Pathophysiological Characterization of Non-obese Type 2 Diabetes Mellitus

by

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A thesis submitted to the
Academy of Scientific & Innovative Research
for the award of the degree of
DOCTOR OF PHILOSOPHY
in
SCIENCE

Under the supervision of
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December 2020-21

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I dedicate this thesis to all the patients and

community health workers…..

without whom none of this work would have been possible

ACKNOWLEDGEMENT

This doctoral thesis has been possible through the support of an entire team of people starting from patients, healthy volunteers, community health workers, social mobilizers to colleagues, doctors and scientists. First of all, I would be like to thank Dr. Partha Chakrabarti, Principal Scientist, Cell Biology and Physiology Division, CSIR-IICB for his support as my guide and mentor during the PhD work. I can never forget his belief in me which has helped me to continue my work confidently and independently over the last 4 years. He was always present not only as my guide but also as my friend in times of need. His encouragement to think and try in an out-of-the-box manner has made my PhD journey really enjoyable.

The next person without whom this work would have been impossible is Mr. Nilangshu Gain, Secretary, SWANIRVAR. My work involved a great deal of participation from the rural Indian community. It required regular management of human resources for almost 3 years. Nilangshu Gain was the person who taught me the tiny details of community program management and helped me learn something that is absent in the academic books.

As my collaborators in this thesis work, I can never forget the support received from Dr. Viswanathan Mohan (Madras Diabetes Research Foundation, Chennai), Dr. Sujoy Ghosh (Institute of Post Graduate Medical Research and Education, Kolkata) and Dr. Om Tantia (Institute of Laparoscopic Surgery, Saltlake) without whom such a clinical work with diverse methodology wouldn't have been possible. My sincere gratitude to Prof. Pabitra Mitra (IIT Kharagpur) and Prof. Olaf Wolkenhauer (University of Rostock, Germany) who took their time over several occasions to discuss and teach me data science and machine learning approaches, something that was entirely new to me. I am also grateful to their students Saikat Biswas and Saptarshi Bej both of whom worked with me on several occasions in this thesis work. These four people helped me to keep hold of my interest in data science.

The entire team of CSIR-IICB was always by me to provide the best suggestions they could regarding my thesis work. My thanks to my Doctoral Advisory Committee members, Dr. Arun Bandyopadhyay, Dr. Dipyaman Ganguly, Dr. Jayati Sengupta, Dr. Sanjay Dutta and Dr. Debabrata Biswas for providing me their guidance to my research question on several occasions. My thanks to all of my lab mates of Metabolic Diseases Laboratory for supporting me in the lab work including all my seniors Mainak Da, Dipsikha Di, Titli Di, Moumita Di, Sougata Da and juniors Saheli, Pratiti, Ankita, Tanushree and Abhisekh. I would like to specially thank Mrs Dipika Ray and Sujay

for meticulously working with me with the clinical samples. Last but not least, my sincere gratitude to Debojyoti who has not only helped me know the various facets of modern science but also provided me the courage to explore some of those new areas on my own.

This work is based on clinical samples and collecting samples along with clinical data required lots of efforts of multiple healthcare workers. I am grateful to all those health workers of SWANIRVAR and all those patients and healthy volunteers without whom this work would have remained a dream only. Research requires time and resource both. Being a clinical study with epidemiological and experimental components both, this study would have been impossible without the generous funding support received from several agencies. First of all, I would like to thank Indian Council of Medical Research (ICMR) who has supported me by providing the Research Fellowship for the entire period. I would be like to thank both the social organizations, Indo-Global Social Service Society (IGSSS) and The Hope Foundation, Kolkata (THF) for providing the support for field workers involved in the epidemiological component. I would like to specially thank Mr. Subrata Roy and his organization "Kalyani Roy Memorial Trust" for supporting the community clinic of North 24 Parganas in its initial stage. I am also indebted to Father Franklin Menezes and "Seva Kendra Calcutta" for providing me the social support and networking in building the cohort of Haldia, East Midnapore. The experimental work wouldn't have been possible without the support of Department of Biotechnology, West Bengal (WBDBT) and Indian Council of Medical Research. Last but not least I would like to mention the omnipresent role of Council of Scientific and Industrial Research (CSIR) for providing a suitable scientific environment and technical resources for successfully completing this thesis work.

As somebody said to me, PhD thesis is just the beginning of the journey. I am not sure whether I get this totally but while I was writing the thesis, I could come up with several relevant questions with which I would like to spend my upcoming life. As this thesis gets submitted, the happy ending of being awarded the highest academic degree also pushes me to an unknown research path. I am not sure whether I shall be able to find proper space for my passion in biomedical research but I am sure that all those teachings which I have acquired over the last 4 years will help me in some way to discover my niche in Indian research.

Jit Sarkar

Date: 08-12-2020

Jit Sarkar

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DPP4	Dipeptidyl Peptidase 4
T2DM	Type 2 diabetes
ASCVD	Atherosclerotic Cardiovascular Disease
CHF	Congestive Heart Failure
GLUT4	Glucose transporter 4
K_{ATP}	ATP sensitive potassium channel
AMPK	5'AMP- activated protein kinase
GDM	Gestational diabetes mellitus
MODY	Maturity-onset diabetes of the young
NAFLD	Non-alcoholic fatty liver diseases
GLP-1	Glucagon like peptide-1
GIP	Glucose dependent insulinotropic polypeptide
FFA	Free fatty acid
TNF-α	Tumour necrosis factor-alpha
WHO	World Health Organization
TZD	Thiazolidinediones
DEXA	Dual-energy X-ray absorptiometry
VAT	Visceral Adipose Tissue
SAT	Subcutaneous Adipose Tissue
BMI	Body Mass Index
PPAR-γ	Peroxisome proliferator-activated receptor-γ
HbA1c	Glycated haemoglobin
HOMA	Homeostatic model assessment
IGT	Impaired Glucose Tolerance

IFG	Impaired Fasting Glucose
RP	Reserve Pool
RRP	Readily Releasable Pool
T2DM	Type 2 Diabetes Mellitus
OGTT	Oral Glucose Tolerance Test
NFHS-4	National Family Health Survey-4
CAB	Clinical, Anthropometric and Biochemical
UMAP	Uniform Manifold Approximation and Projection
DBSCAN	Density-based spatial clustering of applications with noise
IDF	International Diabetes Federation
ADA	American Diabetes Association
FBS	Fasting Blood Sugar
MGB	Mini-Gastric Bypass
GCK	Glucokinase gene
HNF1A	Hepatonuclear Factor gene
RCAD	Renal cysts and diabetes
FPLD	Familial partial lipodystrophy
CGL	Congenital generalized lipodystrophy
IR	Insulin resistance
SGLT2	Sodium-glucose co-transporter-2
PCOD	Polycystic Ovarian Disease
IRS	Insulin Receptor Substrate
PI3K	Phosphatidylinositol 3-kinase
SREBP	Sterol regulatory element-binding protein
MAPK	Mitogen-activated protein kinase

PTP Protein tyrosine phosphatases

MHO Metabolically Healthy Obese

LAR Leptin-Adiponectin ratio

UKPDS United Kingdom Prospective Diabetes Study

NGT Normal Glucose Tolerance

VDCC Voltage dependent Ca^{2+} channels

ATP Adenosine Tri-phosphate

ADP Adenosine Bi-phosphate

cAMP Cyclic Adenosine Mono-phosphate

DAG Diacylglycerol

IP_3 Inositol 1.4.5-triphosphate

GSIS Glucose stimulated insulin secretion

WC Waist circumference

EDTA Ethylenediamine tetraacetic acid

SE Standard error of the mean

MUNO Metabolically Unhealthy Non-obese

HPLC High performance liquid chromatography

DHS Demographic and Health Survey

AIS AIDS indicator survey

SPA Service provision survey

MIS Malaria indicator survey

KIS Key indicator survey

GAM Generalized Additive Modelling

t-SNE t-Stochastic Neighbourhood Embedding

REVIEW OF LITERATURE

1. Type 2 Diabetes Mellitus

1.1 The Diabetes Epidemic and Type 2 Diabetes (T2DM)

Around 4.2 million deaths in 2019 have been attributed to diabetes and its complications, an equivalent to 1 death per 8 seconds [1]. In 2020, the global diabetes population stands at 463 million a number which is predicted to rise to 700 million by 2045 [1]. Though it is more prevalent among the developed countries [2], developing countries are reporting an increasing prevalence too. Urban populations in the developing countries have an increased prevalence of diabetes but studies have shown the rural populations in those countries to report a gradually increasing prevalence of diabetes too [3,4]. The widespread and global nature of diabetes has given it a true "pandemic" status with the diabetic population expected to steeply rise across the entire globe (Table-1.1).

Table 1.1: Total number of diabetic people (20-79 years age group) in the year 2019 and expected numbers in 2030 and 2045 across the International Diabetes Federation (IDF) regions and the entire world.

	Year- 2019	Year- 2030	Year- 2045	% Increase
World	463 million	578 million	700 million	51%
North America & Caribbean	48 million	56 million	63 million	33%
South & Central America	32 million	40 million	49 million	55%
Africa	19 million	29 million	47 million	143%
Europe	59 million	66 million	68 million	15%
South-East Asia	88 million	115 million	153 million	74%
Western Pacific	163 million	197 million	212 million	31%
Middle East & North Africa	55 million	76 million	108 million	96%

Though the increase in the diabetic population is predicted to take place in all the countries globally, quite interestingly the largest increase in diabetic population is predicted to take place in those countries where the economy is moving from low to middle income status [1].

Diabetes is defined as a clinical condition where blood glucose level gets raised beyond a limit either due to inadequate production of insulin or due to ineffective use of insulin by peripheral

tissues. In this context it must be mentioned that cut-offs for blood glucose level also exist for situations where the stage of diabetes hasn't been reached which is termed as "Pre-diabetes". Pre-diabetes includes 2 conditions, Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) where the blood glucose levels in fasting state (no calorie intake for a minimum of 8 hours) and post-prandial (2 hours after 75 gm oral glucose load) state are over the normal limits but below the diabetic limits. However different cut-offs for IFG and IGT have been suggested by different medical organizations (Table 1.2) [5,6] which quite interestingly have predicted the development of Diabetes differently thereby questioning the correctness and relevance of different cut-offs [7].

Table 1.2: WHO and ADA criteria for diagnosis of diabetes and pre-diabetes

	Impaired Fasting Glucose (IFG)	Impaired Glucose Tolerance (IGT)	Diabetes Mellitus
World Health Organization (WHO)	Fasting Plasma Glucose is 110-125 mg/dl & Post-prandial Plasma Glucose below 140 mg/dl	Fasting Plasma Glucose is below 126 mg/dl & Post-prandial Plasma Glucose is 140-200 mg/dl	Fasting Plasma Glucose ≥ 126 mg/dl or Post-prandial Plasma Glucose ≥ 200 mg/dl or Glycated Haemoglobin (HbA1c) ≥ 6.5%
American Diabetic Association (ADA)	Fasting Plasma Glucose is 100-125 mg/dl & Post-prandial Plasma Glucose below 140 mg/dl	Fasting Plasma Glucose is below 126 mg/dl & Post-prandial Plasma Glucose is 140-200 mg/dl	Fasting Plasma Glucose ≥ 126 mg/dl or Post-prandial Plasma Glucose ≥ 200 mg/dl or Glycated Haemoglobin (HbA1c) ≥ 6.5%

Diabetes Mellitus is a heterogenous condition as per the underlying pathology. Broadly it has been classified into 6 categories by the WHO as follows:

1. Type 1 Diabetes
2. Type 2 Diabetes
3. Hybrid forms of diabetes (Ketosis prone type 2 diabetes etc.)
4. Other specific types (Monogenic diabetes, Drug or chemical induced, Infections etc.)
5. Unclassified diabetes
6. Hyperglycaemic first detected during pregnancy

Type 2 Diabetes (T2DM) accounts for 90-95% of diabetes with a great majority present in low and middle income countries. Insulin resistance is the underlying pathology of T2DM [8] where insulin action on peripheral tissues gets compromised resulting in increased glucose levels in the blood. T2DM is therefore is condition of relative insulin deficiency and is present among overweight and obese people. A brief description of different forms of diabetes [9] is mentioned in the table below-

Table 1.3: Different types of diabetes (WHO classification)

Type	Description
Type 1 Diabetes Mellitus (T1DM)	Immune mediated β-cell destruction leading to absolute insulin deficiency
Type 2 Diabetes Mellitus (T2DM)	Insulin resistance followed by β-cell dysfunction, associated with obesity
Hybrid forms of diabetes	
Slowly evolving immune-mediated diabetes of adults (latent autoimmune disease in adults)	Slowly progressing immune-mediated diabetes (positive for glutamic acid decarboxylase autoantibody etc.) among adults (age over 35 years) in the presence of obesity and retaining greater β-cell function compared to T1DM
Ketosis-prone type 2 diabetes	Non-immune ketosis-prone diabetes with a transient β-cell secretory defect during initial presentation but later

	followed by recovery of insulin secretory capacity during diabetes remission
Other specific types of diabetes	
Monogenic diabetes of β-cell function	Due to monogenic defects in β-cell function (GCK MODY, HNF1A MODY, HNF1B RCAD etc.)
Monogenic diabetes of insulin action	Due to monogenic mutations in the insulin receptor in the absence of obesity (LMNA FPLD, BSCL2 CGL etc.)
Diseases of the exocrine pancreas	Underlying process diffusely damaging the pancreas like Fibrocalculous pancreatopathy, Pancreatitis, Neoplasia, Cystic fibrosis etc.
Endocrine disorders	Diseases with excessive secretion of insulin antagonizing hormones namely Cushing's syndrome, Acromegaly, Phaechromocytoma, Glucagonoma etc.
Drug or chemical-induced diabetes	Due to drugs impairing insulin secretion or insulin action like Glucocorticoids, Thiazides, Thyroid hormone, Alpha-adrenergic agonists, Beta-adrenergic agonists etc.
Infection-related diabetes	Due to particular viral infections like Cytomegalovirus, Congenital rubella etc. causing β-cell destruction
Uncommon specific forms of immune-mediated diabetes	Several forms of diabetes in particular immunological diseases with a different pathology leading to T1DM like Insulin autoimmune syndrome, Stiff man syndrome etc.
Other genetic syndromes associated with diabetes	Genetic syndromes with an increased incidence of diabetes like Down's syndrome, Friedreich's ataxia, Hungtington's chorea, Prader-Willi syndrome, Turner's syndrome etc.
Unclassified diabetes	When a newly diagnosed case of diabetes can't be classified into any category, a temporary category of "unclassified diabetes" is given

Hyperglycaemia first detected during pregnancy	
Diabetes mellitus in pregnancy	Diabetes defined by the same criteria as in non-pregnant persons
Gestational diabetes mellitus	Diabetes defined by recommended glucose cut-off values lower than that of diabetes

GCK = glucokinase gene, HNF1A = hepato-nuclear factor gene, MODY = maturity-onset diabetes of the young, RCAD = renal cysts and diabetes, FPLD = familial partial lipodystrophy, CGL = congenital generalized lipodystrophy

1.2 Natural History of disease in T2DM

In the natural history of T2DM, pancreatic β-cell dysfunction in the background of Insulin Resistance (IR) results in the clinical manifestation of the disease [8,10,11]. However whether the series of pathological events occur in this linear fashion (Weight gain, IR and then β-cell dysfunction) remain a matter of debate [12,13]. This is of particular importance to low and middle income countries where β-cell dysfunction, observed even at mild dysglycemia, plays a greater role than IR in T2DM prediction [13].

Insulin Resistance is the primary pathology in T2DM which depends on age, sex and obesity status [14]. Increase in IR causes pancreatic β-cells to secrete increased levels of insulin known as compensatory hyperinsulinemia [15]. Compensatory hyperinsulinemia helps the body to maintain normoglycemia in the presence of IR. It is the inability of β-cells to sustain this increased production of insulin in the background of IR which leads to a gradual increase in plasma glucose levels culminating into T2DM [8]. Hence, T2DM is preceded by a phase called Pre-diabetes when neither fasting nor post-prandial blood glucose levels cross their respective cut-offs for being categorized as T2DM. However, as already mentioned the cut-offs for T2DM differ between guidelines of different organizations [7] thereby raising a debate regarding when to initiate T2DM management.

The stage of Pre-diabetes constitutes 2 groups- Impaired Fasting Glucose (IFG) and Impaired Glucose Tolerance (IGT) [6]. Clinically, IFG and IGT may be present together or in isolation, all of them categorized under Pre-diabetes. IFG has been hypothesized to be the result of predominant β-cell dysfunction whereas IGT the result of predominant Insulin Resistance [16,17]. Prevalence of Pre-diabetes groups, IFG and IGT, have been seen to differ between population groups [1] pointing out to the possibility that normoglycemia doesn’t proceed to T2DM via a similar sequence of pathological events in all. Prevalence of IFG is higher than IGT among Asian Indians, reported in

the ICMR-INDIAB study, suggesting β-cell dysfunction to play a predominant role in T2DM among this group [18].

Though β-cell dysfunction in the presence of IR causes T2DM, the timing of both the events in the natural history of T2DM is controversial [19–22]. Some studies have reported IR to be present a decade earlier before T2DM sets in [22,23]. The literature of β-cell dysfunction is more controversial in this respect with some reporting that the event takes place in the Pre-diabetic phase and some reporting it to occur even at the stage of normoglycemia [19,24].

1.3 Diagnosis of T2DM

T2DM is a global health problem at present which has gradually evolved with modern lifestyle characterised by sedentary life, increased intake of processed foods and reduced physical activity. As insulin resistance is the underlying pathology of T2DM, most people having T2DM are overweight or obese [25,26]. However people who are not obese by BMI criteria may have higher proportion of body fat in the form of visceral adiposity thereby predisposing them to T2DM [27]. Interestingly, certain Asian populations from low and middle income countries [28,29] as well as people from their ethnicity but living in high-income countries [30,31] may have T2DM even without any significant obesity.

T2DM develops over a long period with an initial period of absolute insulin excess followed by gradual reduction of insulin level. The clinical symptoms of T2DM are excessive thirst, blurred vision, frequent urination, lack of energy, fatigue, sudden weight loss and in some cases constant hunger [1]. However all these symptoms may not be that prominent and a great majority may present symptomless. Due to this asymptomatic and insidious nature of T2DM, a large proportion of this diseases remain undiagnosed. This case of undiagnosed T2DM poses significant burden to the society as untreated T2DM continue to cause the complications like neuropathy, retinopathy and

nephropathy is addition to the general loss of quality life among the affected [32,33]. Below is given the top 10 countries with highest number of undiagnosed T2DM in 2019 (Table 1.4)-

Table 1.4: Top 10 countries in 2019 for adults (20-79 years) with undiagnosed diabetes.

Rank	Country	Number of people with undiagnosed diabetes (in millions)	Proportion undiagnosed
1	China	65.2 (60.8-81.6)	56.0
2	India	43.9 (35.5-54.9)	57.0
3	United States of America	11.8 (10.2-13.6)	38.1
4	Pakistan	8.5 (3.5-13.3)	43.8
5	Indonesia	7.9 (6.8-8.5)	73.7
6	Brazil	7.7 (6.9-8.6)	46.0
7	Mexico	4.9 (2.8-5.9)	38.6
8	Egypt	4.8 (2.6-5.5)	54.4
9	Bangladesh	4.7 (3.9-6.0)	56.0
10	Germany	4.5 (3.7-5.0)	47.6

95% confidence interval given in parentheses for number of people with undiagnosed diabetes (in millions)

Considering the symptomless and insidious nature of T2DM as well as the undiagnosed proportion of T2DM, routine screening of adults beyond a certain age limit is required to diagnose T2DM. Adults in the 5th decade of their life and 5-10 years earlier for certain population groups (South Asians) [34] should be routinely screened for T2DM using OGTT (criteria given in Table 1.2). Screening cut-offs for other risk factors like BMI should be different for different sexes [35,36]. However as we enter the era of Precision medicine, a need for personalised screening criteria is needed for T2DM diagnosis and management both [37–43].

