, HDL-2 levels and the HDL-2/HDL-3 ratio independently correlated positively with HDL-C and negatively with TG levels. HDL-3 concentration also positively correlates with both HDL-C and TG levels. This phenomenon may contribute to an impaired reverse cholesterol transport and to attenuated anti-atherogenic activity of HDL in patients predisposed to CMR (Lagos et al., 2009; Athyros et al., 2011).

Therefore, liver fat content correlates well with different components of atherogenic dyslipidemia and also explains why liver fat content in obesity seems to be a better marker of metabolic derangement and CVD risk than visceral obesity per se (Taskinen and Boren, 2015).

2.3 Existence of residual risk

For management of LDL-C, BP and glycaemia, standard treatment methodology is available. However, in predisposed patients, the “residual risk” of vascular events still persists (Pathak et al 2015). The current guidelines on cardiovascular risk reduction advocates use of 3-hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins) for managing dyslipidemia where a raised LDL-C level is considered to be the primary target in the management (NCEP ATP III, 2001; Grundy et al., 2004; Smith et al., 2006; Graham et al., 2007; Baigent et al., 2010). The gains from CVD

prevention over the last 4 decades are being challenged by a global epidemic of obesity and T2DM. Epidemiological data from the USA and UK show an unfavourable trend in CVD mortality in younger men and women (35 to 44 years), related to the obesity and T2DM epidemic (Mooradian, 2009; Athyros et al., 2011). Though statins hold a mainstay in management of CVD, trials demonstrated the relative risk reduction of 25-30% in cardiovascular events. It is not very clear, if lowering LDL levels beyond the specified limits would result in further residual risk reduction or whether decrement in risk is independent of lower LDL thresholds and attributable to secondary features, such as elevated fasting and postprandial TG-rich lipoprotein and decreased HDL-C levels (Joshi et al., 2015). Several studies such as Framingham, PROCAM and Arbiter 6-HALTS study suggest that risk reduction may pertain to a strategy of addressing residual dyslipidemia beyond LDL, and inclusive of treating/addressing the combination 'atherogenic dyslipidemia profile', that is the combination of a high TG and low HDL level (Hancu, 2012).

2.4 Determinants of cardiometabolic risk

The determinants can be risk determinant, risk factor or risk predictor depending on its causality. A risk determinant is defined as the variable that is directly or indirectly associated with outcome, regardless of the postulated causality. In case of a risk factor, the association with outcome is presumed to be causal and is the case of a risk predictor; the variable predicts the outcomes without necessarily conveying causality. A risk marker reflects not only the presence of a risk factor but also some form of susceptibility of the organism to the detrimental effects of this risk factor. A risk marker is associated with the disease (statistically) but needs not be causally linked, and it may be a measure of disease process itself (Vasan, 2006).

Conventional risk factors like high LDL-C, low HDL-C, high TG, hypertension, hyperglycemia and smoking can partly explain excess of CVD risk in individuals with T2DM (Martin-Timon et al., 2014). Ethnic variation in susceptibility to these CV risk factors have also led to identification of new emerging risk factors such as postprandial TG, ApoB, ApoA1, non-HDL cholesterol, plasminogen activator inhibitor (PAI)–1. These

have helped reclassify intermediate patient's risk for major CHD events, demanding more aggressive risk reduction. These emerging risk factors are measurable, improve CV risk prediction, and also assist clinician in making decisions concerning patients at increased risk (Gupta et al., 2013; Upadhyay, 2015). These are discussed below:

2.4.1 Postprandial triglycerides

Due to lifestyle changes, people spend most of their time in postprandial state. Postprandial lipaemia refers to the state of lipid metabolism between food intake and the post-absorptive state (Pirillo et al., 2014; Garcia-Rios et al., 2015). Postprandial hypertriglyceridemia is commonly seen in patients with insulin resistance, obesity and premature CVD (Sottero et al., 2015). It occurs even in subjects who are normolipidaemic in the fasting state. Reduced or slow postprandial clearance increases the residence time of chylomicron remnants in the circulation. This leads to entrapment of chylomicron remnants within the sub-endothelial space and the simultaneous stimulation of inflammatory reactions. Further, postprandial hypertriglyceridemia is also linked to increased oxidative stress, a contributor to endothelial dysfunction (Tiihonen et al., 2015).

Again, insulin resistance is supposed to be the underlying cause for resultant postprandial hypertriglyceridemia due to impaired clearance of TG-rich VLDL and chylomicrons as shown in figure 3. Antilipolytic effect of insulin in adipose tissue is diminished in insulin resistance, thereby increasing postprandial FFA levels. Mechanism underlying postprandial hypertriglyceridemia also includes a reduction in lipolytic enzyme activity, i.e. LpL and HTGL, increased production and higher plasma levels of apoC-III (an inhibitor of LpL) and the defective suppression of hepatic VLDL secretion (Athyros et al., 2011). LpL, HTGL and CETP govern the clearance of TG-rich lipoproteins. LpL is the predominant TG lipase and is responsible for hydrolyzing TG in chylomicrons and VLDL, whereas HTGL is both a phospholipase and a TG lipase and plays an important role in HDL metabolism and in the conversion of VLDL to LDL (Chatterjee and Sparks, 2011). With increased levels of VLDL and chylomicrons, they compete for the same LpL- and receptor-mediated TG clearance pathways. Thus, non-

suppressed VLDL secretion reduces the clearance of chylomicrons and their remnants. Postprandial hypertriglyceridemia also promotes the formation of small dense LDL and HDL particles due to increased residence time allowing for CE and TG exchange through CETP (K Nakajima et al 2011).

A number of studies suggest that postprandial TG concentrations are more strongly associated with CVD risk factors and mortality than the respective fasting values (Miller et al., 2011).

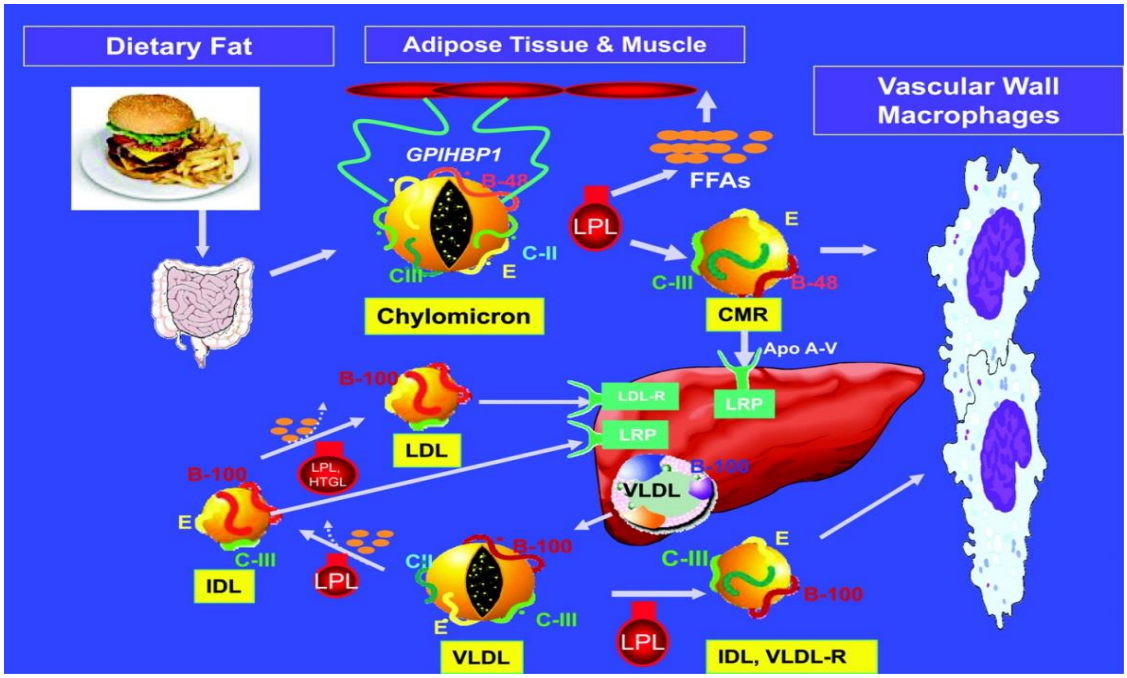


Figure 3: Post prandial triglyceride metabolism

Apo A-V indicates apolipoprotein A-V; CMR, chylomicron remnant; FFAs, free fatty acids; HTGL, hepatic triglyceride lipase; IDL, intermediate-density lipoprotein; LDL, low-density lipoprotein; LDL-R, low-density lipoprotein receptor; LPL, lipoprotein lipase; LRP, LDL receptor–related protein; VLDL, very low-density lipoprotein; and VLDL-R, very low-density lipoprotein receptor. (Miller M et al., 2011)

Two study reports specifically addressed above issues by comparing, fasting and non-fasting TG for predictability of future CV events. The first report was derived from the Women's Health Study cohort, in which 26,509 initially healthy American women were followed for 11-years for MI, stroke, coronary revascularization procedures, and CV death. In analysis, both fasting and non-fasting TG were associated with future CV risk

after adjustments were made for age, BP, smoking status, and hormone-replacement therapy. Among the fasting participants, further adjustment for total cholesterol (TC) and HDL-C markedly weakened this association. These data were consistent with prior work; however, non-fasting TG maintained a strong independent relationship with future CV events in fully adjusted analyses: The hazard ratios [95% confidence interval (CI)] for increasing tertiles of non-fasting TG were 1.0 (referent), 1.44 (0.90 –2.29), and 1.98 (1.21–3.25) (P for trend = 0.006). Moreover, in analyses stratified by time since the last meal, TG concentrations measured 4hr postprandially had the strongest association with CV events, with a fully adjusted hazard ratio (95% CI) for the highest to the lowest tertile of 4.48 (1.98 –10.15) (P for trend <0.001). The second report derived from a prospective cohort of 7,587 women and 6,394 men in Copenhagen with 26 years of follow-up. In this study, non-fasting TG was also found to significantly predict future vascular events in both sexes after multivariate analysis. In subgroup analyses, these effects were somewhat greater for women than for men and were consistent for the endpoints of MI, ischemic heart disease, and total mortality. Moreover, peak TG and remnant lipoprotein cholesterol concentrations were observed 4hr after the last meal, the same time frame for which the maximal predictive value was observed in the Women's Health Study data. In the Copenhagen data, the highest risks were observed among the individuals with the very highest postprandial TG concentrations.

Above data is not only derived from typical Western populations in the US and Europe but also extends to otherwise low-risk populations in which overt hyperlipidemia is less prevalent. In this regard, non-fasting TG has been prospectively associated with increased vascular risk in Japanese men and women, even after adjustment for both TC and HDL-C. Similarly, in an Asia Pacific Cohort Studies Collaboration study that included data from 26 cohorts, non-fasting TG concentrations were a more potent predictor of incident vascular events than were fasting TG (Mora et al., 2008; Ridker, 2008).

2.4.2 Apolipoprotein B and non-HDL cholesterol

In T2DM, dyslipoproteinemia is an important and common risk factor for CHD and also a major contributor to the proatherogenic profile of the disease (Ahmad et al., 2007). Several recent studies have found that non–HDL-C and apoB perform better than LDL-C in CVD risk prediction, both on- and off-treatment, as well as in subclinical CVD risk prediction. ApoB is found in chylomicrons, VLDL, intermediate density lipoprotein (IDL), LDL, and lipoprotein(a) particles. Since each of these particles contain a single apoB molecule, measurements of apoB represent the total burden of particles considered most atherogenic. Reports suggest that once LDL-C is lowered, apoB may be a more effective way to assess residual CVD risk and to determine the need for medication adjustments (Brunzell et al., 2008). There is a strong correlation (r >0.80) between levels of apoB with that of non-HDL-C. Because levels of apoB represent all proatherogenic particles, the replacement of fasting plasma lipids with apoB to assess CVD risk has been supported by many. An advantage of measuring apoB as compared to lipids is that fasting may not be necessary because changes in apoB-100 after eating are minimally different than those measured in the fed state. Table 1 and 2 show the reference ranges of apoB relative to LDL-C levels (Eckel et al., 2014).

Table 1: Reference ranges of apoB levels relative to LDL-C levels

Risk	ApoB	LDL-C
High risk: CHD or CHD risk equivalent	< 90 mg/dL	< 100 mg/dL
Moderate risk: ≥2 risk factors	< 110 mg/dL	< 130 mg/dL
Low risk: 0-1 risk factors	< 130 mg/dL	< 130 mg/dL

The performance of non–HDL-C compared with apoB, however, has been a point of debate. Although both offer the practical benefits of accuracy independent of TG level and prandial state, non–HDL-C proves to be the better marker of choice, given established cut points with safe and achievable goals, no additional cost, and quick time to result with an easy mathematical calculation (Ramjee et al., 2011). Non-HDL-C (TC minus HDL cholesterol) reflects the concentration of cholesterol within all lipoprotein particles currently considered atherogenic. In individuals with

hypertriglyceridemia, there is an increase in VLDL-C. Therefore, in statin treated individuals, achieving LDL-C targets but with persistently elevated TG, especially those with insulin resistant conditions, non-HDL may be the best means of assessing and managing residual CV risk (Fruchart et al., 2013). The ATP III has proposed non-HDL-C levels as a secondary goal of therapy after targeting LDL-C levels.

Table 2: ADA/ACC consensus report treatment goals in patients with cardiometabolic risk and lipoprotein abnormalities

Risk	ApoB	LDL-C	Non-HDL-C
Highest-risk patients: Known CVD or diabetes mellitus plus ≥1 additional major CVD risk factor	<80 mg/dL	<70 mg/dL	<100 mg/dL
High-risk patients: ≥2 CVD risk factors but no diabetes mellitus or known CVD or diabetes melitus but no other major risk	<90 mg/dL	<100 mg/dL	<130 mg/dL

2.4.3 Apolipoprotein A1

The abnormal plasma apolipoprotein levels are consistent with the high prevalence of obesity and diabetes. It is synthesized mainly in the liver and to some extent in the small intestine. Unlike ApoB, ApoA1 is inversely related to CV risk (Riediger et al., 2010; Dodani et al., 2012). ApoA1 helps in removing excess cholesterol from tissues and incorporating it into HDL-C for reverse transport to the liver, thus manifesting anti-atherogenic effects. Several studies have shown that apoB increases significantly only in females, whereas, apoA1 decreases significantly in both genders in patients with metabolic derangements (Erdeve et al., 2010; Dodani et al., 2012). Evidence suggests that apoA1, is a better predictor of heart disease than HDL-C levels (Riediger et al., 2010).

2.4.4 Plasminogen activator inhibitor-1

Generally, before the development of T2DM or CVD, the precursor condition, metabolic syndrome often develops. In certain individuals, MS is associated with vascular inflammation, which can lead to increased clotting, rupture of vulnerable plaque, and vascular injury and subsequently to the development of CVD and acute events such as MI or stroke. MS is strongly associated with low levels of insulin sensitivity and higher degrees of insulin resistance, which act in concert to foster inflammation and in turn impaired fibrinolysis or dysfibrinolysis. Inflammation transforms normal hemostasis or fibrinolysis toward dysfibrinolysis which is the propensity to form thrombi and this pathway also may lead to rupture of vulnerable plaque. Individuals with elevated plasma inflammatory biomarkers and biomarkers of dysfibrinolysis exhibit vascular inflammation and are at greater risk for developing thrombi or plaque rupture (Appel et al., 2009). PAI-1 is a major physiological inhibitor of tissue-type (tPA) and urokinase-type plasminogen (uPA) activators. It also possesses several other roles in human physiology. PAI-1 belongs to the family of serine protease inhibitors (SERPINs), and it is an inhibitor of intravascular fibrinolysis and cell-associated proteolysis (Huotari et al., 2010). Elevated circulating levels of C-reactive protein (hsCRP), insulin, TG and various cytokines have been known to stimulate the abdominal adipocytes and foster excess release of PAI-1, which is indicative of impaired fibrinolysis (Appel et al., 2009). Under normal physiological conditions, PAI-1 is synthesized by the liver, smooth muscle cells, adipocytes and platelets. However, in pathological conditions like atherosclerosis, endothelial cells and other inflammatory-stimulated cells secrete notable amounts of PAI-1(Huotari et al., 2010). Moreover, several studies have reported strong association between liver steatosis and PAI-1 levels (Barbato et al., 2009). As PAI-1 is an independent and true component of MS, it can be a very useful marker while studying the association between markers of CMR.

The interrelationship of the above mentioned CMR determinants with respect to CVD risk is complex. In particular, it is not yet possible to infer any causality of any these new risk markers. Indeed, liver fat accumulation may be cause or consequence of the

MS and postprandial dyslipidemia may contribute the development and progression of fatty liver, but also, once a fatty liver has evolved; postprandial dysmetabolism may aggravate, especially in a patient with T2DM. A better understanding of these interrelationships and their relative contribution to CVD is of importance to design strategies, which will lower CVD risk in populations at risk.

2.5 Body fat distribution and cardiometabolic risk

2.5.1 Abdominal adiposity and metabolic risk

Obesity can be measured using a number of methods, most routinely done and common one is BMI. BMI is an estimate of generalized obesity; however, it is the distribution of fat for example, visceral as opposed to subcutaneous fat– that is more important in terms of disease risk (Barnett, 2008). Long ago it was noted that upper body or the android adiposity was associated with more deranged metabolic profile than the gynoid type of obesity (Hanley and Wagenknecht, 2008). Subsequently, much research has been conducted that reinforces the notion that abdominal obesity exerts deleterious effects on development of CVD, T2DM and their respective metabolic precursors.

Waist circumference has been acknowledged by many studies to be the most practical way to determine abdominal fat levels (Shen et al., 2006). Nevertheless, abdominal fat can be divided into three different components: visceral, subcutaneous and retroperitoneal. The differences are important, as it is visceral fat (the fat within the abdominal cavity and is therefore stored around a number of internal organs such as liver, pancreas and intestine) that is most strongly linked to an increased risk of CVD and T2DM (Hung et al., 2014). Having recognized importance of knowledge on body fat distribution to better predict the associated comorbidity, considerable advancements have been made in imaging techniques over last two decades directed towards estimation and quantification of various abdominal adipose tissue depots. This has allowed researchers to better understand and correlate independent associations of these various fat depots with health risk.

2.5.1.1 Waist circumference as a measure of abdominal obesity

BMI cut-offs for identifying body composition classifications vary with racial populations. For Caucasians, BMI of 18.5-24.9 is typically classified normal weight; 25-29.9 is overweight, and a BMI ≥30 is considered obese (Chiu et al., 2011). In 2004, WHO expert report clearly revealed that Asian populations have different associations between BMI, percentage of body fat, and CMR than Caucasians. It also concluded that the proportion of Asians with a high risk of T2DM and CVD is substantial at BMIs lower than the existing WHO cut-off point for overweight (≥25 kg/m^2) (WHO, 2004). Thus, the guidelines for obesity and overweight based on BMI for Asian Indians were revised through discussions by a Prevention and Management of Obesity and Metabolic Syndrome group. The revised guidelines categorize the patients as underweight (BMI <18.5 kg/m2), normal or lean BMI (18.5–22.9 kg/m2), overweight (23.0 –24.9 kg/m2) and obese (≥25 kg/m2) respectively using values lower than the ethnic specific BMI previously advocated for Asian Indians (Low et al., 2009; Misra et al., 2009).

Similar to BMI related data, CV morbidities occur at lower value of waist circumference in Asian Indians (Misra and Khurana, 2011) and most of the researchers felt a need to revise international guidelines for waist circumference for South Asians (refer to table 3). It is important to note that International Diabetes Federation (IDF) and NCEP, ATP III in their recent definitions of the MS have taken the ethnic-specific cut-off points for waist circumference into consideration (Misra and Shrivastava, 2013).

While, BMI may be misleading in certain situations, such as in individuals with a high proportion of lean muscle mass, waist circumference is a more accurate measure of the distribution of body fat and has been shown to be more strongly associated with morbidity and mortality. Thus, waist circumference is not only a reflector of abdominal fat but also improves the sensitivity of predicting CMR versus BMI alone. Moreover, it has been clearly reported that in general, men and women with high waist circumferences above the National Institute of Health cut off points have significantly higher risk of hypertension, T2DM, dyslipidemia and the MS (Dagan et al., 2013). Results from large international cardiometabolic study (INSPIRE ME IAA study) done in

4,504 patients recruited from 29 countries showed frequent discordance between BMI and waist circumference, driven by the substantial variability in visceral fat for a given BMI. Within each BMI category, waist circumference was cross-sectionally associated with visceral fat, liver fat and CMR factors (Nazare et al., 2015). When it comes to a waist circumference as an anthropometric measure of abdominal fat accumulation, it is unclear how abdominal obesity relates to dyslipidemia and insulin resistance; however, visceral adiposity has been identified as a key component in the deterioration in the metabolic profile (Parikh and Mohan, 2012)

Table 3: Cut offs of BMI (obesity) and waist circumference (abdominal obesity) for Asian Indians vs International criteria

Variable	**Consensus guidelines for Asian Indians[a]**	**Prevalent International**
Generalized obesity (BMI cut-offs in kg/m^2)	Normal: 18.0–22.9 Overweight: 23.0–24.9 Obesity: >25	Normal: 18.5–24.9[b] Overweight:
Abdominal obesity (Waist circumference cut-offs in cm)	Men: >90[c] Women: >80[c]	Men: >102[d] Women: >88[d]

Notes: [a]From Consensus guidelines for Asian Indians (Misra A et al., 2009); [b]According to World Health Organization guidelines (Einhorn D, 2003); [c]Both as per Consensus Guidelines for Asian Indians (Misra A et al., 2009) and International Diabetes Federation (Available online: http://www.idf.org/ metabolic-syndrome accessed on 29 November 2012); [d]According to Modified National Cholesterol Education Program, Adult Treatment Panel III guidelines (NCEP, 2002)

2.6 Visceral adiposity as a key responsible factor for metabolic derangement

2.6.1 Etiopathogenesis of visceral fat accumulation

Evidence suggests that visceral adiposity, one of the most important CMR factors originates from a chronic imbalance between energy intake and expenditure (Bays and Ballantyne, 2006). The spill over theory explains that the excess of energy in the form of FFA or TGs is spilled from the saturated adipocytes to the visceral depots and other ectopic sites predisposing one to a higher CMR (refer to figure 4).

Studies of the measurement of abdominal adiposity using imaging modalities, i.e. MRI and CT have commonly reached the conclusion that the amount of visceral adipose fat and not that of subcutaneous abdominal fat is a dominant correlate of metabolic abnormalities observed in overweight/obese patients. Pathogenesis of visceral fat accumulation is primarily explained on the basis of insulin resistance and hyperinsulinemia.

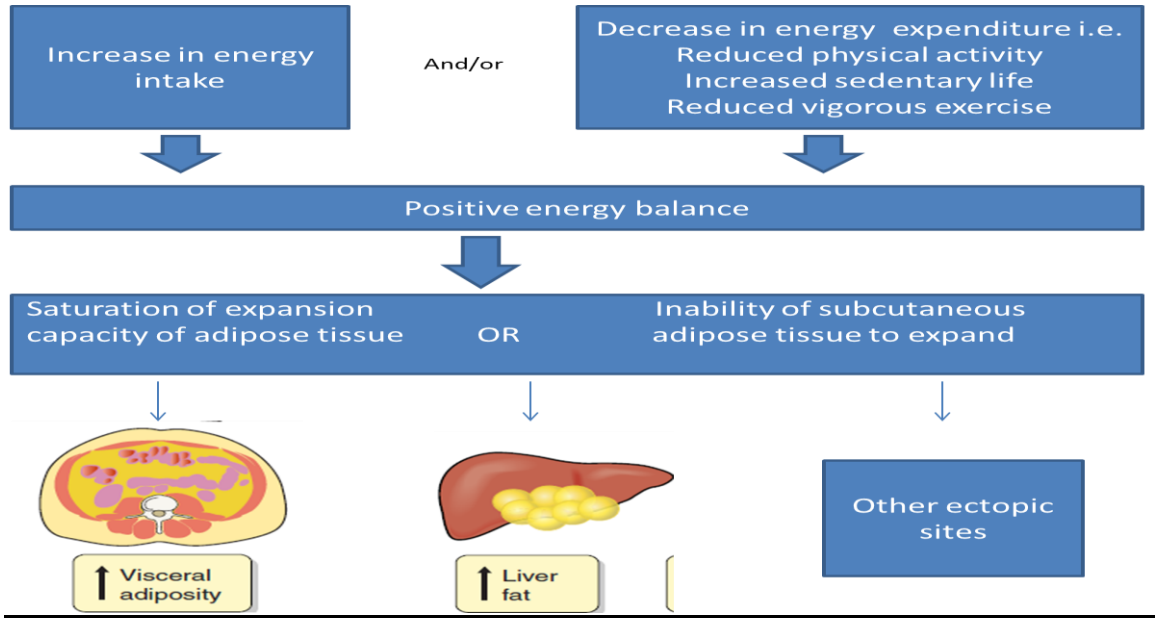


Figure 4: Etiology of visceral fat accumulation

Visceral fat accumulation, which probably underlies the development of insulin resistance, may be involved in onset of diverse disease as explained by following two viewpoints:

Excess energy is stored in the visceral adipocytes in the form of TGs, which are hydrolysed to FFA and glycerol when energy is needed or in starvation. This reaction is known to be more active in visceral fat than in subcutaneous fat. The release of FFA and glycerol corresponds to the volume of visceral fat accumulation and released FFA and glycerol flow directly into liver through portal vein. FFA entering liver stimulates fat synthesis and suppresses insulin catabolism, which results in onset of peripheral hyperinsulinemia. Large amount of glycerol, entering the liver via adipocyte specified

glycerol channel “Aquaporin adipose” (AQPap), gets converted to glucose by glycerokinase and released from the liver (refer figure to 5) (Matsuzawa, 2002; Despres et al., 2008).

Secondly, visceral adipose tissue is not only an energy storage tissue, but also a metabolically active organ, secreting hormones, cytokines and growth factors, collectively called as adipocytokines. It is believed that anti-atherosclerotic adipocytokines like leptin, adiponectin and proatherosclerotic, pro-inflammatory cytokines, such as tumour necrosis factor alpha (TNF-α), PAI-1 co-operatively regulate metabolic and cardiovascular homeostasis at local and remote sites. Visceral adiposity perturbs this homeostasis thus resulting in atherosclerotic CVD (Matsuzawa, 2002; Grundy et al., 2005; Chen et al., 2008; Shimabukuro, 2009)

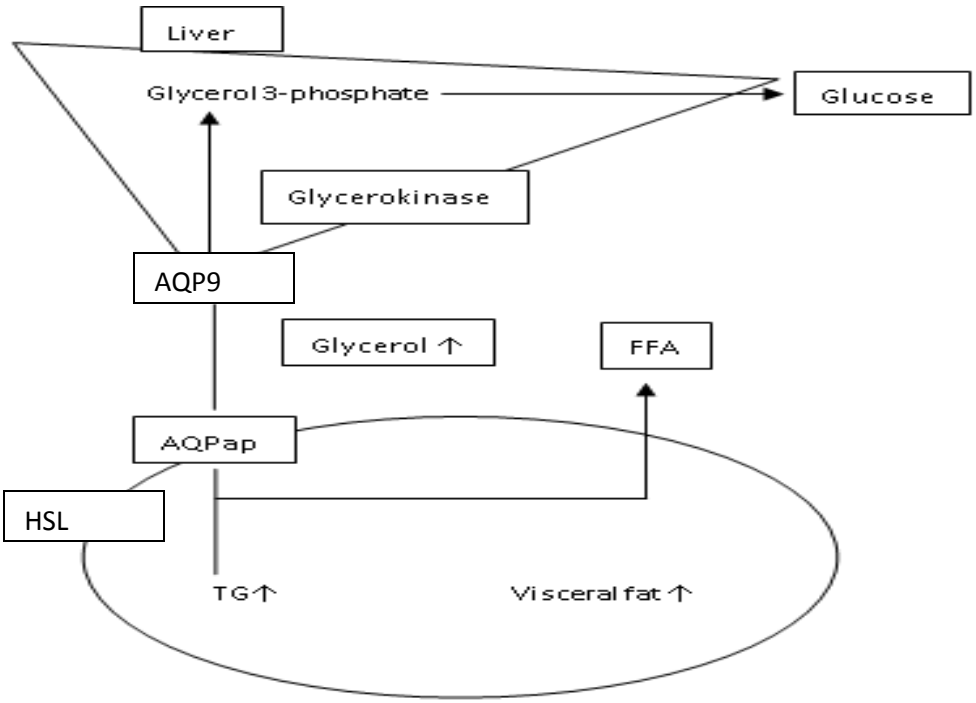


Figure 5: Visceral fat accumulation leading to increased release of FFA and glycerol supporting the portal theory.