1.4 Management of T2DM

T2DM is a multi-factorial disease [44–47] and its management requires a multi-pronged approach which aims to modify all the behavioural risk factors and add suitable glucose-lowering agents to achieve the glycemic target. Obesity being the most important risk factor for T2DM, the most important aim in T2DM management is BMI reduction [6]. Energy restriction through dietary modification and increased physical activity is suggested for weight loss. Pharmacological treatment and Metabolic surgery are the next options. As mentioned in Table 1.5, all these options depend on the BMI category in which the patient falls. However the cut-off for the BMI categories differ for some population groups [48,49].

Table 1.5: Treatment options for obesity among T2DM patients as per different BMI categories

Treatment	BMI category				
	25.0-26.9 (23.0-26.9*)	27.0-29.9	30.0-34.9 (27.5-32.4*)	35.0-39.9 (32.5-37.4*)	≥ 40.0 (≥ 37.5*)
Dietary modification and physical activity	+	+	+	+	+
Pharmacological treatment		+	+	+	+
Metabolic surgery			+	+	+

**BMI cut-off for Asian Americans, +Treatment indicated for the BMI group*

Besides management of obesity, glucose-lowering drugs remain the mainstay of T2DM management [6]. The objective of glucose-lowering drugs is to keep the blood glucose levels within normal limits both in fasting state and after meals. The primary physiological action of these drugs varies widely and also shows difference in terms of efficacy, weight change, hypoglycaemic event,

cardiovascular benefits achieved and cost. Below is mentioned the various drug groups used in glycemic management in T2DM.

A. Biguanides: The most used, cost-effective and popular drug for T2DM management is Metformin [50–53]. Unless contraindicated, it is the first preferred drug in treatment if T2DM. It works by increasing insulin sensitivity in peripheral tissues and also reducing hepatic glucose production through activation of AMP kinase. It shows high efficacy and is weight neutral. It provides potential cardiovascular benefit [54] in Atherosclerotic Cardiovascular Disease (ASCVD) and doesn't cause any hypoglycaemic events.

B. Sulfonylureas: The 2nd generation sulfonylureas are Glimepiride, Glipizide and Glyburide. They work by increasing insulin secretion by closing K_{ATP} channels on β-cell plasma membrane [55–57]. Sulfonylureas also show high efficacy but causes weight gain and hypoglycaemic events. They don't show any cardiovascular benefit in ASCVD and Congestive Heart Failure (CHF).

C. Thiazolidinediones: Pioglitazone and Rosiglitazone are examples of thiazolidinediones which increases insulin sensitivity by activating the nuclear factor PPAR-γ [58–60]. Though similar to metformin in terms of efficacy and risk of hypoglycaemic events, they cause weight gain. As far as cardiovascular effects are concerned, they provide potential benefit in ASCVD but is associated with increased risk of CHF.

D. DPP4 inhibitors: These drugs glucose dependently increase insulin secretion and decrease glucagon secretion by inhibiting Dipeptidyl Peptidase-4 activity and thereby by increasing post-prandial levels of incretin hormones (GLP-1 and GIP) [61–63]. They are known as gliptins with Sitagliptin, Vildagliptin, Teneligliptin used commonly. Their efficacy is intermediate but they don't cause hypoglycaemic events as their action is glucose dependent. Effect on weight change is neutral as is cardiovascular effects in ASCVD.

E. GLP-1 receptor agonists: These drugs (Exenatide, Liraglutide, Albiglutide etc) also increase insulin secretion and decrease glucagon secretion glucose dependently by activating GLP-1

receptors in the intestine [64,65]. They also reduce β-cell apoptosis thereby maintaining insulin secretion [66,67]. They also slow the rate of gastric emptying thereby slowing the rapid increase in post-prandial glucose level. They show high efficacy and cause weight loss. No hypoglycaemic events occur in using them. Although they are neutral ASCVD and CHF, liraglutide provides cardiovascular benefit in ASCVD.

F. SGLT2 inhibitors: The most recent introduction in T2DM management are SGLT2 inhibitors (Canagliflozin, Dapagliflozin) which act by blocking glucose reabsorption by the kidney via inhibition of SGLT2 in the proximal nephron [68,69]. Though their efficacy is intermediate, they provide excellent cardiovascular benefit in ASCVD and CHF both [70,71]. They cause weight loss and aren't associated with hypoglycaemic events.

G. Insulins: The first drug used in the treatment of diabetes is Insulin. As expected, they activate the insulin receptors thereby increasing glucose disposal from the bloodstream and suppressing hepatic glucose production [72]. They have the highest efficacy but causes weight gain and hypoglycaemic events considerably. There are five categories of commercial insulin available depending on their duration of action [73]-

- Rapid-acting analogs- Lispro, Aspart, Glulisine
- Short-acting analogs- Human Regular
- Intermediate-acting analogs- Human NPH
- Basal insulin analogs- Glargine, Detemir, Degludec
- Premixed insulin products- NPH/Regular 70/30, 70/30 aspart mix, 75/25 lispro mix, 50/50 lispro mix

Pharmacological therapy in T2DM depends on the HbA1c levels and target. Guidelines of diabetic associations exist with the treatment algorithm to be initiated and followed for glycaemic control [6,74]. The most common HbA1c target set for T2DM control is 7%. However a more strict target

of 6.5% may be used if that can be achieved without any significant number of hypoglycaemic events.

2. Underlying pathophysiology in T2DM

2.1 Obesity and Insulin Resistance

Obesity is the most important risk factor for T2DM [75–77], the connecting link between them being insulin resistance [78]. Besides T2DM, insulin resistance is also associated with other non-communicable diseases namely Hypertension, Coronary Artery Disease, Hyperlipidaemia, Atherosclerosis, Poly-cystic Ovarian Disease (PCOD), Non-alcoholic Fatty Liver Disease etc [79,80]. Insulin is an anabolic hormone that regulates several aspects of Adipose tissue biology. It acts on adipocytes to stimulate glucose transport and triglyceride synthesis as well as to inhibit lipolysis [81]. The effects of insulin on adipocytes with the signalling pathways are depicted in Figure 2.1.

Insulin resistance denotes resistance of peripheral tissues (adipose and skeletal muscles) to take up glucose and further metabolise it. Another aspect of insulin resistance is impaired suppression of hepatic glucose production [79]. The underlying pathology of insulin resistance may result from impaired insulin signalling in the peripheral tissues or from down-regulation of insulin responsive glucose transporter GLUT4. Insulin binding to its receptor and the downstream cellular events may be impaired in general with some tissue specific alterations responsible for insulin resistance [82,83] (Table 2.1).

Table 2.1: Defects in cellular signalling pathways for adipose and skeletal muscles in Insulin resistance

Tissue	Cellular defect
Adipose tissue	• Down-regulation of GLUT4 • Reduction of IRS-1 expression
Skeletal muscle	• Impaired PI3K activity in association with IRS-1 and IRS-2
Both adipose and skeletal muscle	• Impairment in insulin binding to receptor • Reduced receptor phosphorylation and tyrosine kinase activity • Reduced phosphorylation of Insulin Receptor Substrates (IRS)

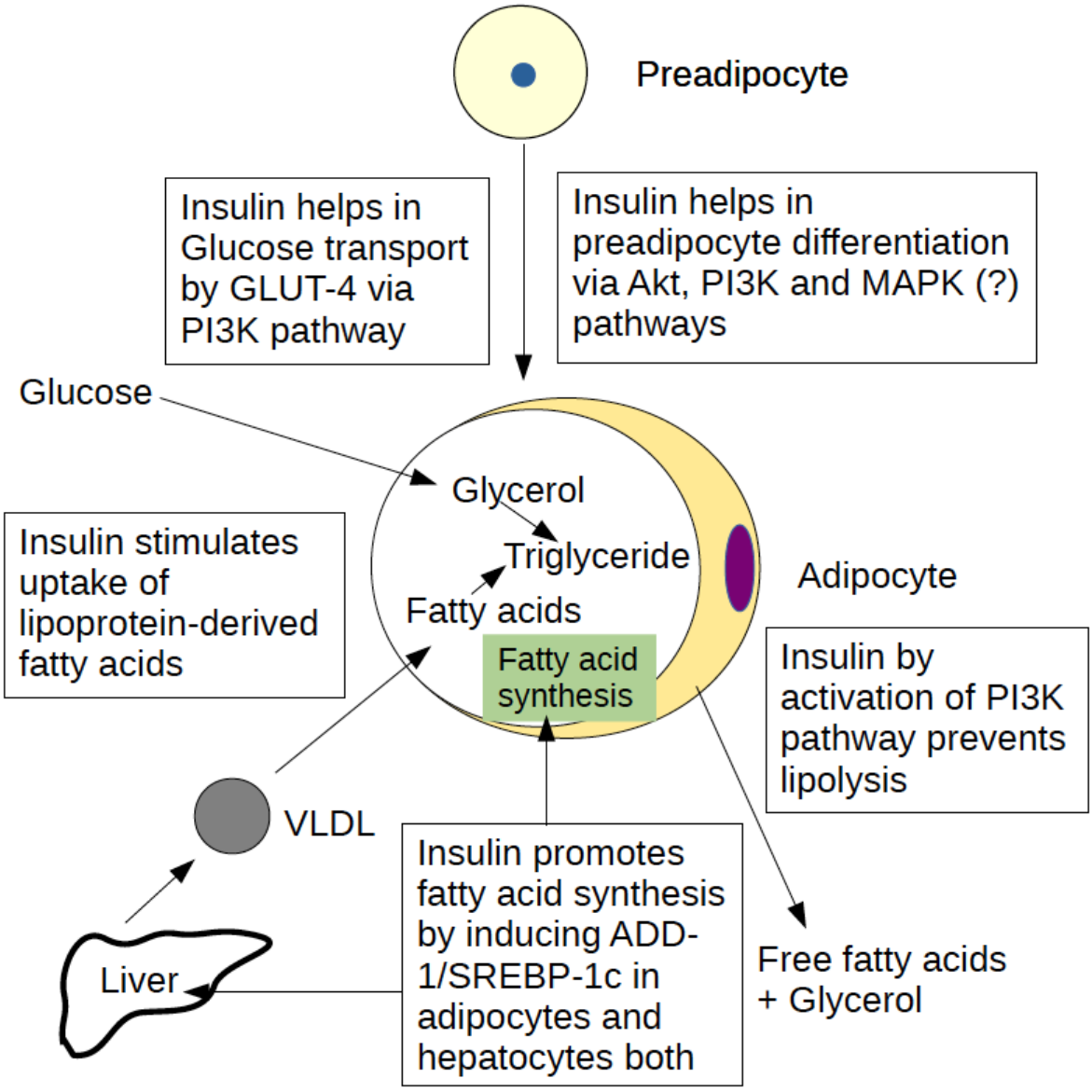


Figure 2.1: Pleotropic effects of Insulin in promoting adipose storage through increased pre-adipocyte differentiation, increased lipogenesis and reduced lipolysis.

Mechanisms of signalling defects in the context of insulin resistance may be increased expression and activity of protein tyrosine phosphatases (PTP), reduced expression of insulin signalling molecules in obesity [84–88] or due to impaired translocation, docking or fusion of GLUT4 vesicles with the plasma membrane [89].

Though obesity and insulin resistance are positively associated in all ethnic groups, the location of fat mass is important in determining the degree of insulin resistance. Both Visceral Adipose Tissue (VAT) and Intracellular Hepatic Fat are considered to be the key contributors of insulin resistance [90,91]. On the contrary, Subcutaneous Adipose Tissue (SAT) is known to be relatively insulin sensitive [92]. Adiposity pattern and distribution, being different between males and females with females having more SAT than males for similar Body Mass Index (BMI), females are more insulin sensitive than males for the same BMI [36,93]. This has been confirmed in studies where males have been reported to show increased T2DM risk at lower BMI than females [35]. However, with increasing BMI, the degree of VAT and SAT proliferation is different between both the sexes with relatively higher SAT proliferation seen among females [36]. Accordingly sex difference in insulin resistance should be less severe among the non-obese population group confirmation of which needs investigation. BMI is the most common marker of obesity extensively used in epidemiological and clinical settings for the ease of its measurement [24,94,95]. However, for long studies have questioned its ability to correlate with T2DM risk especially in some ethnic groups [96]. As T2DM is positively correlated with VAT in particular, Waist Circumference (WC), which reflects abdominal (hence visceral) obesity, has been suggested to be a better marker for T2DM risk assessment [97]. Sex differences in adipose distribution makes the use of BMI more questionable as for the same BMI, females have lower VAT than males. Increased SAT in the gluteo-femoral region in females even has a protective effect as SAT is far more Insulin Sensitive and stores excess nutrients and doesn't spill over Free Fatty Acids (FFAs) in the blood stream thereby reducing T2DM risk [97]. Fat mass content for the same BMI also varies between different ethnic groups

[94]. For the same BMI, individuals of Asian origin has been reported to contain more fat mass [96,98]. thereby increasing their susceptibility to T2DM. Also the pattern of fat deposition is different for particular populations such as Asians whre a pear-shaped fat distribution (Central obesity) is observed in contrast to the apple-shaped distribution seen among the Caucasians.

Another very important aspect in the relationship between obesity and T2DM is the role of adipokines [99]. Adipokines are adipose-derived hormones that act on other organs thereby playing important roles in metabolism. Both harmful [100] and protective adipokines have been reported in the study of T2DM, the most studied being Leptin and Adiponectin respectively [101–103]. Increased adiposity leads to an increase in Leptin-Adiponectin Ratio (LAR) and is positively associated with T2DM [104]. Adiponectin increases Insulin Sensitivity and therefore is a key player in the Metabolically Healthy Obese (MHO) [105–107]. Another important adipokine in the context of T2DM is Adipsin, known to protect β-cell function [108,109]. The perfect balance between protective and harmful adipokines is essential to maintain a metabolically healthy state.

2.2 Pancreatic β-cell dysfunction in T2DM

Pancreatic β-cell dysfunction needs to be present in the background of insulin resistance for manifestation of T2DM [110]. While understanding the role of insulin resistance has been largely explored, the role of β-cell dysfunction largely remained unexplored for a long period of time. The United Kingdom Prospective Diabetes Study (UKPDS) was the first to identify the progressive nature of β-cell dysfunction among T2DM patients [111]. In the UKPDS study as well as the Belfast diet intervention, progressive deterioration of glycaemic control with a paralleled deterioration of β-cell function was observed [112], that too without any change in insulin sensitivity [113].

Insulin secretion form pancreatic β-cells is a relatively complex phenomenon. Insulin secretion follows an oscillatory pattern even in the post-absorptive phase. Two oscillatory patterns of insulin secretion exists- ultradian oscillations observed every 1-2 hours [114] and more rapid oscillations

observed every 10-15 minutes [115]. It has also been noted that pulsatile secretion of insulin is more effective in reducing blood glucose levels than continuous insulin secretion [116]. Experiments with

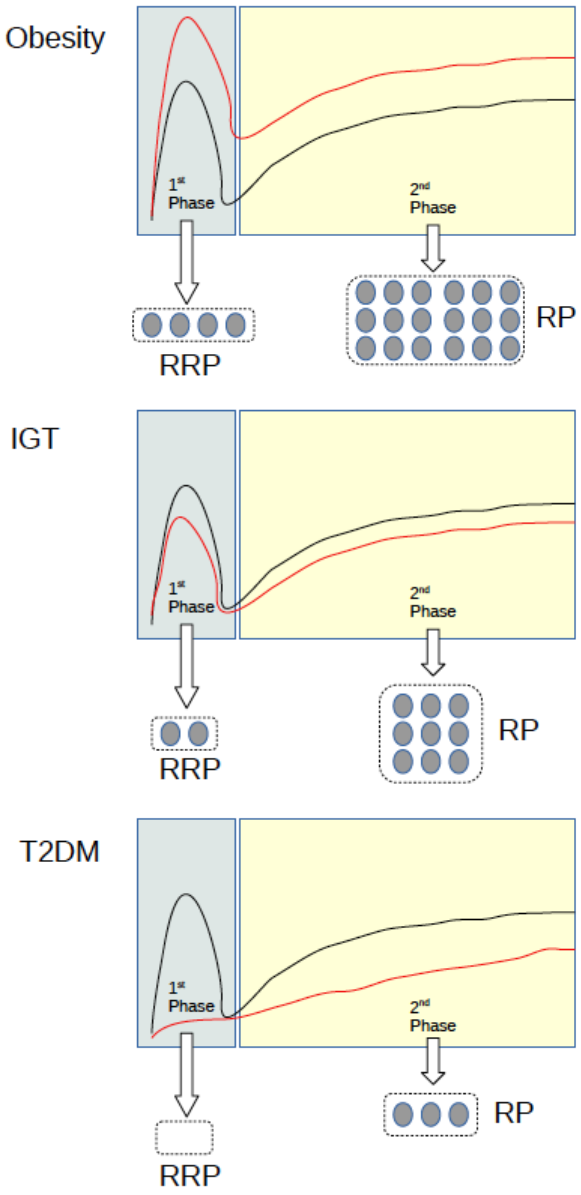


Figure 2.2: First and second phase of insulin secretion in Obesity, Impaired Glucose Tolerance (IGT) and T2DM with the reduction in Reserve pool (RP) and Readily releasable pool (RRP) of Insulin granules

isolated islets and hyperglycaemic clamps have shown that glucose induced insulin secretion occurs in two phases- an initial phase developing rapidly but lasting only a few minutes (5-10 minutes) followed by a sustained phase [117]. A loss of secretion in the first phase is required in Impaired Glucose Tolerance (IGT) and early stages of T2DM whereas both phases show reduced secretion in later stages of T2DM [118] (Figure 2.2). β-cell contain 2 pools of secretory insulin granules depending on their competence to get released, a reserve pool (RP) and a readily releasable pool (RRP) consisting of around 5% of the granules. The first phase of insulin secretion has been proposed to occur because of the release of granules form the RRP while the sustained second phase secretion occurs from the RP [118].

The relation between insulin secretion and insulin sensitivity is essentially hyperbolic [119]. As long as insulin secretion can compensate for reduced insulin sensitivity, the state of normoglycemia is retained. Glucose intolerance is only observed by the insulin secretory defect in the first phase and not by obesity itself [120]. Obesity, itself, has been reported to be a state of primary insulin hypersecretion [121]. Obesity has been shown to be positively associated with β-cell function even after adjusting for IR. Autopsy studies with T2DM patients and healthy controls have reported the β-cell mass to increase by 8-fold in T2DM within the obese group compared to only 3-fold in the non-obese group [122]. Given that obese individuals have increased β-cell function (i.e. increased β-cell secretory reserve) after adjustment for IR compared to the non-obese, whether the later experiences a faster decline in β-cell function thereby rapidly progressing towards T2DM from Normal Glucose Tolerance (NGT) remain unknown. As early intervention in T2DM patients to reach good glycemic control reduces insulin secretory demand and restricts further β-cell dysfunction [123,124], finding the time window of β-cell dysfunction is particularly important in T2DM prevention and progressive deterioration.

Glucose transport into β-cells followed by an increased ATP/ADP ratio and closure of K_{ATP} channels leads to the opening of voltage-dependent Ca^{2+} channels (VDCC). This results in an intracellular

increase in calcium ions thereby triggering insulin secretion [125]. Thus, insulin secretion from the β-cells occur via multiple intracellular signals Ca^{2+}, ATP, cAMP, DAG (Diacylglycerol) and IP_3 (Inositol 1.4.5-triphosphate) [126]. However pathway independent of K_{ATP} channels exist that also helps in insulin secretion [127]. Glucose stimulated insulin secretion (GSIS) occurs in potentiated by incretin hormones by activation of cAMP signalling in the β-cells [128,129]. Beside potentiation of GSIS, cAMP also acts in regulation of insulin granule dynamics for both phases of insulin secretion [130,131].

The relationship between Insulin Sensitivity and Insulin Secretion is curvilinear in fashion [8]. Reduction in Insulin Sensitivity causes the body to increase Insulin Secretion thereby maintaining the normoglycaemic state. Only when further decrease in Insulin Sensitivity doesn't accompany a corresponding increase in Insulin Secretion does an individual enter the Pre-diabetic state and then to T2DM.

2.3 Incretin defect in T2DM

Insulin secretion from β-cells is larger during oral glucose feeding compared to intravenous glucose challenge due to the augmenting effect of incretin hormones (GLP-1 and GIP) on insulin secretion [132–134]. Glucose-dependent insulinotropic polypeptide (GIP) is secreted from the K-cells located in the duodenum of small intestine [135]. GIP level rises on oral food intake which act on β-cells to stimulate glucose-dependent insulin secretion via G-protein coupled receptors. Another hormone GLP-1 secreted from the L-cells located in the distal ileum and colon acts on the GLP-1R receptor in pancreatic islets and also act similarly for insulin secretion [66,136]. Besides potentiating glucose induced insulin secretion, GLP-1 functions in numerous other ways (Table 2.2) from inhibiting gastric emptying to preventing β-cell apoptosis [137–140]. β-cell sensitivity to GLP-1 has been found to associate with insulin sensitivity in humans [141]. Reduction in incretin levels known as incretin defect is a major cause of T2DM [142,143] though controversy exists on whether incretion

defect is the cause or effect of T2DM [142,144]. As such enhancing the incretin effect has been one of the choices in achieving glycaemic control by the usage of GLP-1 agonists etc. [145–148]. Quite interestingly, after metabolic surgery for T2DM treatment incretin levels as well as their effect are seen to increase in association with glycaemic control [149,150]. However the mechanism still remain to be deciphered.

Table 2.2: Actions of GLP-1 on different organs

Organ	Physiological function of GLP-1
Stomach	Decreases Gastric Emptying
Pancreas	Increases Insulin biosynthesis
	Increases β-cell proliferation
	Decreases β-cell apoptosis
	Increases Insulin secretion
	Decreases Glucagon secretion
Muscle	Increases Insulin sensitivity
Liver	Decreases glucose production
Heart	Increases Cardioprotection
	Increases Cardiac output
Brain	Increases Neuroprotection
	Reduced appetite

Both GIP and GLP-1 contain alanine at position-2 which makes it an excellent substrate for the enzyme Dipeptidyl Peptidase-4 (DPP4) [63,151]. Increased DPP4 levels therefore cause a reduction in incretin levels and is predictive of T2DM [152–154]. Identification of increased DPP4 levels as an underlying pathology for T2DM has given rise to a class of anti-diabetic agents known as DPP4 inhibitors [155,156]. Interestingly DPP4 inhibitors have been seen to achieve better glycaemic control among Asian populations [61,62] questioning the possibility of the underlying pathology of T2DM to be different between populations.

DPP4 is shed from different tissues in the human body [157] and its pathophysiological role depends on its tissue origin [158]. Hepatic DPP4 has been seen to induce adipose inflammation [159] and increase insulin resistance [160] while endothelial DPP4 has been observed to control the incretin actions [161].

3. T2DM among Non-obese adults

3.1 Prevalence of T2DM among Non-obese populations

Though T2DM has been known to be an obesity-associated phenomenon, epidemiological studies over the last two to three decades has been reporting T2DM in non-obese populations [162,163] particularly from low and middle-income countries and tropical Asian populations [164]. The percentage of T2DM population in Asian and African countries falling in the non-obese range varies between 24 to 80% in comparison to a mere 10% in the US [163]. This gives rise to the concern that T2DM and Obesity can't be entirely unified and certain aspects of both the diseases remain individualized [77]. The proportion of normal and underweight individuals in the T2DM population in some of the countries are mentioned below (Table 3.1)

Table 3.1: Proportion of Normal and Underweight people among those with T2DM in selected countries

Country	Name of the study	Survey period	Proportion of non-obese people among those with T2DM
India	ICMR-INDIAB Study	2008-2015	51.5%
China	Prevalence and Ethnic Pattern of Diabetes and Prediabetes in China	2013	43%
Vietnam	Prevalence of type 2 diabetes in middle aged women in Northern Vietnam	2010	80%
	Prevalence, Perception, and Factors Associated with Diabetes Mellitus in Vietnam	2014	59%
Korea	Korean National Health and Nutrition Examination Survey	2010-2012	52%
Zambia	Diabetes Mellitus in Zambia and the Western Cape province of South Africa	2010	24%
Uganda	Prevalence and Correlates of Diabetes Mellitus in Uganda	2014	66%

Presence of such a significant population of non-obese T2DM individuals has pointed to the possibility of a different hypothesis for T2DM in this group. Today over half of the T2DM population belong to the low and middle-income countries [1] putting a significant burden of mortality and morbidity among those countries as well as globally. The next section discusses some of the important pathophysiological hypotheses of T2DM in non-obese populations.

3.2 Pathophysiology of T2DM in Non-obese populations

As already mentioned defect in pancreatic β-cell secretion in the background of insulin resistance results in the manifestation of T2DM [8]. Obesity leads to insulin resistance which is mostly driven by inflammation [91,165]. The most common marker for obesity in Body Mass Index (BMI) which is height (in centimetres) divided by weight (in kilograms) squared [94]. However adipose tissue distribution is different across populations and ethnicities [166–169] pointing out that visceral adipose tissue specifically is responsible for insulin resistance [170]. Though waist circumference serves as a marker for visceral obesity [97], its use hasn't still been wide in clinical practice. Hence measuring the distribution of visceral adipose tissue in large epidemiological studies remain a challenge in developing countries. Besides the harmful effects of adipose tissue, there are several protective effects for preventing T2DM too. Adiponectin [106,107,171] and Adipsin [108,109,172] are two well characterised adipokines which increase insulin sensitivity and promote insulin secretion respectively thereby preventing T2DM. Thus it may be the case that non-obese populations have a derangement of the balance between harmful and protective effects of adipose tissue, thereby resulting in the phenotype of non-obese T2DM.

Hyperinsulinaemia is a normal physiological response to insulin resistance [173,174]. Inability to maintain sufficient insulin secretion with decreasing insulin sensitivity causes T2DM [15]. However ethnic differences have been found in studies done to explore the hyperbolic relationship between insulin sensitivity and insulin secretion [175]. Caucasian population are located in the middle of the

hyperbola while African and Asian populations lie at extremes [175]. People from Asian origin has been reported to have genetically predisposed β-cell defect with visceral adiposity showing no independent association with T2DM [177]. Even there has been proposition that insulin resistance isn't a necessary component of T2DM [12]. Such severe degree of β-cell dysfunction has been suggested to occur due to genetic reasons, environmental factors [124] and intra-uterine or childhood malnutrition [178–180]. It is therefore a possibility that the journey from normoglycaemia to T2DM via the pre-diabetic stage is different for the non-obese population (Figure 3.1). However studies need to be done focussed on the non-obese population to reveal the underlying dynamics of non-obese T2DM.

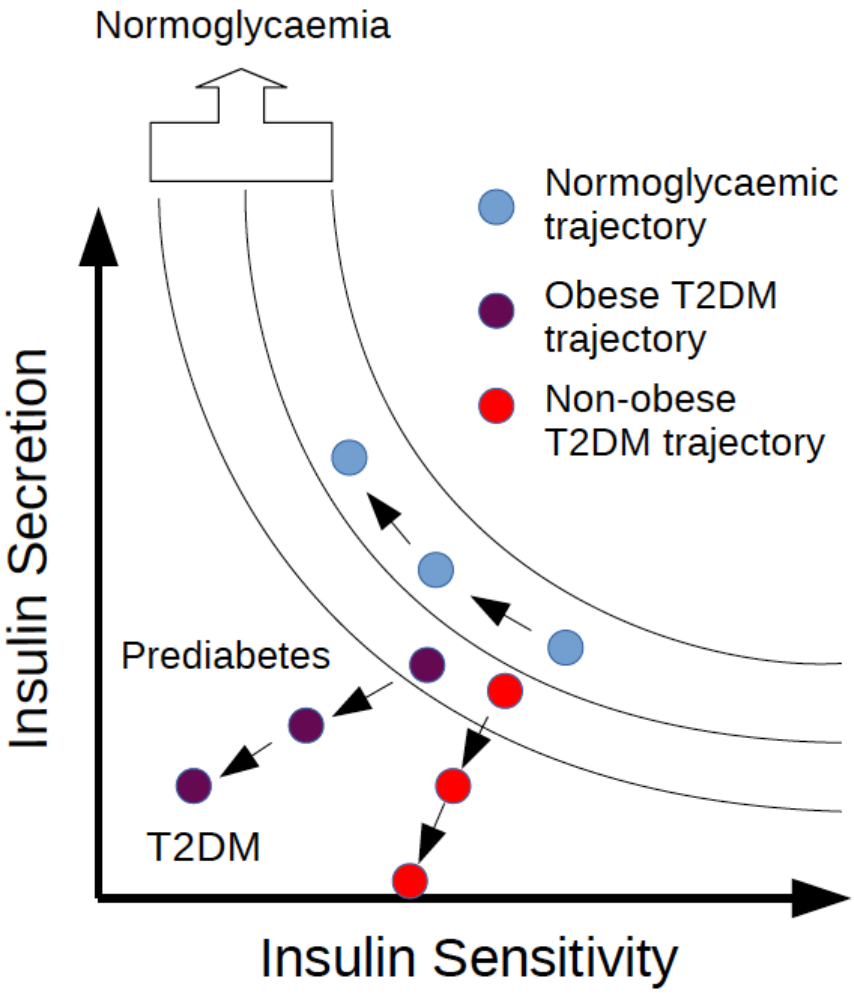


Figure 3.1: Possible trajectories of obese and non-obese individuals from normoglycemia to T2DM

3.3 Treatment of T2DM in Non-obese populations

Guidelines for treatment of T2DM remain the same for obese and non-obese populations. Given the difference in underlying pathology of T2DM, a uniform therapeutic guideline may not be appropriate for all populations (Asians, Africans, Caucasians etc.). Treatment for T2DM may be more precise in the upcoming era of personalised medicine [39]. Treatment of T2DM is a long-term (essentially lifelong) process and thus availability of resources and cost of treatment is important for T2DM patients in low and middle income countries [181]. A nation-wide study in India reports over 75% of the T2DM patients to have uncontrolled glycaemia [182]. A systemic review reports a wide variability in HbA1c reduction among T2DM patients using different classes of anti-diabetic medications [185]. Genetic variants has been identified to be associated with T2DM [184] which again differ between ethnicities and populations [185,186]. Certain variants are associated with the development of insulin resistance [187] and certain with β-cell dysfunction [188,189]. As the relative contribution of β-cell dysfunction and insulin resistance is thought to be different in non-obese T2DM, finding a different therapeutic guideline based on evidence will be more rational and fruitful in terms of glycemic control and prevention of diabetic complications.

3.4 Future perspective

As already discussed non-obese individuals mayn't follow the same course where Insulin Secretion has a greater effect on T2DM. It may be the case that Insulin Secretion defect occurs in non-obese individuals on mild or even no reduction of Insulin Sensitivity making the hyperbolic curves different in the non-obese group compared to the obese group. Generating the hyperbolic curves for the 3 states of glycaemic status- NGT, Pre-diabetes and T2DM in non-obese group is essential from the perspective of understanding the disease as well as designing cut-offs for screening and

management. Given that we are entering the era of precision medicine [40,43], drawing the precise natural history should also be a major task to be undertaken.

Recent evidence on glycemic control among diabetes patients has been shown to be very poor in India, the Diabetes capital of South-East Asia. Less than 25% of the Indian diabetic population have proper control of the disease thereby increasing the burden of co-morbid diseases, diabetes-related complications and mortality among them [182]. As discussed, T2DM, being a multi-factorial disease, has a complex pathology, thereby requiring a complex treatment guideline. There are a plethora of risk factors for T2DM ranging from obesity, smoking, alcohol intake, physical activity to dietary pattern (both macro and micro-nutrients) [46,190–192] all of which need to be properly addressed for successful management of the disease. Identification of risk factors specific to the non-obese T2DM phenotype is thus important for T2DM management among the non-obese.

T2DM, thought to be a homogenous disease is recently being suggested to be a disease mix according to the underlying pathology [193,194]. Whether such clusters exist based on disease phenotypes and epidemiological parameters is also an important task for cluster-driven T2DM prevention and management. It may be a possibility that T2DM among non-obese adults is a particular cluster with its certain differences from others. Keeping in view the aforesaid gaps in the literature of non-obese T2DM the following experimental work was undertaken with the following broad objectives:

1. Whether the relative contribution of Insulin resistance and β-cell dysfunction differ in non-obese and obese T2DM.

2. Whether insulin and incretin response differ between non-obese and obese T2DM.

3. Whether there are epidemiological risk factors for T2DM specific to the non-obese population group.

4. Whether there exist any cluster with its unique features among T2DM patients associated with the non-obese group.

EXPERIMENTAL WORK

4. Compensatory hyperinsulinaemia and Insulin Resistance in non-obese T2DM patients

4.1 Introduction

Obesity is the most important risk factor for Type 2 Diabetes Mellitus (T2DM) [40,75,77,78,90]. Weight gain is the first hit in the pathogenesis of T2DM [8,90] causing Insulin Resistance (IR) thereby inducing hyperinsulinemia with β-cells producing higher levels of insulin to maintain normal blood glucose levels [8,10,15,23,81,195]. Progressive and gradual failure of β-cells to sustain the increased insulin production leads to T2DM [8,110]. Studies have reported insulin levels to increase with obesity even in the absence of IR [121] making obesity a state of primary insulin hypersecretion.

Association of T2DM with obesity and insulin resistance is clinically as well as pathophysiologically well established. But the distinct and understudied non-obese T2DM phenotype with predominant β-cell dysfunction evidenced in middle and low income countries has attracted recent attention only [13,163,196]. In this context, whether hypersecretion of insulin with a corresponding increase in weight occurs in the non-obese population group remains unknown. The overt prevalence of non-obese individuals among T2DM group lying in the range of 51.5% in India to 80% in Vietnam asks for an in-depth examination of compensatory hyperinsulinemia within the non-obese population.

Conducting experiments on a population cohort spread over 8 villages in 2 districts of West Bengal (Figure 4.1) under a Community-based intervention program “From Food Security to Nutrition Security” run by SWANIRVAR, we aimed to investigate whether the compensation in insulin levels in response to an increase in body weight is different between non-obese and obese groups.

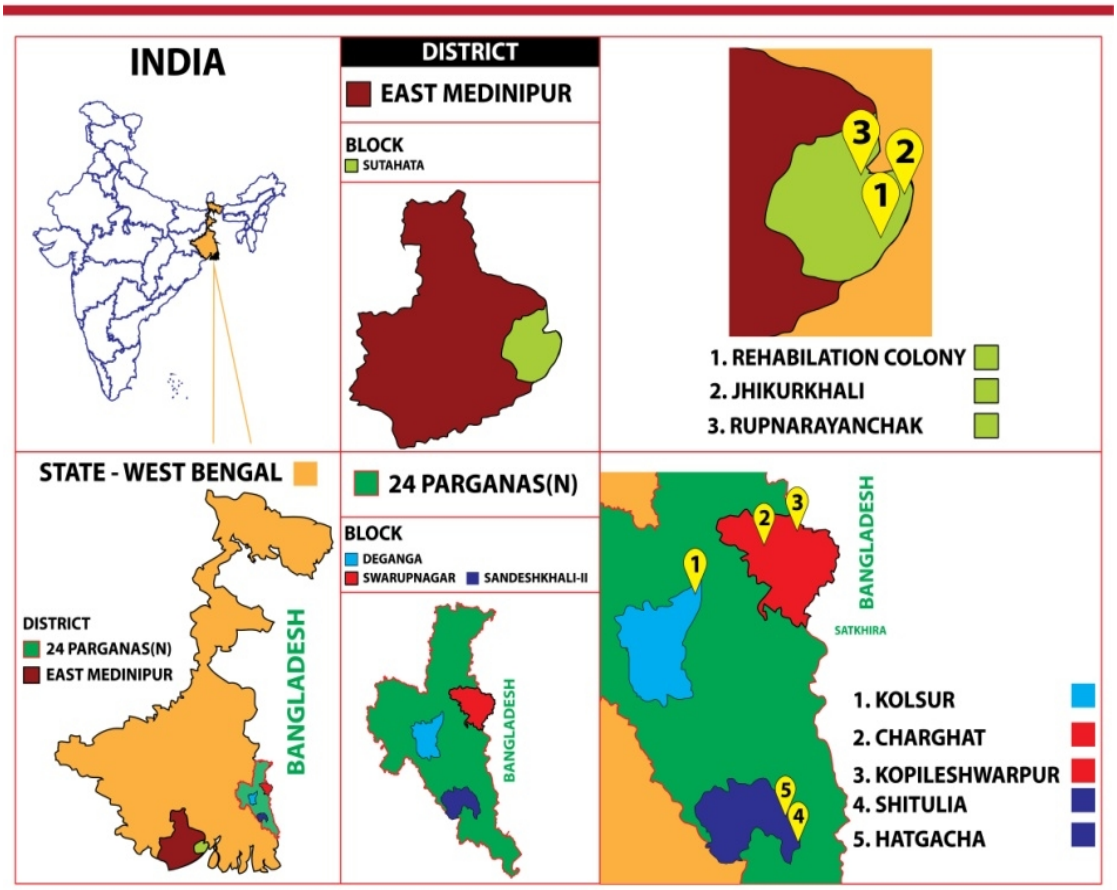


Figure 4.1: 6 villages in 2 districts of West Bengal where the Community-based intervention program "From Food Security to Nutrition Security" is run by SWANIRVAR.

4.2 Materials and methods

4.2.1 Study Design

This is a cross-sectional study covering 650 individuals (64% females, 62% non-obese with BMI < 25) spread over all the 8 villages. Villages were selected from a Community-based Metabolic Health Screening Program named "From Food to Nutrition Security" run by a not-for-profit organization, SWANIRVAR. The program renders both preventive and curative healthcare services to selected families and individuals. The preventive aspect consists of giving balanced diet charts to

individuals and following them up at quarterly intervals for dietary compliance. The curative aspect consists of a clinic to provide healthcare checkup and regular medications for primary health problems including T2DM, Hypertension and Ischaemic heart diseases. Investigations and medication history data of all the patients are regularly captured (once in 2 week) in a digital database.

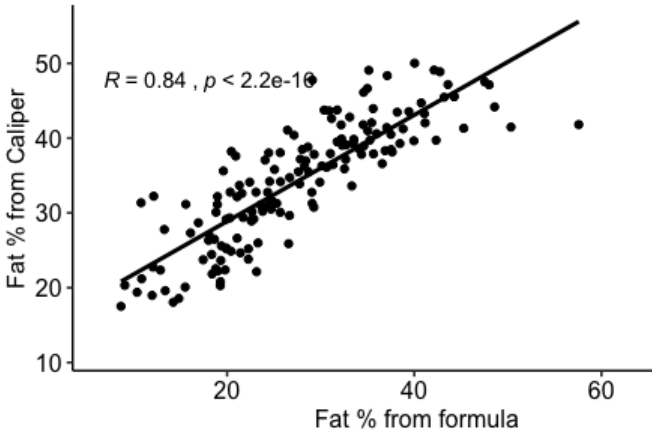
4.2.2 Patient Recruitment

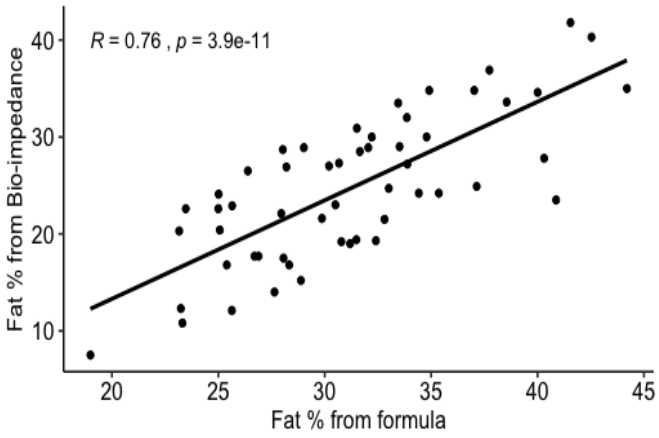
Subjects were recruited from the program from January 2017 to September 2018 for this study. Only naively diagnosed T2DM patients before initiating any anti-diabetic agents were recruited for sample collection in addition to healthy controls. Subjects were classified as T2DM as per the criteria of American Diabetes Association (ADA) [6]. All the subjects were subdivided into non-obese and obese groups based on body mass index (BMI) with an obesity cut-off of 25. The study was approved by human ethics committee of CSIR-IICB, Kolkata (Council of Scientific and Industrial Research-Indian Institute of Chemical Biology). All the subjects gave written informed consent.