2.6.2 Factors influencing visceral fat accumulation

Although net positive energy balance in the body is the key factor responsible for visceral fat accumulation following spill over hypothesis, the propensity of visceral fat

accumulation during excess energy states varies from one individual to other. Such difference may involve multiple factors as given below;

a. **Age:** It is the confounding factor for the accumulation of adipose tissue in the body. (Hunter et al., 2010). Mobilisation and storage of excess energy varies across different age groups. It has been investigated for young males, female to middle aged females; the surplus energy is preferentially stored in subcutaneous adipose tissues except in few genetically susceptible individuals where there might be visceral fat accumulation (Lanska et al., 1984; Enzi et al., 1986).

Increased visceral adipose tissue deposition with age is particularly significant among men and postmenopausal women who, on average, have up to twice the amount of visceral adipose tissue than premenopausal women (Kotani et al., 1994).

b. Gender: Studies have shown that, males tend to accumulate adipose tissue in the upper body (trunk, abdomen), whereas women usually accumulate adipose tissue in the lower body (hips, thighs) (Kvist et al., 1988). Sex hormones might be involved in regulating the typical gender differences in regional body fat distribution.

Studies by Despres and Lamarche found that, among both men and women a value of 100 cm^2 was associated with significant alterations in CVD risk profile and that a further deterioration of the metabolic profile was observed when values greater than 130 cm^2 of visceral adipose tissue. (Despres et al, 1990; Lamarche, 1993). In a study on healthy subjects, value above 110 cm^2 for visceral abdominal fat area was associated with an increased risk of CHD in pre and postmenopausal women whereas; males with abdominal visceral fat cross-section areas measuring more than 131 cm^2 were clearly at an increased risk for CAD. (Hunter et al., 1994; Williams et al., 1996).

c. Ethnicity: Ethnicity is a factor which contributes to fat accumulation as some populations may be prone to accumulate adipose tissue in the subcutaneous adipose depots, while other to accumulate adipose tissue in the visceral cavity. (Katzmarzyk et al., 2011). Asians are reported to have a higher body fat content at lower BMI values compared with Caucasians (Sandeep et al., 2010). It has been reported that despite similar level of total adiposity, Caucasians have more visceral adipose tissue than

African Americans (Conway et al., 1997; Razak et al., 2007; Camhi et al., 2011), whereas Asians and Indian Asians seem prone to visceral fat accumulation despite lower total adiposity values compared with individuals from other ethnic backgrounds (Kadowaki et al., 2006).

d. Nutritional factors: Some meta-analyses on the topic of sugar-sweetened beverages, obesity, and CMR have shown that increased consumption of such beverages is likely to be associated with obesity, metabolic alterations, and the development of T2DM (Malik et al., 2010). Stanhope et al. studied the effect of fructose diet on CMR; by giving overweight adults either glucose-sweetened or fructose-sweetened beverages for 10 weeks. He observed significant increase in total body fat in both groups, but significant increase in visceral fat in the group consuming fructose. These findings suggest that fructose consumption might specifically promote visceral fat accumulation (Stanhope et al., 2009; Pollock et al., 2012).

e. Sedentary lifestyle/Physical inactivity: Sedentary lifestyle has emerged as a unique risk factor for chronic disease morbidity and mortality (Saunders et al., 2013). In a study conducted on 276 subjects suggests that change sedentary lifestyle is not associated with longitudinal changes in visceral adiposity in men and women except on waist circumference and thus not contribute as a primary factor development of CMR (Cart, 2012).

2.6.3 Imaging tools for estimating visceral fat area and methodological concerns

Although waist circumference is a useful diagnostic tool, it cannot distinguish visceral from subcutaneous fat accumulation. With development of radiological imaging techniques, such as CT and MRI, visceral fat can be accurately quantified. However, there are few methodological concerns while utilizing these radiological techniques for visceral fat quantification as discussed below:

With the involvement of radiation in CT scans, its use in the subjects of large cardiometabolic studies has been restricted to a small number of scans. Most studies performed with CT have used single measures either at L4-L5 level or at the umbilicus. Using CT, visceral fat estimate at various abdominal levels found to be well correlated,

hence it was concluded that the location of abdominal scan does not have influence over magnitude of association between visceral fat and comorbidities.

Table 4: Visceral fat area cut offs predicting increased cardiovascular risk in various studies

Study	CT		MRI	Gender		Obesity		Visceral fat area (cm^2)
	Umbilicus	L4-L5		Males	Females	Present	Absent	
Despre`s and Lamarche		+		+	+	+	+	≥130
Hunter et al.		+		+		+	+	≥131
Williams et al.		+			+	+	+	≥110
Anderson et al.		+	+	+	+	+	+	≥132[a]
Matsuzawa et al.	+			+			+	≥133[b]
Saito et al.	+			+		+	+	≥100[c]
					+	+	+	≥90[c]
Lottenberg et al.	+			+	+	+	+	≥107

a Chinese T2DM; b Non-obese CHD Japanese patients; c Japanese subjects

However, MRI based studies using multiple slices have shown that there is substantial variation in the visceral fat area across different abdominal scan location, i.e. from L1-L2 to L4-L5. Various studies have used different abdominal sections to determine visceral fat estimate, not allowing for proper validation across studies. However, L4-L5 has been a popular location to perform the abdominal scan partly due to reports that it is best predictor of total body adiposity and partly because it is proximal to measures taken at the umbilicus. Table 4 lists the number of studies estimating visceral fat area providing details on quantification method and population under study. Most studies involving L4-L5 as abdominal scan location, yielded visceral fat area cut off of around

≥130 cm^2 to predict CV risk as indicated by significant deterioration of metabolic profile (Wajchenberg, 2000; Tchernof and Despres, 2013).

2.7 Fat accumulation in liver

Liver, one of the major sites of ectopic fat storage, plays a pivotal role in maintaining energy homeostasis during fasting-fed transitions & in buffering carbohydrate load by suppression of hepatic glucose production and promoting liver glycogen deposition (Savage et al., 2007).

Accumulation of fat in liver along with portal inflammation in advanced stages is termed as non-alcoholic steato-hepatitis (NASH) and may progress to hepatic fibrosis and even cirrhosis. Fatty liver is known to be associated with various metabolic abnormalities, but not much information about the association between metabolic disease and severity of fatty liver is available (Gaharwar et al., 2015; Lonardo et al., 2015; Mikolasevic et al., 2015).

The prevalence of non-alcoholic fatty liver disease (NAFLD) in general Indian population falls between 9-32% and approximately one fourth of the urban population is affected (Kalra et al., 2013). A case control study showed that Asian Indians in north India with NAFLD have higher adiposity, fasting hyperinsulinemia, MS and glucose intolerance than those without NAFLD (Misra and Shrivastava, 2013). Interestingly, in a recent study in Indians, it was found that metabolic profile of overweight/obese NAFLD patients was similar to that of lean NAFLD patients, and that NAFLD was consistently associated with deranged metabolic profile across the study participants (Kumar et al., 2013). Further, there are studies, which evaluated hepatic gluconeogenesis pathway in non-diabetic Asian Indian males having NAFLD using in vivo (31P) phosphorous magnetic resonance spectroscopy (MRS) and correlated it with anthropometry and surrogate marker of insulin resistance. Interestingly, non-obese non-diabetic subjects with NAFLD showed more derangements of hepatic gluconeogenesis enzymes than non-obese subjects without NAFLD. In a comparative study in USA, South Asians had higher hepatic TG levels, which were associated with lower adiponectin levels than white Caucasians. It is possible; therefore, that Asian Indians have greater TG

deposition in liver than white Caucasians, which may be related to higher magnitude of insulin resistance or inherent genetic tendency (Misra and Shrivastava, 2013).

2.7.1 Pathogenesis of fat accumulation in liver

Accumulation of fat in liver disturbs normal liver functioning (Despres and Lemieux, 2006) and it over produces components of MS, i.e. hyperglycemia /hypertriglyceridemia (Yki-Jarvinen, 2014) and is thus associated with CMR.

2.7.1.1 Sources of liver fat in normal and insulin resistant states

There are mainly three pathways through which liver derives TGs.

i) Uptake of albumin bound FFA in the plasma or circulation: In peripheral insulin resistance, there is increased FFA circulation due to failure of insulin to inhibit lipolysis at an adipose tissue level (Karpe et al., 2011). Interestingly, not visceral adiposity but the upper body non visceral adiposity drains the majority of FFA flux to the liver, mainly via portal vein (Nielsen et al., 2004; Saponaro et al., 2015). Various in-vitro and in-vivo studies have shown that FFA entering the liver hepatocytes stimulates apoB secretion thereby aiding in assembly and secretion of VLDL.

ii) Uptake of remnants of TG rich lipoprotein: TGs in large TG rich lipoproteins, i.e. VLDL and chylomicron in hydrolysed by LpL and are further taken up by the liver with their remaining TG. In insulin resistance, increased FFA levels stimulate apoB secretion thereby fostering secretion of VLDL and chylomicron as well (Adiels et al., 2008). Moreover, in insulin resistance, LpL activity is modestly reduced leading to reduced lipolysis of VLDL and chylomicron TG (Cohn et al., 2004). Due to this inefficient lipolysis, TG enriched VLDL and chylomicrons are taken up by the liver. Secondly, it has also been suggested that fatty acids derived from the hydrolysis of chylomicron TG, if escape its re-esterification in adipose tissue (spill over), are eventually taken by the liver in a manner similar to the uptake of albumin bound fatty acids and could stimulate secretion of apoB100. This spill over theory has added further complexity in

understanding the association between postprandial lipemia and liver fat accumulation (Barrows et al., 2005; Miles and Nelson, 2007).

iii) Raised de novo lipogenesis: De novo lipogenesis increases in the obese and insulin resistant state. During the state of positive energy balance in liver, there is increased glucose uptake in the liver (glucose uptake in liver is not insulin dependent). Glucose is converted into fatty acids through de novo lipogenesis. Insulin is known to promote de novo lipogensis through stimulation of sterol response element binding protein (SREBP-1c) expression (Zivkovic et al., 2007). SREBP-1c is a transcriptional factor which regulates almost all genes involved in fatty acid and TG synthesis. The pathophysiology underlying deranged glucose homeostasis, increased VLDL secretion and liver fat accumulation is the selective insulin resistance where insulin fails to suppress hepatic gluconeogenesis while its effect of stimulating de novo lipogenesis is retained (Rametta et al., 2013; Zhang and Liu, 2014).

2.7.1.2 Liver fat disposition in a normal and insulin resistant state

Liver can dispose its TGs by various pathways and the interferences in them in an insulin resistant state have been discussed below:

i) Fatty acid oxidation: It is the mitochondrial catabolic pathway leading to degradation of TGs and fatty acids. It generally occurs in fasting state or carbohydrate starvation, where beta oxidation of fatty acids produces acetyl-CoA which gets converted to ketone bodies within liver mitochondria. The ketone bodies are then released and taken up by other tissues like brain, muscle or heart where they are converted back to acetyl-CoA to serve as an energy source (Saponara et al., 2015). There are numerous studies reporting the relation between alteration of hepatic fatty oxidation and liver fat content. However, there is lack of evidence that reduced fatty oxidation causes an increase in VLDL secretion or increased liver fat accumulation in insulin resistant state.

ii) VLDL assembly within the liver and its secretion: Normally, assembly of VLDL involves two steps. The first step involves translocation of lipid poor apoB-100 into lumen of endoplasmic reticulum which is mediated by microsomal transfer protein (MTP) (Zhou et al., 1998; Fisher et al., 2001). Fatty acid mediated activation of apoB secretion takes place at this stage which is independent of their role as a substrate for core lipid synthesis (Zhang et al., 2004). The second step is the addition of TGs as core lipid and its transport from endoplasmic reticulum membrane to its lumen forming lipid droplets. Later, the fusion of lipid droplets and apoB-100 takes place forming nascent VLDL. The size of VLDL secreted depends on step two (Fisher et al., 2001; Sparks and Dong, 2009).

   Insulin targets apoB-100 degradation at least partly through phosphoinositol (PI)3-kinase, thereby can directly affect apoB secretion. However, in insulin resistant state there is lack of responsiveness to insulin over time and the increased fatty acid/hepatic TG mediated stimulation of apoB secretion which in turn increases VLDL assembly and secretion state (Sparks and Dong, 2009).

iii) Liver fat accumulation in insulin resistance: It has been reported that the TGs derived from de novo lipogenesis increases VLDL particle size without increasing number of particles secreted, and de novo lipogenesis is increased in insulin resistant state (Choi and Ginsberg, 2011).

2.7.2 Non-invasive methods of liver fat estimation

Liver fat is a meaningful marker of, and a contributor to, both hepatic and systemic morbidity and mortality. Liver steatosis is reversible with intervention, and reduction in liver fat may diminish many of its associated risks. Therefore, there is need, in both clinical and research arenas, to detect its presence and to assess its severity (Reeder and Sirlin, 2010).

Liver biopsy is currently the gold standard for estimation of liver fat content (Lee and Park, 2014). Liver fat accumulation or steatosis is typically graded on a scale of 0-3 depending on the number of hepatocytes with intracellular vacuoles of fat (Idilman et al., 2013).

Grade 0 (normal) = less than 5%

Grade 1 (mild) = between 5 to 33%

Grade 2 (moderate) = between 33 to 66%

Grade 3 (severe) = greater than 66%

However, liver biopsy comes with its limitation. In addition to be an expensive affair, liver biopsy is an invasive procedure which brings discomfort to the patients and is associated with possible complications like bleeding (Betzel and Drenth, 2014). One of the studies involving liver biopsy in patients with NASH reported an overall complication rate of 10%, with 1–3% of post-biopsy patients requiring hospitalization, and an overall mortality rate of 0.01% (Gaidos et al., 2008). Moreover, the heterogeneity of fat deposition in the liver (Deacrie et al., 2011) may limit the reliability of results, as only approximately 1/50000th part of the liver becomes available for histological analysis leading to sampling error (Ellis and Mann, 2012). This calls for optimization and maximal utilization of non-invasive methods to determine liver fat content.

Overview of non-invasive methods

Varieties of non-invasive (radiological) methods are being used to determine liver fat content in vivo, i.e. transabdominal ultrasonography (USG), CT, and MRI and MRS (Lin et al., 2015). However, these methods have their own advantages and drawbacks as discussed and tabulated in table 5:

2.7.2.1 Transabdominal Ultrasonography

Transabdominal USG has been used widely in recent years, which has led to a great increase in findings of non-alcoholic fatty liver (Wu et al., 2014). USG is widely available, involves low examination cost, and is safe and free of radiations (Valls et al., 2006).

Evaluation of fatty changes in liver using USG is based on the echogenicity of liver. Normally, liver parenchyma has homogenous texture and echogenicity similar to that of renal cortex and spleen. However, with the fat accumulation in the liver, its echogenicity becomes higher than the renal cortex and spleen (Singh et al., 2013).

Performance of USG in determining liver fat content has been variable across different studies having a sensitivity (Se) and specificity (Sp) range of 60%–95% and 84%–100% (Charatcharoenwitthaya and Lindor, 2007). In addition, the USG method is also highly an operator dependent resulting in low reproducibility.

2.7.2.2 Computed Tomography

Like USG, CT is also widely used and easy to perform (Reeder and Sirlin, 2010). It involves ionizing radiation and measures tissue density as a function of attenuation. As the attenuation value of fat is much lower than that of soft tissue, liver fat accumulation lowers the attenuation of liver parenchyma (Piekarski et al., 1980; Kodama et al., 2007).

The Se and Sp of unenhanced CT in detection of moderate to severe liver fat content (>30% histologically) have been reported to be 73%–100% and 95%–100%, respectively (Charatcharoenwitthaya and Lindor, 2007).

The main limitation of CT in determining liver fat content is its inability to detect a mild degree of hepatic steatosis. In addition, several other conditions and factors may interfere with the observed liver densities, such as edema. These issues, combined with the dependence on ionizing radiation, render unenhanced CT for the assessment of liver fat content a clinically unacceptable method (Lee and Park, 2014).

2.7.2.3 Magnetic Resonance Imaging and magnetic resonance spectroscopy

Both, MRI and MRS are highly accurate and reproducible for measuring liver fat content. MRS is more advanced application and is regarded as the gold standard technique for non-invasive estimation of liver fat content (Lee and Park, 2014; Williams and Taylor-Robinson, 2016). Unlike CT and USG, which evaluate liver fat content through proxy parameters (echogenicity and attenuation), MRI and MRS can more directly measure liver fat content. MRI and MRS both measure proton density fat fraction, i.e. amount of protons bound to fat divided by amount of all protons in the liver, including those bound to water and fat. Despite these advantages, high cost of the MR examination restricts its use in routine practice. In the histological range of

liver fat content of 5-10%, Se of greater than 85% and Sp of almost 100% has been reported. Despite this relative superior performance, which is achieved without the use of ionizing radiation, MRI is not as widely used as one would expect. The main reasons are the high costs per examination, the dependence on full patient cooperation, and some contraindications for the examinations such as a pacemaker implant or patient claustrophobia.

In contrast to MRI, MR spectroscopy resolves the observed signal into a frequency spectrum, providing biochemical information (Mehta et al., 2008). Proton (1H) MRS is currently by far the most promising and most sensitive non-invasive method to assess liver fat content (Adams and Lindor, 2007). The main advantage of 1H MRS is its extremely high sensitivity also at very low liver fat content. In an optimal setup, even liver fat content as low as 0.5 % can be reliably detected (Machann et al., 2006). Since the method does not rely on ionizing radiation and thus is safe for the patient, it is a potentially ideal tool for screening patients at risk for NAFLD. Nevertheless, 1H MRS is mainly used in academic institutions for research purposes because a great deal of expertise and experience are required for practical implementation of the method and reliable data analysis.

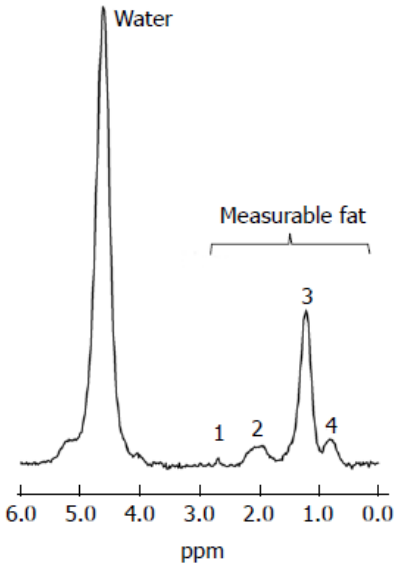


Figure 6: Different frequencies of water and fat peaks in MRS. Water appears as single peak at 4.7 ppm, whereas fat appears as four peaks, dominant methylene (CH2) peak at 1.3 ppm (3), a methyl (CH3) peak at 0.9 ppm (4), and α-olefinic acid and α-carboxyl peak at 2.1 ppm (2), and a diacyl peak at 2.75 ppm (1); Proton density fat fraction can be calculated as (Sum of fat peaks) / (Sum of fat peaks + water peaks)

Table 5: Comparative evaluation of radiological tests for determining liver fat content

Tests	Advantages	Limitations	Applicability
USG (intensity of echogenicity)	Readily available, easy to perform and less expensive	Se increases with increase in fat infiltration, limited accuracy in mild hepatic steatosis. Operator dependent. Provides qualitative assessment	Used only for initial screening of steatosis instead of quantification or assessment of treatment response
Unenhanced CT (Infiltration in liver parenchyma ↑, the attenuation ↓)	Quick and accurate. Widely available and easy to perform	Involves radiation hazard. Provides Qualitative assessments. Expensive then USG. Presence of iron impacts attenuation Insensitive if <33% fat	Detecting moderate to severe hepatic steatosis
MRI (chemical shift imaging)	Entire liver can be imaged Can be used at both 1.5 and 3 T Highly accurate & reproducible	T2 effects increase amount of signal loss. Without T2 correction, liver fat content can be miscalculated. Long scan acquisition time High cost	Follow up of response after treatment in practice or clinical trial
(^{1}H) MRS (gold standard for noninvasive fat quantification)	Highly accurate and reproducible, Correlates well with biopsy results. High Se 87-100% for determination of fatty liver and quantification of liver fat	Inflammation and fibrosis not easily detected using MRS. High cost. Long scan acquisition time. Expertise required for data acquisition and analysis	Follow up of response after treatment in practice or clinical trial

2.7.3 Liver fat, a consequence or cause of insulin resistance

2.7.3.1 Liver fat as a consequence of insulin resistance

Several animal models support this concept (Hebbard and George, 2011). Ota *et al*. investigated the role of insulin resistance in the development of hepatic steatosis and steatohepatitis. They developed high fat diet insulin resistance in methionine and choline-deficient rats with diet-induced NASH and with obesity/diabetes in the background. Their results showed that obesity, diabetes and high fat diet accelerated not only steatosis but also inflammation and fibrosis in the liver. Moreover, they demonstrated a beneficial effect of pioglitazone—a drug that improves insulin resistance—on steatohepatitis pathology in this model. This supports a causal role of insulin resistance in liver fat accumulation (Ota et al., 2007).

A direct causal relation between insulin resistance and hyperinsulinemia is known. Further, insulin may directly increase liver fat accumulation as seen in patients with metastatic insulin-secreting tumors (insulinomas) or with pancreatic islet cell transplants (Bhargava et al., 2005).

More evidence that insulin resistance causes steatosis derives from patients with V-Akt Murine Thymoma Viral Oncogene Homolog 2 (AKT2) mutations (Semple et al., 2009). In normal liver, insulin signaling inhibits glucose production and promotes fatty acid synthesis (Saltiel and Kahn, 2001). In contrast, in patients with AKT2 mutations or hyperinsulinemia, the inhibitory effect of insulin on glucose production is diminished, whereas the stimulatory effect of insulin on liver lipogenesis is retained or increased (Brown and Goldstein, 2009; Semple et al., 2009). In fact, hyperinsulinemia activates the transcriptional factor SREBP-1 promoting lipogenic enzyme gene expression in spite of insulin resistance. Furthermore, high levels of insulinemia may also contribute to TG accumulation in the liver through the suppression of Forkhead Box 01 (Fox) a2 transcription factor, which promotes fatty acid oxidation. Hyperglycemia can also stimulate lipogenesis by activating the carbohydrate response element binding protein (ChREBP) resulting in the transcription of genes involved in glycolysis and lipogenesis (Fruci et al., 2013).

2.7.3.2 Liver fat: Cause of Insulin Resistance

The notion that excess TG in liver causes insulin resistance is also debated. Evidence that liver steatosis may cause development of insulin resistance come from certain animal models. For instance, mice with targeted overexpression of LpL in the liver develop liver-specific steatosis associated with liver-specific hepatic insulin resistance. Rats with high-fat diet induced hepatic steatosis to undergo hepatic insulin resistance before obesity develops and circulating adipocytokines increase. Further evidence supporting the role of intrahepatic lipid accumulation in mediating hepatic insulin resistance comes from the treatment of high-fat diet rats with low doses of 2,4-dinitrophenol (DNP). DNP, by promoting mitochondrial energy uncoupling and preventing liver fat accumulation, protects rats from hepatic insulin resistance. Moreover, FFAs, which are associated with the development of liver steatosis, are inductors of insulin resistance via activation of protein kinases. Indeed, it has been shown that hepatic protein kinase-c (PKC) isoforms are involved in hepatocyte insulin resistance by inhibiting insulin signaling in human liver biopsy samples. Samuel *et al*. showed that PKCε silencing by antisense oligonucleotide, leads to significant reduction in intra-hepatic TGs, hepatic insulin-resistance and fasting plasma insulin concentrations. PKCε silencing also restored insulin receptor substrate (IRS)-2 phosphorylation and protein-serine threonine kinase activity. Activation of hepatic PKCε, was the best predictor of insulin resistance (Fruci et al., 2013).

2.8 Independent association of liver and visceral fat to cardiometabolic risk

Several studies using CT & MRI/MRS tool have reported direct and independent correlation between visceral and liver fat content while subcutaneous adipose tissue has not been related to liver fat content (Gastaldelli and Basta,2010), reinforcing the prevailing dogma that visceral fat has deleterious metabolic effects (Fabbrini et al., 2009). However, if visceral fat was a major contributor to metabolic risk, visceral adipose tissue should be the major source of systemic FFA flux to the liver via portal vein, which is not the case. Visceral fat contributes to only 15% of the total systemic FFA whereas the majority of FFA is contributed by non-splanchnic adipose tissue. This

observation raises the doubt over considering visceral adipose tissue being major contributor of metabolic abnormalities (Patel and Abate, 2013). There is a causal link between liver fat content and metabolic dysfunction. High liver fat content causes hepatic insulin resistance leading to fasting hyperglycemia, loss of post-prandial suppression of gluconeogensis and dyslipidemia. Increased lipid availability prevents local degradation of apoB in hepatocytes leading to overproduction of TG rich VLDL particles. Thus, a high liver fat content can, by itself, largely explain the hyperinsulinemic, hyperglycemic, hypertriglyceridemic, and elevated apoB dysmetabolic state independent of contribution from visceral adipose tissue. Moreover, liver dysfunction arising from steatosis also releases pro-atherogenic and pro-coagulant proteins such as fibrinogen, CRP and PAI-1 (Després, 2012; Ghosh, 2014).

With a view to explore the pathophysiology of CMR, causal link between various ectopic fat depots and cardiometabolic dysfunction needs to be established. Reports indicate that visceral fat contributes most to the obesity related metabolic abnormalities. It has been established that visceral fat correlates directly with liver fat and an increase in liver fat is associated with similar metabolic abnormalities as linked to visceral fat content. Although visceral and liver fat are connected metabolically and both are associated with CMR factors, it is important to know their independent contribution to metabolic disturbances and in turn CV risk. Table 6 & 7 summarize the list and results of studies exploring independent association of visceral and liver fat with various metabolic risk factors.

In 2003, Nguyen-Duy et al evaluated fasting blood glucose and lipid parameters in 162 overweight/obese male Caucasians. It was found that, both visceral and liver fat carries independent health risk. However, visceral fat is a stronger correlate of metabolic risk than liver fat (Nguyen-Duy et al., 2003). Results from the similar study in 293 overweight/obese male Caucasians was published in 2007, where visceral fat was found to have independent correlation with metabolic markers after adjusting other fat depots whereas liver fat did not (McMillan et al., 2007). Kuk et al examined

mortality as the outcome measure in 291 males (predominantly white) and found that visceral fat was the only fat measure independently predictive of mortality risk (Kuk, et al., 2006). Although, these studies provided a link between visceral fat distribution and CMR, it is noteworthy that these studies had relatively small sample size and included only male participants. Thus, whether these observations can be extended across gender was not evident. Recently, large cohort studies in a community-based sample like Framingham Heart Study (sample = 2589) and the AGES-Reykjavik Study (sample = 2495), using CT technology extensively across both genders, generated robust and convincing evidence on metabolic risk associates.