4.2.3 Sample Collection and Anthropometric measurements

All blood samples have been collected in Sodium Fluoride/Na_2 EDTA vials (BD Vacutainer, NJ, USA) after overnight fasting for 8-10 hours. Plasma was separated in the field offices of SWANIRVAR and then transported to the laboratory for long term storage at -80ºC. Height, Weight and Waist circumference were measured as anthropometric parameters. Body Mass Index (BMI) was calculated form height and weight. Waist circumference (WC) was measured midway between the lowest point of ribcage and the highest point of Iliac crest as a marker of central obesity. Body fat percentage was calculated from the formula as follows: (0.13 × age in years) + (1.5 × BMI in kg/ m^2) – 23.5 (for men) or -11.5 (for women) [94]. This formula was validated in 3 subsamples

obtained from the cohort with body fat percentage measured by Harpenden skinfold caliper (n = 153), Bioelectrical Impedance Analyzer (n = 54) and Dual Energy X-ray Absorptiometry (DEXA) scan (n = 33) where we found strong correlations in all the three cases (Figure 4.2) with body fat percentage calculated from this formula. Blood pressure was measured for the subjects recruited from the community based program by digital sphygmomanometer (Model: HEM-8712, OMRON HEALTHCARE Co. Ltd. Kyoto, Japan) in sitting condition. All the measurements were done by a single trained person.





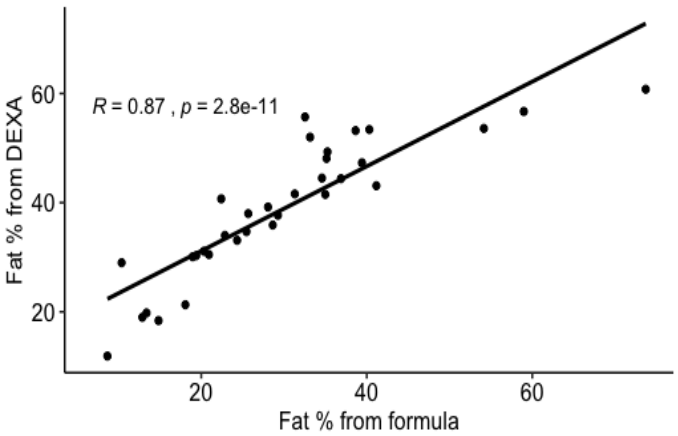


Figure 4.2: Correlation of body fat percentage calculated using Gallagher's formula with body fat percentage obtained using Skinfold Calliper, Bio-impedence Analyzer and DEXA Scan in random sub-samples of the community cohort

4.2.4 Biochemical Analysis

Plasma Glucose was measured by Glucose Oxidase method, Total Cholesterol by Cholesterol Oxidase method, Total Triglycerides by Lipase/GPO-PAP method. Plasma Insulin (Merck Millipore, MA, USA) & Plasma leptin (R&D Systems, MN, USA) levels were measured by ELISA. Homeostatic model assessment (HOMA) [197] was done to calculate HOMA-IR for insulin resistance and HOMA-B for β-cell function as per the formula: HOMA-IR = (Fasting Insulin × Fasting Glucose) / 22.5; HOMA-B = (20 × Fasting Insulin) / (Fasting Glucose − 3.5).

4.2.5 Statistical analysis

Data have been summarized as mean and standard error of the mean (SE). Shapiro-Wilk's W test was performed to assess normality of the variables. Numerical variables are compared between groups by independent-samples two-sided Student's t-test or Man-Whitney U test as appropriate. Categorical variables are tested using Chi-square test. Age and sex adjusted mean and standard error is also reported for the subjects. Age, sex and fasting plasma glucose adjusted mean and standard error is also reported for obese and non-obese T2DM subjects separately. Adjustments are done for each variable by linear modelling using the *lsmeans* package in R. Partial correlation has been calculated between adiposity parameters and markers of insulin secretion and resistance after adjusting for age, sex and fasting plasma glucose. *p*-value less than 0.05 is considered to be statistically significant. Statistical analysis has been performed in RStudio (Version 1.1.447) [198].

4.3 Results

4.3.1 Non-obese T2DM patients have fasting insulin level comparable to non-obese healthy controls

The cohort had 175 subjects diagnosed with T2DM while other 475 were normoglycameic. Fasting plasma glucose, total cholesterol, triglyceride, insulin and leptin levels were measured in all 650 individuals. The summary values of clinical and biochemical parameters in both groups is given in Table 4.1. Subjects were grouped into non-obese (BMI < 25) (T2DM=98, healthy=307) and obese (BMI ≥ 25) (T2DM=77, healthy=168) and sub-group analyses between T2DM and healthy were done within each BMI group. Comparison was also done between non-obese and obese sub-groups within the T2DM group.

Age was significantly increased in T2DM compared to the healthy in both obese and non-obese groups whereas sex difference was noted between T2DM and healthy in the non-obese group only. Though both body mass index (BMI), waist circumference and body fat (%) all were increased in T2DM compared to healthy within both the groups, body fat percent showed a modest increase only within the non-obese BMI group. A decrease in leptin levels among the non-obese T2DM was found compared to the non-obese healthy (15.26 ± 3.2 vs 21.18 ± 1.24, p = 0.001) which was lost after age and sex adjustment (Table 4.2); thereby suggesting that leptin levels before adjustment was due to increased leptin secretion in females.

Compensatory hyperinsulinemia was found in obese T2DM compared to the obese healthy group (9.34 ± 0.8 μU/ml vs 6.05 ± 0.37 μU/ml, p < 0.001). However, no such event of compensatory hyperinsulinemia was found in non-obese T2DM compared to the non-obese healthy group (5.83 ± 0.65 μU/ml vs 4.29 ± 0.16 μU/ml, p = 0.5) suggesting that insulin secretory defect at diagnosis occurs to a greater extent in non-obese T2DM (Figure 4.3). Fasting insulin levels in the non-obese

T2DM increased after adjustment of age and sex but the increase was by 26% within the non-obese group compared to 57% in the obese group (Table 4.2).

Table 4.1: The subject characteristics of healthy and T2DM groups in the community cohort and their biochemical parameters

	Non-obese (BMI < 25)			Obese (BMI ≥ 25)			Non-obese DM vs Obese DM	Non-obese Non-DM vs Obese Non-DM
VARIABLE	Non-DM	DM	P value	Non-DM	DM	P value	P value	P value
N (Male/Female)	307 (102/205)	98 (53/45)	< 0.001	168 (57/111)	77 (21/56)	0.373	< 0.001	0.957
Age (yrs)	42.98 ± 0.89	50.11 ± 1.24	< 0.001	42.65 ± 0.9	46 ± 1.04	0.011	0.015	0.856
Adiposity parameters								
BMI (kg/m^2)	21.07 ± 0.15	21.8 ± 0.24	0.023	28.96 ± 0.38	30.7 ± 0.71	0.03	< 0.001	< 0.001
WC (cms)	78.96 ± 0.59	84.18 ± 0.85	< 0.001	94.71 ± 0.91	100.83 ± 1.71	0.001	< 0.001	< 0.001
Body Fat (%)	22.68 ± 0.38	23.79 ± 0.71	0.055	35.24 ± 0.7	39.13 ± 1.14	0.004	< 0.001	< 0.001
Leptin (ng/ml)	21.18 ± 1.24	15.26 ± 3.2	0.001	42.85 ± 3.95	58.05 ± 10.14	0.323	< 0.001	< 0.001
Metabolic parameters								
FBS (mg/dl)	86.89 ± 0.68	195.23 ± 6.45	< 0.001	94.88 ± 1.09	176.86 ± 5.83	< 0.001	0.028	< 0.001
TG (mg/dl)	111.09 ± 5.34	167.48 ± 11.9	< 0.001	118.65 ± 4.65	154.64 ± 8.45	< 0.001	0.751	0.001
TC (mg/dl)	160.95 ± 2.41	178.86 ± 5.86	0.007	161.25 ± 3.78	185.83 ± 7.76	0.002	0.466	0.69
SBP (mm Hg)	125.8 ± 1.56	130.15 ± 2.92	0.083	129.85 ± 1.57	128.95 ± 3.12	0.465	0.801	0.003
DBP (mm Hg)	77.97 ± 0.84	81.19 ± 1.54	0.082	83.62 ± 1.05	81.29 ± 1.69	0.135	0.988	< 0.001
Fasting Insulin (μU/ml)	4.29 ± 0.16	5.83 ± 0.65	0.5	6.05 ± 0.37	9.34 ± 0.8	< 0.001	< 0.001	< 0.001
HOMA-IR	0.94 ± 0.04	2.81 ± 0.34	< 0.001	1.47 ± 0.1	3.96 ± 0.36	< 0.001	0.001	< 0.001
HOMA-B	76.55 ± 2.91	19.33 ± 2.5	< 0.001	75.67 ± 4.07	36.19 ± 3.76	< 0.001	< 0.001	0.672

Data represented by means ± SE. Non-obese are individuals with BMI < 25. p-value < 0.05 considered statistically significant

Table 4.2: Age and sex adjusted subject characteristics of healthy and T2DM patients in the community cohort and biochemical parameters

	Non-obese (BMI < 25)			Obese (BMI ≥ 25)		
VARIABLE	Non-DM	DM	P value	Non-DM	DM	P value
Adiposity parameters						
BMI (kg/m^2)	21.2 ± 0.16	22.1 ± 0.29	0.01	28.9 ± 0.44	30.9 ± 0.72	0.019
WC (cms)	80.7 ± 0.58	84.1 ± 1.06	< 0.005	96.1 ± 1.05	101.3 ± 1.74	0.011
Body Fat (%)	21.6 ± 0.33	24.1 ± 0.6	< 0.001	34.3 ± 0.72	38.1 ± 1.19	0.006
Leptin (ng/ml)	17.9 ± 1.33	18.8 ± 3.19	0.78	35.4 ± 4.58	45.5 ± 13.19	0.471
Metabolic parameters						
FBS (mg/dl)	87.6 ± 1.99	197.5 ± 3.64	< 0.001	95.7 ± 2.47	175.5 ± 4.07	< 0.001
TG (mg/dl)	113 ± 6.09	177 ± 11.16	< 0.001	122 ± 5.42	165 ± 8.82	< 0.001
TC (mg/dl)	160 ± 2.77	184 ± 5.13	< 0.001	158 ± 4.68	191 ± 7.61	< 0.001
SBP (mm Hg)	128 ± 1.42	127 ± 3.24	0.632	130 ± 1.68	124 ± 3.17	0.133
DBP (mm Hg)	79.4 ± 0.85	81.9 ± 1.9	0.23	83.4 ± 1.21	78.9 ± 1.99	0.056
Fasting Insulin (µU/ml)	4.28 ± 0.24	5.39 ± 0.44	0.029	6.03 ± 0.45	9.47 ± 0.75	< 0.001
HOMA-IR	0.95 ± 0.1	2.51 ± 0.19	< 0.001	1.46 ± 0.17	3.94 ± 0.27	< 0.001
HOMA-B	74.7 ± 2.73	18.6 ± 4.99	< 0.001	74 ± 3.76	37.6 ± 6.21	< 0.001

Data represented by means ± SE. Non-obese are individuals with BMI < 25. p-value < 0.05 considered statistically significant.

4.3.2 Non-obese T2DM patients have lower β-cell secretion and insulin resistance compared to obese T2DM patients

On comparing within both the T2DM groups, we found non-obese T2DM to have lower insulin resistance (measured by HOMA-IR) (2.81 ± 0.34 vs 3.96 ± 0.36, p = 0.001) and lower β-cell secretion (measured by HOMA-B) (19.33 ± 2.5 vs 36.19 ± 3.76, p < 0.001) than the obese T2DM group (Figure 4.3). Findings were same between two groups after adjusting for age, sex and fasting

plasma glucose (Table 4.3). There was a reduction of HOMA-B by 4-fold with T2DM in the non-obese group in contrast to a 2-fold decrease in the obese group. No difference was found in β-cell secretion in obese healthy compared to non-obese healthy in spite of the former having gained significantly higher weight and showing increased insulin resistance compared to the later.

Thus obesity associated insulin resistance gets compensated by increased β-cell secretion among healthy obese subjects. The degree of compensatory hyperinsulinemia at diagnosis is more dampened in the non-obese T2DM suggesting a severe impairment of β-cell secretion.

Table 4.3: Age, sex and FBS adjusted subject characteristics of non-obese and obese T2DM subjects in the community cohort and their biochemical parameters.

VARIABLE	Non-obese (BMI < 25) DM	Obese (BMI ≥ 25) DM	P value
Adiposity parameters			
BMI (kg/m^2)	22 ± 0.46	30.3 ± 0.6	< 0.001
WC (cms)	84.4 ± 1.16	100.4 ± 1.61	< 0.001
Body Fat (%)	24.3 ± 0.81	37.4 ± 1.05	< 0.001
Leptin (ng/ml)	16.4 ± 4.84	34.1 ± 10.69	0.136
Metabolic parameters			
TG (mg/dl)	170 ± 10.2	155 ± 14.6	0.409
TC (mg/dl)	179 ± 5.9	191 ± 8.33	0.248
SBP (mm Hg)	129 ± 2.88	124 ± 4.18	0.312
DBP (mm Hg)	81.8 ± 1.54	79.2 ± 2.26	0.339
Fasting Insulin (μU/ml)	5.64 ± 0.69	9.09 ± 0.9	0.003
HOMA-IR	2.56 ± 0.32	4.05 ± 0.42	0.005
HOMA-B	20 ± 2.75	33.1 ± 3.58	0.004

Data represented by means ± SE. Non-obese are individuals with BMI < 25. p-value < 0.05 considered statistically significant.

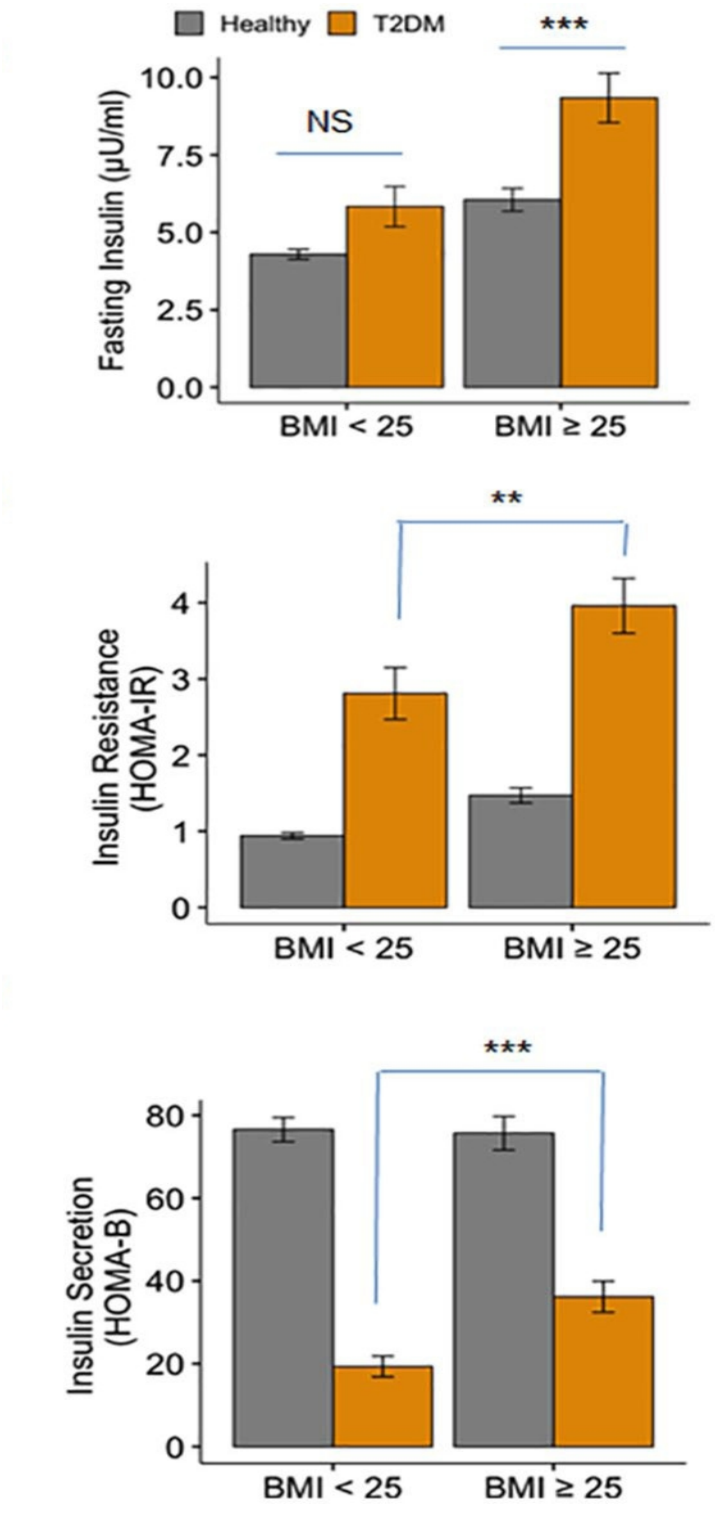


*Figure 4.3: Difference in Fasting Insulin, Insulin Resistance and Insulin secretion between T2DM and healthy sub-groups within both non-obese and obese groups in the community cohort. NS: Non-significant, $p < 0.01$**, $p < 0.001$****

4.3.3 Adiposity parameters don't correlate with Insulin level, β-cell secretion and insulin resistance in non-obese T2DM population

As obesity independently causes an increase in insulin secretion irrespective of IR, it was examined if insulin level, insulin resistance and insulin secretion increase with increments in adiposity parameters across all the groups in our study population. Partial correlations were calculated between three adiposity parameters (BMI, WC and Leptin) and fasting insulin, HOMA-IR and HOMA-B after adjustment of age, sex and fasting plasma glucose. Fasting insulin, HOMA-IR and HOMA-B all increased with a corresponding increase in adiposity parameters in the healthy obese group. Parameters were found to more strongly correlate with fasting insulin, HOMA-IR and HOMA-B in the obese T2DM group. The lowest was the correlation between BMI and HOMA-B ($r = 0.48$, $p < 0.001$) and the highest between Leptin and Fasting Insulin ($r = 0.66$, $p < 0.001$) (Table 4.4). This confirms the presence of compensatory hyperinsulinemia with increased body weight in the obese group in both case of presence and absence of T2DM. However none among BMI, WC and leptin correlated with fasting insulin, HOMA-IR or HOMA-B in the non-obese T2DM group. Correlation was weak in the healthy non-obese group (Table 4.4).

Table 4.4: Age, Sex and FBS adjusted correlations of fasting insulin, HOMA-B & HOMA-IR with adiposity markers in the community cohort

Obese (BMI ≥ 25)						
	Obese healthy			Obese T2DM		
VARIABLE	Fasting Insulin	HOMA-IR	HOMA-B	Fasting Insulin	HOMA-IR	HOMA-B
BMI (kg/m^2)	0.38 (< 0.001)	0.38 (< 0.001)	0.32 (< 0.001)	0.54 (< 0.001)	0.54 (< 0.001)	0.48 (< 0.001)
WC (cms)	0.38 (< 0.001)	0.39 (< 0.001)	0.31 (< 0.001)	0.54 (< 0.001)	0.48 (< 0.001)	0.57 (< 0.001)
Leptin (ng/ml)	0.42 (< 0.001)	0.44 (< 0.001)	0.27 (0.006)	0.66 (< 0.001)	0.66 (< 0.001)	0.59 (< 0.001)
Non-obese (BMI < 25)						
	Non-obese healthy			Non-obese T2DM		
VARIABLE	Fasting Insulin	HOMA-IR	HOMA-B	Fasting Insulin	HOMA-IR	HOMA-B
BMI (kg/m^2)	0.13 (0.024)	0.12 (0.034)	0.12 (0.034)	0.08 (0.444)	-0.002 **(**0.986**)**	0.14 (0.185)
WC (cms)	0.09 (0.15)	0.08 (0.211)	0.1 (0.091)	0.09 (0.393)	0.02 (0.828)	0.14 (0.2)
Leptin (ng/ml)	0.13 (0.044)	0.12 (0.064)	0.1 (0.108)	-0.01 **(**0.956**)**	0.01 (0.948)	-0.02 (0.91)

Non-obese are individuals with BMI < 25. p-value < 0.05 considered statistically significant.

4.4 Discussion

Basal plasma insulin level doesn't increase in non-obese T2DM patients but increases in obese T2DM compared to the obese healthy group. Moreover this increase occurs linearly with an increase in all adiposity markers (BMI, WC and leptin) in healthy and T2DM within the obese group. Though correlations are weakly present within the non-obese healthy group, they are absolutely absent in the non-obese T2DM group. Compensatory hyperinsulinemia is therefore absent in non-obese T2DM. Impaired basal insulin secretion is with isolated impaired fasting glucose (IFG). So this finding seem to explain the higher proportion of pre-diabetic patients with IFG in the South Asian population [13,18]. Decrease in the basal insulin may be caused due to reduced β-cell mass in the non-obese T2DM which has been previously reported in autopsy studies. The studies report that β-cell apoptosis increases 10-fold in obese T2DM in comparison to a mere 3 fold in non-obese T2DM. [122,199].

Obesity is a state of primary insulin hypersecretion and hence BMI exerts a positive effect on insulin secretion irrespective of insulin resistance status. Increased BMI is associated with an increase in β-cell mass amounting to 10-30% increase for every 10 kg of body weight [121]. As adiposity parameters in this study showed strong correlation with fasting insulin and insulin secretion (HOMA-B value) in the obese group, presence of adequate basal β-cell function among the obese T2DM group at the time of diagnosis is confirmed. On the other hand, absence of this correlation in the non-obese group reveals the predominance of impaired β-cell function in the non-obese T2DM group at the time of diagnosis. The classical pathway of T2DM is insulin resistance followed by impaired insulin secretion [8,163]. As the non-obese group displays severely β-cell dysfunction at diagnosis, insulin secretory defect rather than insulin resistance may have been the predominant pathology which starts long before the disease manifests among non-obese individuals.

This study had strengths and limitations. T2DM patients were recruited over a long period and only those diagnosed for the first time without receiving any anti-diabetic medication were recruited for sample collection. The prime limitation was to use HOMA modeling to calculate insulin secretion and resistance. However use of other indices in epidemiological studies is still challenging in developing countries. Moreover the fat compartments and body fat distribution in the subjects remain unadjusted as body fat distribution also regulates insulin resistance.

4.5 Summary of the work

Severe basal β-cell dysfunction in non-obese T2DM at diagnosis gives a call to revisit the therapeutic guidelines and screening criteria for T2DM in this group. Epidemiological evidence accumulating from the low and middle income countries is helping us to appreciate the distinct Metabolically Unhealthy Non-obese (MUNO) phenotype. Unexpectedly, this MUNO group exhibits higher risk of mortality from cardiovascular events, a phenomenon termed as the 'Obesity Paradox' [95,200,201]. Hence prospective studies need to be done to quantify the degree, timing and duration of β-cell dysfunction in the natural history of T2DM in this group detection of which will help arrest the pathogenesis at an earlier stage and provide better preventive and therapeutic options in the context of β-cell revival.

However secretion of insulin from β-cells increase after meals via stimulation by glucose, aminoacids and gut-derived hormones called incretins. Whether this ability of β-cells to secrete insulin in the post-prandial state is defective too among the non-obese T2DM group is reported in the next chapter.

5. Insulin and Incretin Response during OGTT in non-obese T2DM patients

5.1 Introduction

Insulin response after meals is impaired in T2DM. Impairment in first phase of insulin release leads to the pre-diabetic state and impairment of both phases leads to T2DM [118]. Reduction in the readily releasable pool of insulin granules causes impairment of the first phase whereas reduced reserve pool is responsible for impairment of the second phase. Glucose is the most important stimulant of insulin release from the pancreatic β-cells, the phenomenon termed as Glucose-induced Insulin Secretion (GSIS). Another very important and relevant stimulant of insulin release are the gut-derives peptide hormones known as incretins [135]. Incretin hormones, namely GLP-1 and GIP, not only potentiates GSIS but also plays role in prevention of β-cell apoptosis [132,139,202]. An important regulator of the incretin effect on β-cell secretion in the enzyme Dipeptidyl Peptidase-4 (DPP4/CD26), a widely expressed single pass type II transmembrane protein with a very short cytosolic tail and having a unique exopeptidase activity [203]. DPP4 cleaves N-terminal dipeptides from many substrates including incretin hormones GIP and GLP-1, thereby impairing insulin secretion from β-cells. Hence DPP4 inhibitors are clinically used as 'incretinergic' drugs in treatment of T2DM [63].

Interestingly, plasma DPP4 activity show contradictory reports of reduction [204,205] and increment [152,206] in T2DM patients. From the context of obesity, DPP4 is an adipokine connecting obesity to T2DM [151]. However from the therapeutic aspect, DPP4 inhibitors report better glycemic control for the Asian population with large population of non-obese T2DM [61,62]. So understanding whether obesity associated DPP4 contributes to T2DM among non-obese individuals remain an open ended question.

Using the aforementioned community cohort and two hospital cohorts, we aimed to investigate the difference in DPP4-Incretin-β-cell axis between non-obese and obese T2DM patients.

5.2 Materials and methods

5.2.1 Study Design

123 (M=49 & F=74) newly diagnosed T2DM patients were recruited from Department of Endocrinology & Metabolism of Institute of Postgraduate Medical Education and Research (IPGME&R), Kolkata. 74 healthy controls (M=39 & F=35) healthy controls were taken from the community cohort mentioned in the previous chapter. Sampling was done from these two populations in a cross-sectional manner. Another hospital based cohort was developed from Department of Surgery, ILS Hospitals, Saltlake where 63 obese subjects (T2DM=27; non-T2DM=36) were followed up in a prospective manner over 6 weeks. This was a prospective study.

5.2.2 Patient Recruitment

For all the individuals, the criterion for T2DM was set as per the American Diabetes Association (ADA) [6]. Subjects were subdivided into obese and non-obese groups based on Body Mass Index (BMI) < 25 [196]. The cross-sectional study was approved by human ethics committee of IPGME&R hospital and all subjects gave written informed consent. Subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) were collected from 43 (T2DM=23; non-T2DM=20) patients during sleeve gastrectomy, mini-gastric bypass, herniectomy or cholecystectomy surgeries. For the prospective study, 20 subjects (T2DM=4; non-T2DM=16) undergoing Mini Gastric Bypass (MGB) bariatric surgery were followed up over 4-6 weeks in whom blood samples and anthropometric measurements were done before and after the surgery. This study was approved by human ethics committee of ILS Hospitals, Saltlake and all the subjects gave written informed consent.

5.2.3 Sample collection and Anthropometric measurements

All blood samples were collected in Sodium Fluoride/Na_2 EDTA vials (BD Vacutainer, NJ, USA). Plasma was separated and stored at -80ºC for long term storage. Anthropometric measurements were taken during sample collection. For SAT and VAT collection, the tissue was collected during the surgery and immediately suspended in RNA later solution so as to fully immerse the tissue. It was then collected to the laboratory for storage at -80ºC.

5.2.4 Oral Glucose Tolerance Test

39 T2DM patients underwent Oral Glucose Tolerance Test (OGTT) after an overnight fasting for 8-10 hours. 75 grams of anhydrous glucose mixed in 200 ml of water was fed to them and blood samples (2 ml) were collected every 15 mins at 8 time-points over the next 2 hours. Samples were immediately mixed with Protease Inhibitor and DPP4 Inhibitor (within 15-20 seconds) and centrifuged at 4ºC separating the plasma for GLP-1 measurement.

5.2.5 Biochemical Analysis

Plasma Glucose was measured by Glucose Oxidase method, Total Cholesterol by Cholesterol Oxidase method, Total Triglycerides by Lipase/GPO-PAP method. Glycated hemoglobin (HbA1c) was measured by HPLC (D10 Hemoglobin analyzer, Bio-Rad, Hercules, CA, USA). Plasma Leptin (RayBiotech, Norcross, GA, USA), Insulin (Merck Millipore, MA, USA), DPP4 (R&D Systems, MN, USA) and GLP-1(Active and Total) (Merck Millipore, MA, USA) levels were measured by ELISA. Homeostatic model assessment (HOMA2) designed by Diabetes Trials Unit, The Oxford Centre for Diabetes, Endocrinology and Metabolism was used to estimate insulin resistance (HOMA2 IR) from all fasting venous samples.

5.2.6 Gene expression analysis

Total cellular RNA was isolated from 50-100 mg homogenized adipose tissue samples using TRIzol reagent (Invitrogen). cDNA was synthesized from 1000 ng of total RNA using cDNA synthesis kit

(Roche). *DPP4* gene expression was analyzed by quantitative PCR (LightCycler 96 real time PCR, Roche) using SYBR Green master mix (FastStart Universal SYBR Green Master, Roche) with following primers-forward 5'AAGTGGCGTGTTCAAGTGTG3' and reverse 5'GGCTTTGGAGATCTGAGCTG3'. Relative gene expression was analyzed by ΔΔCt method and normalized by 18S RNA.

5.2.7 Immunoblotting

VAT and SAT samples were homogenized in cell lysis buffer (50 mM TrisHCl pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA) containing 1% Triton X100 and protease inhibitor cocktail (Roche). Equal amount of total tissue lysates were separated by SDS-PAGE, transferred into Immobilon-P PVDF membrane (Millipore, Bangalore, India), and were probed with primary antibodies against CD26 (DPP4) (Abcam, Cambridge, UK) and β-actin (Sigma) followed by HRP tagged secondary antibody (Genei, Bangalore, India). DPP4 expression was visualized by enhanced chemiluminescence using LuminataClassico Western HRP substrate (Millipore, St Charles, MO USA) and band intensity was normalized with β-actin using NIH Image J software.

5.2.8 DPP4 enzyme assay

DPP4 activity in plasma and in tissue lysates was assayed as described earlier [3]. Briefly, DPP4 activity was determined as the rate of 7-amino-4-methylcoumarin (AMC) cleavage per minute per mL from the synthetic substrate H-glycyl-prolyl-AMC (Sigma Aldrich, St. Louis, MO, USA). AMC fluorescence (excitation/emission - 380/460 nm) was measured in a plate reader (Synergy H1 multi-mode microplate reader; Biotek, Winooski, VT, USA).

5.2.9 Statistical analysis

Statistical analysis was performed in RStudio (Version 1.1.447) and data are represented in GraphPad Prism 5 software (La Jolla, CA, USA). Descriptive summary of the data have been represented by mean and standard deviation. 95% confidence interval has been presented where relevant. Shapiro-Wilk's W test was performed to assess normality. Numerical variable have been

compared between groups by independent-samples t-test or Mann-Whitney U test as appropriate. For paired analysis, paired t-test was used. Pearson's correlation coefficient 'r' has been calculated to explore association between variables. Power was calculated at the significance level of 0.05 for the difference in DPP4 level between obese and non-obese T2DM groups. Sex adjustment was done using *lsmeans* package in R. Correlation coefficients between all the parameters was calculated by *Hmsic* package in R. *p*-value less than 0.05 was considered to be statistically significant.

5.3 Results

5.3.1 Non-obese T2DM patients have reduced Insulin Response during OGTT in comparison to obese T2DM patients

Subjects undergoing OGTT were divided into 2 groups based on BMI- Non-obese and Obese. Plasma glucose and insulin was measured at all the 8 time-points 0, 15, 30, 45, 60, 75, 90 and 120 minutes. Comparison was done at each time point. No difference in glucose levels was observed at all the 8 time-points during OGTT. However there was significant reduction in insulin levels in the non-obese groups compared to the obese at all the 8 time-points (Figure 5.1).

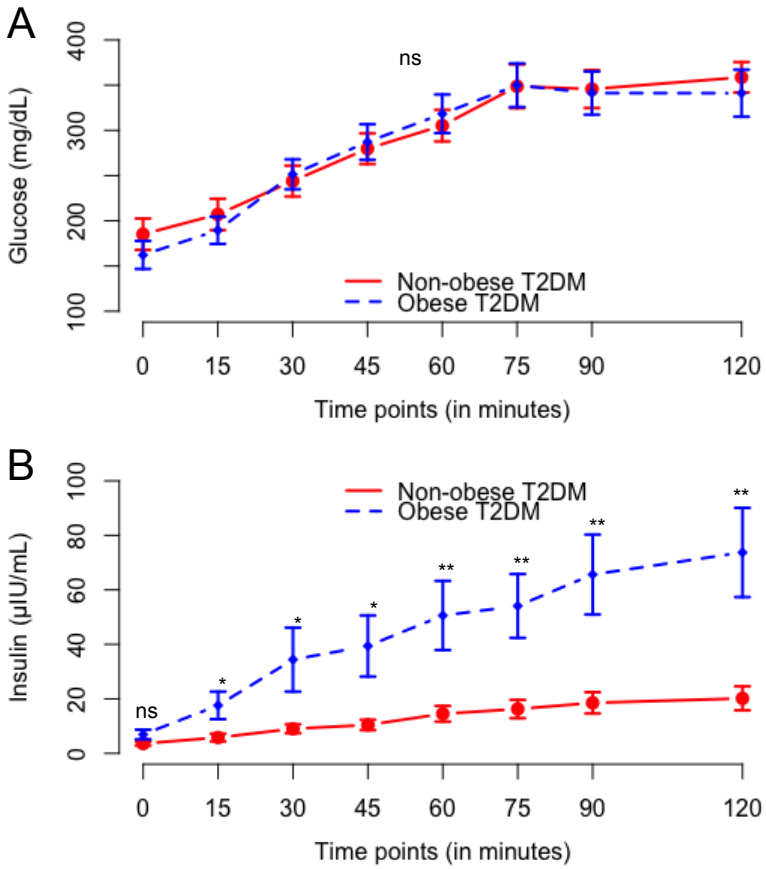


*Figure 5.1: Comparison between Glucose (A) and Insulin (B) levels at all the 8 time-points between non-obese and obese T2DM during OGTT. NS: Non-significant, p < 0.01**, p < 0.001****

5.3.2 Non-obese T2DM patients show similar GLP-1 response during OGTT compared to obese T2DM patients

As GLP-1 potentiates GSIS, the next step was to see whether GLP-1 response in non-obese and obese T2DM patients was different as that could explain the reduced insulin response during OGTT. Both active and total (active and inactive) GLP-1 were measured for that purpose. There was no significant difference between both active and inactive GLP-1 response between non-obese and obese T2DM groups (Figure 5.2).

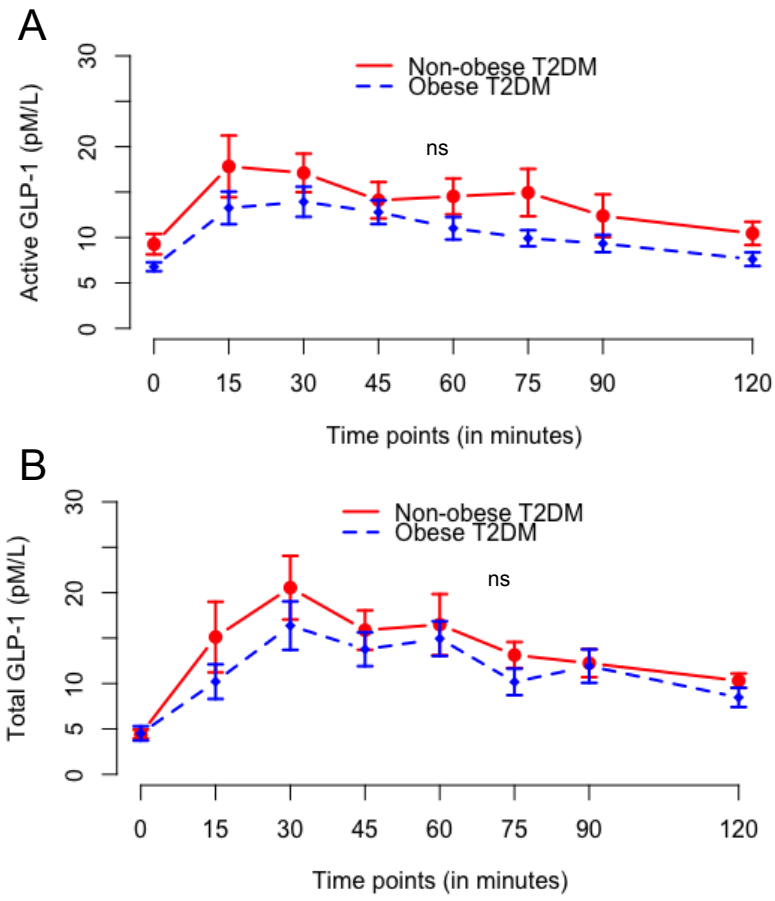


*Figure 5.2: Comparison between Active GLP-1 (A) and Total GLP-1 (B) levels at all the 8 time-points between non-obese and obese T2DM during OGTT. NS: Non-significant, $p < 0.01$**, $p < 0.001$****

5.3.3 Plasma DPP4 activity is similar in non-obese and obese T2DM patients

The mean values of different clinical and biochemical parameters (Fasting plasma DPP4 levels, DPP4 activity, insulin and glucose) in T2DM and non-diabetic subjects are depicted in Table 5.1. Plasma DPP4 concentrations and activity both were increased in T2DM patients compared to control subjects irrespective of BMI distribution (Figure 5.3). Next the T2DM population was divided into obese (n=52) and non-obese (n=71) sub-groups (Table 5.2). Higher plasma DPP4 levels was present in obese T2DM patients (Figure 5.3), but no difference in DPP4 activity was there between these groups (Figure 5.3). DPP4 levels were higher in the obese group than the non-

obese group even after adjusting for sex (p = 0.037). Interestingly, DPP4 activity and DPP4 levels correlated significantly only in the non-obese T2DM group with no such correlation in the obese group (Figure 5.3).

Table 5.1. The subject characteristics of healthy and T2DM patients and biochemical parameters

	Healthy	T2DM	P value
N (Male/Female)	74 (39/35)	123 (49/74)	
Age (years)	39.34±9.92	45.8±8.13	<0.001
BMI (kg/m^2)	27.34±8.82	25.6±6.04	0.62
WC (Waist Circumference) (cms)	97.43±23.84	93.89±15.3	0.746
FBS (Fasting Blood Glucose) (mg/dl)	88.92±10.22	162.58±49	<0.001
HOMA2 IR	1.41±0.91	1.39±1.33	0.998
HOMA2 %B	118±56	47.4±37.4	<0.001
TG (Triglycerides) (mg/dl)	135.49±73.89	156.67±89.91	0.0847
TC (Total Cholesterol) (mg/dl)	173.28±39.89	187.87±48.65	0.032
DPP4 activity (nmol/min/ml)	17.24±12.09	22.34±15.53	0.009
DPP4 concentration (μg/ml)	41.78±30.01	53.38±30.01	0.017

Data represented by means ± SD. Healthy subjects are individuals without T2DM. p-value ≤ 0.05 considered statistically significant.