In the Framingham heart study, both visceral and liver fat were significantly associated independently with lipid-glucose traits (Speliotes et al., 2010). In the AGES-Reykjavik study, independent association of visceral and liver fat estimates with metabolic syndrome differed across the levels of obesity (BMI <25, 25-29.9 and ≥30 kg/m^2). For visceral fat, significant ($p<0.01$) correlation was found in females across all obesity levels, but association diminished with increased BMI. In males, only overweight group showed significant ($p<0.01$) correlation. For liver fat, no correlation was found in normal weight group; however significant ($p<0.001$) independent correlation observed in overweight & obese groups in females (odds ratio 1.38 & 1.45) and for overweight & obese males odds ratio of 1.38 ($p=0.01$) and 1.27 ($p=0.10$) were observed (Kim et al., 2011). Jackson heart study enrolled over 2000 African-Americans, who underwent CT to examine the independent correlation of visceral and liver fat with CMR. It was found that both liver fat and visceral fat were independent correlates of CMR, but associations were stronger for visceral than for liver fat (Liu, et al., 2011).

Results from studies using CT are consistent with respect to demonstrating strong correlation between visceral fat and various metabolic markers while not so assertive for liver fat. However, with the availability of MRS, various studies have been carried out in the same line showing very strong association between liver fat content and CMR profile predicting T2DM and CVD (Mehta et al., 2008).

A study including both male and females carried out in 2009 observed that when groups were matched for liver fat values, no difference in insulin sensitivity was observed between normal and high visceral fat subgroups (Fabbrini et al., 2009). Further, it showed a strong independent association between liver fat content and metabolic markers that included lipid parameters, whereas visceral fat did not correlate independently (Adiels, et al., 2006; Hoenig, et al., 2010). In 2011, results from a study in relatively larger sample size of 356 including both genders were published. Both visceral and liver fat found to have significant independent correlation with TG, HDL-C, fasting blood glucose & insulin except for association between visceral fat & fasting blood glucose (Kotronen, et al., 2011).

Thus, irrespective of the radio-diagnostic tool employed (whether MRI or CT), most of the studies clearly demonstrate strong metabolic connection between these fat depots (visceral & liver fat). However, while exploring individual contribution of these fat depots to CMR, those studies which employed CT as the radio-diagnostic tool for fat estimation showed strong independent correlation between visceral fat and the metabolic markers. Whereas studies employing MR technique provided contrasting results, i.e. liver fat having significant independent association with various metabolic markers. But the studies done so far using MRI method had relatively small sample size than those employing CT techniques. Further, it is worth noting that the results of studies using CT technique enrolling large sample size (AGES-Reykjavik study and Framingham heart study) showed results in line with MRI based study results, i.e. liver fat being stronger correlate of CMR.

Table 6: Studies using CT to determine independent association of visceral and liver fat with cardiometabolic risk factors

Article	Subjects	Tool	Metabolic parameters	Independent association of Liver fat	Independent association of Visceral fat	Conclusion
(Jackson Heart Study) Liu J et al 2011	**Sample size:** 2882 **Gender:** 35% Males & 65% Females **Condition:** Community based samples **Ethnicity**: African-Americans	CT	Fasting glucose, TG, high density lipoproteincholesterol (HDL-C), systolic/diastolic BP (SBP/DBP),MS, diabetes	Significant association with impaired glucose, TG, HDL-C, hypertension, diabetes, MS. Association persisted after adjustment of visceral fat with exception for impaired glucose and hypertension	Visceral fat associated significantly with all cardio metabolic traits ($p<0.0001$). In regression analysis, association of visceral fat with TG, HDL-C, MS & impaired glucose significantly greater than fatty liver	Fatty liver and visceral fat are independent correlates of CMR but associations are stronger for visceral fat than fatty liver
(AGES-Reykjavik Study) Kim LJ et al 2011	**Sample size:** 2495 **Gender:** 879 Males & 1616 Females **Condition:** Community based samples with no history of coronary artery disease (CAD), Diabetes or liver disease **Ethnicity**: Caucasian	CT	MS	No correlation in normal weight groups. However, significant ($p<0.001$) independent correlation in overweight/obese groups in women (OR=1.38 & 1.45). And for overweight/obese men OR= 1.38 ($p=0.01$) & 1.27 ($p=0.10$)	In women, significant ($p<0.01$) correlation in normal/overweight/obese groups (OR= 2.78, 1.63, 1.43). However, association diminished with increased BMI. In men, only overweight group showed significant ($p<0.01$) correlation, OR=1.69.	Visceral & liver fat have independent association with metabolic risk however for visceral fat, association is more significant at lower levels of obesity whereas for liver fat it is more significant at higher levels
(Framingham Heart Study) Speliotes EK et al 2010	**Sample size:** 2589 **Gender:** Male (49%) & female (51%) **Condition:** Community based samples **Ethnicity**: Primarily Caucasian	CT	MS, glucose related parameters (fasting glucose, HOMA-IR, adiponectin); lipid related: TG, HDL-C, TC, hypertension & BP	Statistically significant (all $p<0.001$) association of fatty liver with: Diabetes (OR 1.64, 95% CI 1.11-2.41) IGT (OR 1.58, 95% CI 1.21-2.07) IR (OR 2.79, 95% CI 2.14-3.65),MS (OR 1.95, 95% CI 1.48-2.56), TG & HDL. While association with SBP & DBP attenuated ($p>0.05$) on adjusting for visceral fat	In multivariate analysis, visceral fat remained significantly ($p< 0.0001$) associated with all metabolic correlates (lipid, glucose traits and SBP/DBP)	After adjustment of visceral fat, fatty liver remained significantly associated with lipid and glucose traits but the association diminished for SBP and DBP
McMillan KP et al 2007	**Sample size:** 293 **Gender:** Males **Condition:** Healthy **Ethnicity**: Caucasian	CT	Glucose, TG, TC, HDL-C	Liver fat was significantly ($p < 0.05$) associated with TC & TG independent of Subcutaneous fat, age and CRF but not after control for visceral fat.	Visceral fat remained significantly ($p< 0.01$) associated with all metabolic risk factors after control for liver fat, subcutaneous fat	Visceral fat but not liver fat is associated with metabolic risk independent of other fat depots
Kuk JL et al 2006	**Sample size:** 291 **Gender:** Males **Condition:** (97 decedents & 194 control) **Ethnicity**: Caucasian	CT	All-cause mortality	After adjustment of other fat measures (visceral and subcutaneous fat), liver fat was not found to be significant predictor mortality (OR=0.87; $p=0.55$)	Visceral fat was the only significant (OR=1.81; $p<0.05$) predictor of mortality adjusting for other fat measures	Visceral fat was the only fat measure independently predictive of mortality risk.
Nguyen-Duy TB et al 2003	**Sample size:** 162 **Gender:** Male **Condition:** Overweight/Obese **Ethnicity**: Caucasian	CT	Fasting glucose, TG, TC, LDL-C, HDL-C, TC/HDL-C	Liver fat was a significant ($p \leq 0.05$) correlate of fasting glucose and TG	Visceral fat was a significant ($p \leq 0.01$) correlate of TG, HDL-C & TG/HDL-C after control for liver fat.	Visceral fat is a stronger correlate of metabolic risk in overweight obese men than liver fat

Table 7: Studies using MRI/MRS to determine independent association of visceral and liver fat with cardiometabolic risk factors

Article	Subjects	Tool	Metabolic parameters	Independent association of Liver fat	Independent association of Visceral fat	Conclusion
Kotronen A et al 2011	**Sample size:** 356 **Gender:** 186 Males & 170 Females **Condition:** No acute or chronic disease other than obesity **Ethnicity:** Caucasian	MRI /MRS	TG, HDL-C, blood pressure, fasting glucose and insulin, liver enzymes (ALT, AST)	Significant ($p<0.001$) independent correlation of liver fat with TG, HDL-C, fasting glucose and insulin, liver enzymes	Significant ($p\leq0.001$) independent correlation of visceral fat with TG, HDL-C & Insulin	Both Liver fat and visceral fat had significant independent correlation with metabolic risk parameters. However, Liver fat and NOT visceral fat correlated significantly with fasting glucose and liver enzyme levels
Hoenig MR et al 2010	**Sample size:** 43 **Gender:** 35 Males & 8 Females **Condition:** High risk vascular cohort **Ethnicity:** Caucasian	MRI /MRS	MS	Liver fat independently associated with MS. Odds ratio 1.17. Liver fat of >4.0% identified MS with 84% sensitivity & 82% specificity	Visceral fat did not contribute to MS under logistic regression analysis	Liver fat is associated with metabolic syndrome independent of visceral fat.
Fabbrini E et al 2009	**Sample size:** 31 **Gender:** Males & Females **Condition:** Obese subjects **Ethnicity:** Caucasian	MRI /MRS	Hepatic, skeletal and adipose tissue insulin sensitivity & hepatic VLDL-TG secretion rate as determined using euglycemichyperinsulinemic clamp procedure	When matched for visceral fat values, hepatic, skeletal and adipose tissue insulin sensitivity was found to be lower (41, 36 and 13%)while hepatic VLDL-TG secretion rate was double in subjects with higher than normal liver fat content.	When matched for liver fat values, no difference in insulin sensitivity & hepatic VLDL-TG secretion rate observed between normal and higher visceral fat sub groups	Liver fat associated with metabolic derangement independent of visceral fat
Adiels M et al 2006	**Sample size:** 28 **Gender:** Males **Condition:** 10 Diabetic & 18 Non-diabetic = 18 **Ethnicity:** Caucasian	MRI /MRS	Fasting glucose, Insulin, HOMA-IR, Adiponectin, TG, ApoB	In multiple regression analysis, significant correlation with VLDL, TG and ApoB production rates.	No significant correlation with lipid variables found in multiple regression analysis	Liver fat, and not visceral fat is an independent correlate of lipid variables
Westerbacka J et al 2004	**Sample size:** 132 **Gender:** 66 Males & 66 Females **Condition:** Healthy **Ethnicity:** Caucasian	MRI / MRS	Fasting Insulin, TG, C-peptide, LDL-C, HDL-C, Adiponectin	Significant ($p<0.001$) correlation with fasting insulin and TG	Did not correlate significantly with metabolic markers. But has significant ($p<0.001$) independent correlation with liver fat.	Liver fat, but not visceral fat independently associated with visceral fat. No genderdifference in metabolic markers(insulin, TG, HDL-C &adiponectin)observed forsimilar amount of visceral
Lindros AS et al 2002	**Sample size:** 30 **Gender:** Male **Condition:** Healthy **Ethnicity:** Caucasian	MRI /MRS	Fasting insulin, TG, HDL-C, SBP, in vivo insulin sensitivity of glucose rate of production (Ra), rate of utilization (Rd) & serum FFA	Group with low and high liver fat content showed significant ($p<0.05$) difference in fasting insulin, TG, HDL-C,SBP. Further, insulin suppression glucose (Ra)and of serum FFA was significantly ($p<0.05$) impaired in high compared to low liver fat group	Visceral fat correlation with metabolic risk parameters was not studied instead at the same level of visceral fat; subjects were divided into group of High and low liver fat content.	Liver fat independently associated with features of insulin resistance and other metabolic risk

Westerbacka et al in their MR imaging study done in 132 apparently healthy (66 males and 66 females) subjects to explore the gender difference in markers of CV risk found that despite twice as much subcutaneous fat in women, amount of visceral and liver fat were comparable to that in males. No gender difference in metabolic markers (TG, HDL-C, Insulin and adiponectin) was observed for similar amounts of visceral and liver fat. Further, multiple linear regression analysis revealed that visceral fat was significantly associated with liver fat independent of subcutaneous fat. Liver fat and not visceral fat independently is predictive of variation in fasting serum insulin levels (Westerbacka et al., 2004).

With the advancement in imaging tools (MRS), the imaging studies reinforced the causal link between liver fat content and the risk of T2DM and CAD surpassing earlier notion of visceral fat being the primary target to treat obesity related metabolic dysfunction and CV risk.

CHAPTER 3 AIMS AND OBJECTIVES

3.1 Gap in existing research and study objectives

Lack of evidence in Indian perspective

Asians Indians are a high risk ethnic group for T2DM, MS and CVD; and have a unique phenotype called as the "Asian Indian Phenotype" (Sandeep et al., 2010). Studies have shown that visceral fat area is useful for quantifying the obesity related CV risk. Also, it has been postulated that hepatic fat percent may better identify the at-risk patient than visceral fat area and that hepatic fat percent is associated with metabolic risk factors independently of visceral fat area (Hoenig et al., 2010). Irrespective of the tool employed (MR or CT); most studies described were carried out in Caucasians, Whites or Western population. Thus, it is not reasonable to generalize the result of these studies across different ethnicities and races. No such comparative study has been done in Indian population.

Further, tests and procedures involving estimation of visceral/hepatic fat (using imaging modalities) and postprandial TG clearance (fat load test) are not reported to have been performed in people in India. Also, so far no published literature can be found providing reasonable estimates of these parameters in context to Indian subjects.

Need for indices estimating obesity related CV risk – Visceral adiposity index

It is well known that metabolic diseases such as diabetes and CVD are majorly prevalent in obese patients than among normal weight individuals. In this regard, visceral adiposity dysfunction (VAD) has been found to be a major link with a cluster of diabetogenic, atherogenic, prothrombotic and proinflammatory metabolic abnormalities (Despres et al., 2006). VAD causes release of different bioactive molecules and hormones, such as adiponectin, leptin, tumour necrosis factor, resistin and interleukin 6 (IL-6). Due to its anatomic location and peculiar metabolic, hyperlipolytic activity, the expanded visceral adipose depot is considered to be an independent component of CMR (Despres et al., 2006). It is well established that T2DM significantly increases the risk of CVD and that merely treating hyperglycemia does not eliminate all the CV risk (Gerich et al., 2007). Gastaldelli et al explored metabolic effects of visceral fat accumulation in T2DM using two specialized

techniques MRI and euglycemic insulin clamp. They observed significant negative impact of visceral fat over glycemic control through a decrease in peripheral insulin sensitivity and an enhancement of gluconeogenesis. Further, while ethnicity, gender, age, duration of diabetes, and obesity (as body mass index) together explained only 25% of HbA1c variability, the inclusion of visceral fat in their model raised the explicable HbA1c variability to 45%. According to their model, HbA1c is predicted to be 0.8% higher for each 50-cm^2 increment in visceral fat area. These estimates confirmed that an accurate measurement of visceral fat is an important part of clinical phenotyping and has rather direct consequences for the metabolic control of patients with T2DM (Gastaldelli et al., 2002). Since then it has been demonstrated that obese diabetic patient with raised metabolic abnormalities like high insulin resistance and atherogenic dyslipidemia associated with an excess visceral adiposity are predisposed to higher CVD risk (Despres et al., 2012).

Indian diabetes federation recommends CT & MRI for assessing visceral fat accumulation (Klopfenstein et al., 2012; Oh et al., 2013). In the interim, the available literature supports visceral fat thresholds of 100 cm^2 below which disturbances of glucose, insulin and lipid metabolism are uncommon. Secondly, a level of 130 cm^2 often detects the metabolic abnormalities representing an increased risk group (Hunter et al., 1994; Rankinen et al., 1999; Hunter et al., 2010).

With a view to identify a routinely applicable indicator of VAD having higher Se and Sp than classical parameters (such as waist circumference, BMI and lipids), Amato et al in 2010 came up with visceral adiposity index (VAI). It is a gender-specific mathematical index based on simple anthropometric [BMI and waist circumference] and metabolic [TG and HDL-C] parameters, as a presumed surrogate marker of adipose tissue function and distribution, independently linked to insulin sensitivity and CMR in the general population. Although VAI was modeled in Caucasian population, several studies confirm its validity in other races. The application of the VAI in patients with T2DM, and general population has produced interesting results, which have led to the hypothesis that the VAI could be considered a marker of adipose tissue dysfunction (Amato et al., 2010; Amato et al., 2014; Amato et al., 2014).

Several studies have been carried out in different races (Chinese, Sicilian, Japanese and Caucasians) to explore and validate VAI cut offs in determining metabolic risk. To date, no such published data is available in context to Indian population (Kumpatla et al., 2012). A study from India carried out in 600 subjects showed a proportional increase in VAI values with glucose tolerance levels, suggestive of metabolic derangement with an increase VAI. Therefore, present study will explore VAI cut off for predicting visceral adiposity dysfunction in T2DM Indian patients and to evaluate whether VAI could become a surrogate marker for visceral MRI scanning.

To addresses the research gaps highlighted above, the study was carried out to achieve following objectives:

- Evaluating the relative contributions of visceral and liver fat to cardiometabolic risk in type 2 diabetic patients

- Exploring the differences in metabolic markers and postprandial triglyceride clearance within type 2 diabetic patients based on liver fat content

- Studying the difference in metabolic markers and postprandial triglyceride clearance within type 2 diabetic patients based on gender

- Exploring visceral adiposity index as a predictor of visceral adiposity dysfunction and evaluating its performance in predicting hepatic insulin resistance in Indian type 2 diabetics

CHAPTER 4 MATERIAL AND METHODS

4.1 Entry criteria

The sample size of the study was not based on formal calculation, but the research work was carried out using the primary data from the participants recruited into the human metabolic studies from selected primary and secondary care centres in Ahmedabad, Delhi and Kolkata. The study included the background analysis to observe the said differences in anthropometric measurements and metabolic markers between the 48 healthy control and 81 T2DM patients.

The present research analyzed the data obtained from healthy controls (BMI 18.5 – 23 kg/m^2) in the age range 30-65 years without existence of any surgical or medical condition, which may compromise the conduct or outcome of the study. Further, T2DM patients were selected based on following inclusion and exclusion criteria:

Inclusion criteria

- Ethnicity Indian
- Male and females in age range of 30-65 years (both inclusive)
- Overweight/obese (BMI 23-35 kg/m^2)
- Waist circumference of ≥80 cm in females and ≥90 cm in males
- non-smokers
- For diabetes group, the participant could be newly diagnosed or known diabetic, not on any antidyslipidemic medications.

Exclusion criteria

- Participants with history of type 1 diabetes mellitus,
- Positive for pregnancy
- Any significant history of endocrine, cardiovascular, renal or hepatic disease
- Other causes of chronic liver disease were excluded based on reported alcohol, drug history and hepatitis serology test data.
- Participants with alcohol intake >20 ml/day ethanol and any individuals with a history of alcohol excess were also excluded.

All participants gave written informed consent, and ethical approval was obtained from the respective local ethics committee.

Objective I:

Aim: To determine relative contributions of visceral and liver fat to cardiometabolic risk in type 2 diabetic patients

Subjects: 81 overweight/obese T2DM patients. The diabetic patients consisted of 48 males and 33 females. All lab values are based on analysis of samples obtained after an overnight fast.

Measurements and design: Demographic details of all participants were obtained. Each participant had undergone anthropometric measurements, laboratory biochemical assessments of metabolic parameters, quantitative estimation of liver fat by ^{1}H-MRS and visceral and subcutaneous fat estimation by a single slice MRI at L4-L5 level. Anthropometric measurements included estimation of BMI and waist circumference. Laboratory assessments involved estimation of glycemic parameters (including fasting insulin and HOMA-IR calculation), lipid profile (including apoA1 and apoB) and PAI-1.

To study a contribution of liver fat to CMR, participants were divided into two groups based on liver fat content determined by ^{1}H-MRS as low liver fat (<5.56 %) and high liver fat group (≥5.56%). Anthropometric and metabolic parameters of participants were compared. Further, independent association of liver fat to CMR was evaluated after adjusting for age, visceral fat and BMI. Similarly, independent association of visceral fat to CMR was evaluated after adjusting for age, liver fat and BMI.

Objective II:

Aim: To examine whether liver fat accumulation is a predictor of delayed post prandial TG clearance and metabolic risk in a subset of overweight/obese type 2 diabetic patients

Subjects: Of 81 diabetic patients, the patients who had consented for and underwent standardized fat load test procedure were considered for analysis. Thus, data from 76 patients was evaluated, which included 45 males and 31 females.

Measurements and design: Postprandial fat meal responses were examined following consumption of standardized fat meal equivalent to 30 g fat/m^2 body surface area. TG levels were estimated before (fasting) and after standardized fat meal consumption. Area under the TG curve was determined for each participant using trapezoid method. Difference in area under the curve (AUC) for postprandial TG and incremental AUC (iAUC) levels was evaluated in low and high liver fat groups.

Objective III:

Aim: To explore gender differences in metabolic parameters and postprandial TG clearance in overweight/obese type 2 diabetic patients

Subjects: 81 overweight/obese T2DM patients. The diabetic patients consisted of 48 males and 33 females. All lab values are based on analysis of samples obtained after an overnight fast. For postprandial TG clearance, of 81 diabetic patients, the patients who had consented for and underwent standardized fat load test procedure were considered for analysis. Thus, data from 76 patients was evaluated, which included 45 males and 31 females.

Measurements and design: To evaluate gender difference in metabolic parameters, dataset was divided based on gender. Also, to minimize a contribution of postmenopausal metabolic changes in females, gender difference was evaluated in participants aged ≤ 51 years. Postprandial fat meal responses were examined following consumption of standardized fat meal equivalent to 30 g fat/m^2 body surface area. TG levels were estimated before (fasting) and after standardized fat meal consumption. Area under the TG curve was determined for each participant using trapezoid method. Difference in postprandial TG AUC and iAUC levels was evaluated across gender and age group ≤51 years.

Objective IV:

Aim: Explore VAI cut off to determine VAD and evaluating its performance in predicting hepatic insulin resistance in overweight/obese type 2 diabetic patients

Subjects: 81 overweight/obese T2DM patients. The diabetic patients consisted of 48 males and 33 females. All lab values are based on analysis of samples obtained after an overnight fast.

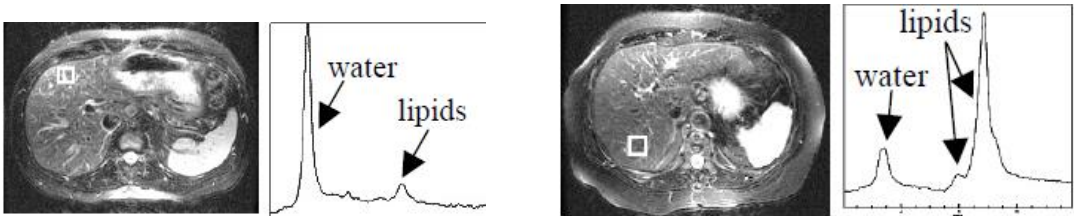
Measurements and design: All subjects had undergone anthropometric measurements, laboratory biochemical assessments of metabolic parameters, quantitative estimation of liver fat by ^{1}H-MRS and visceral and subcutaneous fat estimation by MRI. Anthropometric measurements included estimation of BMI and waist circumference. Laboratory biochemical assessments involved estimation of glycemic parameters (including fasting insulin and HOMA-IR calculation), lipid profile (including apoA1 and apoB) and PAI-1. In addition to this, VAI was derived using waist circumference, BMI, TG and HDL-C values of the participants.

VAI cut off to predict VAD was determined using receiver operating curve (ROC) analysis and considering visceral fat area of ≥130 cm^2 as a marker for VAD. Diabetic participants were divided into two groups based on VAI cut off as VAD absent and VAD present group respectively. The difference in metabolic profile between the groups was evaluated. Further, the correlation between VAI and hepatic insulin resistance (HOMA-IR) was also determined.

4.2 Methods

4.2.1 Estimation of liver fat content

Liver fat content was quantitatively determined using proton MRS performed using 3 Tesla MRI set up (Philips/Siemens). ^{1}H-MRS is a sensitive method considered as the gold standard for liver fat estimation. The MRS Procedure was based on a standard protocol (Exam Card) shared with the radio-diagnostic centers. A voxel of 20 X 20X 20 mm was positioned in the right hepatic lobe, avoiding inclusion of the diaphragm and edges of the liver, but also vascular and biliary structures. Voxel size and time for acquisition were standardized for all subjects. The liver ^{1}H-MR spectra was evaluated using jMRUI software for lipid and water peak areas and the results were expressed as percentage contents of lipids (refer to figure 7).



(A) **(B)**

Figure 7: Lipid and water peaks obtained from a normal liver (A) and a fatty liver (B)

Liver fat content was calculated as:

100*Lipid peak area / (Lipid peak area + water peak area)

The liver MRS scans so obtained from respective radio diagnostic centres were analyzed and the results were reported by central lab, i.e. Academic Medical Centre, Amsterdam.

Considerations: 1H-MRS has proven to be a very sensitive non-invasive method to detect liver fat content and has shown to correlate well with liver biopsy results (refer to figure 8) (Werven et al., 2009)**.** To date, MR imaging, particularly 1H-MRS, has been by far the most promising and most sensitive non-invasive method to assess liver fat content (Werven et al., 2010)**.**

Steatosis as defined by biopsy according to a number of affected hepatocytes: S1 (5–33%, ‘‘mild’’), S2 (33–66%, ‘‘moderate’’), S3 (>66%, ‘‘severe’’). Considering non-invasive methods as an alternative to liver biopsy, ^{1}H- MRS cut-off value for the detection of any steatosis grade corresponds to data from Szczepaniak et al. who determined a hepatic fat percent of 5.56% (or 55.6 mg/g liver tissue) to define fatty liver (Szczepaniak et al. 2005; Karlas et al., 2014).

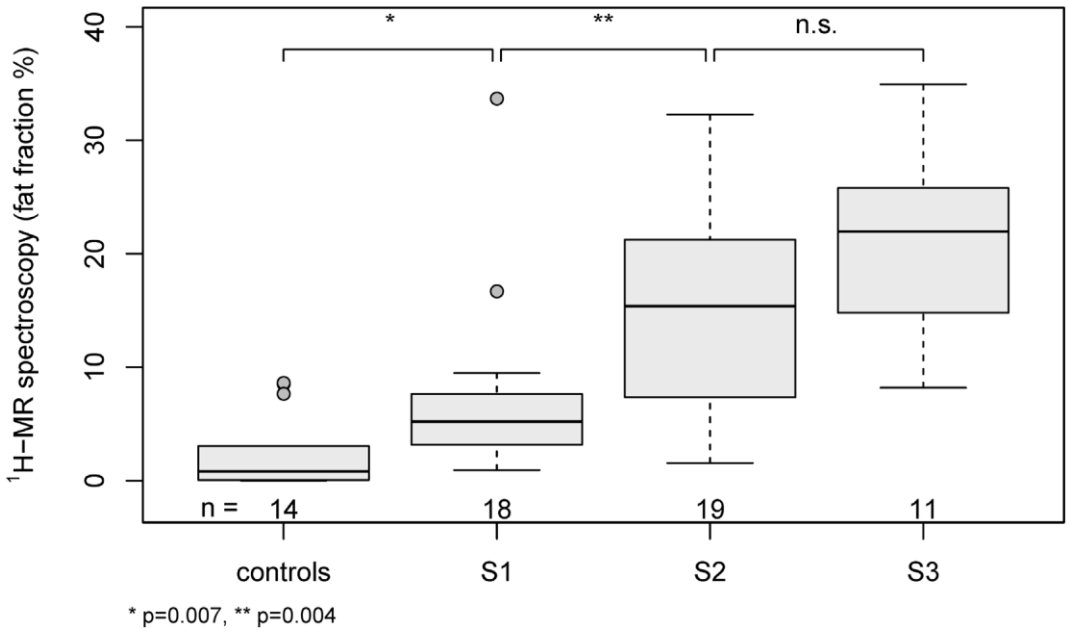


Figure 8: Correlation between biopsy defined grading of liver fat (S1, S2 & S3) and non-invasive 1H-MRS employed liver fat estimation.

Liver fat percent cut off of 5.56% is validated in various large population studies and the results have been consistent. Thus this cut off has been employed in our study.