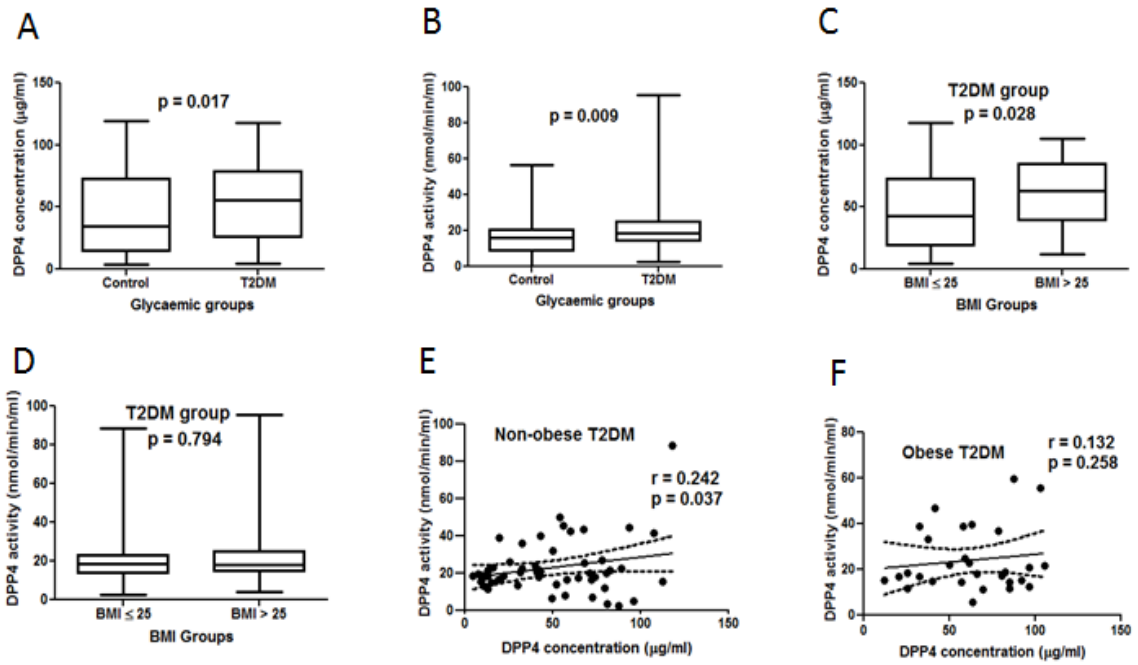


Figure 5.3. Plasma DPP4 concentrations and activity in obese and non-obese T2DM groups. Boxplots showing plasma DPP4 concentrations (A) and DPP4 activity (B) between T2DM and healthy individuals. Plasma DPP4 concentration (C) and DPP4 activity within T2DM group grouped by BMI (D). Correlation between plasma DPP4 concentration and activity in non-obese (BMI ≤ 25) (E) and obese (BMI > 25) (F) T2DM patients. The boxplots represent the values as means ± SEM, pearson's correlation coefficient expressed as 'r'. P < 0.05 was considered statistically significant. p-values calculated by two-tailed test for boxplots (A-D) and by one-tailed test for correlation plots (E-F). 95% confidence interval plotted by dotted lines in correlation plots.

Table 5.2. The subject characteristics non-obese and obese T2DM patients and biochemical parameters

	Non-obese	Obese	P value
N (Male/Female)	71 (32/39)	52 (17/35)	
Age (years)	46.8±8.91	44.42±6.78	0.085
BMI (kg/m^2)	22.05±2.1	30.46±6.3	<0.001
WC (Waist Circumference) (cms)	86.16±7.53	104.99±16.81	<0.001
FBS (Fasting Blood Glucose) (mg/dl)	168.42±45.83	154.6±52.42	0.014
HOMA2 IR	1.18±0.75	1.56±0.96	0.084
HOMA2 %B	36.5±21.4	56.1±43.3	0.022
TG (Triglycerides) (mg/dl)	165.84±103.23	143.5±65.18	0.4
TC (Total Cholesterol) (mg/dl)	181.07±51.56	197.63±42.82	0.067
DPP4 activity (nmol/min/ml)	21.2±13.73	23.81±17.62	0.794
DPP4 concentration (µg/ml)	48.4±30.98	62.49±26.27	0.028

Data represented by means ± SD. p-value ≤ 0.05 considered statistically significant.

Table 5.3. The biochemical parameters of pre- and post MGB surgery patients

	Pre-MGB	Post-MGB	P value
N (Male/Female)	20 (5/15)	20 (5/15)	
Diabetic/Non-diabetic	20 (4/16)	20 (1/19)	
BMI (kg/m^2)	43.33±5.53	39.77±5.13	<0.001
FBS (Fasting Blood Glucose) (mg/dl)	106.04±21.55	93.54±20.84	0.004
HOMA2 IR	2.35±0.67	1.95±0.54	0.019
HOMA2 %B	132.44±47.38	150.16±41.74	0.041
DPP4 activity (nmol/min/ml)	15.66±6.16	17.55±4.51	0.084
DPP4 concentration (µg/ml)	64.99±24.56	74.78±21.17	0.163

Data represented by means ± SD. p-value ≤ 0.05 considered statistically significant. Pre-MGB refers to the status before Mini Gastric Bypass and Post-MGB 4-6 weeks after Mini Gastric Bypass.

5.3.4 Adipose tissue derived DPP4 activity is similar between obese T2DM and obese healthy group

Assessment of DPP4 gene expressions, protein levels and enzymatic activities in the VAT and SAT depots from obese patients was done next. Samples were taken during abdominal surgery from T2DM patients (n=20) or without T2DM (n=23) having comparable BMI (38±11 vs 42±10, p= 0.224) and leptin levels (36147.75±33624.77 pg/ml vs 24707.27±29473.27 pg/ml, p= 0.297). DPP4 protein levels, gene expressions and activity were significantly increased in VAT compared to SAT (Figure 5.4). Same parameters were compared for the fat depot between non-diabetic and T2DM groups. DPP4 gene expression was higher in VAT of T2DM patients (Figure 5.4) but no difference in either VAT or SAT DPP4 enzymatic activity (Figure 5.4) was present between these two groups.

5.3.5 Patients undergoing Mini-Gastric Bypass surgery doesn't show reduction in DPP4 levels or activity in spite of glycaemic control

Finally a follow-up study with 20 obese patients (T2DM=4 and non-T2DM=16) undergoing MGB was done. Fasting plasma samples were collected before and after 4-6 weeks of the surgery and several biochemical assays were performed thereafter and are shown in Table 5.3. There was a significant difference in reduction of BMI ($p < 0.001$), FBS ($p=0.004$) and insulin resistance ($p=0.019$) before and after the surgery (Table 5.3 and Figure 5.5), But no difference in plasma DPP4 activity ($p=0.084$) and levels ($p=0.163$) was present after the surgery (Table 5.3 and Figure 5.5). Thus weight reduction did not accompany any reduction of plasma DPP4 activity.

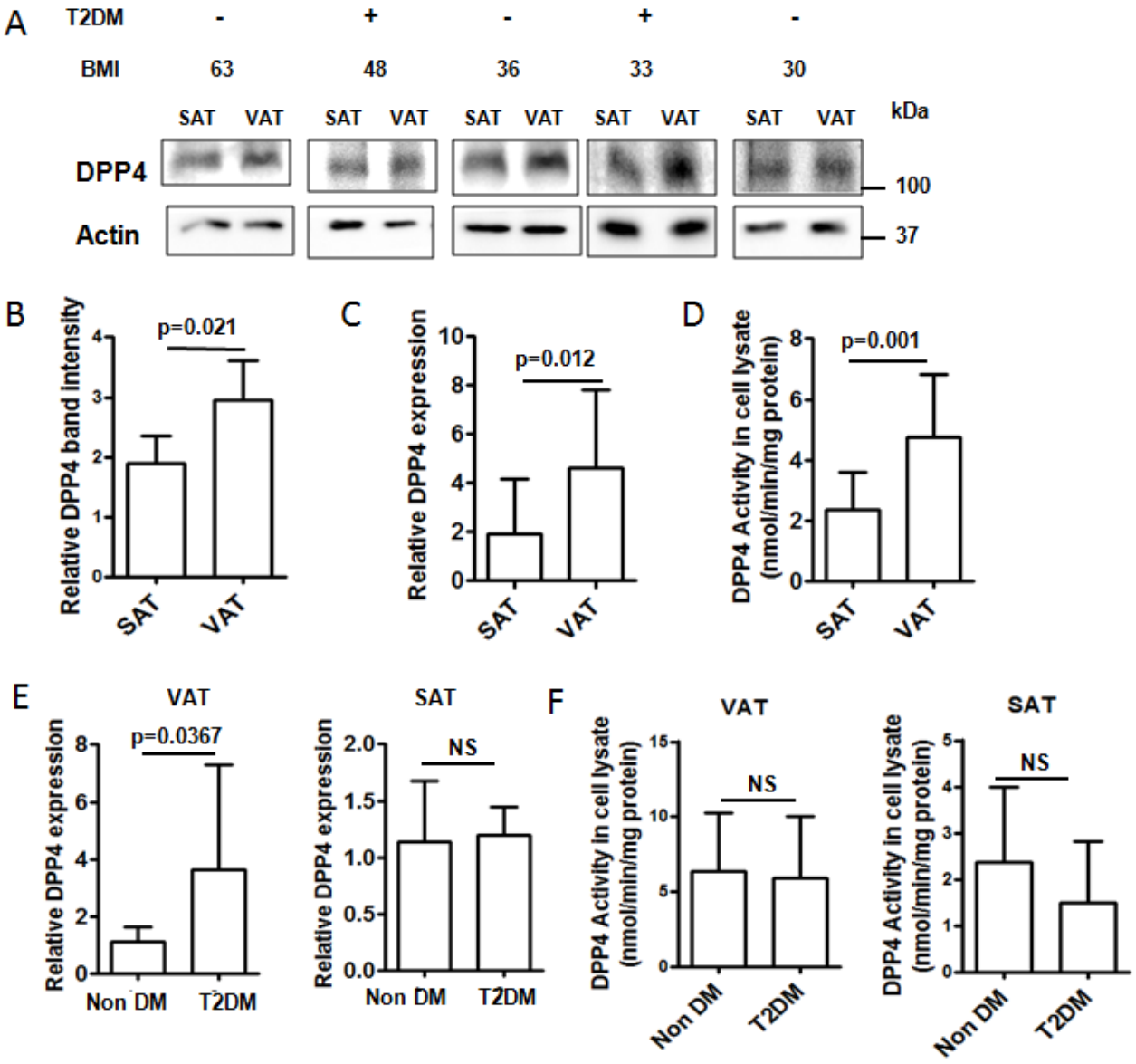


*Figure 5.4. DPP4 protein levels, activity and expression in adipose tissue from obese patients. A. DPP4 protein levels in SAT and VAT analyzed by Western blot (N=13). B. Densitometric analysis of DPP4 protein levels normalized to actin. C. Relative DPP4 gene expressions of SAT and VAT tissue. D. DPP4 activity in SAT and VAT homogenate. E-F. Adipose samples from T2DM (N=20) and non-diabetic (N=23) patients were analyzed for DPP4 activity and relative gene expression. All panels: Data are represented as mean ±SD, *p<0.05.*

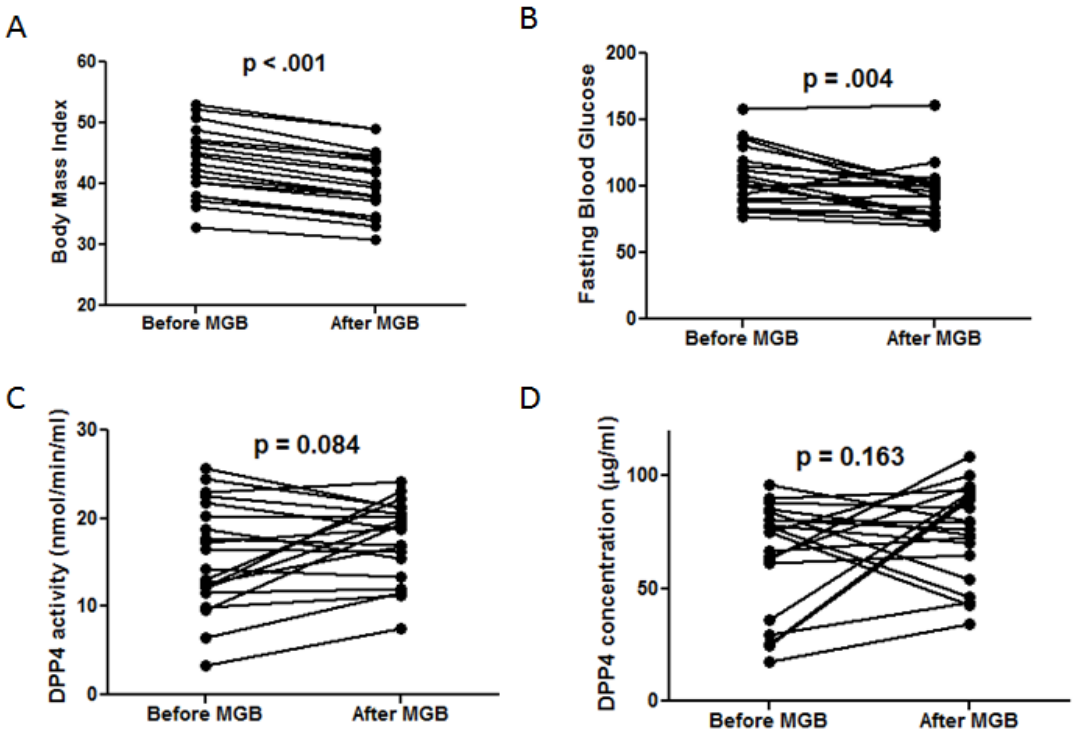


*Figure 5.5. Four to six weeks follow up of plasma DPP4 concentrations and activity in obese patients underwent mini gastric bypass surgery. Lineplots showing change in body mass index (A), fasting blood glucose (B), DPP4 Activity (C) and DPP4 concentration (D) before and after Mini Gastric Bypass bariatric surgery. All panels: two-tailed t-test was performed with *p<0.05.*

5.4 Discussion

Insulin response during OGTT is relatively more impaired among non-obese T2DM compared to the obese T2DM group though glucose levels were comparable between both the groups for all the 8 time-points after glucose feeding. Reduced insulin response among the non-obese T2DM group was however not accompanied with any reduction in GLP-1 levels. This points out that the pronounced impairment of glucose induced insulin secretion among non-obese T2DM isn't a result of reduced GLP-1 response. Similar findings have been reported in another study [207] thereby confirming our result. To next see whether DPP4 activity gets increased thereby rapidly reducing the GLP-1 levels, DPP4 level and activity was measured in non-obese and obese T2DM patients.

Plasma DPP4 level was increased in the obese T2DM group compared to the non-obese T2DM group putatively due to the contribution of adipose tissue in determining the plasma DPP4 level. However this increase in plasma DPP4 level in the obese T2DM group had no effect on the plasma DPP4 activity as plasma DPP4 activity was comparable between obese & non-obese T2DM groups. DPP4 has been suggested to be the connecting link between obesity and T2DM. However as mentioned earlier, DPP4 inhibitors has been reported to cause greater reduction in HbA1c among Asian populations with increased proportion of non-obese T2DM patients. Experiments with SAT and VAT depots collected only from the obese subjects showed adipose tissue derived DPP4 activity to be similar between T2DM and non-T2DM subjects. Finally the follow-up study with patients undergoing MGB surgery pointed out that weight loss (majorly a reduction in adipose tissue mass) does not accompany reduction in plasma DPP4 activity.

Results agree with earlier studies exploring the possible association between plasma DPP4 activity and glycemic control [206]. DPP4 activity is a predictor for the onset of insulin resistance, pre-diabetes, and T2DM independent of BMI [153]. However adipose derived DPP4 doesn't contribute to increased DPP4 activity with obesity. Adipose specific DPP4 knockout have reported the non-essential role of adipose derived DPP4 for circulatory DPP4 activity, GLP-1 levels and glucose

homeostasis, thus corroborating with this results [158]. Structural evidence suggest that catalytically active DPP4 exists as higher order homodimeric or homotetrameric quaternary state [208,209]. Discerning the tissue specific DPP4 production and their structural heterogeneity impacting T2DM needs to be studied in future.

5.5 Summary of the work

Non-obese T2DM patients have relatively severe β-cell secretory defect compared to obese T2DM patients as observed during OGTT. This difference can't be attributed to reduced incretin levels as GLP-1 levels were comparable between both groups during OGTT. On the other hand, though obesity is associated with increased DPP4 protein levels in the plasma, non-obese and obese T2DM patients show comparable DPP4 activity to cleave Active GLP-1. Also reduction in obesity doesn't accompany any reduction in DPP4 activity. In summary, non-obese T2DM is characterized by impaired compensatory hyperinsulinaemia in the basal state and reduced insulin response after feeding in spite of comparable DPP4 activity & GLP-1 levels.

Thus β-cell secretory defect is relatively more in non-obese T2DM. However if there are certain risks factors that modifies the impact of β-cell dysfunction on T2DM needs to be known. This is important for designing specific criteria for T2DM screening for different socio-demographic groups as early identification of β-cell dysfunction can even prevent its progress towards T2DM and preserve its function [210,211] via clinical treatment or through simple measures like calorie restriction. The next chapter reports identification of risk factors using two of the largest epidemiological T2DM datasets of India.

6. Non-obese males at high risk of T2DM with increased impact of β-cell dysfunction

6.1 Introduction

Insulin Resistance (IR) due to obesity is the primary pathology in the natural history of Type 2 Diabetes Mellitus (T2DM) [8,81]. However, hyperglycaemia occurs only when pancreatic β-cell dysfunction occurs in presence of IR [10]. Though obesity is the most important risk factor for T2DM, studies from the low and middle income countries have been reporting a great majority of T2DM patients to be non-obese [163]. Several of those studies have even showed that β-cell dysfunction has an increased impact on T2DM in this non-obese group [13].

However T2DM is also a multifactorial disease with several predictors independently contributing to disease risk [78,190,191,212]. Interactions between the predictors (Age, Sex, Body Mass Index, Hypertension etc.) within themselves make their relationship with the outcome (T2DM) more complex. Considering the possibility of such non-linear relationship between the predictors and the outcome, machine learning approach [213,214] was used on 2 epidemiological datasets of India to identify risk factors for T2DM unique to the non-obese group. Machine learning models are known as black-box models because the internal logic of modeling remains confined within itself. Thus interpretability of machine learning models has remained a challenge for long. As it was needed to study the relationship of predictors with the outcome, partial dependence plots [215,216] were used which have been designed to provide an interpretability to black-box machine learning models. Having discovered the risk factors for T2DM limited to the lower BMI group, investigation was carried out in the community cohort to see whether those risk factors specific to the non-obese group modify the impact of β-cell dysfunction on T2DM.

6.2 Materials and methods

6.2.1 Study Design and Dataset preparation

Machine learning models were first built on the National Family Health Survey-4 (NFHS-4) and Clinical, Anthropometric and Biochemical (CAB) Survey 2014 dataset to identify risk factors. Next the community cohort dataset (discussed in Chapter -3) was taken to see whether the risk factor modifies the impact of β-cell dysfunction on T2DM among non-obese individuals.

National Family Health Survey-4 (NFHS-4) dataset:

The NFHS-4 dataset was downloaded from The Demographic & Health Surveys (DHS) Program website (*https://www.dhsprogram.com/*). The DHS contains all the datasets from a total of 425 surveys conducted in 83 countries starting from the year 1985 to 2019. The major survey types implemented by DHS are Demographic & Health Survey (DHS), AIDS Indicator Survey (AIS), Service Provision Survey (SPA), Malaria Indicator Survey (MIS), Key Indicators Survey (KIS), Other Quantitative Surveys and Qualitative Research Surveys. Demographic and Health Surveys (DHS) are nationally conducted at the household level to collect data on a wide range of indicators for population, health and nutrition. There are 2 types of DHS- Standard DHS and Interim DHS. Standard DHS are conducted every 5 years with large sample sizes whereas Interim DHS are conducted in between 2 consecutive Standard DHS with smaller sample sizes. While the former focuses on a wide range of topics like anemia, family planning, child health, education, nutrition, unmet needs, women empowerment etc, the later is conducted to collect data on key performance health indicators like anthropometry, mortality rates, HIV knowledge etc. The variables in the DHS datasets are uniformly coded to maintain similarity across all the countries. The Standard DHS was first conducted in India in the year 1992-93 and after that India has successfully completed 4

Standard DHS till 2016. The last survey conducted in India in the year 2015-16 belonged to the DHS Phase-VII and is nationally known as the National Family Health Survey-4 (NFHS-4).

NFHS-4 is the fourth version of national health survey conducted under the supervision of Ministry of Health and Family Welfare, Government of India with the International Institute for Population Sciences (IIPS), Mumbai serving as the main nodal agency for all the surveys (ref). The sampling procedure followed in NFHS-4 was of stratified two-stage sampling covering all the 640 districts of India. The survey was successfully conducted with 601,509 households. In those interviewed households 112,122 men and 699,686 women could be successfully interviewed. Four survey questionnaires (Household Questionnaire, Woman's Questionnaire, Man's Questionnaire and Biomarker Questionnaire) were implemented in 17 local languages to collect information on basic demographic information, socio-economic parameters, family planning issues, nutritional status, health indicators, contact with community health workers etc. Uniqueness of the NFHS-4 study was that it collected data on Diabetes status and performed a Random Blood Glucose for individuals (15-54 years) using a finger-stick blood specimen. As a result, the biomarker measurements and tests besides anthropometric measurements like anemia testing, blood pressure measurement, blood glucose testing and HIV testing were included in the survey.

For dataset preparation and cleaning, the 3 questionnaires were merged- Woman's Questionnaire, Man's Questionnaire and Biomarker Questionnaire. The first two contained information about background characteristics (location, age, sex, religion, social group, literacy, wealth status etc), nutritional practices, addictions and co-morbidities while the bio-marker questionnaire contained information on height, weight, blood pressure and random blood glucose. A unique code was generated for all individuals in all the 3 questionnaires by appending the Country code and phase, Cluster number, Household number & Line number. The 3 datasets were joined by the unique code to prepare a single dataset of 810,971 individuals consisting of all men and women between 15-54 years of age. Pregnant women were next excluded to discard the possibility of Gestational Diabetes

Mellitus. Individuals with missing diabetic and blood pressure status were also excluded. BMI, age and haemoglobin level were taken as continuous variables and the rest as categorical variables. Outliers were removed separately for all the 3 continuous variables to obtain the final dataset with 610498 individuals (526678 females and 83820 males).

Clinical, Anthropometric and Biochemical (CAB) Survey 2014 dataset:

The CAB Survey 2014 dataset was downloaded from the Open Government Date (OGD) Platform India website (*https://data.gov.in/*). The Clinical, Anthropometric & Biochemical Survey 2014 was conducted under the supervision of Ministry of Health and Family Welfare (MoHFW), India to supplement the Annual Health Survey (AHS) by collecting data on nutritional status and lifestyle diseases namely diabetes, hypertension & anemia. The survey was conducted on a sub-sample of AHS in all Empowered Action Group (EAG) states namely Bihar, Chhattisgarh, Jharkhand, Madhya Pradesh, Odisha, Rajasthan, Uttarakhand & Uttar Pradesh and in the state of Assam. Information was collected on anthropometric parameters like height and weight for all the adults and children over 1 month age. For adults above 18 years, fasting blood glucose, hemoglobin level, blood pressure and utilization of iodized salt were measured.

The sample design adopted in AHS is a uni-stage stratified simple random sample without replacement except in case of larger villages in rural areas (population over or equal to 2000 as per 2001 census) where a 2-stage stratified sampling was applied. The sampling units are Census Enumeration Blocks (CEBs) in urban locations and villages in rural areas. CAB was conducted on a subsample of 12 sample units per district in all the 284 districts except for 2 districts of Uttarakhand due to administrative reasons. All eligible members of alternate households were surveyed to cover a total population of 1650000.

The fasting blood glucose prevalence was taken to decide the sample size at the district level in the CAB survey. Taking the prevalence of DM as 4% across districts and permissible level of error to

be 10 percentage relative standard error, the sample size was calculated. Only adults above 18 years were selected in our analysis. For our analysis, the final dataset obtained had 616681 individuals (333647 females and 283034 males). Variables common to both survey datasets (namely BMI, Sex, Age, hemoglobin level, Place of residence and Hypertensive status) were selected for analysis in this present study. BMI, age and hemoglobin level were taken as continuous variables and the rest as categorical variables.

Study definitions:

For the NFHS-4 dataset, individuals were categorized as having diabetes (T2DM) if they fulfilled any of the following criteria-

- Self-reported to have diabetes
- Random blood glucose ≥ 126 mg/dl with last food and last drink other than water both taken more than 10 hours earlier
- Random blood glucose ≥ 200 mg/dl with last food and last drink other than water both taken more than 2 hours earlier.

For the CAB survey dataset and in the community health cohort, individuals were categorized as T2DM if they had fasting blood glucose levels above or equal to 126 mg/dl.

Individuals in the NFHS-4 and CAB Survey dataset were considered to be Hypertensive if they had a systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg or if they gave a history of taking anti-hypertensive medications.

Sample collection

All blood samples in the Community Cohort were collected in Sodium Fluoride/Na_2 EDTA vials (BD Vacutainer, NJ, USA). Samples were centrifuged immediately after collection, transported to the laboratory and stored at -80° C for long-term storage.

Biochemical measurements

Plasma was used for biochemical measurements with reagents from Randox Laboratories Ltd. (County Antrim, UK). Plasma Glucose was measured by Glucose Oxidase method. Plasma Insulin (Merck Millipore, MA, USA) was measured by ELISA. HOMA modelling was done as follows to calculate the insulin sensitivity and insulin secretion indices from all fasting venous samples-

- HOMA-IR (Insulin Resistance)= [Fasting insulin (μIU/mL) × Fasting glucose (mM)] / 22.5
- HOMA-B (Insulin Secretion)= [Fasting insulin (μIU/mL) × 20] / [Fasting glucose (mM) – 3.5]

6.2.2 Machine Learning analysis

Random Forest classification algorithm [217] was implemented in the NFHS-4 and CAB Survey datasets independently using the *randomForest* package in R keeping T2DM as the outcome. Datasets were randomly split into training and test dataset containing 80% and 20% of the total samples respectively. The random forest model was tuned by increasing the number of trees to 2000. For evaluating the model performance, Area under the ROC curve was calculated on the test datasets. The number of trees were limited to 2000 as beyond that there was no increase in model performance beyond that. As there was class imbalance in the datasets, we used Random Forests where we could pick up equal number of positive and negative classes for each tree. So for each tree we picked up the non-DM cases and equal number of DM cases randomly without replacement. After the model was generated, we investigated the relationship of the predictors with the outcome via partial dependency plots. Partial dependency plots were used to determine the marginal effect of all the predictors on T2DM classification. Summary statistics of the variables for NFHS-4 and CAB Survey is given in Table 6.2 and Table 6.3 as mean and standard error of the mean.

Foe the Community Cohort, we investigated Age, Sex, BMI, HOMA-IR and HOMA-B and their relationship to T2DM risk. The non-obese and obese group was analysed separately. Descriptive summary of the data have been represented by mean and standard error of the mean (SE) in Table-

6.1. Shapiro-Wilk's W test was performed to assess normality of the variables. Numerical variables have been compared between groups by independent-samples two-sided Student's t-test or Man-Whitney U test as appropriate. Categorical variables have been tested using Chi-square test. Generalized Additive Modelling (GAM) [218] using the *mgcv* pacakge was implemented to capture the non-linear relationship of T2DM risk with rest of the predictors. The model can be expressed as follows: $h(\mu) = s_1(x_1) + s_2(x_2) + ... + s_p(x_p)$, where μ is the distributed mean of the outcome, h a link function, x_i the predictors and $s_i(.)$ a smooth function estimated from the data. Finally the dataset was divided into non-obese and obese group based on the BMI cut-off of 25 and logistic regression models were built with T2DM as the outcome. Study design and the methodology are shown in Figure 6.1. All statistical analyses were performed in RStudio (Version 1.2.1335).

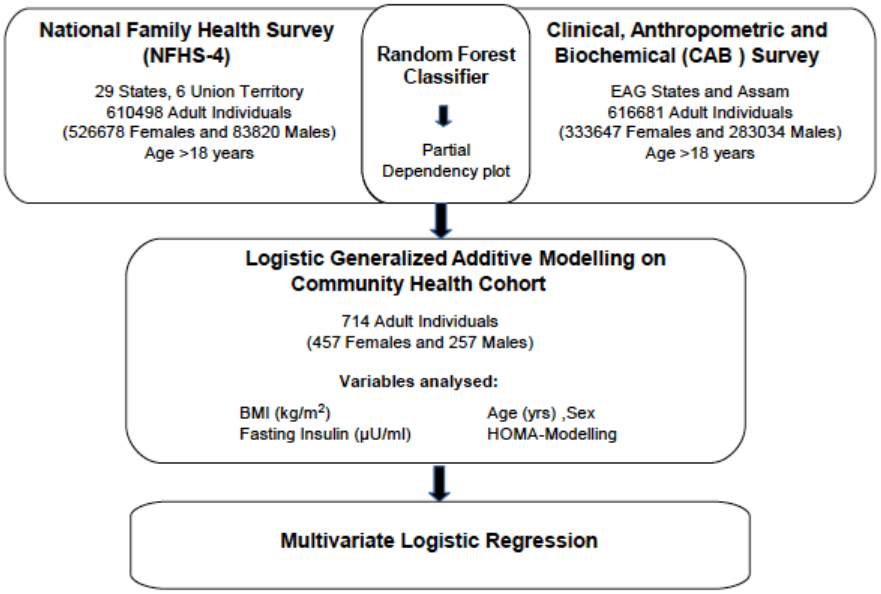


Figure 6.1. Methodological workflow of NFHS-4 and CAB Survey 2014 dataset followed by analysis on the community cohort

6.3 Results

6.3.1 Males are at higher T2DM risk towards the lower BMI range

Random forest classification models were built independently for the NFHS-4 and CAB Survey training datasets (containing 80% of the data) and the models validated over the test datasets (containing 20% of the data). Being an imbalanced dataset [219] with low case (T2DM) frequency (1.76 % in NFHS-4 were T2DM and 3.59 % in CAB Survey were T2DM), we used the Area Under the ROC Curve (AUROC) value as the model performance metric. The AUROC value for NFHS-4 and CAB Survey test datasets were found to be 0.77 and 0.72 respectively. For NFHS-4, the specificity and sensitivity at the best threshold value in the test dataset was 74% and 69% respectively. For CAB Survey, the specificity and sensitivity at the best threshold value in the test dataset was 64% and 69%. After fitting the models, we calculated the partial dependency of T2DM on BMI and each variable separately (Age, Sex, Hemoglobin level, Place of residence and Hypertensive status). Bi-variate plots were generated for this purpose to determine the marginal effect of BMI and the other predictors on T2DM classification (Figure 6.2, 6.3, 6.4). Interestingly, after examining all the bivariate partial dependency plots, we found age and sex to have a differential effect on T2DM across the BMI range. While the impact of age on T2DM was more in the higher BMI range (Figure 6.2), sex was found to be the only risk factor limited to the lower BMI group (Figure 6.4). Here, males had higher T2DM risk than females in the lower BMI range with no difference observed in the higher range (Figure 6.4). This particular trend was similar in both the NFHS-4 and CAB Survey datasets, thereby confirming the validity of this finding.

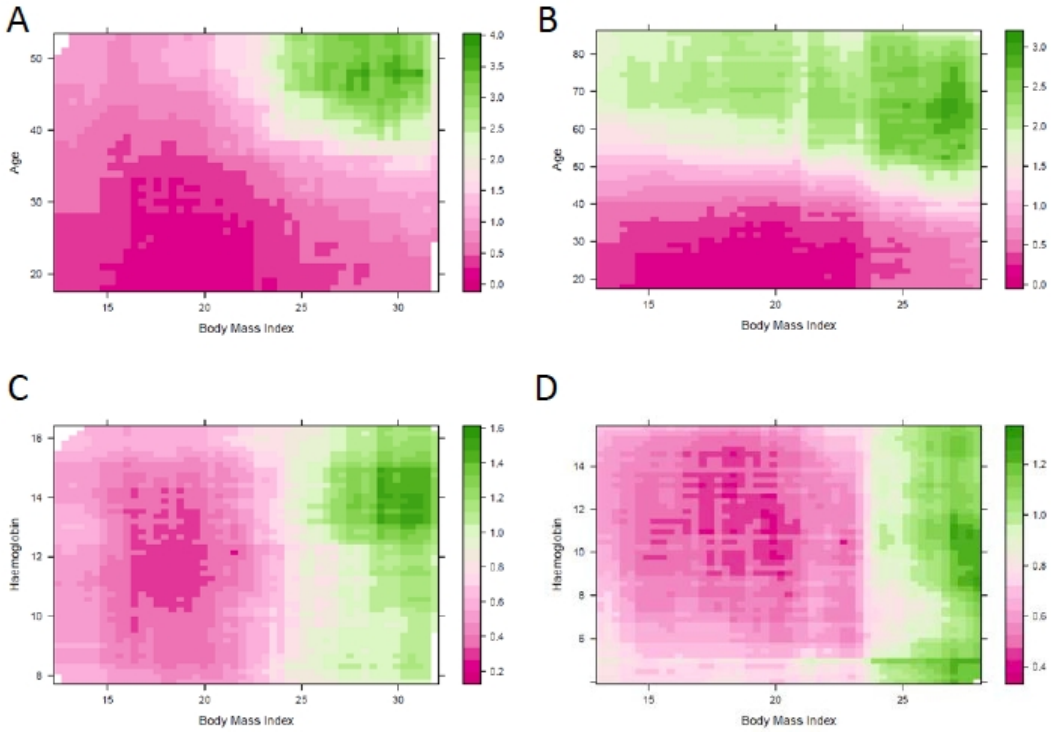


Figure-6.2. Bivariate Partial Dependency Plots of T2DM risk for BMI and continuous variables. (A-D) Partial Dependence Plot based on Random Forest classifier showing Type 2 Diabetes Mellitus (T2DM) dependence on Body Mass Index (BMI) and Age in National Family Health Survey-4 (NFHS-4) (A) and Clinical, Anthropometric and Biochemical Survey (CAB Survey) dataset (B), Partial Dependence Plot based on Random Forest classifier showing Type 2 Diabetes Mellitus (T2DM) dependence on Body Mass Index (BMI) and Haemoglobin in National Family Health Survey-4 (NFHS-4) (C) and Clinical, Anthropometric and Biochemical Survey (CAB Survey) dataset (D).

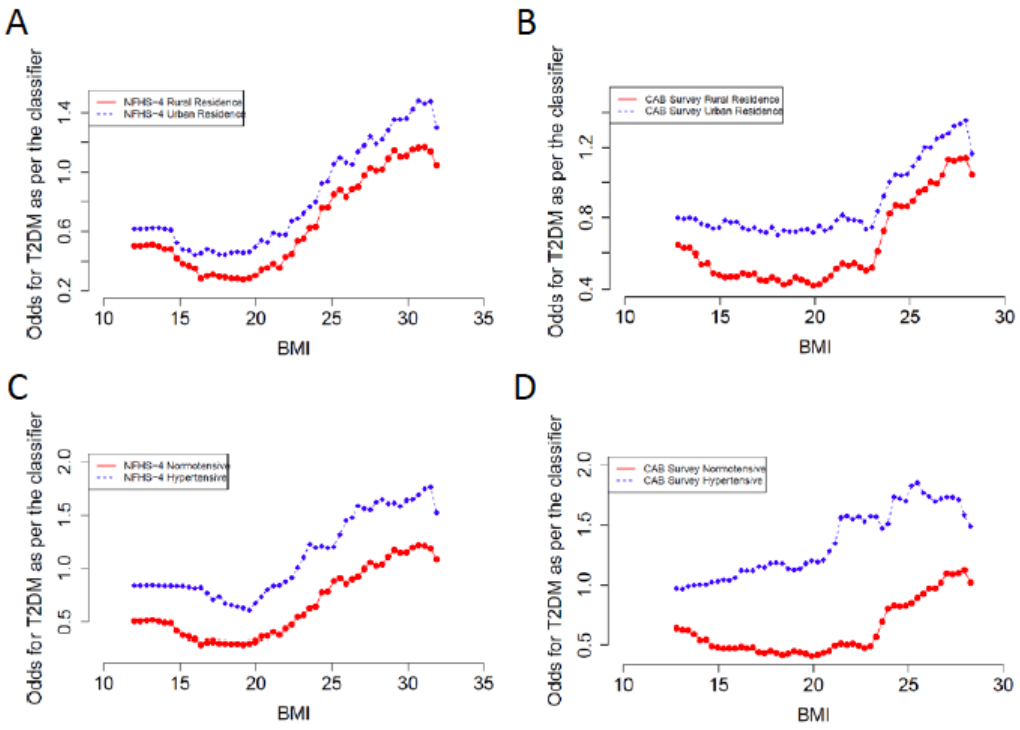


Figure-6.3. Bivariate Partial Dependency Plots of T2DM risk for BMI and other categorical variables. (A-D) Partial Dependence Plot based on Random Forest classifier showing Type 2 Diabetes Mellitus (T2DM) dependence on Body Mass Index (BMI) for rural and urban residence in National Family Health Survey-4 (NFHS-4) (A) and Clinical, Anthropometric and Biochemical Survey (CAB Survey) dataset (B), Partial Dependence Plot based on Random Forest classifier showing Type 2 Diabetes Mellitus (T2DM) dependence on Body Mass Index (BMI) for normotensive and hypertensives in National Family Health Survey-4 (NFHS-4) (C) and Clinical, Anthropometric and Biochemical Survey (CAB Survey) dataset (D).

Table 6.1. The subject characteristics of Non-T2DM and T2DM individuals in the community cohort dataset

	Non-obese (BMI < 25)			Obese (BMI ≥ 25)		
VARIABLE	Non-T2DM	T2DM	P value	Non-T2DM	T2DM	P value
N (Male/Female)	115/234	67/63	< 0.001	45/99	30/61	0.895
Age (yrs)	44.59 ± 0.8	49.7 ± 0.98	< 0.001	43.24 ± 0.94	47.57 ± 0.93	0.003
BMI (kg/m^2)	20.96 ± 0.14	21.76 ± 0.2	0.004	27.75 ± 0.21	28.37 ± 0.29	0.038
HOMA-IR	0.7 ± 0.03	1.71 ± 0.18	< 0.001	1.03 ± 0.06	2.8 ± 0.23	< 0.001
HOMA-B	54.76 ± 2.07	13.93 ± 1.7	< 0.001	55.84 ± 2.73	29.25 ± 2.58	< 0.001

Data represented by means ± SE. p-value < 0.05 considered statistically significant. BMI- Body Mass Index, HOMA-IR: Insulin Resistance marker, HOMA-B: Insulin Secretion marker

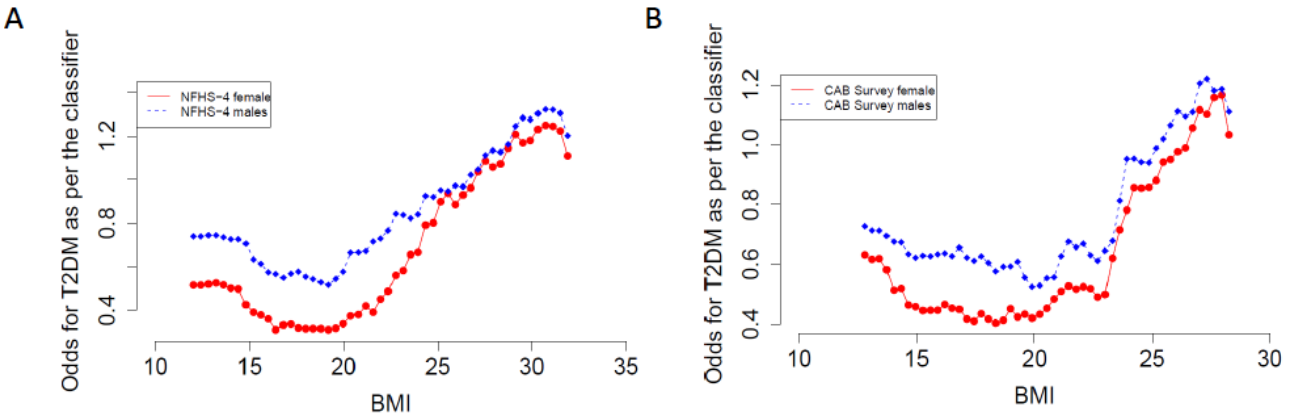


Figure 6.4. Sex differences in T2DM risk across BMI range. (A, B) Partial Dependence Plot based on Random Forest classifier showing Type 2 Diabetes Mellitus (T2DM) dependence on Body Mass Index (BMI) for both sexes in National Family Health Survey-4 (NFHS-4) (A) and Clinical, Anthropometric and Biochemical Survey (CAB Survey) dataset (B).