4.2.2 Estimation of visceral and subcutaneous fat area

Abdominal fat measurement was done by using single slice axial measurement at the lumbar level of L4-L5 region using 3-Tesla MRI. As that for liver MRS, MRI procedure was also based on standard protocol. Prior to scanning, participants were coached through a deep breathing exercise, as the participants had to hold their breath for the duration of the scan, approximately 18 seconds. The scan acquisition was prescribed from the saggital scout such that image plane is passed through the centre of the vertebral disc between the L4 and L5 vertebrae (refer to figure 9 and 10).

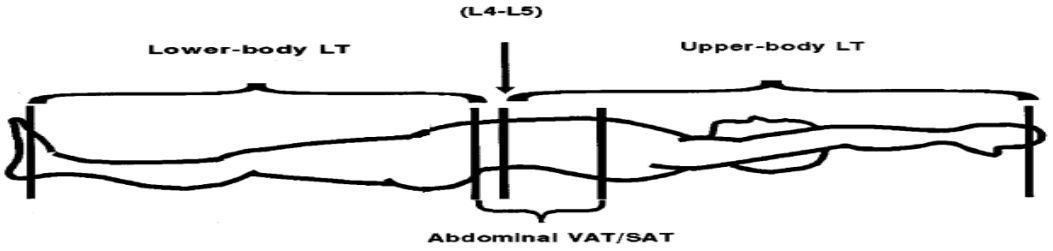


Figure 9: Single slice axial measurement at the level of L4-L5

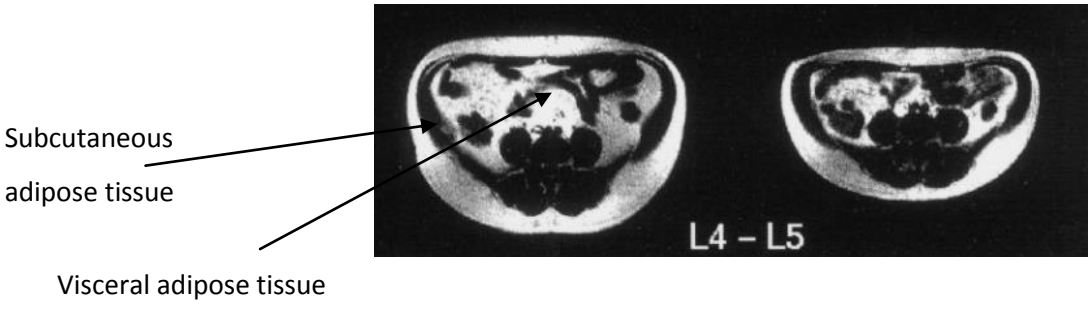


Figure 10: Showing visceral and subcutaneous fat area. Adipose tissue appears white and non-adipose tissue appears black.

The selected radio-diagnostic centers acquired the scans and shared the same in DICOM format (files compatible with a commercial image analysis software program). The scans were analyzed using slice-O-matic software Version 4.3, (TomoVision, Montreal, Qc, Canada) for estimation of visceral and subcutaneous fat area (as shown in figure 10).

Abdominal fat segmentation was done based on intensity histogram. A threshold was chosen at the local minimum between the low intensity first peak, representing the muscle and background pixel intensities, and the higher intensity peak, representing the fat pixel intensities. Fat area was then tagged automatically based on this threshold. The fat segment was further divided into subcutaneous and visceral compartments by drawing the contours. The non-fat components of the segmentation, such as the spinal cord and stray single isolated islands of fat, were omitted manually by carefully drawing region of interest.

The results (visceral and subcutaneous fat area) were to be expressed as square of millimeter (mm^2).

Visceral fat area for each participant was converted into cm^2 unit and was also coded ordinal such that visceral fat area <130 cm^2 was classified as absence of VAD while visceral fat area ≥130 cm^2 represented presence of VAD.

4.2.3 Anthropometric assessments - estimation of weight, height, BMI and waist circumference

All anthropometric measurements were carried out by trained medical/paramedical personnel.

Height was measured in a standing position without shoes to the nearest 0.5 cm using the portable Leicester height measure. During the measurement, patients were asked to take a deep breath and look straight ahead with the head upright, whilst ensuring heels remained on the floor and together.

Weight was measured to the nearest 0.1 kg using standardized instrument. Patients were asked to wear light clothing and to remove their shoes prior to measurement.

BMI was calculated using the equation: BMI = body weight (kg) ÷ $height^2$ ($meter^2$).

Waist circumference was measured using adjustable tape midway between the lower rib margin and the iliac crest. The step wise procedure is as follows:

1. The person stands with the feet shoulder width apart. The arms hang on each side of the body at an angle towards outside.
2. Mark bony landmarks of the right and left last rib margin.
3. Mark bony landmarks of the right and left iliac crest.
4. Mark mid-distance between the last rib margin and the top of the iliac crest of the two sides.
5. Place the tape horizontally directly on the skin with respect to both mid-distance landmarks.
6. Suggest the patient to relax and breathe normally (abdominal muscles should not be contracted). Ask the patient to take 2 or 3 normal breaths. Measure the waist circumference (to the nearest millimetre) at the end of a normal expiration.

4.2.4 Derived parameters or index

Homeostasis model assessment

Insulin Resistance was estimated using HOMA-IR. It is an index based on fasting glucose and fasting insulin values. Formula as given below:

HOMA-IR = fasting glucose (mmol/L) X fasting insulin (μU/ml)/22.5

Non-HDL Cholesterol

Non-HDL cholesterol was derived by subtracting HDL-C from TC

Visceral adiposity index

VAI score was calculated using the following gender-specific equations, when TG levels expressed in mmol/l and HDL-C levels expressed in mmol/l:

Males: VAI = (WC/ 39.68 + (1.88 × BMI)) X (TG/1.03) X (1.31/HDL)

Females: VAI = (WC/ 36.58 + (1.89 × BMI)) X (TG/0.81) X (1.52/HDL)

4.2.5 Biochemical assessments

All participants underwent biochemical assessments. All metabolic parameters were measured from venous blood samples using standard methods in the central research laboratory.

Blood samples were analyzed for:

- Fasting plasma glucose, fasting serum Insulin and HbA1C
- Metabolic Parameters
    - Lipid Profile: TC, HDL-C, LDL-C, Fasting TG, apoB, apoA1, apoB/A1 ratio. All lipid parameters were estimated in serum samples.
    - Inflammatory marker PAI-1 in plasma
    - Post fat load serum TG estimation

Fat Load Test to study post fat meal clearance

Fat load test was performed to measure postprandial lipid kinetics.

Method: Standardised fat load was administered after an overnight fast. After collection of sample for baseline measurement, a high fat meal equivalent to 30 gm fat/m^2 body surface area was given.

Amul Cream was used as a fat source; the composition of cream is as follows:

<u>Per 100 ml of cream:</u>

Total fat: 25 gm (saturated fat=16 gm)

Carbohydrate- 3.2 gm

Sugar – 0 gm

Protein – 2 gm

Fat per ml = 0.25 gm

Amount (ml) of Cream to be ingested by a subject was calculated as:

= (30 * Body Surface Area) / 0.25

The preparation was to be consumed within 5 min of start of meal.

[Example: If a subject has weight 80 kg and height 165 cm, then body surface area will be 1.94 m^2. For this body surface area, the amount of Amul Cream will be calculated as:

= (30*1.94)/0.25

= 232 ml]

The serum samples were analyzed for TG levels at time points were 0 (pre meal), 2hr, 3hr, 4hr, 5hr post meal.

4.2.5.1 Analytical procedures for estimation of metabolic parameters

All metabolic parameters were analyzed at central lab.

Glucose concentrations in plasma samples were estimated with Hexokinase method using Cobas Integra 400 plus – Roche analyzer. Fasting insulin concentration in serum was measured by electrochemilluminescence method using Cobas Integra 400 plus – Roche analyzer. HbA1C was estimated in K2 EDTA whole blood through high performance liquid chromatography using D10 BioRad analyzer.

All lipid parameters were analyzed in serum samples. TC was measured with CHOD-POD enzymatic method using Cobas Integra 400plus- Roche analyzer. Both fasting and postprandial TG was measured with GPO-PAP method using Cobas Integra 400plus-Roche analyzer. HDL-C and LDL-C was measured with homogenous enzymatic colorimetric method using Cobas Integra 400plus-Roche analyzer. apoB and apoA1 were measured with Immunoturbidimetery method using Cobas Integra 400plus-Roche analyzer.

PAI Type-I was measured in citrated plasma through enzyme linked immunosorbant assay (ELISA) method using Da-Vinci Quottro analyzer.

4.2.6 Statistical analyses (Objective I, II, III, and IV)

In all analysis, p-value of less than 0.05 was considered statistically significant. Statistical analyses for all objectives were carried out using SPSS 17.0 for Windows (SPSS, Chicago, IL) and online GraphPad software. Line chart and bar diagrams were plotted using Microsoft excel 2010. Shapiro-Wilk's test was used to explore a distribution of each variable and logarithmic transformation with variables representing non-normal distribution.

Objective I:

Participants were divided into two groups based on their liver fat content (< or ≥ 5.56%). Non-normally distributed data were used after logarithmic (base 10) transformation. If distributed normally, data are shown as means ± SD, whereas non-normally distributed data are shown as a median (25% percentile, 75% percentile). The unpaired Student's t test was used to compare mean values between groups. For non-parametric data, Man Whitney test was applied to compare median values between the groups.

To study independent association of liver fat to CMR factors, multivariate analysis of covariance (MANCOVA) was used to adjust for age, visceral fat and BMI. Similarly, Independent association of visceral fat was studied for two cut offs, i.e. 100cm^2 and 130cm^2 after adjusting for age, liver fat and BMI as covariate.

Objective II:

Data are presented as mean ± SD. The AUC and iAUC for postprandial TG levels were calculated according to the trapezoid rule. Missing values for a given time-point were imputed by interpolation. Both AUC and iAUCs were compared between subjects with low liver fat (<5.56%) and high liver fat (≥5.56%) content using unpaired student's t-test. AUCs and iAUCs of low liver fat and high liver fat groups were also compared graphically using line plots and bar diagram respectively.

Objective III:

Participants were initially divided based on their gender, and metabolic parameters were compared between males and females. Similarly, gender wise comparison was

made in the age group <51years. If distributed normally, data are presented as mean ± SD, while non- normally distributed data are shown as a median followed by the interquartile range (25^{th} and 75^{th} percentiles) for continuous variables and as proportions for categorical variables. Difference between the groups were analyzed using unpaired t-tests or Mann–Whitney U tests was used for continuous variables, depending on whether relevant distributional assumptions were met.

Objective IV:

If distributed normally, data are presented as mean ± SD, while non- normally distributed data are shown as a median followed by the interquartile range (25^{th} and 75^{th} percentiles) for continuous variables and as proportions for categorical variables. To assess the ability of a variable to discriminate between patients with and without VAD, ROC curves were constructed for VAI. In addition, for VAI, we measured a number of other diagnostic statistics: the Se, Sp, positive likelihood ratio and negative likelihood ratio at various cut-points. Statistical comparisons of patients with and without VAD were taken for all demographic variables, unpaired t-tests or Mann–Whitney U tests was used for continuous variables, depending on whether relevant distributional assumptions were met. Linear regression analysis was performed to evaluate the merit of VAI cut point in predicting hepatic insulin resistance.

CHAPTER 5 RESULTS

5.1 Characteristics of diabetic versus healthy participants

Sample size of the study was 129. The data was collected from the participants who belonged to overweight/obese T2DM group (n=81) and healthy control group (n=48). Initially, inferential analysis was used to ascertain whether there was any significant difference between diabetic group and healthy control group in terms of anthropometric, clinical and biochemical metabolic parameters.

Table 8: Comparison between diabetic and healthy control group based on the anthropometric, clinical and biochemical metabolic parameters.

Parameters	Group	
	Controls (C) N = 48 (M:F 35:13)	**Diabetic (D) N = 81 (M:F 46:35)**
Anthropometric parameters		
Age (years)	34.5 (31, 41.5)	52.0 (45.5, 57)**
BMI (Kg/m^2)	21.8 (20.3, 22.6)	27.5 (25.4, 30.5)**
Waist circumference (cm)	80.6 ± 6.2	97.5 ± 9.4**
Clinical & Biochemical parameters		
Visceral fat (cm^2)	70.2 ± 50.3	148.6 ± 50**
Liver fat (%)	1.1 (0.6, 1.7)	4.7 (2.7, 9) **
Fasting glucose (mg/dl)	93.9 (84.8, 108.6)	132.1 (105.8, 172.7) **
HbA1C (%)	5.5 (5.2, 5.7)	7.4 (6.5, 8.5) **
Fasting insulin (µU/ml)	7.3 (4.3, 10.7)	17.3 (10.2, 40.2) **
HOMA-IR	1.7 (0.94, 2.6)	5.4 (2.61, 14.9) **
Total cholesterol (mg/dl)	163 ± 38.5	195.4 ± 40.5 **
Triglyceride (mg/dl)	81.2 (60.6, 100.6)	177.2 (114.3, 242.6) **
LDL-C (mg/dl)	98.5 ± 30.3	108.7 ± 29.8
HDL-C (mg/dl)	41 (33.9, 46.5)	40.8 (35.4, 47.3)
Non HDL-C (mg/dl)	120.9 ± 35.0	152.9 ± 39.9 **
Apo B (mg/dl)	75.1 (55, 99.6)	83.6 (69.6, 107.8)*
Apo A1 (mg/dl)	118.3 ± 29.7	132.6 ± 23.4*
Apo (B/A1) ratio	0.7 (0.5, 0.9)	0.62 (0.53, 0.85)
Visceral Adiposity Index	1.13 (0.9, 1.8)	3.1 (1.7, 4.5) **

Data are presented as mean ± SD for normally distributed data and median (interquartile) for non-normal distribution. Consequently, continuous variables compared using the unpaired t-tests or Mann-Whitney U tests depending on whether data met the relevant distributional assumptions. BMI= Body Mass Index; HbA1C- Haemoglobin A1C; HOMA-IR- Insulin Resistance Index; LDL-C=Low Density Lipoprotein Cholesterol, HDL-C= High Density Lipoprotein Cholesterol. **p<0.01, *p<0.05, M = Males, F = Females

Table 8 presents the comparison between diabetic and healthy control group based on the anthropometric, clinical and bio-chemical metabolic parameters. In this study, 48 participants were in healthy control while 81 were in diabetic group. There was a significant difference in the anthropometric parameters between diabetic and healthy control group with respect to their age (p<0.001), BMI (p<0.001) and waist circumference (p<0.001). In fact, diabetic subjects were older (D=52.0 years > C=34.5 years) than control group subjects. In addition, diabetic group subjects had significantly higher BMI (D=27.5kg/m^2 > C=21.8kg/m^2) and waist circumference (D=97.5cm > C= 80.6cm) compared to control group subjects. The amount of differences in age, BMI and waist circumference between diabetic group and healthy control group participants were 17.5 years, 5.7kg/m^2 and 16.9cm respectively.

Likewise, when it is considered the clinical and bio-chemical metabolic parameters of subjects, diabetic group and healthy control group differed significantly (p<0.01) in terms of visceral fat level, liver fat level, fasting glucose level, HbA1C level, fasting insulin level, HOMA-IR, TC level, TG level, non-HDL-C level and VAI. Diabetic group subjects had significantly higher visceral fat (D=148.6cm^2 > C=70.2cm^2), liver fat (D=4.7% > C=1.1%), fasting glucose (D=132.1mg/dl > C=93.9mg/dl), HbA1C (D=7.4% > C=5.5%), fasting insulin (D=17.3µU/ml > C=7.3µU/ml), HOMA-IR (D=5.4 > C=1.67), TC (D=195.4mg/dl > C=163.0mg/dl), TG (D=177.2mg/dl > C=81.2mg/dl), non-HDL-C (D=152.9mg/dl > C=120.9mg/dl) and VAI (D=3.1 > C=1.13) than healthy control group subjects. The amount of differences in visceral fat, liver fat, fasting glucose, HbA1C, fasting insulin, HOMA-IR, TC, TG, non-HDL-C and VAI between diabetic and healthy control group subjects were 78.4cm^2, 3.6%, 38.2mg/dl, 1.9%, 10.0µU/ml, 3.73, 32.4mg/dl, 96.0mg/dl, 32.0 mg/dl and 1.97 respectively.

In addition, diabetic group and healthy control group varied significantly (p<0.05) with respect to apoB and apoA1. Diabetic group subjects had higher apoB (D=83.6mg/dl > C=75.1mg/) and apoA1 (D=132.6mg/dl > C=118.3mg/dl) than healthy control group subjects. In addition, the amount of differences in apoB and apoA1 were 8.5mg/dl and 14.3mg/dl respectively. However, both group had almost a similar level of LDL-C (p>0.05), HDL-C (p>0.05) and apoB/A1 ratio (p>0.05).

5.2 Determining metabolic differences within type 2 diabetic patients based on gender

Of 81 T2DM patients, 48 patients were males and 33 patients were females. To understand the gender wise baseline characteristics, unpaired t test or Mann-Whitney U test was applied to evaluate a difference between male and female diabetic patients in terms of level of anthropometric, clinical and biochemical metabolic parameters. Further, considering the fact that menopause is a state of hormonal turbulence with a possibility of weight gain, visceral obesity and development of MS in females; age of ≤51 years was considered based on literature to rule our post-menopausal females. Consecutively, unpaired t test or Mann-Whitney U test was applied in male (n=18) and female (n=17) diabetic patients group that aged ≤51 years.

5.2.1 Differences in anthropometric, clinical and biochemical parameters between male and female diabetic patients (overall)

Table 9 presents the comparison between male and female diabetic patients based on the levels of anthropometric, clinical and biochemical metabolic parameters. In the study, 48 patients were males, and 33 patients were females.

When considered the anthropometric parameters of patients, there was a significant (p<0.001) difference between male and female diabetic patients with respect to waist circumference. Male diabetic patients had significantly higher waist circumference (M=101.52cm > F=91.21cm) compared to female diabetic patients. Hence, the amount of difference in waist circumference between male and female diabetic patients was 10.31cm. However, BMI and age were comparable (M=51.81, F=50.96) in male and female diabetic patients.

Similarly, when considered the clinical and biochemical metabolic parameters of patients, male and female diabetic patients highly differed with respect to HDL-C level (p=0.004<0.01) and apoA1 (p=0.001<0.01). Female diabetic patients possessed higher HDL-C level (M=37.5mg/dl < F=42.90mg/dl) and apoA1 (M=124.15mg/dl < F= 143.33mg/dl) compared to male diabetic patients. Hence, the amounts of difference in HDL-C and apoA1 between male and female diabetic patients were 5.4mg/dl and 19.18mg/dl respectively. However, there was no statistically significant difference

between male and female diabetic patients with respect to the levels of liver fat (p=0.219 >0.05), PAI-1 (p=0.408 >0.05), fasting glucose (p=0.777 >0.05), fasting insulin (p=0.526 >0.05), HbA1C (p=0.841 >0.05), HOMA-IR (p=0.590 >0.05), TC (p=0.219 >0.05), non-HDL-C (p=0.649 >0.05), LDL-C (p=0.537 >0.05), TG (p=0.825 >0.05), apoB (p=0.799 >0.05), subcutaneous fat (p=0.199 >0.05) and visceral fat (p=0.075 >0.05). Hence, it may be concluded that male and female diabetic patients were similar with respect to most of the metabolic parameters (liver fat, PAI-1, fasting glucose, fasting insulin, HbA1C, HOMA-IR, TC, non-HDL-C, LDL-C, TG, apolipoprotein, subcutaneous fat and visceral fat).

Table 9: Comparison between male and female type 2 diabetic participants based on anthropometric, clinical and biochemical metabolic parameters.

Parameters	**Gender**	
	Males (n=48)	**Females (n=33)**
Anthropometric parameters		
Age (years)	51.81 ± 8.63	50.96 ± 7.51
BMI (kg/m^2)	28.06 ± 3.07	27.96 ± 3.28
Waist circumference (cm)	101.52 ± 8.88	91.21 ± 6.46**
Clinical & Biochemical parameters		
Liver fat (%)	6.25 (2.82, 10.77)	3.80 (2.05, 8.15)
PAI-1	28.16 (12.55, 48.05)	30 (18.44, 47.56)
Fasting glucose (mg/dl)	133.35 (110.39, 172.59)	132.08 (97.29, 215.90)
Fasting insulin (µU/ml)	18.27 (10.55, 40.98)	17.25 (8.57, 39.28)
HbA1C (%)	7.65 ± 1.38	7.72 ± 1.59
HOMA-IR	6.65 (2.78, 13.69)	4.68 (2.25, 16.78)
Total cholesterol (mg/dl)	189.45 ± 43.75	201.01 ± 37.22
HDL-C (mg/dl)	37.50 (34.25, 44.67)	42.90 (38.80, 51.50)**
Non HDL-C (mg/dl)	149.96 ± 42.75	154.16 ± 37.49
LDL-C	106.00 ± 31.47	110.24 ± 28.39
Triglycerides (mg/dl)	181.80 (114.25, 227.03)	167.90 (110.65, 268.50)
Apo A1 (mg/dl)	124.15 ± 19.66	143.33 ± 23.31**
Apo B (mg/dl)	83.70 (67.92, 115.35)	80.80 (69.10, 102.20)
Subcutaneous fat (cm^2)	269.51 ± 91.83	299.91 ± 119.15
Visceral fat (cm^2)	157.03 ± 54.03	136.85 ± 41.85

Data are presented as mean ± SD for normally distributed data and median (interquartile) for non-normal distribution. Consequently, continuous variables compared using the unpaired t-tests or Mann-Whitney U tests depending on whether data met the relevant distributional assumptions. M=Male, F=Female **p<0.01

5.2.2 Differences in anthropometric, clinical and biochemical parameters between male and females diabetic patients (Age ≤51 years) – to exclude post-menopausal females

Considering the fact that menopause is a state of hormonal turbulence with a possibility of weight gain, visceral obesity and development of MS, age of ≤51 years was employed based on literature to rule our post-menopausal females. Consecutively, unpaired t test or Mann-Whitney U test was used in male (n=18) and female (n=17) diabetic patients group that aged ≤51 years.

Table 10: Comparison between male and female diabetes mellitus participants who aged ≤ 51 years, based on anthropometric, clinical and biochemical parameters.

Parameters	**Gender**	
	Males (n=18)	**Females (n=17)**
Anthropometric parameters		
Age (years)	42.99 ± 6.62	45.05 ± 4.66
BMI (kg/m^2)	27.67 ± 2.96	27.57 ± 3.48
Waist circumference (cm)	102.96 ± 8.08	90.06 ± 6.14**
Clinical & Biochemical parameters		
Liver fat (%)	10.45 (6.05, 12.60)	2.70 (1.60, 6.55)**
PAI-1	38.93 (22.71, 52.88)	26.27(22.14, 49.59)
Fasting glucose (mg/dl)	145.97 (106.88, 190.92)	120.20 (96.14, 171.00)
Fasting insulin (µU/ml)	20.90 (17.18, 51.04)	17.25 (10.75, 36.49)
HbA1C (%)	7.62 ± 1.51	7.65 ± 1.62
HOMA-IR	8.15 (4.60, 22.89)	4.75 (2.52, 12.95)
Total cholesterol (mg/dl)	188.37 ± 49.09	191.82 ± 29.55
HDL-C (mg/dl)	35.70 (30.05, 43.53)	42.00 (38.25, 52.85)*
Non-HDL (mg/dl)	152.433 ± 46.07	145.05 ± 33.67
LDL-C (mg/dl)	100.84 ± 30.88	101.32 ± 25.62
Triglycerides (mg/dl)	222.55 (147.22, 268.60)	167.90(108.05, 268.50)
Apo A1 (mg/dl)	123.67 ± 22.90	145.40 ± 24.98**
Apo B (mg/dl)	78.70 (66.52, 117.87)	78.70 (66.75, 92.30)
Subcutaneous fat (cm^2)	278.35 ± 78.95	292.15 ± 113.52
Visceral fat (cm^2)	165.58 ± 51.39	134.22 ± 44.08

Data are presented as mean ± SD for normally distributed data and median (interquartile) for non-normal distribution. Consequently, continuous variables compared using the unpaired t-tests or Mann-Whitney U tests depending on whether data met the relevant distributional assumptions.*M=Male, F=Female; **p<0.01 *p<0.05*

In the 81 diabetic patients, 46 patients were in above 51 year age group, and 35 patients were in below 51 year age group. Among the 35 patients, 18 patients were males and 17 patients were females.

Table 10 shows the comparison between male and female diabetic patient group that aged ≤51 years, based on the levels of anthropometric, clinical and biochemical metabolic parameters. Initially, when considered the anthropometric parameters, there was a significant (p<0.01) difference between male and female diabetic patients who aged below 51 years, based on waist circumference. Male diabetic patients who aged below 51 years had higher waist circumference (M=102.96cm > F=90.06cm) compared to females. The amount of difference in waist circumference between male and female diabetic patients who aged below 51 years was 12.90cm. However, BMI level and age were similar in male and female diabetic patients who aged below 51 years.

Similarly, when considered the clinical and biochemical metabolic parameters, male and female diabetic patients who aged below 51 years, varied significantly in terms of liver fat level (p=0.002 <0.01), HDL-C (p=0.013 <0.05) and apoA1 (p=0.011 <0.01). Female diabetic patients who aged below 51 years had higher HDL-C level (M=35.70mg/dl < F=42.00mg/dl) and apoA1 (M=123.67mg/dl < F=145.40mg/dl) than the males. While males who aged below 51years possessed significantly higher liver fat level (M=10.55% > F=4.70%) compared with females. The amount of difference in HDL-C, apoA1 and liver fat between male and female diabetic patients who aged below 51 years, were 6.3mg/dl, 21.73mg/dl and 5.85% respectively.

Finally, there was no significant difference between male and female diabetic patients who aged below 51 years, with respect to the levels of PAI-1 (p=0.741 >0.05), fasting glucose (p=0.428 >0.05), fasting insulin (p=0.166 >0.05), HbA1C (p=0.955 >0.05), HOMA-IR (p=0.121 >0.05), TC (p=0.804 >0.05), non-HDL-C (p=0.594 >0.05), LDL-C (p=0.961 >0.05), TG (p=0.306 >0.05), apoB (p=0.644 >0.05), subcutaneous fat (p=0.678 >0.05) and visceral fat (p=0.062 >0.05). Hence, male and female diabetic patients who aged below 51 years possessed an almost similar level of PAI-1, fasting glucose, fasting

insulin, HbA1C, HOMA-IR, TC, non-HDL-C, LDL-C, TG, apoB, subcutaneous fat and visceral fat.

Hence, tables 9 and 10 suggest that though the difference in metabolic parameters in T2DM patients across gender was more when considered age group ≤51 years; there was no significant difference in the metabolic parameters and visceral fat area across the gender except for HDL, ApoA1 and liver fat levels.

5.2.3 Difference in postprandial triglyceride clearance based on gender (overall)

A total of 76 diabetic patients who underwent fat load test were considered for evaluation, of which 45 patients were males and 31 patients were females. Unpaired t test was used to compare whether male and female patients showed similar post prandial TG clearance. Post prandial clearance was evaluated based on AUC obtained from TG concentrations at various time point post fat load administration. Both AUC and incremental AUC were considered for analysis.

Moreover, of these 76 diabetic pateints, 33 patients aged ≤ 51 years. Again unpaired t-test was applied between male and female patients who aged ≤ 51 years to compare whether male and female patients showed similar post prandial TG clearance (AUC and iAUC) minimising the confounding effect of menopause in females.

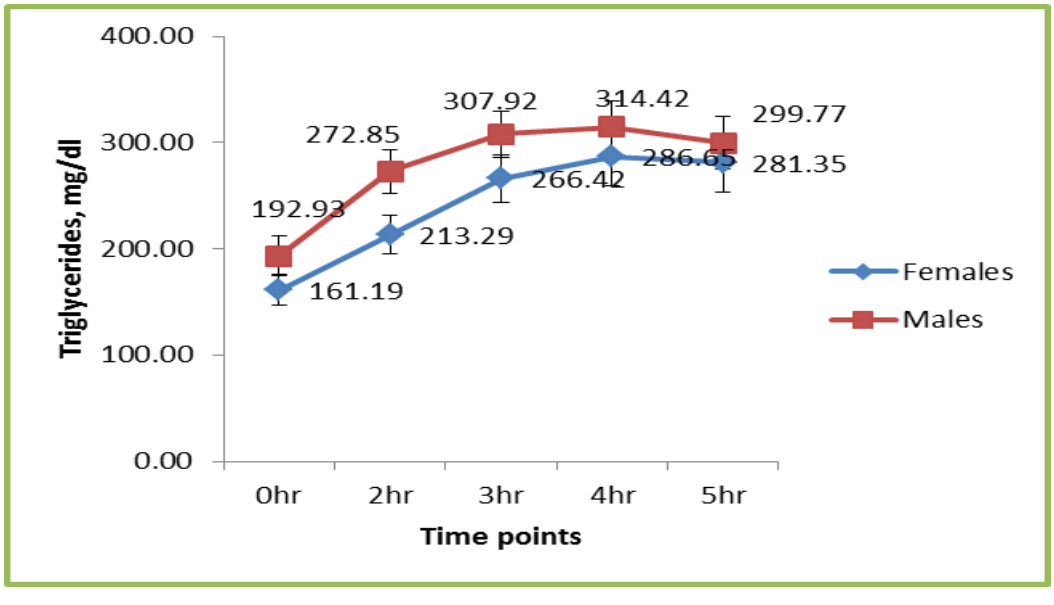


Figure 11: Postprandial triglyceride response (AUC) in males and females (Overall, N=76)

Figure 11 revealed that male diabetic patients had higher postprandial TG AUC level compared to female diabetic patients. However, the difference in AUC did not meet statistical significance. From figure 11, initially male diabetic patients had TG AUC level 192.93 mg/dl while female diabetic patients had 161.19 mg/dl. TG AUC levels regularly increased among male and female diabetic patients for the time-points 2hr, 3hr and finally achieving its peak at 4hr. After 5 hours, male diabetic patients possessed TG AUC level 299.77 mg/dl while female diabetic patients had 281.35 mg/dl.

When considered the post-prandial TG AUC level increment of male patients, an increment was 79.92 mg/dl for the 0^{th} hour to 2^{nd} hour. Similarly, for the 2^{nd} hour to 3^{rd} hour and 3^{rd} hour to 4^{th} hour the increments were 35.07 mg/dl and 6.5 mg/dl respectively. However, for the 4^{th} hour to 5^{th} hour, a decrement level was 14.65mg/dl. Analogously when considered the females post-prandial TG AUC level increment, an increment was 52.10 mg/dl for the 0^{th} hour to 2^{nd} hour. For the 2^{nd} to 3^{rd} hour and 3^{rd} to 4^{th} hour, increments of TG AUC level were 53.13 mg/dl and 20.23 mg/dl respectively. However, the decrement of TG AUC was observed for the time hours 4^{th} to 5 hours (5.3 mg/dl). The peak for TG AUC of both male and female patients was observed at 4 hours post meal. This suggests that, males had relatively higher postprandial TG response and similar time-point of peak levels compared to females.

Further, the difference in TG levels between male and female patients, at the 0^{th} hour was 31.74mg/dl, at 2^{nd} hour was 59.56mg/dl. At 3^{rd}, 4^{th} and 5^{th} hours the differences of triglyceride levels between male and female patients were 41.50mg/dl, 27.77mg/dl and 18.42mg/dl respectively. It showed that male patients had rapid increment in triglyceride level than female patients within 2^{nd} hour of fat meal consumption.

Table 11: Comparison between male and female diabetic patients based on the postprandial triglycerides AUC level (Overall, N=76).

	Gender		**p-value**
	Males (n=45)	**Females (n=31)**	
Post prandial TG AUC Level	1375.98 ± 687.59	1178.61 ± 571.25	0.19

Data are presented as mean ± SD for normally distributed data, Continuous variable compared using the unpaired t-tests ** p <0.01, * p <0.05, M=Males, F=Females

Table 11 provides the mean comparison between male and female diabetic patients based on the postprandial TG AUC level. The significance value (p=0.192 >0.05) specifies that male and female diabetic patients had an almost similar level of postprandial TG AUC. Male and female diabetic patients' postprandial TG AUC levels were 1375.98 mg/dl and 1178.61 mg/dl respectively.

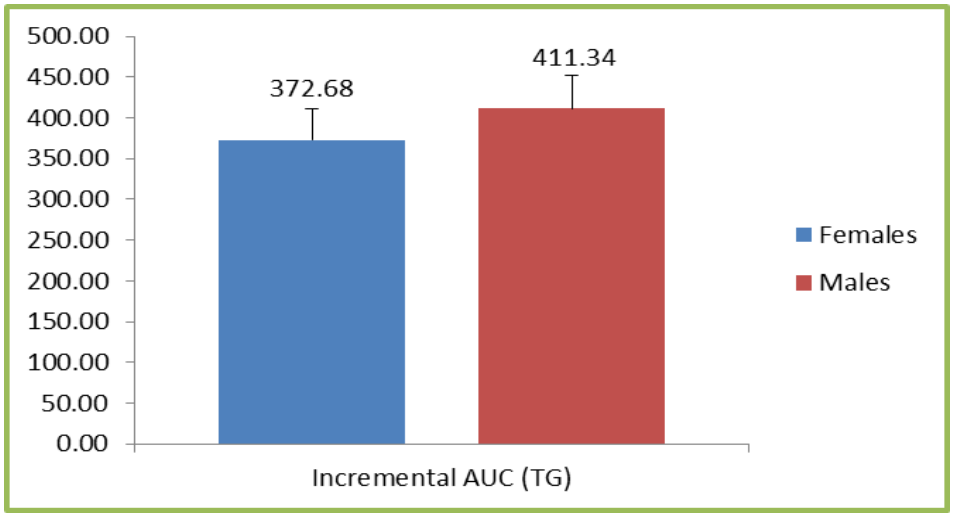


Figure 12: Post-prandial incremental triglyceride response (iAUC) in males and females (overall)

Figure 12 was drawn based on the average value of postprandial TG iAUC level. The chart exhibited that the male diabetic patients had postprandial TG iAUC level 411.34mg/dl while for female diabetic patients, it was 372.68mg/dl. Hence, male diabetic patients had higher postprandial TG iAUC level compared to female diabetic patients. The amount of difference in post-prandial TG iAUC level between male and female diabetic patients was 38.66 mg/dl.

Table 12: Comparison between male and female diabetic patients based on the postprandial triglycerides iAUC level.

	Gender		**p-value**
	Males (n=45)	**Females (n=31)**	
Post prandial TG iAUC level	411.34 ± 268.86	372.68 ± 212.63	0.51

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests. ** p <0.01, * p <0.05, M=Males, F=Females

Table 12 shows the mean comparison between male and female diabetic patients with respect to postprandial TG iAUC level. The significance value (p>0.05) indicates that male and female diabetic patients did not vary significantly based on postprandial TG iAUC level. The postprandial TG iAUC levels of male and female diabetic patients were 411.34mg/dl and 372.68mg/dl respectively.

5.2.4 Difference in postprandial triglyceride clearance based on gender (Age ≤51 years) – to exclude post-menopausal females

Of 76 diabetic pateints who underwent fat load test, 33 patients aged ≤ 51 years. Again unpaired t-test was applied between male and female patients who aged ≤ 51 years to compare whether male and female patients showed similar postprandial TG clearance (AUC and iAUC) minimising the confounding effect of menopause in females.

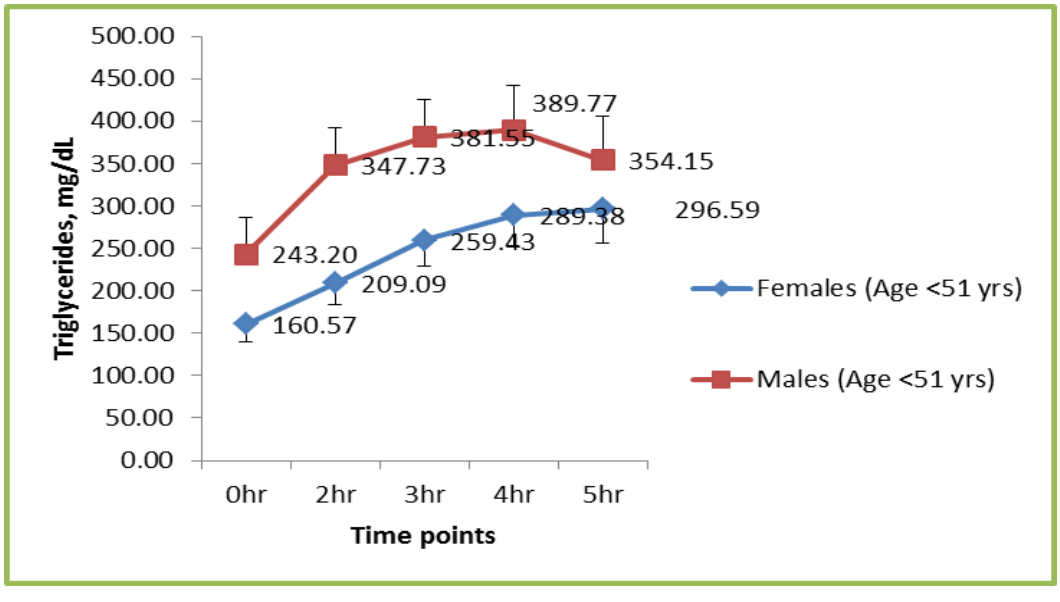


Figure 13: Post-prandial triglyceride response (AUC) in males (n=17) and females (n=16) aged ≤ 51 years

Study comparing postprandial response of male and female diabetic patients aged ≤ 51 years included 17 males and 16 females. The line chart (Figure 13) indicated that initially in fasting state, male diabetic patients less than 51 years had TG level 243.20 mg/dl while female diabetic patients less than 51 years retained only 160.57 mg/dl. TG levels regularly increased for the time 2hr, 3hr and 4hr among the male and female diabetic patients who aged less than 51 years. After 5 hours, male diabetic patients

possessed TG level of 354.15 mg/dl while female diabetic patients had 296.59 mg/dl. Overall male diabetic patients, who aged less than 51 years, had higher postprandial TG AUC level compared with female diabetic patients who aged less than 51 years at various time periods.

When considered the post-prandial TG AUC level increment of male patients, an increment was 104.53mg/dl for the 0^{th} hour to 2^{nd} hour. Similarly, for the 2^{nd} hour to 3^{rd} hour and 3^{rd} hour to 4^{th} hour the increments were 33.80mg/dl and 8.24mg/dl respectively. However, for the 4^{th} hour to 5^{th} hour, a decrement level was 35.62mg/dl. The peak for triglycerides AUC level of male patients who aged less than 51 years was perceived during 3 to 4 hours.

Analogously when considered the post-prandial TG AUC level increment of female patients, an increment was 48.52mg/dl for the 0^{th} hour to 2^{nd} hour. For the 2^{nd} to 3^{rd} hour and 3^{rd} to 4^{th} hour, increments of TG AUC level were 50.34mg/dl and 29.95mg/dl respectively. However, the decrement of TG AUC was observed for the time hours 4^{th} to 5 hours (7.29mg/dl). The peak for TG AUC level of female patients who aged less than 51 years was observed during 4 to 5 hours. This suggests that, males aged less than 51 years had significantly higher postprandial TG response compared to females. However, males showed faster clearance of fat compared to females. Peak TG concentration in blood achieved at 3 to 4 hours in males while females showed plateau having its peak TG concentration at 5^{th} hours post fat meal consumption. This indicates that female diabetics aged less than 51 years had relatively delayed postprandial clearance compared to males.

Further, the difference between male and female patients who aged below 51 years, at 0^{th} hour the difference of TG AUC was 82.63mg/dl, at 2^{nd} hour the difference of TG was 138.64mg/dl, at 3^{rd},4^{th} and 5^{th} hours the difference of TG were 122.10mg/dl, 100.39mg/dl and 57.56mg/dl respectively. It revealed that male patients who aged less than 51 years had rapid TG level increment than female patients who aged less than 51 years for the 2^{nd} hour.

Table 13: Comparison between male and female diabetic patients who aged ≤ 51 years, based on the postprandial triglycerides AUC level.

	Gender	
	Males (n=17)	**Females (n=16)**
Post prandial TG AUC level	1711.00 ± 895.50	1179.00 ± 563.90*

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests.** p <0.01, * p <0.05, M=Males, F=Females

Table 13 presents the mean comparison between male and female diabetic patients who aged below 51 years, based on the postprandial TG AUC level. From the significance value (p<0.05), male and female diabetic patients who aged below 51 years, varied significantly in terms of postprandial TG AUC level. Male diabetic patients possessed higher postprandial TG AUC level (M=1711.00mg/dl > F=1179.00mg/dl) compared with female diabetic patients who aged below 51 years. Hence, the amount of difference in postprandial TG AUC level between male and female diabetic patients who aged below 51 years was 532.00mg/dl.

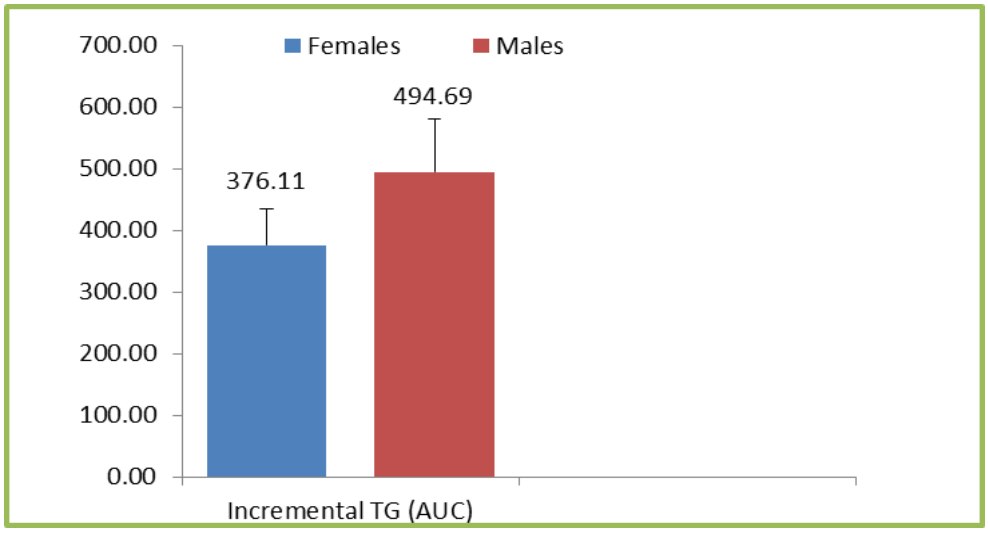


Figure 14: Postprandial incremental triglyceride response (iAUC) in males and females (Age ≤51 years)

Male diabetic patients had the postprandial TG iAUC level 494.69mg/dl while for female diabetic patients; it was 376.11mg/dl. The bar chart (Figure 14) revealed that male diabetic patients had higher postprandial TG iAUC level compared to female

diabetic patients. Hence, the amount of difference in post-prandial TG iAUC level between male and female diabetic patients was 118.58mg/dl.

Table 14: Comparison between male and female diabetic patients who aged ≤ 51 years, based on the postprandial triglycerides iAUC level.

	Gender		p-value
	Males (n=17)	Females (n=16)	
Post prandial TG iAUC	494.69 ± 355.04	376.11 ± 233.32	0.27

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests. ** p <0.01, * p <0.05, M=Males, F=Females

Table 14 provides the mean comparison between male and female diabetic patients who aged below 51 years, based on the postprandial TG iAUC level. The significance value (p >0.05) specifies that there was no significant difference between male and female diabetic patients, in terms of postprandial TG iAUC level.

5.2.5 Difference in body fat distribution based on gender and its contribution to postprandial triglyceride clearance

To examine the difference in pattern of body fat distribution across gender and to study importance of visceral fat and liver fat accumulation to the gender difference in postprandial TG clearance, men and women were matched for various fat depots. First, BMI was matched and corresponding differences in subcutaneous, visceral and liver fat were observed. Subsequently, each fat measure was matched and differences in other fat depots were studied across gender.

5.2.5.1 Difference in body fat distribution

Table 15 shows body fat distribution of males and females. Matched for BMI consists of 20 male and 20 female participants. When compared males and females who were matched for BMI, males had significantly ($p<0.01$) higher visceral fat (M=159.1 > F=125.6) and liver fat (M=7.8 > F=3.5) compared to females. However, males had lower to similar subcutaneous fat (M=278.9 < F=286.2) compared to females.

Likewise, matched for subcutaneous fat and matched for visceral fat group contain 24 participants (12 males and 12 females) and 26 participants (13 males and 13 females)

respectively. When compared males and females who were matched for subcutaneous fat, males and females had comparable BMI levels (M=28.44 < F=28.59), while males had though not significant but relatively higher visceral fat (M=154.60 > F=143.84) and liver fat (M=8.15 > F=4.93) compared with females. Analogously when males and females were matched for visceral fat, females had higher BMI (M=27.45 < F=28.76) and subcutaneous fat (M=250.57 < F=311.24) compared to males. While the difference between liver fat values reduced with males having higher levels compared to females (M=7.8 > F=6.0).

Table 15: Body fat distribution in men and women matched for A) BMI, B) Subcutaneous fat, C) Visceral fat & D) Liver fat.

Variables	**Males**	**Females**	
Matched for BMI			**P-value**
No. of subjects (N)	20	20	
BMI (kg/m^2)	27.7 ± 2.9	27.8 ± 2.9	NA
Visceral fat (cm^2)	159.1 ± 39.4	125.6 ± 37.3	0.009**
Subcutaneous fat (cm^2)	278.9 ± 88.2	286.2 ± 93.0	0.8
Liver fat (%)	7.8 ± 5.6	3.5 ± 2.7	0.004**
Matched for Subcutaneous fat			
No. of subjects (N)	12	12	
BMI (kg/m^2)	28.4 ± 2.8	28.6 ± 2.9	0.865
Visceral fat (cm^2)	154.6 ± 54.3	143.8 ± 35.7	0.571
Subcutaneous fat (cm^2)	321.9 ± 70.6	322.9 ± 71.1	NA
Liver fat (%)	8.2 ± 7.0	4.9 ± 6.4	0.241
Matched for Visceral fat			
No. of subjects (N)	13	13	
BMI (kg/m^2)	27.5 ± 3.1	28.8 ± 3.7	0.341
Visceral fat (cm^2)	151.7 ± 29.2	152.5 ± 29.3	NA
Subcutaneous fat (cm^2)	250.6 ± 72.0	311.2 ± 115.4	0.121
Liver fat (%)	7.8 ± 4.2	6.0 ± 4.4	0.297
Matched for Liver fat			
No. of subjects (N)	15	15	
BMI (kg/m^2)	27.8 ± 3.2	27.7 ± 3.3	0.934
Visceral fat (cm^2)	156.1 ± 41.6	147.9 ± 44.6	0.607
Subcutaneous fat (cm^2)	267.2 ± 91.9	271.4 ± 109.8	0.91
Liver fat (%)	7.4 ± 5.5	7.5 ± 5.4	NA

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests.** p <0.01, * p <0.05

Matched for liver fat levels contain 30 patients (15 males and 15 females). In contrast to above findings, when males and females were matched for liver fat values, BMI and subcutaneous fat levels became comparable between the gender and the difference in visceral fat area also diminished. Yet males had higher visceral fat area than females (M=156.1 > F=147.9).

This suggests that males tend to have higher visceral and liver fat values compared to females at a same level of BMI and subcutaneous fat and females tend to have higher subcutaneous fat than males even when both genders are matched for visceral fat. However, liver fat explicitly explains the variance in body fat distribution across the gender. It was observed that when males and females were matched for liver fat levels, the variance in visceral and subcutaneous fat between the genders diminished with subcutaneous fat levels becoming similar.

Further, above results predicts that the predisposition of males to higher CMR can be attributed to the higher levels of visceral and liver fat accumulation in them.

5.2.5.2 Difference in postprandial triglyceride clearance when matched for various fat measures

Mean TG AUCs of males and females were compared while matching the groups for various fat measures. as shown in table 16.

Table 16: Difference in TG AUCs across gender when matched for different fat measures.

Parameters	Males	Females	P-value
Matched for subcutaneous fat			
N	12	12	
TG AUC	1372.19 ± 995.40	1096.26 ± 666.48	0.43
Matched for visceral fat			
N	13	13	
TG AUC	1394.94 ± 587.42	1250.26 ± 647.94	0.55
Matched for liver fat			
N	15	15	
TG AUC	1363.92 ± 847.68	1411.06 ± 651.13	0.86

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests.** p <0.01, * p <0.05 M=Males, F=Females

In previous sections, it was observed that gender difference exists in postprandial TG clearance and that males have higher TG AUCs compared to females. The difference became significant when post-menopausal females were excluded, i.e. age group of ≤51 years was considered. Thus, to elucidate the plausible role of body fat distribution in postprandial TG clearance pattern across gender, following analysis was carried out.

First, while matching males and females for subcutaneous fat, although the difference in TG AUCs reduced compared to that observed previously in section 5.2.3, the difference was still noticeable (M=1372.19 > F=1096.26). Secondly, males and females were matched for visceral fat levels and the difference in TG AUCs further declined (M=1394.94 > F=1250.26). Lastly, males and females were matched for liver fat content, the difference in TG AUCs almost nullified or reversed to some extent (M=1363.92 < F=1411.06) with p-value 0.865. These results indicated that both visceral and liver fat explains the severity of metabolic derangement and it negatively influences postprandial TG clearance in T2DM patients. Refer table 16.

Figures 15, 16 and 17 below shows the gender differences in TG AUCs when matched for different fat measures. The bars between the AUC curves of males and females represent the magnitude of difference. It is evident from the figures that when matched of visceral fat levels the magnitude of difference is reduced compared to the difference observed when matched for subcutaneous fat. Similarly, at a same level of liver fat level, the difference in TG AUCs is nullified and to some extent, is reversed with females showing delayed TG clearance as compared to males.

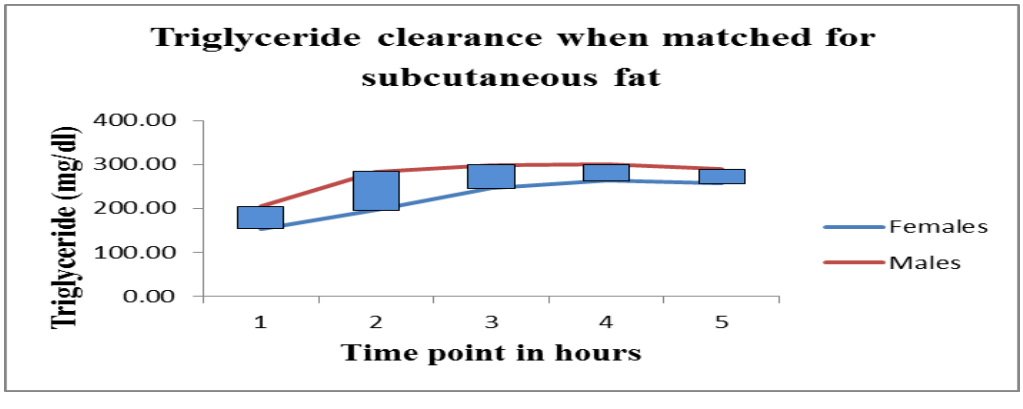


Figure 15: Gender difference in postprandial triglyceride clearance when matched for subcutaneous fat levels

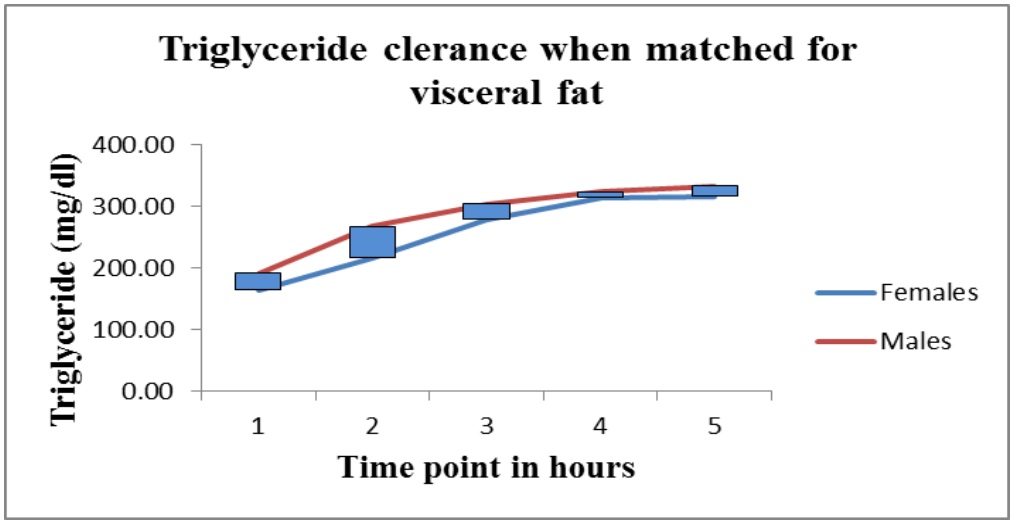


Figure 16: Gender difference in postprandial triglyceride clearance when matched for visceral fat levels

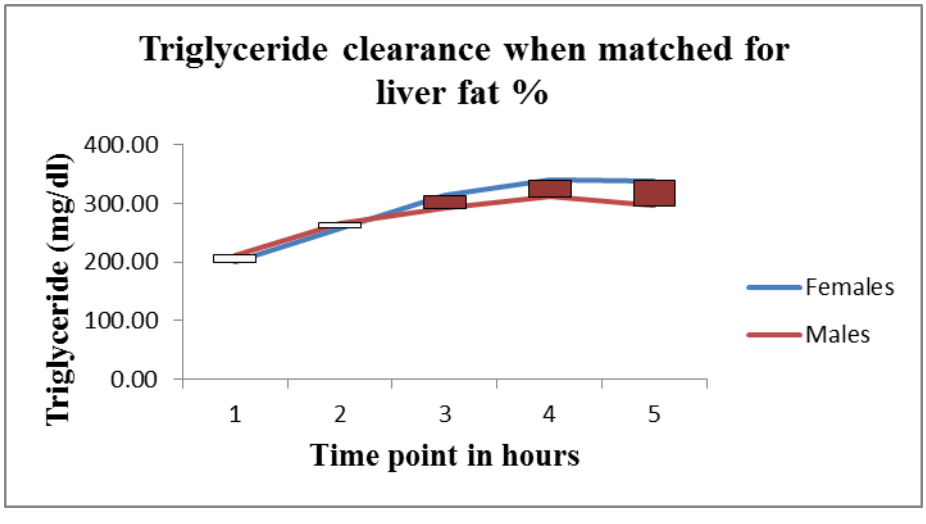


Figure 17: Gender difference in postprandial triglyceride clearance when matched for liver fat levels

5.3 Determining metabolic differences within type 2 diabetic patients based on liver fat content

5.3.1 Differences in anthropometric, clinical and biochemical parameters between type 2 diabetic patients with low liver fat content and high liver fat content

81 diabetic patients were segregated based on their liver fat level as determined using MRS. In this case, 43 patients had low liver fat (< 5.56%, n=43) and remaining 38 patients had high liver fat (≥5.56%, n=38). Unpaired t test or Mann Whitney U test was performed to evaluate a difference in metabolic parameters between the two groups segregated based on defined liver fat level.

The purpose of the analysis is to determine whether any significant difference exists within diabetic patients when segregated based on low and high liver fat group in terms of anthropometric, clinical and biochemical metabolic parameters.

Table 17 presents the comparison between diabetic patients who had low liver fat and high liver fat, based on the levels of anthropometric, clinical and biochemical metabolic parameters. When considered the anthropometric parameters, the diabetic patients in low and high liver fat groups were significantly distinct in terms of their waist circumference level (p=0.047 <0.05). The diabetic patients who had high liver fat possessed higher waist circumference (H=99.53cm > L=95.37cm) compared with the diabetic patients who had low liver fat. The amount of difference in waist circumference between low and high liver fat groups was 4.16cm. However, there was no statistically significant difference in BMI level and age between the diabetic patients in low and high liver fat group.

Table 17: Comparison between diabetic patients, who had low and high liver fat, based on anthropometric, clinical and biochemical parameters**.**

Parameters	**Levels of liver fat**	
	Low Liver Fat (n=43)	**High Liver Fat (n=38)**
Anthropometric parameters		
Age (years)	52.97 ± 7.88	49.77 ± 8.22
BMI (kg/m^2)	27.81 ± 2.96	28.26 ± 3.35
Waist circumference(cm)	95.37 ± 9.37	99.53 ± 9.14*

Clinical & Biochemical parameter		
Liver fat (%)	2.70 (1.60, 3.80)	9.90 (7.45, 13.58)**
PAI-1	28.91 (13.96, 47.42)	28.71 (15.93, 50.5)
Fasting glucose (mg/dl)	125.60 (97.91,	142.12 (113.04, 206.47)*
Fasting insulin (µU/ml)	11.24 (8.16, 30.15)	24.82 (16.83, 61.27)**
HbA1C (%)	7.43 ± 1.23	7.97 ± 1.66
HOMA-IR	3.54 (2.09, 12.88)	11.41 (4.26, 27.17)**
Total cholesterol (mg/dl)	188.19 ± 34.97	200.92 ± 47.16
HDL-C (mg/dl)	43.60 (36.50, 52.40)	37.50 (33.78, 43.15)**
Non HDL-C (mg/dl)	142.29 ± 34.98	162.28 ± 44.05*
LDL-C (mg/dl)	103.39 ± 27.60	112.63 ± 32.46
Triglycerides (mg/dl)	133.2 (102.2, 207.3)	212.2(146.47, 288.59)**
Apo A1 (mg/dl)	135.21 ± 23.43	128.29 ± 22.49
Apo B (mg/dl)	81.50 (67.20, 97.30)	83.65 (71.52, 116.82)
Subcutaneous fat (cm^2)	278.61 ± 100.75	285.61 ± 109.22
Visceral fat (cm^2)	132.99 ± 132.99	166.69 ± 50.71**

Data are presented as mean ± SD for normally distributed data and median (interquartile) for non-normal distribution. Consequently, continuous variables compared using the unpaired t-tests or Mann-Whitney U tests depending on whether data met the relevant distributional assumptions. **p<0.01; *p<0.05: L-Low liver fat group, H-High liver fat group

Analogously when considered the clinical and biochemical metabolic parameters, the diabetic patients in low and high liver fat group, significantly differed on the liver fat level (p=0.001 <0.01), fasting glucose level (p=0.037 <0.05), fasting insulin level (p=0.005 <0.01) insulin resistance as represented by HOMA-IR (p=0.004 <0.01), HDL-C level (p=0.002 <0.01), non-HDL (p=0.026 <0.05), TG level (p=0.001 <0.01) and visceral fat level (p=0.002 <0.01). Diabetic patients who had high liver fat, retained significantly (p<0.01) higher fasting insulin (H=24.81µU/ml > L=11.24µU/ml), HOMA-IR (H=11.41 > L=3.54), TG (H=212.20mg/dl > L=133.20mg/dl) and visceral fat (H=166.69cm^2 > L=132.99cm^2) compared to the diabetic patients who had low liver fat. In addition, the diabetic patients who had high liver fat possessed significantly higher fasting glucose (H=142.12mg/dl > L=125.60mg/dl) and non-HDL-C (H=162.28mg/dl > L=142.29mg/dl) than the diabetic patients who had low liver fat. Hence, the amount of difference in fasting insulin, HOMA-IR, TG, visceral fat, fasting glucose and non-HDL-C between low and high liver fat's diabetic patients were 13.57µU/ml, 7.87, 79.0mg/dl,

33.7cm^2, 16.52mg/dl and 19.99mg/dl respectively. However, the diabetic patients in low and high liver fat group, retained almost similar levels of PAI-1 (p=0.906 >0.05), HbA1C (p=0.095 >0.05), TC (p=0.168 >0.05), LDL-C (p=0.170 >0.05), apoA1 (p=0.180 >0.05), apoB (p=0.332 >0.05) and subcutaneous fat (p=0.765 >0.05).

5.3.2 Differences in postprandial triglyceride clearance between type 2 diabetic patients with low liver fat content and high liver fat content

Postprandial TG clearance was determined from fat load test carried out using standardized fat meal. TG levels were estimated in the blood before consumption of the standardized fat meal and subsequently 2, 3, 4 and 5 hours postprandial. From a total of 81diabetic patients, data of 76 patients who underwent fat load test were considered for analysis. Among the 76 patients, 39 patients had low liver fat and 37 patients had high liver fat. Postprandial TG AUC and iAUC levels were measured among the patients who had low and high liver fat at distinct time periods. In order to compare the patients who had low and high liver fat based on postprandial TG AUC and iAUC levels, unpaired t test was performed in this section.

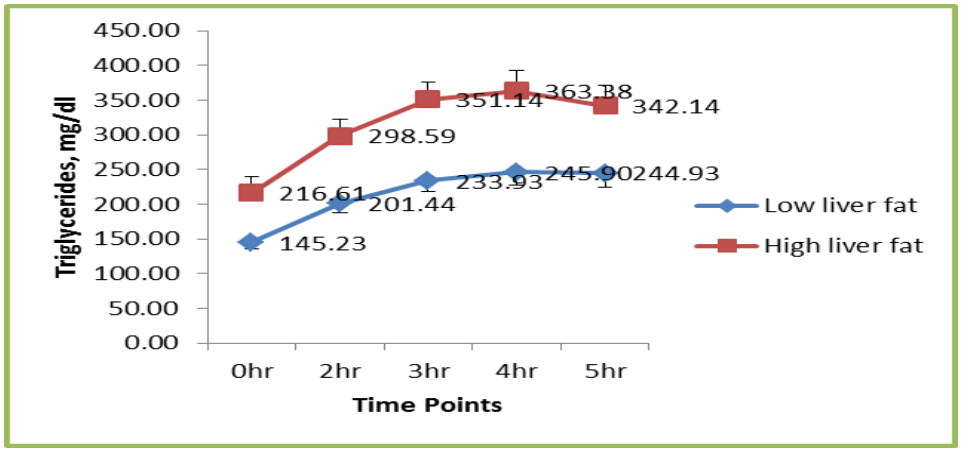


Figure 18: Postprandial TG response (AUC) in low and high liver fat groups

The line chart strongly revealed that the diabetic patients, who had high liver fat, possessed significantly higher postprandial TG AUC level compared to the diabetic patients who had low liver fat at all time periods (Figure 18 & Table 18). From the figure 18, at 0 hours, diabetic patients with high liver fat had mean TG level of 216.61mg/dl while diabetic patients with low liver fat retained only 145.23mg/dl. TG

levels gradually increased for the 2hr, 3hr and 4hr among the low and high liver fat diabetic patients.

When considered diabetics with high liver fat, an increment of TG level was 81.98mg/dl for the 0^{th} to a 2^{nd} hour. Likewise, for the 2^{nd} to a 3^{rd} hour and 3^{rd} to a 4^{th} hour were 52.55mg/dl and 12.24mg/dl, respectively.

In the case of diabetics with low liver fat, an increment of TG was gradual where 0^{th} to 2^{nd} hour an increment was 56.21mg/dl, 2^{nd} to a 3^{rd} hour (32.49mg/dl) and 3^{rd} to a 4^{th} hour (11.97mg/dl). The peak for TG of a diabetic with high and low liver fat patients was observed during 3 to 4 hours.

Further, the difference between diabetic patients with low and high-liver fat, at the 0^{th} hour the difference of TG AUC was 71.38mg/dl. Likewise, at the 2^{nd}, 3^{rd}, 4^{th} and 5^{th} hours the differences of TG AUC between low and high liver fat diabetic patients were 97.15mg/dl, 117.21mg/dl, 117.48mg/dl and 97.21mg/dl respectively. It specified that diabetic with high liver fat patients had higher TG AUC level than diabetic with low liver fat patients.

Table 18: Comparison between the diabetic patients, who had low and high liver fat, based on the postprandial triglycerides AUC level.

	Levels of liver fat	
	Low liver fat (n=39)	**High liver fat (n=37)**
Post prandial TG AUC level	1053.12 ± 433.40	1550 ± 735.28**

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests **p<0.01 L=Low liver fat, H=High liver fat

Table 18 shows the mean comparison between diabetic patients who had low and high liver fat, based on the postprandial TG AUC level. The significance value (p <0.01) strongly revealed that there was the highly significant difference between the diabetic patients who had low and high liver fat, with respect to postprandial TG AUC level. Diabetic patients with high liver fat, had significantly higher postprandial TG AUC (H=1550.00mg/dl > L=1053.12mg/dl) compared with the diabetic patients with low liver fat. Hence, the amount of difference in postprandial TG AUC level among the diabetic patients who had low and high liver fat was 499.88mg/dl.

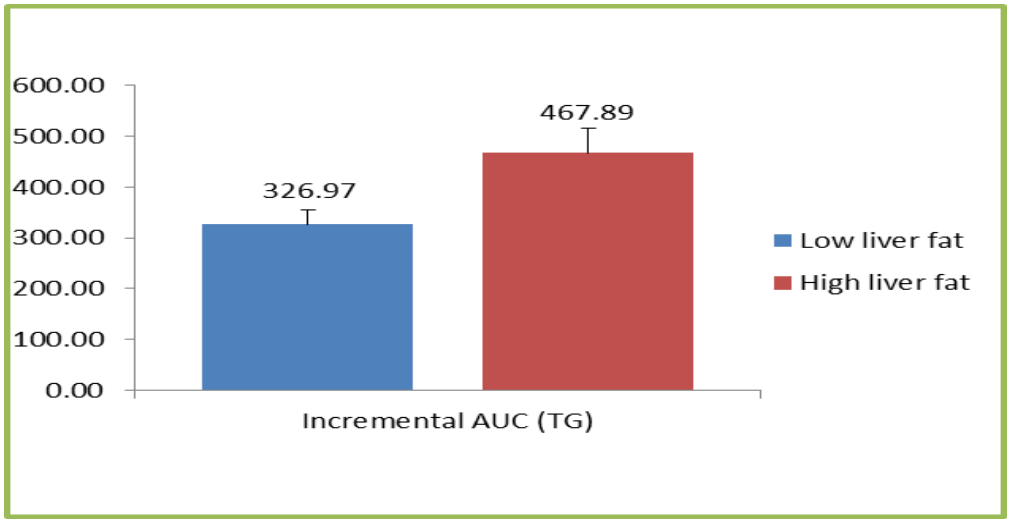


Figure 19: Incremental Postprandial TG response (iAUC) in low and high liver fat groups

Figure 19 shows that the diabetic patients with high liver fat retained the postprandial TG iAUC level 467.89mg/dl while the diabetic patients who had low liver fat possessed relatively low iAUC level of 326.97mg/dl. The difference in post-prandial TG iAUC level between low and high liver fat diabetic patient group was 140.92mg/dl.

Table 19: Comparison between the diabetic patients who had low and high liver fat based on the postprandial triglycerides iAUC level**.**

	Levels of liver fat	
	Low liver fat	**High liver fat**
Post prandial TG iAUC	326.97 ± 177.92	467.89 ± 287.89**

Data are presented as mean ± SD for normally distributed data, continuous variables compared using the unpaired t-tests.**p<0.01 L=Low liver fat, H=High liver fat

Table 19 presents the comparison between the diabetic patients who had low and high liver fat, in terms of postprandial TG iAUC level. Diabetic patients who had high liver fat possessed significantly ($p<0.05$) higher postprandial TG iAUC level (H=467.89mg/dl > L=326.97mg/dl) compared to the diabetic patients who had low liver fat.

Overall, these data reveals that the diabetic patients with high liver fat have relatively higher postprandial TG peaks and thus have delayed TG clearance. Resultantly, this group is in a more hypertriglyceridemic state as compared to diabetics with low liver fat.

5.4 Determining independent association of liver fat and visceral fat to cardiometabolic risk in type 2 diabetic patients

5.4.1 Relative contribution of liver fat and visceral fat to metabolic derangements in type 2 diabetic patients.

T2DM patients were divided based on low and high visceral fat area. The high visceral fat area category refers to those greater than or equal to 130cm^2 of visceral fat area as quantified using single slice MRI at L4-L5 level. These groups were further divided based on liver fat levels. Low liver fat was defined as liver fat percentage of <5.56% and high liver fat as ≥5.56% as determined using liver MRS.

Histograms of mean (SD) values for fasting insulin, HOMA-IR, fasting glucose, HDLc, non HDL-C and TG were plot as shown in figures 20 (a-f) below.

Comparing the difference in metabolic parameters between the low and high liver fat groups within diabetic patients having low visceral fat area (< 130 cm^2) –

There was no difference in fasting insulin levels between the low and high liver fat group. Insulin resistance was found to be higher in high liver fat group; however, the difference was not significant. Further, there was significant ($p<0.05$) difference in fasting glucose ($p=0.031$), non HDL-c ($p=0.002$) and TG ($p< 0.003$) levels between low and high liver fat group. High liver fat group had significantly high fasting glucose (174.25 mg/dl > 123.35 mg/dl), non HDL-c (199.18 mg/dl > 145.7 mg/dl) and TG (289.49 mg/dl > 143.89 mg/dl) compared to low liver fat group. In addition, high liver fat group had low HDL-c levels compared to low liver fat group (42.48 mg/dl < 50.45 mg/dl); however, the difference was not significant.

Comparing the difference in metabolic parameters between the low and high liver fat groups within diabetic patients having high visceral fat area (≥ 130 cm^2) –

High liver fat group showed a more deranged profile in terms of all metabolic parameters compared to low liver fat group. However, none of the differences were significant except for TG levels ($p=0.027$) between the group. High liver fat group showed higher levels of fasting insulin (50.38 µU/ml > 35.74 µU/ml), HOMA-IR (21.45 > 15.48), fasting glucose (160.53 mg/dl > 143.19 mg/dl), non HDL-c (152.44 mg/dl >

139.33 mg/dl) and TG (230.69 mg/dl > 160.56 mg/dl) compared to low liver fat group. Again, high liver fat group had low HDL-c levels compared to low liver fat group (37.62 mg/dl < 41.93 mg/dl); however, the difference did not achieve significance.

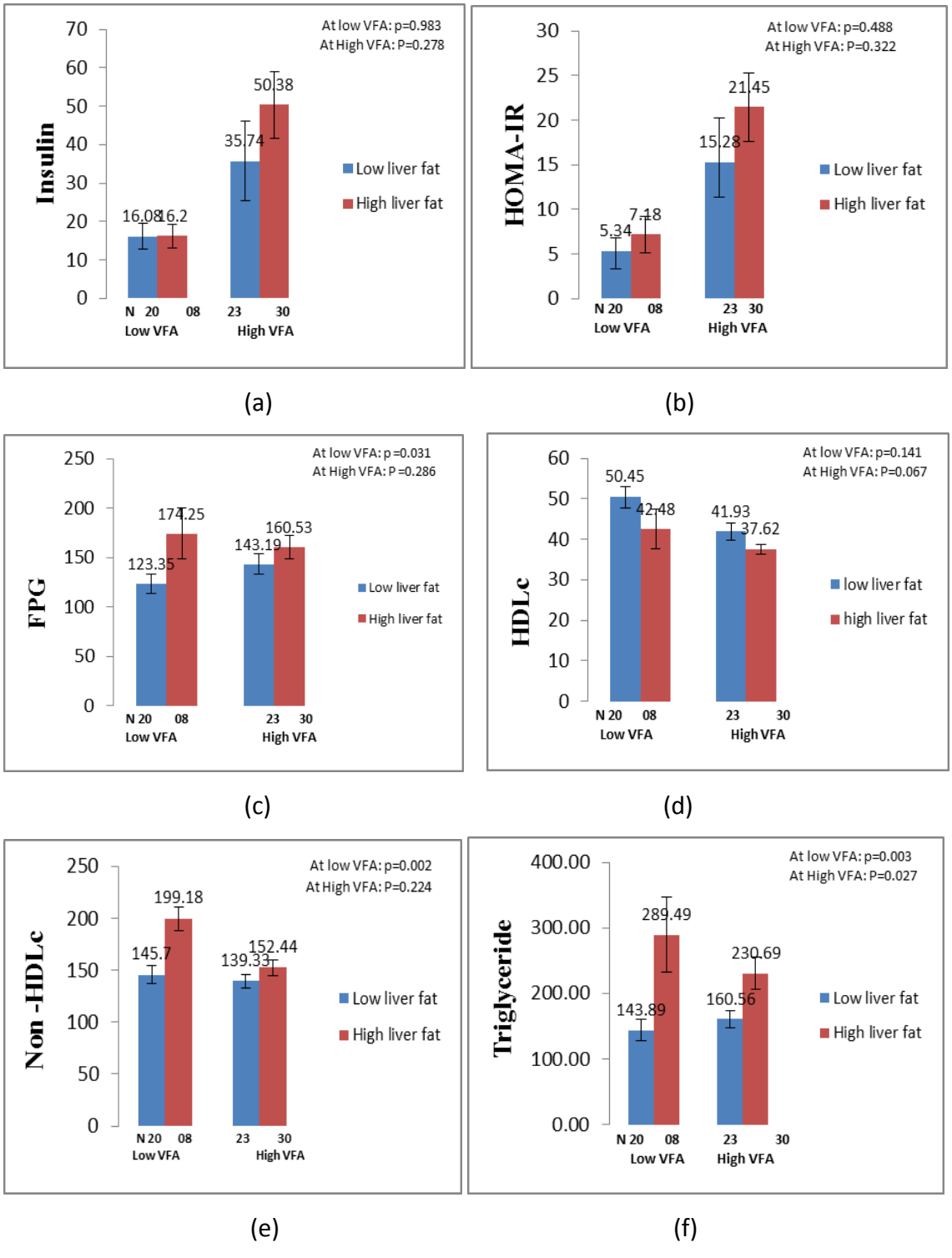


Figure 20 (a-f): Relative contribution of visceral and liver fat content to metabolic derangements in type 2 diabetic patients

5.4.2 Determining independent association of liver fat and visceral fat to cardiometabolic risk in type 2 diabetic patients.

To determine independent association of liver fat and visceral fat to CMR, MANCOVA was used to assess the statistical differences on multiple continuous dependent variables (metabolic parameters) by independent variables (liver fat and visceral fat respectively) while controlling for covariates.

5.4.2.1 Determining independent association of liver fat to cardiometabolic risk with visceral fat as covariate

Fatty liver was defined as liver fat percentage ≥ 5.56%. Fatty liver was considered independent variable exerting its impact on dependent variables, i.e. metabolic parameters while different models were chosen keeping age, visceral fat and BMI as covariates. Table 20 shows the level of significance achieved for association of liver fat to various metabolic parameters.

Table 20: Determining independent association of liver fat to cardio metabolic risk.

Dependent Trait	**Model 1: Fatty Liver+Covariates***		**Model 2: Fatty Liver +Covariates****		**Model 3: Fatty Liver+ variates*****	
	P Value	**Group P value**	**P Value**	**Group P value**	**P Value**	**Group P Value**
Ln_HDL-C	**0.005**	**0.008**	**0.029**	**0.04**	**0.067**	0.109
ApoA1	0.18		0.434		0.474	
Ln_TG	**0.000**	**0.001**	**0.001**	**0.006**	**0.001**	**0.007**
Ln_ApoB	0.242		0.093		**0.001**	
Non HDL-C	**0.024**		**0.010**		**0.010**	
LDL-C	0.127		0.083		**0.056**	
Total	0.148		**0.046**		**0.040**	
Ln_Glucose	**0.033**	**0.039**	0.091	0.278	0.118	0.288
Ln_Insulin	**0.008**		0.182		0.189	
Ln_HOMA_IR	**0.004**		0.095		0.108	
HbA1c	0.087		0.081		0.075	
TG_AUC	**0.001**	**0.003**	**0.002**	**0.006**	**0.005**	**0.015**
TG_iAUC	**0.017**		**0.020**		**0.031**	

Log transformation (Ln) was applied to parameters not normally distributed. * Age covariate, ** VF covariate, ***Age, BMI and VF covariate. Significance levels were calculated using multivariate analysis of covariance (MANCOVA) test.

Table 20 shows the independent association of liver fat to CMR using MANCOVA. In the model, fatty liver was considered as an independent variable while risk factors

such as HDL-C, apoA1, TG, apoB, non-HDL-C, LDL-C, TC, fasting glucose, fasting insulin, HOMA-IR, and HbA1c were considered as dependent variables. In model 1, age was covariate, while model 2, age was replaced with visceral fat and in the model 3, age, BMI and visceral fat were considered as covariates.

The findings showed that there was a statistically significant association of fatty liver with HDL-C when controlled for age (p = 0.005) and visceral fat (p = 0.029). Indicating that, liver fat is a predictor of HDL-C levels independent of visceral fat and age. However, when age, visceral fat and BMI were simultaneously controlled, HDL-C became insignificant (p=0.067>0.05). Association of liver fat to ApoA1 levels was not significant across model 1 (p=0.180), model 2 (p = 0.434) and model 3 (p=0.474).

Fatty liver was a strong and independent predictor of TG as observed from table 20. There was significant association of fatty liver with TG after controlling for age (p=0.000) and visceral fat (p=0.001). Such association remained same even after simultaneously controlling for age, visceral fat and BMI (p=0.001). These findings illustrated that fatty liver was an independent predictor of TG. This trend was also observed for non-HDL-C, where significance remained same, in fact, improved after adjusting for various confounders. As shown in the table 20, there was a significant association between fatty liver and non-HDL-C (p=0.024) after controlling for age. When covariate age was replaced with visceral fat, the significance further strengthened to p= 0.010. A similar trend was observed after simultaneously controlled for age, visceral fat and BMI, where the level of significance (p=0.010) remained same.

Interestingly, when covariate age was controlled in model 1, LDL-C (p=0.127), TC (p=0.148) and apoB (p=0.242) failed to show any association with fatty liver. After replacing age with covariate visceral fat, the association of liver fat with LDL-C, apoB and TC improved. The level of significance for TC became significant with p= 0.046 while that for LDL-C was p=0.083 and for apoB was p=0.093. Further, when adjusting for age, visceral fat and BMI as covariate, the association became statistically significant for apoB (p=0.001). The trend remained same (significant) for TC when simultaneously controlled for visceral fat and BMI (p=0.040). In the case of LDL-C

(p=0.056), there was a further improvement in the level of significance, although it did not achieve significance.

Overall, liver fat is an independent predictor for lipid parameters, i.e. TG, apoB, non-HDL-C, LDL-C and TC when adjusted for age, BMI and visceral fat. All these parameters are known to be metabolic markers of CV risk.

Liver fat was significantly associated with fasting glucose (p=0.033), fasting insulin (p=0.008) and HOMA-IR (p=0.004) after controlling for age. However, these risk factors became insignificant, when age was replaced with visceral fat. This indicates that visceral fat perhaps is a stronger predictor of fasting glucose (p=0.091), fasting insulin (p=0.182) and HOMA-IR (p=0.095) than fatty liver. Even after adding age, visceral fat and BMI, there was no improvement in the significance level. Association of liver fat with HbA1C remained similar and non-significant across all the models.

5.4.2.2 Determining independent association of visceral fat to cardiometabolic risk with liver fat as covariate

Two visceral fat levels, i.e. 100 cm^2 and 130 cm^2 were considered for determining its independent association with CMR. These diagnostic thresholds have been selected based on available literature. Below visceral fat threshold of 100 cm^2, disturbances of glucose, insulin and lipid metabolism are uncommon. Secondly, a level of 130 cm^2 often detects the metabolic abnormalities representing an increased risk group. Visceral fat was considered independent variable exerting its impact on dependent variables, i.e. metabolic parameters while different models were chosen keeping age, liver fat and BMI as covariates. Tables 21 and 22 shows the level of significance achieved for association of visceral fat to various metabolic parameters.

Table 21 and 22 present the level of significance achieved for association of visceral fat with CMR independent of age, liver fat and BMI. In the models, visceral fat 100cm^2 and visceral fat 130cm^2 were considered as independent variables while risk factors such as HDL-C, apoA1, TG, apoB, non-HDL-C, LDL-C, TC, fasting glucose, fasting insulin, HOMA-IR and HbA1C were considered as dependent variables. In model 1, age was covariate,

while model 2, age was replaced with liver fat and in the model 3, age, BMI and liver fat were considered as covariates.

Table 21: Determining independent association of visceral fat (≥100 cm^2) to cardio metabolic risk.

Dependent Trait	**Model 1:Visceral Fat (≥100cm^2)+ Covariates***		**Model 2: Visceral Fat (≥100cm^2) + Covariates****		**Model 3: Visceral Fat (≥100cm^2) + Covariates*****	
	P Value	**Group P value**	**P Value**	**Group P value**	**P Value**	**Group P value**
Ln_HDL-C	**0.018**	**0.031**	0.090	0.198	0.090	0.187
ApoA1	0.286		0.348		0.366	
Ln_TG	0.079	0.129	0.341	0.37	0.265	0.581
Ln_ApoB	0.861		0.740		0.934	
Non HDL-C	0.968		0.671		0.846	
LDL-C	0.700		0.633		0.658	
Total	0.547		0.429		0.580	
Ln_Glucose	0.875	**0.017**	0.655	**0.035**	0.875	**0.017**
Ln_Insulin	**0.001**		**0.006**		**0.001**	
Ln_HOMA_IR	**0.006**		**0.026**		**0.006**	
HbA1c	0.507		0.289		0.507	
TG AUC (n=76)	0.142	0.256	0.456	0.542	0.234	0.296
TG iAUC (n=76)	0.879		0.745		0.801	

Log transformation (Ln) was applied to parameters not normally distributed. * Age covariate, **Ln LF covariate, ***Age, BMI and Ln LF covariate

Visceral fat cut off level of 100 cm^2 was significantly and negatively ($p<0.01$) associated with HDL-C when controlled for age. However, after adjustment for liver fat and BMI in model 2 and model 3, the association became insignificant. Moreover, visceral fat cut off 100cm^2 was not significantly associated with any of the other lipid parameters (including postprandial TG clearance) in any of the three models.

Table 22: Determining independent association of visceral fat (≥130 cm^2) to cardio metabolic risk.

Dependent Trait	**Model 1: Visceral Fat (≥130 cm^2)+ Covariates***		**Model 2: Visceral Fat (≥130 cm^2)+ Covariates****		**Model 3: Visceral Fat (≥130 cm^2)+ Covariates*****	
	P Value	**Group P value**	**P Value**	**Group P value**	**P Value**	**Group P value**
Ln_HDL-C	**0.001**	**0.0006**	**0.012**	**0.029**	**0.015**	**0.038**
Apo_A1	0.123		0.167		0.161	
Ln_TG	0.274	**0.0003**	0.658	**0.003**	0.721	**0.034**
Ln_ApoB	0.064		**0.020**		**0.086**	
Non HDL-C	0.136		**0.014**		**0.032**	
LDL-C	0.107		**0.039**		**0.045**	
Total cholesterol	**0.018**		**0.002**		**0.007**	
Ln_Glucose	0.196	**0.015**	0.778	**0.035**	0.707	0.294
Ln_Insulin	**0.0006**		**0.008**		0.089	
Ln_HOMA_IR	**0.0006**		**0.019**		0.119	
HbA1c	0.559		0.153		0.151	
TG AUC (n=76)	0.820	0.954	0.188	0.409	0.421	0.578
TG iAUC (n=76)	0.959		0.375		0.321	

Log transformation (Ln) was applied to parameters not normally distributed. * Age covariate, **Ln LF covariate, ***Age, BMI and Ln LF covariate

While visceral fat cut off 130cm^2 was significantly associated with HDL-C in all three models, i.e. when controlled for age (p=0.001), liver fat (p=0.012) and age, liver fat and BMI (p=0.015). Visceral fat cut off 130 cm^2 was not significantly associated with any of the other lipid parameters (including postprandial TG clearance) except TC when controlled for age alone (p=0.0175). However, when controlled for liver fat in model 2, the association of visceral fat to lipid parameters became significant for apoB (p=0.020), nonHDL-C (p=0.014), LDL-C (p=0.039) and TC (p=0.002). The association of visceral fat cut off 130 cm^2 remained significant even after controlling for age, liver fat and BMI together in model 3. This indicated that visceral fat is independently associated with lipid parameters (HDL-C, apoB, non HDL-C, LDL-C and TC) when controlled for liver fat and BMI levels. However, visceral fat is not independently associated with TG and postprandial TG clearance.

Further, both visceral fat levels, i.e. 100cm^2 and 130cm^2 were significantly associated with fasting insulin and hepatic insulin resistance index HOMA-IR in all three models (refer tables 21 and 22). This clearly indicates the robust and independent association of visceral fat with insulin resistance/hyperinsulinemia. However, association of visceral fat with fasting glucose and HbA1C levels was not significant in any of the models.

5.5 Exploring visceral adiposity index cut-offs to determine visceral adiposity dysfunction and to evaluate its performance in predicting hepatic insulin resistance in Indian type 2 diabetics

The sample size of the study was 129. The data was collected from the subjects who belong to overweight/obese type 2 diabetes group (n=81) and healthy control group (n=48). VAI is a simple clinical algorithm developed as a surrogate marker for characterizing VAD. Thus, for each participant VAI was derived using BMI, waist circumference, TG and HDL-C, and it was studied against visceral fat area measuring ≥130 cm^2 by MRI as it is associated with higher CMR through raised VAD. VAI cut off was derived using ROC analysis. Based on the derived VAI cut offs, the diabetic group was further classified into two groups (i) DM+VAD present and (ii) DM+VAD absent. Partial correlation analysis was performed for ascertaining the linear relationship between two continuous variables, after controlling one variable.

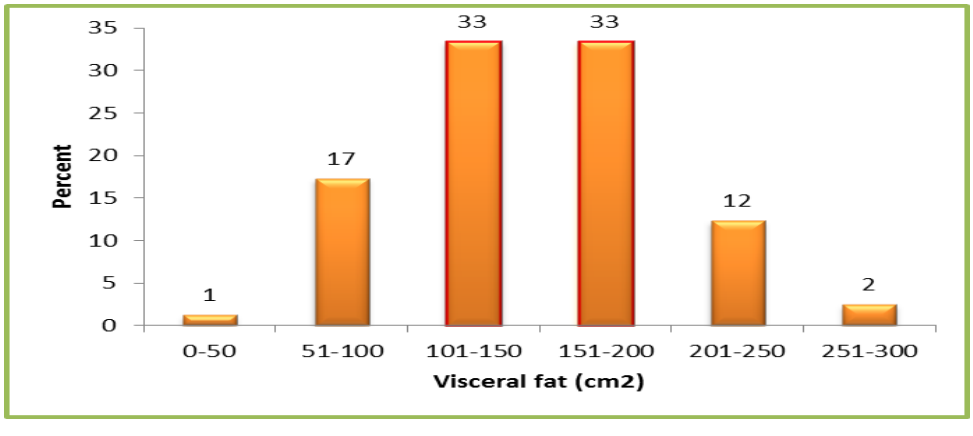


Figure 21: Levels of visceral fat (cm^2) observed in type 2 diabetes patients

The figure 21 shows that majority (33%) of participants in T2DM group had visceral fat level range between 101-150cm^2 and 151-200cm^2 each. 17% subjects had visceral fat

level 51-100cm^2 and 12% possessed visceral fat level 201-250cm^2. Finally, few percent (2%) of the subjects had visceral fat over 251cm^2. The cut-off value of normal healthy range for visceral fat is under 100cm^2. Hence, only 18% of subjects had visceral fat in normal level and rest of the diabetic group subjects had visceral fat in the abnormal range.

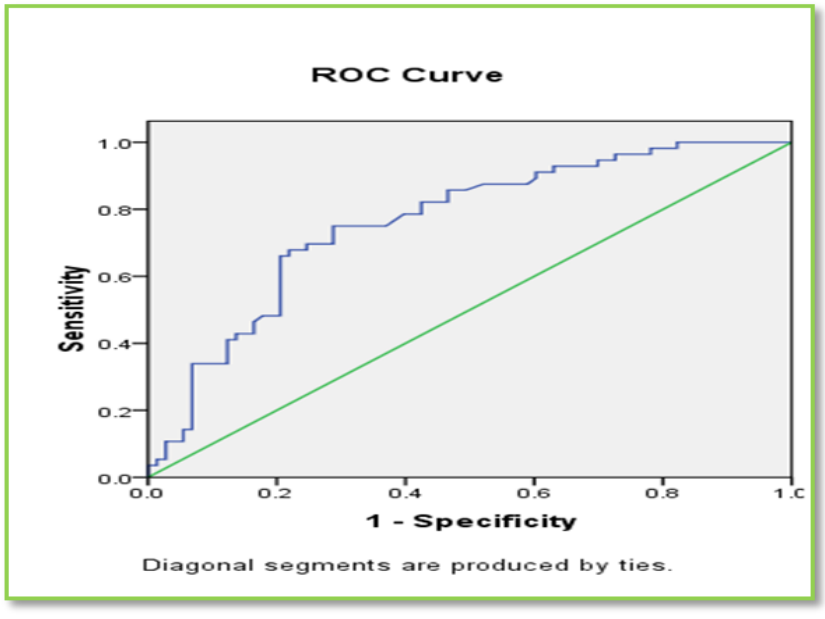


Figure 22: Receiver operating characteristics analysis of visceral adiposity index to predict the absence or presence of Visceral Adiposity Dysfunction as defined by visceral fat value of ≥130 cm^2 on MRI

ROC analysis was conducted to define appropriate cut-off points of VAI in identifying diabetic patients with VAD.The figure 22 revealed that the area under the ROC curve for VAI was 0.761, i.e. the accuracy of test was 76%. Table 23 gives the Se, Sp, positive likelihood ratios and negative likelihood ratios for the range of 0.25 unit intervals for VAI. The optimal cut off point to predict visceral adiposity was 2.0 which yielded a Se of 73.21% with a negative likelihood ratio of 0.38 and Sp of 71.23% with a positive likelihood ratio of 2.55.

Based on the VAI value, the total diabetic group subjects (n=81) were divided into two groups. Those groups were (i) diabetes mellitus without VAD (n=25) and (ii) diabetes mellitus with VAD (n=56). 16 males and 9 females were affected by diabetes mellitus

without VAD and 30 males, and 26 females were affected by diabetes mellitus with VAD.

Table 23: Diagnostic accuracy of VAI at various cut points.

VAI values	Se	Sp	+LHR	-LHR	PPV	NPV
≥ 0.50	100	2.74	1.03	0	44.09	100
≥ 0.75	100	15.07	1.18	0	47.46	100
≥ 1.00	94.64	30.14	1.35	0.18	50.96	88
≥ 1.25	91.07	38.36	1.48	0.23	3.12	85
≥ 1.50	85.71	50.68	1.74	0.28	57.14	82.2
≥ 1.75	78.57	60.27	1.98	0.36	60.7	78.5
≥ 2.00	73.21	71.23	2.55	0.38	66.13	77.6
≥ 2.25	69.64	75.34	2.82	0.4	68.42	76.3
≥ 2.50	66.07	79.45	3.22	0.43	71.15	75.3
≥ 2.75	58.93	79.45	2.87	0.52	68.75	71.6
≥ 3.00	48.21	79.45	2.35	0.65	64.29	66.6
≥ 3.25	42.86	83.56	2.61	0.68	66.67	65.5
≥ 3.50	42.86	86.3	3.13	0.66	70.59	66.3
≥ 3.75	37.5	87.67	3.04	0.71	70	64.6
≥ 4.00	33.93	90.41	3.54	0.73	73.08	64.0
≥ 4.25	30.36	93.15	4.43	0.75	77.27	63.5
≥ 4.50	26.79	93.15	3.91	0.79	75	62.3

Se = sensitivity; Sp = specificity; +LHR = positive likelihood ratio; -LHR = negative likelihood ratio; PPV = positive predictive value; NPV = negative predictive value.

Table 24 shows the comparison analysis between the diabetic patients with and without VAD. When considered anthropometric parameters of patients, diabetes mellitus with and without VAD group were varied with respect to waist circumference (p<0.05). Moreover, the patients who had diabetes mellitus with VAD possessed higher waist circumference than the patients who had diabetes mellitus without VAD (DM+VAD=96cm > DM+VAD absent=92cm). Hence, the amount of difference in waist circumference between the patients who had diabetes mellitus with and without VAD was 4.0cm. However, there was no significant difference between the patients who had diabetes mellitus with and without VAD, based on the BMI (p>0.05) and age (p>0.05). Hence, the patients who had diabetes mellitus with and without VAD did not vary with respect to BMI and age.

Table 24: Difference between diabetic with VAD group and diabetic without VAD group based on the anthopometric, clinical and biochemical parameters.

Parameters	Diabetes group	
	VAI < 2.0 (DM + VAD absent) N = 25 (M:F 16:9)	**VAI ≥ 2.0 (DM+ VAD present)** N=56 (M:F 30:26)
Anthropometric parameters		
Age (years)	53.7 ± 6.6	50 ± 8.5
BMI (Kg/m^2)	26.4 (24.7, 29.4)	28.4 (25.8, 30.5)
Waist circumference (cm)	92 (87.5, 102)	96 (93, 104.8)*
Clinical & Biochemical parameters		
Visceral fat area (cm^2)	129.6 ± 47.8	157.1 ± 49.1*
Fasting glucose (mg/dl)	124.8 (103.1, 138)	138.3 (104.7, 186.9)
HbA1C (%)	7.7 (6.8, 8.4)	7.3 (6.4, 8.6)
Fasting insulin (µU/ml)	10.8 (6.1, 31.1)	20.1 (11.5, 52.5)*
HOMA-IR	3.6 (1.7, 10.1)	7.8 (3.2, 19.1)*
Total cholesterol (mg/dl)	185.7 ± 40.3	199.6 ± 40.3
Triglyceride (mg/dl)	102.2 (78, 118.9)	215 (168.4, 269.4)**
LDL-C (mg/dl)	101 ± 27.50	112.1 ± 30.4
HDL-C (mg/dl)	51.4 (43.7, 57.2)	37.5 (34.8, 42)**
Non HDL-C (mg/dl)	134.9 ± 37.8	160.9 ± 38.4*
Apo B (mg/dl)	78.4 (57.6, 86.6)	86.2 (74, 116.9)
Apo A1 (mg/dl)	142 ± 24.3	128.4 ± 21.9*
Apo lipoprotein (B/A1) ratio	0.55 (0.37, 0.66)	0.69 (0.56, 0.91)*
Visceral adiposity index	1.46 (0.9, 1.6)	3.9 (3.0, 5.6)**

Data are presented as mean ± SD for normally distributed data and median (interquartile) for non-normal distribution. Unpaired t-test or Man-Whitney U test was applied based on normality assumption. DM+VAD=Diabetes Mellitus with VAD, DM+VAD absent=Diabetes Mellitus without VAD; M= Males; F=Females; BMI= Body Mass Index; HbA1C- Hemoglobin A1C; HOMA-IR- Insulin Resistance Index; LDL-C=Low Density Lipoprotein Cholesterol, HDL-C= High-Density Lipoprotein Cholesterol **p<0.01, *p<0.05

Analogously when considered clinical and biochemical parameters of patients, there was a highly significant difference between the patients who had diabetes mellitus with and without VAD in terms of TG level (p<0.01), HDL-C level (p<0.01) and VAI (p<0.01). Here, the patients who had diabetes mellitus with VAD possessed significantly higher TG (DM+VAD=215mg/dl > DM+VAD absent=102.2mg/dl) and VAI (DM+VAD=3.9 > DM+VAD absent=1.46) compared to the patients who had diabetes

mellitus without VAD. Diabetes mellitus without VAD group patients had more HDL-C level than diabetes mellitus with VAD group patients (DM+VAD=37.5mg/dl < DM+VAD absent=51.4mg/dl). Hence, the amount of differences in TG, VAI and HDL-C between the patients who had diabetes mellitus with and without VAD were 112.8mg/dl, 2.44 and 13.9mg/dl respectively. Also, there was significant difference between the patients who had diabetes mellitus with and without VAD, with respect to visceral fat (p=0.05), fasting insulin level (p=0.05), HOMA-IR level (p=0.05), non-HDL-C level (p=0.05), apoA1 level (p=0.05) and apoB/A1 level (p=0.05). Patients who had diabetes mellitus with VAD, retained higher levels of visceral fat (DM+VAD=157.1cm^2 > DM+VAD absent=129.6cm^2), fasting glucose (DM+VAD=138.3 mg/dl > DM+VAD absent=124.8 mg/dl), HOMA-IR (DM+VAD=7.8 > DM+VAD absent=3.6), non-HDL-C (DM+VAD=160.9mg/dl > DM+VAD absent=134.9mg/dl) and apoB/A1 ratio (DM+VAD=0.69 > DM+VAD absent=0.55) compared with the patients who had diabetes mellitus without VAD. Based on the magnitude of HDL-C and Apolipoprotein A1, diabetic patients without VAD possessed more good cholesterol level than the patients with VAD. However, diabetes mellitus with and without VAD group did not differ based on the fasting glucose level (p=0.560), HbA1C (p=0.898), TC (p=0.155), LDL-C (p=0.122) and apoB (p=0.154).

Table 25: Age-adjusted partial correlation coefficient of VAI, BMI and waist circumference with hepatic insulin resistance estimated using HOMA-IR.

Variables	HOMA-IR	
	correlation (r)	p-value
VAI	0.32	0.001**
BMI	0.31	0.001**
Waist circumference	0.35	0.001**

**p<0.01

The correlation coefficient (r value) ranges between -1 and 1. If the r-value is 1, then the relationship between two variables is perfect positive correlation, and if the r value is -1, then the relationship between two variables is a perfect negative correlation.

Table 25 shows the linear relationship between the coefficients of VAI, BMI and waist circumference with HOMA-IR after adjusting the age effect. From this analysis, there

was a positive linear relationship between the following variables namely, VAI vs. HOMA-IR ($p < 0.01$), BMI vs. HOMA-IR ($p < 0.01$) and waist circumference vs. HOMA-IR ($p < 0.01$), after adjusting the participants' age effect. It concluded that if VAI, BMI and waist circumference increases, there is a significant and simultaneous increase in HOMA-IR value.

CHAPTER 6 DISCUSSION

6.1 Background

The prevalence of obesity and related diseases such as T2DM is increasing worldwide, and the Asian Indian population seems to be particularly susceptible to developing T2DM, even at a low BMI (Ramachandran et al., 2012). The rationale for different cut-offs for obesity and abdominal obesity in Asians Indians compared to Caucasians is based on the fact that MS, or parts of the syndrome that are definable as T2DM, leads to an increased CVD risk at a lower waist circumference or BMI in Asians (Misra et al., 2009; Alberti et al., 2006). Of note, the increased prevalence of obesity-related diseases such as T2DM and CVD in Asian Indians is concomitant with an ongoing increase in the prevalence of obesity (Misra and Khurana, 2011).

In 2005, 23.2% of the adult global population was estimated to be overweight (BMI 25.0–29.9 kg/m^2) and 9.8% to be obese (BMI ≥30 kg/m^2), corresponding to 937 million and 396 million people, respectively. In 2030, with unchanged secular trends, the projected numbers for overweight and obese persons will be 2.16 billion and 1.12 billion, respectively (Kelly et al., 2008). There are considerable differences in the prevalence of overweight and obesity in different populations. Abdominal obesity carries a particularly increased risk of CVD (Yusuf et al., 2004) and T2DM and the risk seems to be associated with visceral and not subcutaneous fat accumulation (Erlingsson et al., 2009).

Concomitant with the previously described obesity epidemic is an alarming and global increase in T2DM. As with obesity, the prevalence of T2DM differs largely among but also within countries. Within the same country, large differences in the prevalence of diabetes also exist, as in India where recent studies showed a range from 5% to 20% in different populations (Ramachandran and Snehalatha, 2009). Among four Asian countries, India had the highest prevalence but also, compared to China and Japan, a peak in the prevalence at an age 10 years younger (DECODA Study group, 2003). Asian Indians have a younger onset of diabetes also in comparison to Caucasians (Ramachandran et al., 2004), and a gradually younger age at onset has been seen over

time (Mohan et al., 2007). The number of patients with diabetes in India is expected to increase from 51 million in 2010 to 87 million in 2030 (Atlas IDF, 2009).

The particular susceptibility among Asian Indians to T2DM, MS, and CVD, and increased risk even at low BMI, is of specific interest for both research and health management. Possible mechanisms are multifactorial and not restricted to Asian Indians and probably reflect a quantitative and not a qualitative difference between ethnicities (Misra and Khurana, 2011).

Large population studies utilizing imaging techniques (CT or MRI) showed visceral fat area at the L4-L5 level to be a superior determinant of metabolic risk factors than the subcutaneous fat area after correction for BMI and waist circumference (Carr et al., 2004; Fox et al., 2007). Visceral fat area is considered 'gold standard' for quantifying obesity-related CV risk and has been independently linked to the development of CAD while subcutaneous fat area has not been shown to carry prognostic significance (Fujimoto et al., 1999). However, the relationship between CVD and visceral fat is not straightforward, as racial and ethnic differences exist. African Americans have greater insulin resistance with less visceral fat when compared to Caucasians (Lovejoy et al., 1996). With the increasing use of MRI in lieu of CT for metabolic risk assessment, liver fat percent measured by MRS has also emerged as a significant correlate of metabolic risk factors (Chan et al., 2006). The potential utility of liver fat percent measured by MRS is highlighted by the finding of patients with 'metabolically-benign' obesity, i.e. obese patients with normal insulin sensitivity and lower liver fat percentages compared to insulin-resistant obese individuals (Stefan et al., 2008). This suggests that obese patients with low levels of liver fat may not have metabolic risk factors despite larger amounts of visceral fat compared to lean individuals. We hypothesized that liver fat percent determined by MRS is associated with CMR factors independent of visceral fat area in patients with T2DM. Given the prognostic significance of the metabolic markers (i.e. glycemic parameters, lipid parameters, postprandial TG clearance, PAI-1) in patients with T2DM, we evaluated the association of visceral fat area and liver fat percent with metabolic markers of CMR in high-risk T2DM patients. We sought to identify a useful cut-off value of VAI which would identify patients with VAD and in

turn predict predisposition to higher CMR. VAI would be an easy tool for the evaluation of the CMR in T2DM patients or in other populations, mainly in the absence of an overt MS. Further, this data also suggests that VAI can replace specialized imaging procedures with the advantages of a reduced economic burden and radiation hazard

In order to examine this broader aim, the study included data from 48 healthy and 81 adult patients with T2DM. Data were from participants recruited into human metabolic studies from primary and secondary care (Ahmedabad>Kolkata>Delhi) centres. Data were representative of healthy controls and individuals with diabetes being overweight/obese (BMI 23-35 kg/m^2), with a waist circumference ≥80 cm in females and ≥90 cm in males. For diabetes group, the participants could be newly diagnosed or diagnosed case of diabetes, not on any antidyslipidemic medications. All participants gave written informed consent, and ethical approval was obtained from the respective local ethics committee.

Firstly, the known differences in various anthropometric and metabolic measures of CMR between healthy and T2DM patients were well observed. To further dissect the differences in liver fat content and visceral fat area and study its impact on CMR within T2DM patients, stepwise analysis was carried out.

Known is the gender difference in terms of body fat distribution with males commonly having android type of fat distribution (trunk and upper body or central obesity) and females (most commonly premenopausal) having gynoid type (hips and thighs). While for long it was considered that android type of fat distribution which is associated with greater visceral adiposity is linked to a greater CV risk and that gynoid type is a more protective manifestation. However, results from recent studies indicate a commingling effect of both gynoid and android fat patterns on cardiometabolic dysregulation. It is therefore being advised that subjects who present with both android and gynoid adiposities should be advised of the associated health risks. Both android and gynoid fat accumulations should be considered in developing public health strategies for reducing cardiometabolic disease risk (Okosun et al., 2015). Considering this, difference in liver fat content, visceral fat area, anthropometric measures and

metabolic markers of CMR was studied across gender within the diabetic patient group. Further, considering the fact that menopause is a state of hormonal turbulence with a possibility of weight gain, visceral obesity and development of metabolic syndrome in females; age of ≤51 years was also considered to rule out this confounding effect while exploring obesity related metabolic derangements across gender.

To study contribution of liver fat to CMR, T2DM patients were classified as low (<5.56%) and high liver fat (≥ 5.56%) groups. The cut off of 5.56% liver fat derived using 3Tesla MRS to distinguish patients with and without steatotic liver was based on available literature. Liver fat percent greater than 5.56% corresponds to 55.6 mg/g liver tissue. These values are consistent with prior studies wherein the TG levels in hepatic tissues have been measured chemically in autopsy specimens or in clinical trials in which the effect of pharmacological agents on liver fat content has been monitored using MRS (Szczepaniak et al., 2005)

Finally, VAI cut offs were explored using visceral fat area derived using gold standard technique of MRI in 81 diabetic and 48 healthy individuals. The available literature supports visceral fat thresholds of 100 cm^2 below which disturbances of glucose, insulin and lipid metabolism are uncommon. Secondly, a level of 130 cm^2 often detects the metabolic abnormalities representing an increased risk group. Between visceral fat area of 100 cm^2 and 130 cm^2, the later was chosen as a representative of further cardio-metabolic disturbances in T2DM patients (Rankinen et al., 1999; Hunter et al., 2010).

6.2 Discussion of the findings

This study to our knowledge is the first of its kind from India that provide quantitative estimates of liver fat content and visceral fat area using gold standard MRS and MRI technique respectively. Further, the cut points of the fat measures used in this study to predict CMR are though validated in other ethnicities but have never been tested for its applicability in Asian Indian. This will be first study to estimate and report the contribution of these cut points to predict raised CMR in T2DM patients in India. Understanding how individuals develop metabolic sequelae from their obesity and

their diseases per se helps to target at-risk individuals and guide the development of novel therapeutics to combat the disease. In line with the literature, the study data yet revealed that T2DM patients have significantly deranged CMR profile (dyslipidemia, hyperglycemia, inflammatory marker PAI-1) compared to healthy controls. The background pathophysiological defect in CMR, i.e. abdominal obesity, liver fat content and insulin resistance was significantly highlighted in T2DM patients compared to healthy controls. This background defect is known to have significant impact on the consequent atherogenic dyslipdemic profile in T2DM patients.

6.2.1 Gender differences across study participants

Metabolic profile:

Overall, the metabolic profile of male and female diabetic patients was comparable. Although males had significantly high waist circumference levels compared to females, the difference did not achieve significance with respect to visceral fat and liver fat levels across gender. Also, corresponding metabolic profile was comparable between males and females.

Further, gender difference was also studied for age group ≤51 years in order to discount known post-menopausal metabolic derangement in females. It was observed that in addition to WC, males had significantly higher liver fat levels. Moreover, males had elevated visceral fat, PAI-1, fasting glucose, HOMA-IR and TG levels compared to females but the difference did not reach significance. This may be due to smaller sample size.

Post-prandial triglyceride clearance

TG clearance post consumption of standardized fat meal was studied in T2DM patients. It was found that, in addition to higher fasting TG levels, males had higher post meal peak plasma concentration of TG and displayed overall larger AUC. However, the difference was not significant compared to females. Whereas for age group ≤51 years, it was observed that males had significantly delayed TG clearance with significantly elevated fasting TG, post meal peak plasma TG concentration and larger AUC. These observations were again in line with the published literature where males have been recognized to have delayed fat clearance compared to

premenopausal females and are often linked to elevated visceral adiposity in males. Moreover, it also highlights the postmenopausal metabolic derangements in females which predispose them to a higher CMR nullifying the gender differences.

Body fat distribution

In line with the previously published results of studies in other ethnicities, difference in body fat distribution across gender was observed between Indian male and female T2DM patients. Males and females were matched for BMI, subcutaneous fat, visceral fat and liver fat content respectively and difference in other fat measures was observed across gender.

Matched for BMI, males had significantly higher visceral fat and liver fat content compared to females. However, males had lower to similar subcutaneous fat compared to females. When matched for subcutaneous fat, yet males had relatively higher visceral fat and liver fat compared to females though the difference was not significant. Analogously when males and females were matched for visceral fat, females had higher BMI and subcutaneous fat compared to males. While the difference between liver fat values reduced, males still having higher levels compared to females. In contrast to above findings, when males and females were matched for liver fat values, BMI and subcutaneous fat levels became comparable between the gender and the difference in visceral fat area also diminished.

This suggests that males tend to have higher visceral and liver fat values compared to females at same level of BMI and subcutaneous fat and females tend to have higher subcutaneous fat than males even when both genders are matched for visceral fat. However, liver fat explicitly explains the variance in body fat distribution across the gender. It was observed that when males and females were matched for liver fat levels, the variance in visceral and subcutaneous fat between the genders diminished with subcutaneous fat levels becoming similar.

Further, above results predicts that the predisposition of males to higher CMR can be attributed to the higher levels of visceral and liver fat accumulation in them. This observation was further supported by the finding when postprandial TG clearance AUCs between males and females were compared while matching for different fat

measures. It was found that when matched of visceral fat levels the magnitude of difference in AUCs was reduced compared to the difference observed when matched for subcutaneous fat. Similarly, at same level of liver fat level, the difference in TG AUCs was nullified and to some extent is reversed with females showing delayed TG clearance as compared to males. This indeed confirms strong interlink between liver fat and postprandial fat clearance which renders gender difference insignificant.

6.2.2 Contribution of liver fat to cardio metabolic risk

Although, several studies have reported stronger correlations between CMR factors and visceral adiposity than with liver fat, some other studies have even suggested that the associations between visceral adiposity and diabetogenic and atherogenic metabolic complications could be entirely explained by the concomitant increase in liver fat content. Asian Indian phenotype is recognized to have higher abdominal obesity/visceral adiposity at similar levels of BMI. Moreover, there are ample evidences linking raised visceral adiposity and increased CMR in Indian Population. However, there are no such studies reporting quantitative estimate of liver fat and its association with CMR in Indian T2DM patients.

In our study, liver fat was quantitatively estimated using gold standard MRS technique. Diabetic subjects were divided into low liver fat (< 5.56%) and high liver fat (≥5.56%) groups respectively to study the difference in metabolic markers between the two groups. It was found that diabetic patients in high liver fat group showed significantly deteriorated metabolic profile as represented by significantly elevated fasting glucose, fasting insulin & HOMA-IR levels indicative of hyperinsulinemic and highly insulin resistant state and abnormal lipid profile represented by hypertriglyceridemia & raised non HDL-C level which is a characteristic of diabetic dyslipidemia. These observations were in line with the previously published literature and are suggestive of raised liver fat levels and its role in predisposing Asian Indian Phenotype to high CMR.

6.2.3 To determine whether hepatic fat content is predictor of postprandial triglyceride clearance

In order to understand whether there is any significant impact of increased liver fat content on postprandial lipid metabolism among T2DM patients. Our study findings

showed that liver fat content was associated with postprandial TG levels. It specified that diabetic patients with high liver fat had significantly elevated postprandial TG AUC than diabetic patients with low liver fat content. Overall, these data reveal that the diabetic patients with high liver fat have higher postprandial TG peaks and delayed TG clearance. Resultantly, this group is in prolonged hypertriglyceridemia state as compared to diabetics with low liver fat. Our findings are consistent with the previous study findings. Lipid availability in the liver regulates the assembly and secretion of VLDL particles. It has also been shown in previous studies that liver fat content is one of the best predictors for overproduction of large VLDL1 particles seen in T2DM. Because the components of diabetic dyslipidemia are closely linked to the elevation of VLDL1 particles, liver fat content is being hypothesized to be a major correlate of postprandial triglyceridemia (Søndergaard et al., 2012) Although, our study does not allow drawing conclusions whether increased TG rich lipoprotein production, diminished lipolysis, impeded catabolism, or all of these account for postprandial triglyceridemia. This study results demonstrated that the postprandial triglyceridemia is significantly associated with the liver fat content. To our knowledge, this is perhaps the first ever reported study with respect to Indian T2DM patients.

6.2.4 Independent association of liver and visceral fat to cardiometabolic risk

The study showed that liver fat percent is a predictor for CMR factors especially dyslipidemia independent of visceral fat area among diabetic patients. Liver fat was significantly associated with raised TG, apoB, non HDL-C, TC and postprandial TG clearance when adjusted for age, BMI and visceral fat. These study findings corroborates with the previous studies by Adiels M et al. (2006) and Hoenig MR et al. (2010). Further, liver fat did show significant association with fasting glucose, fasting insulin and HOMA-IR levels when controlling for age alone. However, the significance was lost after adjusting for visceral fat and BMI. Similar finding was observed with HDL-C levels, significance of its association with liver fat was lost when controlling for visceral fat and BMI. Thus, liver fat was strongly and independently associated with atherogenic lipid profile while visceral fat needs to be accounted with liver fat for predicting insulin resistance state.

On the other hand, when adjusting for liver fat values, consistent with previous study findings on association between visceral fat and CMR factors; strong and independent association was observed between visceral fat and fasting insulin levels which also translated with HOMA-IR. Both cut offs of visceral fat area, i.e. 100cm^2 and 130cm^2 were independently associated with hyperinsulinemia and insulin resistance in T2DM patients when adjusted for liver fat levels. However, it is cut off 130cm^2 only which was significantly and independently associated with atherogenic dyslipidemia (i.e. LDL-C, apoB, non HDL-C and TC) when adjusted for liver fat content.

Thus, it can be concluded that visceral fat accumulation is undoubtedly and independently associated with CMR in T2DM patients. Though, the association varies with different thresholds. This study data shows that visceral fat area of ≥130 cm^2 strongly predicts the cardiometabolic abnormalities in T2DM patients in India. However, absence of visceral adiposity does not rule out confrontational nature of liver fat accumulation, as it is independently associated with atherogenic dyslipidemia and postprandial hypertriglyceridemia which serves as a trigger for amplifying insulin resistance in T2DM patients and are considered to be major CV risk determinants. Thus, precise quantification of liver fat and visceral fat using gold standard MRI/MRS techniques provides substantial knowledge on obesity related risk assessment.

In obese patients usually the FFA content is elevated. The visceral fat induces the increase in the free fatty acids. As the levels of the FFA keep increasing they have a negative effect on the lipolytic action of insulin, which in turn increases FFA in circulation. These increased FFA in the plasma further induces the suppression of the insulin function regulated by the hepatic glucose production. This further promotes gluconeogenesis, VLDL synthesis, decreases glucose uptake and insulin resistance (Bray et al., 2009; Pradeepa et al., 2015).

Excess abdominal obesity is associated with increased hepatic fat (Kyrou et al., 2014). In the agreement, the studies have also found a strong relationship between liver fat content with visceral fat volume. Tiikkainen et al., demonstrated that the hepatic fat content is more closely associated with indexes of insulin resistance than generalized obesity among diabetic subjects (Tiikkainen et al., 2002; Tiikkainen et al., 2004). This is

because that fatty liver fails to suppress glucose production in response to insulin in people with and without diabetes (Verges, 2005). However, whether hepatic insulin resistance is a consequence of increased liver fat has been still in the controversy (Gruben et al., 2014; Valenti et al., 2016). In our study, contradictory to the findings of Tiikkainen et al., we observed that visceral fat was more closely associated with insulin resistance among T2DM patients. However, it would be challenging to compare findings of other studies with the present study due to ethnic variation, study population type, different fat estimation techniques and different parameters to classify cardiometabolic outcomes.

So far, visceral fat measures in Indian population have been reported with CT imaging. Further, no studies in Indian population have reported visceral fat cut off to predict CMR. In our study, visceral fat is estimated using gold standard MRI techniques and the cut offs used are based on studies in Caucasians. **(**Rankinen et al., 1999, Hunter et al., 2010)

6.2.5 Exploring VAI cut-offs to determine visceral adiposity dysfunction and to evaluate its performance in predicting hepatic insulin resistance in Indian type 2 diabetics

Although BMI is widely accepted as a simple marker of adiposity in population-based studies and recognized as an instrument to diagnose obesity for all age groups (BMI ≥ 30 kg/m|^2). It should be more properly seen as an index of weight excess, rather than body fatness. Regardless of BMI value, patients with increased intra-abdominal fat usually have an atherogenic lipid profile, high fasting serum glucose, insulin levels and high BP, all metabolic factors participating in the atherosclerotic process. These factors subsequently contribute to the occurrence of CHD, stroke, as well as peripheral vascular diseases. Recent studies showed that it is the type of fat distribution particularly, intra-abdominal and ectopic fat accumulation, rather than BMI, is a significant independent predictor of the insulin resistance and dyslipidemia seen in both MS and diabetes (Despres and Lemieux, 2006; Despres, 2012). According to the findings of our study and the results of previous studies, we suggested that measuring the waist circumference only or measuring waist circumference plus the level of TG are

not enough to predict the short term, and long term risks factors associated with obesity but measuring visceral fat would be comparatively better and more robust. As in this study, it was illustrated that there was significant association of visceral fat (both at 100 cm^2 and 130 cm^2 cut off) with fasting insulin and HOMA-IR after adjustment for liver fat. This indicates that visceral fat has a stronger association with fasting insulin and insulin resistance compared to liver fat. Moreover, visceral fat cut off of 130 cm^2 was significantly associated with atherogenic dyslipidemia independent of liver fat levels. Thus, it is clear from the study results that visceral fat accumulation is a major pathophysiological link for raised CMR in T2DM patients. Reduction in visceral adiposity may serve as promising target in reducing global CMR.

The most compelling and unique finding in our study were identifying cut-off of VAI for VAD and exploring its correlation with insulin resistance. VAI as a simple indicator of VAD was strongly associated with the severity of obesity related CMR. The optimal cut off point for VAI was 2.0 (73.21% Se, 71.23% Sp). This cut-off point of VAI was useful in patients with diabetes in identifying the severity of CMR. The VAI also showed good correlation with hepatic insulin resistance measured using HOMA-IR after adjusting for age.

Using this cut off, 76 percent of the patients were correctly classified with the presence of VAD based on the VAI. This finding is one of considerable importance, as to our knowledge this is the first study to provide the diagnostic ability of VAI among the Indian population. Previous studies that used MRI / CT to quantify visceral fat and liver fat had a similar range of cut-off. Thus, we suggest that the VAI would be an easy tool for the evaluation of the CMR in T2DM or in other populations, mainly in the absence of an overt MS. Further, these data also suggest that the VAI can replace radio imaging procedures with the advantages of a reduced economic burden and a reduced radiation hazard. However, it is necessary to identify the age- and sex-specific cut-off points in the general population for early diagnosis and individualized therapeutic programs in persons at risk for CVD.

6.3 Strength and limitations of the study

To our knowledge, this is the first head-to-head comparison of two strong predictors (liver fat and visceral fat) of obesity-related CV risk measures with respect to Indian population. More importantly, it emphasizes the importance of monitoring visceral and liver fat depots especially in high risk T2DM patients.

So far measurement of abdominal adiposity in India has been reported using CT only. Therefore, this study is the first of its kind to use gold standard MRI for the measurement of abdominal obesity giving precise estimates of subcutaneous and visceral fat area. Thus, it may aid to bring the gold standard here too for more effective measurement thereby effective estimation of CMR. Further to this, there also exists a gold standard for non-invasive measurement of liver fat using MRS. Although this is predominantly so in the western population again; here, the same is reported for use in clinical practice for the first time in the Indian scenario. In addition to this reports of post prandial fat clearance in Indian diabetic patients have not been projected for Indian diabetic patients so far. However, strength of this study is critical use of post-prandial fat clearance as a CMR determinant.

Precise determination of liver fat and abdominal fat involves specialized techniques MRS and MRI. Use of these techniques is limited owing to its high cost, specialization and availability. Current anthropometric measures (like BMI and waist circumference) do reflect on generalized obesity and abdominal obesity but its accuracy and specificity for the concerned fat measures is compromised. The research work explored VAI cut off that best predict adiposity dysfunction and can serve as an effective tool for distinguishing severity of CMR in T2DM patients in routine clinical practice. VAI can replace radio imaging procedures with the advantages of a reduced economic burden and a reduced radiation hazard.

Limitations include, although the study showed independent association of liver fat percent and visceral fat area cut off to be associated with the CMR factors, these findings requires further validation as a marker of obesity-related CV risk and assessment in prospective cohort studies. Being a cross-sectional design, and relatively small sample size predominantly representative of west India, would further limit the

generalizability of the findings. Moreover, the study results are reflective of on overt derangements rather than establishing pathophysiological links through mechanistic models. Further, the study was carried based on few assumptions, i.e. the visceral fat area cut-offs used in this study are based on studies in other ethnicity especially Caucasians. Future research is warranted in this direction through large cohort studies to firm up or validate visceral fat area cut offs in Indians. Another assumption of the study is the age consideration with regard to menopause state in females. With the unavailability of the details on menopause status of females, literature based cut off of 51 years was considered for analysis. Furthermore, blood pressure which is major determinant of CMR could not be included owing to unavailability of data. Unlike exploring VAI cut off as a surrogate for VAD, fatty liver index cut off as a surrogate for liver fat content could not be explored due to unavailability of data on its components.

CHAPTER 7 SUMMARY AND SPECIFIC CONTRIBUTIONS

Summary

Obesity has been considered as a major correlate of cardiometabolic abnormalities for decades and is undoubtedly recognized as a prominent risk factor for increased CVD and diabetes related morbidity and mortality. However, the definition of obesity has undergone sequential refinement; starting with weight to BMI to waist circumference to abdominal fat to visceral fat area. Studies utilizing MRI as a most precise technique for abdominal fat quantification have shown visceral fat area to be the superior contributing factor for cardiometabolic abnormalities compared to all previous obesity determinants, i.e. weight, BMI and waist circumference. These results were also confirmed by large population based studies utilizing CT technique at the L4-L5 (umbilicus). It was also found that unlike Blacks, Asian Indians tend to have greater visceral adiposity at similar levels of total body fat compared to Caucasian and are thus more predisposed to increase CMR. Moreover, it is reported that both diabetes and CAD occur about 10 years earlier among South Asians than in any other population. Visceral fat area thus is considered as a 'Gold Standard' for quantifying obesity related CMR and has been independently associated with CAD. While most studies carried out to characterize Asian Indian Phenotype have utilized CT technique, to our knowledge there are none reported to provide visceral fat estimates of Asian Indians utilizing most precise technique, i.e. MRI. Also, till date no studies have been reported to have explored the applicability of visceral fat area cut off for predicting CMR in Asian Indians.

Moreover, with advancements in imaging modalities for metabolic risk assessment, liver fat as determined quantitatively by MRS has emerged as a significant correlate of metabolic risk factors. The potential utility of liver fat percent measured by MRS is highlighted by the finding of patients with 'metabolically-benign' obesity, i.e. obese patients with normal insulin sensitivity and lower liver fat percentages compared to insulin-resistant obese individuals. This suggests that obese patients with low levels of liver fat may not have metabolic risk factors despite larger amounts of visceral fat compared to lean individuals. Currently, research is directed towards accounting the independent association of liver fat to CMR factors whilst visceral fat as gold standard

for quantifying obesity related CMR. Off late, several studies have published their mixed opinion on the same which can be attributable to the type of fat quantification technique involved. Moreover, most of these studies were carried out in relatively small sample size representative of western population; the results cannot be generalized across different ethnicities. While it is known that Asian Indians tend to have high visceral adiposity than whites at relatively similar levels of BMI and are more predisposed to CV risk, there are no published reports that provide quantitative estimates of liver fat content and its association with CMR with respect to Indian population. Furthermore, contribution of visceral and liver fat to CMR within T2DM patients has not been reported earlier so far.

I. In line with the literature, the study data yet revealed that T2DM patients have significantly deranged CMR profile (dyslipidemia, hyperglycemia, inflammatory marker PAI-1) compared to healthy controls. The background pathophysiological defect in CMR, i.e. abdominal obesity, liver fat content and insulin resistance was significantly highlighted in T2DM patients compared to healthy controls. This background defect is known to have significant impact on the consequent atherogenic dyslipdemic profile in T2DM patients.

II. Further**,** in the sample observational study, the metabolic profile of diabetic patients was comparable across gender with males having a higher waist circumference than the females but no significant difference observed for visceral and liver fat. Also, there was no significant difference in the postprandial TG clearance across the gender. However, to discount the postmenopausal metabolic interferences in females, age group of ≤51 years was considered to study the gender differences in terms of body fat distribution and its influence on metabolic profile between genders. It was observed that the visceral fat area and metabolic derangements in T2DM males became more robust compared to females with significantly higher levels of liver fat content, higher postprandial TG levels and also delayed postprandial TG clearance in males. These observations reveal the predominance of android type fat distribution in males which is representative of higher visceral and liver

fat levels compared to females. However, despite significant differences in liver fat and to extent similar in visceral fat between genders, there was no significant difference observed in other metabolic markers except for postprandial TG clearance. This may be due to small sample size or chance error.

III. Most of the diabetic patients had visceral fat area ≥100cm^2. However, diabetic patient group having high liver fat content (≥5.56%) had significantly deranged cardiometabolic profile compared to low liver fat group.

IV. Test to identify independent association of visceral fat and liver fat to increased CMR in T2DM patients was applied. Where liver fat was found to be significantly associated with TG, nonHDL-C, TC levels and post prandial TG clearance independent of visceral fat area. While, association of liver fat with apoB, LDL-C, insulin resistance and glycemic parameters was not significant when adjusted for visceral fat values. Although, liver fat was significant predictor of insulin resistance and hyperinsulinemia when adjusted for age alone.

On the contrary, both cut offs of visceral fat area, i.e. 100cm^2 and 130cm^2 were independently associated with hyperinsulinemia and insulin resistance in T2DM patients when adjusted for liver fat levels. However, it is cut off 130cm^2 only which was significantly and independently associated with atherogenic dyslipidemia (i.e. LDL-C, apoB, nonHDL-C and TC) when adjusted for liver fat content.

Thus, it can be concluded that visceral fat accumulation is undoubtedly and independently associated with CMR in T2DM patients. Though, the association varies with different thresholds. This study data shows that visceral fat area of ≥130 cm^2 strongly predicts the cardiometabolic abnormalities in T2DM patients in India. However, absence of visceral adiposity does not rule out confrontational nature of liver fat accumulation, as it is independently associated with dyslipidemia and postprandial hypertriglyceridemia which serves as a trigger for amplifying insulin resistance in T2DM patients and

entering the vicious cycle. Thus, quantification of liver fat content using MRS can precisely distinguish fatty from no fatty liver.

V. Further, the research work explored VAI cut off that best predict VAD (visceral fat area ≥130 cm^2) and can serve as an effective tool for determining severity of CMR in T2DM patients in routine clinical practice. VAI cut off of 2.0 had 73.21% Se and 71.23% Sp to predict VAD. This cut-off point of VAI was found to be useful in distinguishing diabetic patients with greater CMR. The VAI also showed good correlation with hepatic insulin resistance measured using HOMA-IR after adjusting the age. Thus, VAI can replace imaging procedures (MRI) with the advantages of reduced economic burden and can be used as screening tool for surveillance of CMR in Indian population

In conclusion, CVD is the end result of a continuous process of atherosclerosis and can be prevented by a reduction in the rate of atherogenesis. Atherogenesis can be prevented by controlling CMR factors like atherogenic dyslipidemia, insulin resistance, hyperglycemia which are associated with body composition and body fat distribution as observed in this study.

Specific Contributions

Increased visceral adiposity is a characteristic of Asian Indian Phenotype and insulin resistance or hyperinsulinemia is a characteristic of T2DM patients. In line with the literature, our study data revealed that visceral fat area had significant independent association with hyperinsulinemia and insulin resistance in T2DM patients. Thus, striving towards a better fat distribution may either delay the progression of diabetes or may delay its occurrence in case of prediabetes

Further, development of dyslipidemia in T2DM patients namely 'diabetic dyslipidemia' or 'atherogenic dyslipidemia' or postprandial lipemia predisposes them to an increased risk of CVD. Our study showed that, liver fat percent was independently associated with the dyslipidemia (raised TG, TC, non HDL-C and diminished HDL-C) and postprandial lipemia in T2DM patients while visceral fat area was a redundant predictor of atherogenic dyslipidemia when adjusted for liver far values. Thus,

interventions that can reduce or prevent liver fat accumulation may address the residual risk in T2DM patients.

To our knowledge, this is the first head-to-head comparison of two strong predictors of obesity-related cardiovascular risk measures with respect to Indian population.

Our study provides the first estimates of prevalence of hepatic steatosis in T2DM patients in urban India. We found that healthy subjects have about four fold less hepatic fat compared to the T2DM patients. Approximately 65% of T2DM patients and about 15% of healthy had percent hepatic fat above 5.56% estimated by MRS.

Study data clearly demonstrated contribution of body fat distribution in predisposing one to a greater CMR. More importantly, it emphasizes the importance of monitoring visceral and liver fat depots especially in high risk T2DM patients. However, its precise determination involves specialized techniques MRS and MRI. Use of these techniques is limited owing to its high cost, specialization and availability. Current anthropometric measures (like BMI and waist circumference) do reflect on generalized obesity and abdominal obesity but its accuracy and specificity for the concerned fat measures is compromised. Identification of a routinely applicable indicator for the evaluation of obesity related metabolic derangments, with higher sensitivity and specificity than classical parameters could be useful for cardiometabolic risk assessment. Amato et al in 2010, came out with VAI, a novel gender-specific index, based on WC, BMI, TG, and HDL-C, indirectly expressing adiposity dysfunction and was strongly associated with the severity of obesity related CMR. However, VAI cut off to predict adiposity dysfunction have never been reported for Indian population and so is the case with T2DM population as well.

The research work explored VAI cut off that best predict adiposity dysfunction and can serve as an effective tool for distinguishing severity of CMR in T2DM patients in routine clinical practice. VAI cut off of 2.0 was obtained having 73.21% sensitivity and 71.23% specificity to predict adiposity dysfunction. This cut-off point of VAI was useful in diabetic patients in identifying the severity of CMR. The VAI also showed good correlation with hepatic insulin resistance measured using HOMA-IR after adjusting the age. Thus, we suggest that the VAI would be an easy tool for the evaluation of the

cardiometabolic risk in T2DM or in other populations, mainly in the absence of an overt metabolic syndrome.

Further, these data also suggest that the VAI can replace radio imaging procedures with the advantages of a reduced economic burden and a reduced radiation hazard. However, it is necessary to identify the age- and sex-specific cut off in the general population for early diagnosis and individualized therapeutic programs in persons at risk for CVD.

FUTURE SCOPE OF RESEARCH

The research work here demonstrated that visceral fat area threshold of 130 cm^2 as evaluated using most reliable non-invasive MRI method is strongly and independently associated with atherogenic dyslipidemia, hyperinsulinemia and insulin resistance. These risk factors have been recognized to be the key players in pathogenesis of cardiometabolic risk. While, visceral fat area cut off of 130 cm^2 is based on several studies carried out in Caucasian and Japanese population, visceral fat area using MRI is being reported here for the first time in sub group representing Indian population. Asian Indians are recognized to have higher visceral adiposity at the similar level of BMI compared to Caucasians. Larger studies are warranted in this direction to identify visceral fat cut offs specific to Asian Indians.

It can be speculated as to whether insulin resistance is a cause or a consequence of fat accumulation in liver and follow-up studies and in vitro studies in this field are therefore needed. The possible mechanisms linking fatty liver to CVD are widely investigated. According to the current knowledge, the best speculation is that an excess of inflamed visceral fat mass leads to increased production of inflammatory cytokines, increased insulin resistance and increased free fatty acid concentrations. These actions lead to impaired liver functions, which result in increased production of inflammatory proteins and coagulation factors. Finally, chronic inflammation and atherogenic dyslipidemia contribute to CVD (Bhatia et al. 2012). It is still poorly understood how active a role a fatty liver plays in the pathogenesis of CVD, or whether fatty liver is only a marker of insulin-resistant state.

Indeed, liver fat percent has been shown to be greater in obese insulin-resistant patient's vs obese insulin-sensitive patients (10.5% vs 5.6%) with the obese insulin-sensitive patients having a carotid intima-media thickness comparable to healthy normal weight individuals (Hoenig et al., 2010). Hence, while the definition of obesity has evolved from weight to body mass index and more recently waist circumference and subsequently visceral fat area, liver fat percent may represent the future determinant of obesity-related cardiovascular risk assessment. Indeed, it is possible

that obese patient with higher amount of visceral fat area but low liver fat percent may have a cardiovascular event rate comparable to normal-weight individuals. While our data are encouraging in showing that both liver fat percent and visceral fat are independently associated with atherogenic dyslipidemia and it is liver fat but not visceral fat which is strongly and independently linked postprandial triglyceridemia (a strong predictor of CVD) may better identify the at-risk patient than visceral fat area, our data set is small and cross-sectional. These findings encourage further investigations in how the two fat depots influence the development of cardiometabolic risk. A large prospective cohort is required to determine if liver fat percent is independently associated with cardiovascular events.

In the clinical setting, VAI could serve as a potential marker in the diagnosis of visceral adiposity dysfunction and liver fat accumulation. Therefore, its potential therapeutic usefulness for the treatment and prevention of fatty liver should be tested. VAI may offer a potential source for treatment of fatty liver disease and fat accumulation in other peripheral tissues.

The most important issue though, is prevention of the epidemic of obesity and of type 2 diabetes in India. The results of various preventive methods are often not encouraging. Much effort therefore has to be spent in search for better and validated preventive methods. The prevention has to take place both at an individual level, and in all of the society to succeed. To quote the German doctor, scientist and politician Rudolf Virchow (1821-1902):

“Medicine is a social science, and politics is nothing but medicine on a large scale. Medicine, as a social science, as the science of human beings, has the obligation to point out problems and to attempt their theoretical solution: the politician, the practical anthropologist, must find the means for their actual solution”.

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APPENDIX

Publications related to the existing research

1. Van der Valk F, Hassing C, Visser M, Thakkar P, Mohanan A, **Pathak K**, Dutt C, Chauthaiwale V, Ackermans M, Nederveen A, Serlie M, Nieudorp M, Stroes E. The effect of a diiodothyronine mimetic on insulin sensitivity in male cardiometabolic patients: a double-blind randomized controlled trial. PLOS ONE 2014; 9(2):1-7
2. **Pathak K**, Mohanan A, Jadhav H, Acharya S. Cardiometabolic risk: independent role of visceral and liver fat. World Journal of Pharmaceutical Research 2015; 4(11)
3. **Pathak K,** Mohanan A, Jadhav H, Acharya S, Mandavia D. Exploring VAI as a predictor of visceral adiposity dysfunction and evaluating its performance in predicting hepatic insulin resistance in Indian type 2 diabetics. International Journal of Pharmacy and Pharmaceutical Sciences 2016; 8(8):297-301
4. **Pathak K,** Mohanan A, Jadhav H. Influence of fatty liver on determinants of cardiometabolic risk independent of visceral fat: Study in Indian type 2 diabetic patients. (under review)

Contribution of Liver Fat and Visceral Fat to Cardiometabolic Risk in Diabetic Patients in India

THESIS

Submitted in partial fulfilment

of the requirements for the degree of

DOCTOR OF PHILOSOPHY

by

KAUSHAL PATHAK

Under the Supervision of

DR. ANOOKH MOHANAN

And co-supervision of

PROF. HEMANT R JADHAV

BITS Pilani

Pilani | Dubai | Goa | Hyderabad

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE, PILANI

2016

FUTURE SCOPE OF RESEARCH

The research work here demonstrated that visceral fat area threshold of 130 cm^2 as evaluated using most reliable non-invasive MRI method is strongly and independently associated with atherogenic dyslipidemia, hyperinsulinemia and insulin resistance. These risk factors have been recognized to be the key players in pathogenesis of cardiometabolic risk. While, visceral fat area cut off of 130 cm^2 is based on several studies carried out in Caucasian and Japanese population, visceral fat area using MRI is being reported here for the first time in sub group representing Indian population. Asian Indians are recognized to have higher visceral adiposity at the similar level of BMI compared to Caucasians. Larger studies are warranted in this direction to identify visceral fat cut offs specific to Asian Indians.

It can be speculated as to whether insulin resistance is a cause or a consequence of fat accumulation in liver and follow-up studies and in vitro studies in this field are therefore needed. The possible mechanisms linking fatty liver to CVD are widely investigated. According to the current knowledge, the best speculation is that an excess of inflamed visceral fat mass leads to increased production of inflammatory cytokines, increased insulin resistance and increased free fatty acid concentrations. These actions lead to impaired liver functions, which result in increased production of inflammatory proteins and coagulation factors. Finally, chronic inflammation and atherogenic dyslipidemia contribute to CVD (Bhatia et al. 2012). It is still poorly understood how active a role a fatty liver plays in the pathogenesis of CVD, or whether fatty liver is only a marker of insulin-resistant state.

Indeed, liver fat percent has been shown to be greater in obese insulin-resistant patient's vs obese insulin-sensitive patients (10.5% vs 5.6%) with the obese insulin-sensitive patients having a carotid intima-media thickness comparable to healthy normal weight individuals (Hoenig et al., 2010). Hence, while the definition of obesity has evolved from weight to body mass index and more recently waist circumference and subsequently visceral fat area, liver fat percent may represent the future determinant of obesity-related cardiovascular risk assessment. Indeed, it is possible

that obese patient with higher amount of visceral fat area but low liver fat percent may have a cardiovascular event rate comparable to normal-weight individuals. While our data are encouraging in showing that both liver fat percent and visceral fat are independently associated with atherogenic dyslipidemia and it is liver fat but not visceral fat which is strongly and independently linked postprandial triglyceridemia (a strong predictor of CVD) may better identify the at-risk patient than visceral fat area, our data set is small and cross-sectional. These findings encourage further investigations in how the two fat depots influence the development of cardiometabolic risk. A large prospective cohort is required to determine if liver fat percent is independently associated with cardiovascular events.

In the clinical setting, VAI could serve as a potential marker in the diagnosis of visceral adiposity dysfunction and liver fat accumulation. Therefore, its potential therapeutic usefulness for the treatment and prevention of fatty liver should be tested. VAI may offer a potential source for treatment of fatty liver disease and fat accumulation in other peripheral tissues.

The most important issue though, is prevention of the epidemic of obesity and of type 2 diabetes in India. The results of various preventive methods are often not encouraging. Much effort therefore has to be spent in search for better and validated preventive methods. The prevention has to take place both at an individual level, and in all of the society to succeed. To quote the German doctor, scientist and politician Rudolf Virchow (1821-1902):

“Medicine is a social science, and politics is nothing but medicine on a large scale. Medicine, as a social science, as the science of human beings, has the obligation to point out problems and to attempt their theoretical solution: the politician, the practical anthropologist, must find the means for their actual solution”.