Table 6.2. The subject characteristics of Non-T2DM and T2DM individuals in NFHS-4 dataset

VARIABLE	Non-T2DM	T2DM	P value
N (Total)	599723	10775	
Sex (Female/Male)	517946/8732	8732/2043	< 2.2e-16
Age (years)	31.8 ± 9.18	39.6 ± 8.32	< 2.2e-16
BMI (kg/m^2)	21.6 ± 3.56	24.2 ± 3.91	< 2.2e-16
Haemoglobin (gm/dl)	12.2 ± 1.65	12.4 ± 1.7	< 2.2e-16
Residence (Rural/Urban)	426198/173525	6193/4582	< 2.2e-16
Hypertension (Normal/Hypertensive)	543491/56232	7909/2866	< 2.2e-16

Data represented by means ± standard deviation for continuous variables and by number of individuals for categorical variables. p-values < 0.05 considered statistically significant.

Table 6.3. The subject characteristics of Non-T2DM and T2DM individuals in CAB Survey dataset

VARIABLE	Non-T2DM	T2DM	P value
N (Total)	594553	22128	
Sex (Female/Male)	322782/271771	10865/11263	< 2.2e-16
Age (years)	39.8 ± 15.8	51 ± 15.6	< 2.2e-16
BMI (kg/m^2)	20.5 ± 2.77	21.1 ± 3.09	< 2.2e-16
Haemoglobin (gm/dl)	9.89 ± 2.21	9.83 ± 2.24	< 0.0001
Residence (Rural/Urban)	488740/105813	16656/5472	< 2.2e-16
Hypertension (Normal/Hypertensive)	511226/83327	14849/7279	< 2.2e-16

Data represented by means ± standard deviation for continuous variables and by number of individuals for categorical variables. p-values < 0.05 considered statistically significant.

6.3.2 Increase in T2DM risk with reduction in β-cell function is more among males in the lower BMI range

β-cell dysfunction in the background of IR causes T2DM. Having observed a sex-dimorphic pattern in T2DM risk with BMI in the lower BMI range, it was studied whether the relationship of T2DM risk with insulin secretory dysfunction (measured by HOMA-B) is different across the BMI range between both the sexes. To do this a Generalized Additive Model (GAM) was employed with T2DM as the outcome. GAMs are non-linear models which consider the outcome as a combination of smooth functions of predictors. As sex was the only risk factor for T2DM unique to the lower BMI group, its was seen whether sex modifies the impact of β-cell dysfunction on T2DM in the lower BMI range only. Hence the following logistic GAM was used across the entire BMI range to model T2DM risk as a function of HOMA-B, Age and Sex after adjusting for Age and HOMA-IR with an interaction introduced among BMI, HOMA-B and Sex:

log [μ/(1- μ)] = s_1 (BMI, HOMA-B, by = Sex) + s_2 (HOMA-IR) + s_3 (Age), where μ is the probability of being T2DM.

The model could explain 83.6% of the variance and had an adjusted R-squared value of 0.89. Age and IR adjusted regression revealed differential trends for both sexes. For females, a reduction in HOMA-B value led to a similar increase on T2DM risk throughout the entire BMI range (denoted by blue lines in Figure 6.5). On the contrary, T2DM risk showed a discrepancy for males with a steep increase in T2DM risk with reduction in HOMA-B only in the lower BMI range (denoted by blue lines in Figure 6.5).

6.3.3 Males have a greater impact of β-cell dysfunction on T2DM only in the non-obese group

Finally, to quantify the sex-dimorphic pattern of insulin secretory defect in predicting T2DM, the dataset was split into non-obese and obese groups and logistic regression models were employed separately. The following model was built including an interaction between Sex and HOMA-B after adjusting for Age and HOMA-IR:

log [μ/(1- μ)] = Age + HOMA-IR + BMI + (HOMA-B * Sex), where μ is the probability of being T2DM.

There was a significant interaction between Sex and HOMA-B ($P_{interaction}$= 0.049) in the model run in the non-obese group. The impact of HOMA-B on T2DM was higher in males (OR for males = 0.54, OR for females = 0.62). However, there was no significant interaction between Sex and HOMA-B in the obese group.

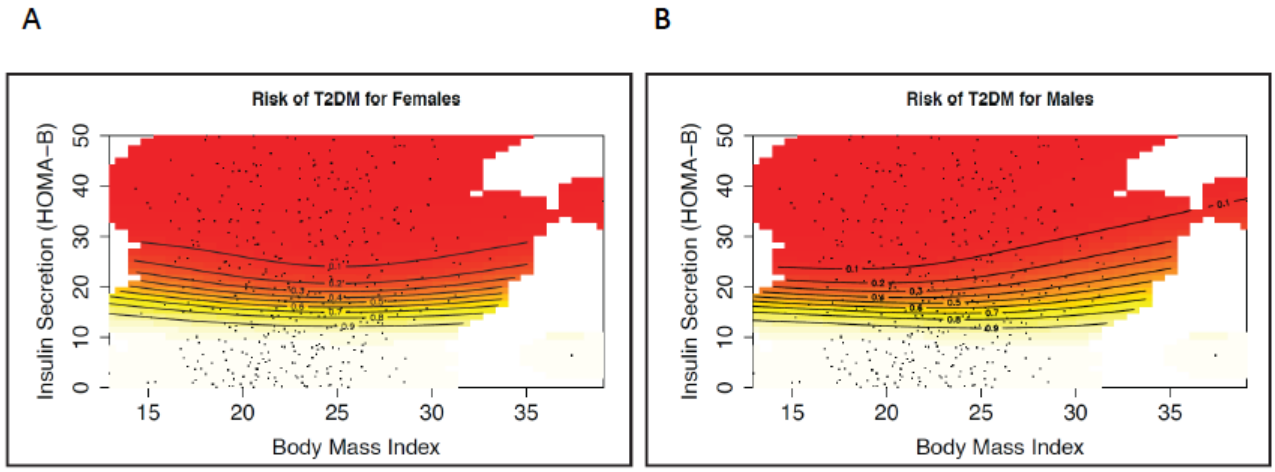


Figure 6.5. Sex differences in the impact of HOMA-B over T2DM across BMI range.

(A, B) Heatmap showing odds of T2DM with shift from red to yellow colour with increasing odds of T2DM for females (A) and males (B) in the Community Health Cohort for change in BMI and insulin secretion (HOMA-B) as per the Logistic Generalized Additive Model (GAM).

6.4 Discussion

Independent analysis on both datasets revealed sex to be the only risk factor for T2DM unique to lower BMI group. T2DM dependence on BMI was higher among males in the lower BMI range with no difference in risk between males and females in the higher range. Further investigation in the community health cohort reported a sex-dimorphic pattern in non-obese T2DM with males showing an increased odds for T2DM for similar β-cell dysfunction. Significant interaction was found between sex and HOMA-B in the non-obese group during logistic regression with an increased impact of HOMA-B on T2DM risk among the males. Thus the impact of β-cell dysfunction on T2DM in higher among the non-obese males. Greater impact of β-cell dysfunction on T2DM among males in the non-obese group justifies our initial finding of sex being a risk factor unique to the non-obese group. Gradual reduction of β-cell function in the background of Insulin Resistance causes an individual to progress from Normal Glucose Tolerance through Pre-diabetes to T2DM [15]. As reduction in HOMA-B has greater impact in causing T2DM among the males from the non-obese group, males showed an increased risk of being T2DM in the lower BMI range in both the epidemiological studies.

β-cell dysfunction has already been reported to have more impact than IR on T2DM among Asian Indians [12,13] where the T2DM population is majorly non-obese [163]. Our finding reporting the impact of β-cell dysfunction to be even greater among non-obese males thereby identify them as a major high-risk group within the non-obese T2DM phenotype. β-cell dysfunction occurs at an earlier stage in the natural history of T2DM even before T2DM sets in [14]. Prospective studies have confirmed insulin secretion defect to be present in the pre-diabetic or even in the normoglycemic state; 3-6 years before T2DM is diagnosed [15,22,23]. As impaired β-cell function can be reversed if identified early in the course of the disease [123,211], this calls for the necessity of a more strict screening criteria for T2DM in non-obese males.

These results also generate the need to understand the sex-dimorphic impact of β-cell dysfunction in the non-obese group. Whole body adipose tissue is distributed in 2 compartments- Subcutaneous adipose tissue (SAT) and Visceral Adipose Tissue (VAT) [90,92,97]. Increase in adiposity accompanies an increase in both visceral and subcutaneous adipose tissue. While VAT is more insulin resistant and less efficient in storing excess nutrients in the form of triglycerides thereby causing increased Free Fatty Acid (FFA) spillover in the bloodstream, SAT is insulin sensitive and effectively stores excess nutrients in the form of triglycerides [92,97]. Imaging studies have shown females to have more SAT than males for comparable BMI [36], thereby making the former more insulin sensitive. Perhaps, the disparity in adipose tissue distribution and insulin sensitivity between males and females is wider in the non-obese group putting non-obese males more at T2DM risk with similar degree of β-cell dysfunction.

Another possibility is the sex difference in adipose tissue function [220]. Inability to expand the adipose tissue depot has been seen to correlate with metabolic dysfunction [169]. Also sex differences in adipose tissue expansion and function has suggested the role of adipose tissue to be different in males and females [166,221]. Inability to expand the adipose tissue depot has been reported to give rise to a dysfunctional adipose tissue among males which may be pronounced in the non-obese group. Dysfunctional adipose tissue in turn may impair the endocrine function of the adipose tissue [99] thereby reducing the production of metabolically protective adipokines like Adipsin [108,109,222] and Adiponectin [105–107] more in the non-obese males putting them at increased T2DM risk.

Obesity has several pathological consequences [90]. But recent epidemiological studies have been reporting lower BMI to have an increased association with mortality events, a phenomenon termed as the “Obesity Paradox” [95,200,201]. As suggested in those studies there may be some correlates within the non-obese population contributing to this paradoxical outcome. Thus, whether the impact

of those correlates is greater among non-obese males is vital from the perspective of gender based prevention as well as therapy.

Though this study was conducted independently in two epidemiological datasets and further investigated in another community health cohort, it has several limitations. One important limitation for this study is to calculate Insulin Resistance and β-cell function from HOMA-modelling in place of the gold standard hyperinsulinemic-euglycemic clamp procedure. The BMI cut-off for non-obese group was considered to below 25 which has been chosen for the sake of quantification in the logistic regression model.

6.5 Summary of the work

To conclude, the findings in identifying non-obese males as a risk group will have implications at the public health and policy level for setting a strict screening criteria in this population in the context of β-cell function. Studies are required to further investigate the sex-dimorphic impact of β-cell dysfunction on T2DM among the non-obese population. This is of great clinical relevance to those countries where majority of the T2DM population belong to the non-obese BMI category and is characterized by the "Obesity Paradox".

As already mentioned, T2DM once considered to be a homogenous diseases entity, is now being considered as a heterogeneous disease with the underlying pathology varying to different extents in the T2DM sub-groups [193,194,223]. However similar analyses hasn't been performed from the epidemiological point of view. To explore if any specific T2DM cluster characterized by normal or underweight BMI exists, a novel unsupervised clustering workflow was applied on an entire T2DM population with a great number of features. The next chapter discuses the clustering and the results with their significance in T2DM management.

7. Non-obese clusters in T2DM population with lower age and belonging to rural residence

7.1 Introduction

T2DM is a multifactorial disease estimated to rise to 629 million cases by 2045 [224,225]. Conceived as a homogeneous entity for long, recent studies report T2DM to be a mix of heterogenous subtypes [193,223]. These studies have reported the underlying pathophysiology of T2DM to vary thereby suggesting the need of a personalized treatment for T2DM. Besides obesity, other factors like age, sex, socio-economic status, place of residence (rural/urban), smoking habit, alcohol intake, food frequency etc. significantly associate with T2DM [226–233], several of which are modifiable in nature and hence are important in T2DM management. However, modification of lifestyle-related factors vary thereby leading to a differential degree of glycemic control in T2DM patients [182]. Glycaemic control and response to glucose-lowering drugs has also been shown to be different among T2DM sub-groups [194]. With the objective of exploring patient sub-populations in the T2DM population based on socio-demographic and lifestyle factors, an unsupervised clustering approach was done on the largest and most comprehensive epidemiological dataset in India, the National Family Health Survey-4 dataset. Clusters were then characterized to identify unique socio-demographic and lifestyle patterns associated with them.

Epidemiological datasets contain a comprehensive set of information ranging from socio-demography, lifestyle, addiction to co-morbid diseases. Variables containing such information are known as *features* in the Machine Learning. 36 such features were used in this study which contained information for each diabetes patient. For the sake of understanding, the features are categorized into three types:

Continuous features: These are features which assume any numeric value from a continuous range. For example, BMI is a continuous feature.

Ordinal features: These are features which assume values from a discrete range and has a sense of order in the values assumed by the feature. For example, let us assume a feature ‘meat consumption by a patient’, assumes values ‘daily’, ‘weekly’ or ‘monthly’. Clearly the range of the feature ‘meat

consumption by a patient' is discrete, since it can assume any one of the three values. There is a sense of order in the values, indicating that daily meat consumption is the highest and daily meat consumption is the lowest, if we want to quantify meat consumption.

Nominal features: These are the features which assume values from a discrete range but lacks any sense of order in the values assumed by the feature. For example, let us assume a feature 'Religion of a patient', assumes values 'Hindus', 'Muslims' or 'Christians'. Clearly the range of the feature 'meat consumption by a patient' is discrete, since it can assume any one of the three values. But there is no sense of order in the possible values assumed by the features. Yet, this feature draws its importance from the fact that lifestyle patterns or diets are largely variable among religious groups.

Such diverse types of features in epidemiological data create multiple challenges for clustering analyses. Conventional application of the state-of-the-art dimension reduction tool Uniform Manifold Approximation (UMAP) was found to be ineffective for our dataset. Continuous features, although smaller in number, had an overpowering effect on the distribution of clusters. To address this, a distributed clustering workflow was designed and implemented, combining different similarity measure settings of UMAP, for clustering continuous, ordinal and nominal features separately. Reduced dimensions from each feature-type-distributed clustering were subsequently integrated to obtain an interpretable and unbiased clustering of the data.

The workflow used in the present study (Figure 7.1) involves investigation of underlying sociodemographic patterns within patient sub-populations using unsupervised learning. Dimension reduction approaches are often used to reduce higher dimensional data to lower dimensions such that in the lower dimensional embedding of the data underlying clusters can be visualized, that is not apparent in the higher dimensions [234]. Several such techniques have been developed over the last few decades. Until recently the dimension reduction technique t-Stochastic Neighbourhood Embedding (t-SNE) was a state-of the-art algorithm providing numerous applications in various fields [235–237]. t-SNE projects high dimensional data to a lower dimension while maintaining the

underlying local manifold structure in a sense that, in a lower dimension t-SNE can cluster points, that are close enough in the latent high dimensional manifold.

With a rigorous mathematical foundation, considerably high speed and easy to use using *scikitlearn* API, UMAP has turned out to be one of the most popular choices among the data scientists [238,239]. As opposed to t-SNE, UMAP uses a graph based manifold approximation mechanism which contributes to preservation of the global as well as local properties of the latent data manifold in a lower dimensional representation of the data. Given some low dimensional representation of the data, a similar process can be used to construct an equivalent topological representation. UMAP builds a graph considering customized neighborhoods for every data points. This graph is a representation of the higher dimensional data manifold. The end result is a patchwork of low-dimensional representations of neighborhoods that groups similar data points on a local scale while better preserving long-range topological connections to more distantly related data points [238]. For the ability of UMAP to preserve the long-range topological connections along with the short-range topological connections and because of its high computational efficiency we choose UMAP for our unsupervised clustering approach. Moreover, UMAP allows a user to specify several similarity measures through the tuning of the metric parameter. This has been critical in our workflow, since our data contains continuous and categorical features and choosing suitable similarity measures for continuous and categorical features is crucial for a meaningful and informative clustering [240].

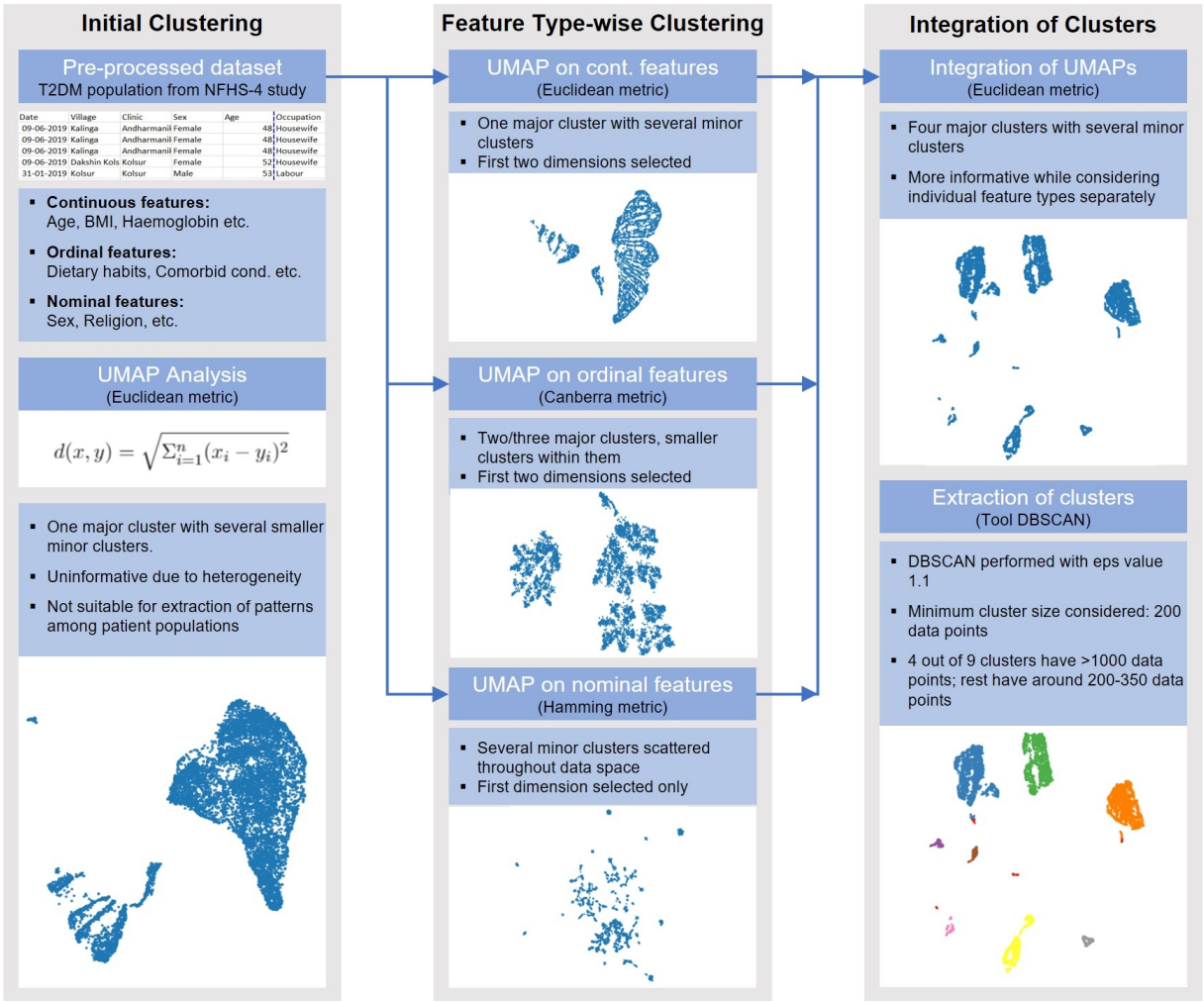


Figure 7.1: Workflow describing the analysis of the T2DM NFHS-4 Dataset

7.2 Materials and methods

7.2.1 Study Design and Dataset preparation

The NFHS-4 dataset was used in the analysis. Description of the dataset has already been given in the previous chapter (*6.2.1 Study Design and Dataset preparation*). As the objective is to identify significant sub-populations in the T2DM population, only T2DM patients were used in analysis the number being 10,125. A total of 36 features were taken in the clustering. Details of the features and their categories are as follows:

1. Co-morbid conditions: This class of features considers the co-morbid diseases among T2DM patients. It is considered whether a T2DM patient had medical conditions such as Asthma, Thyroid disorder, Heart disease, Cancer, Tuberculosis and Hypertension. There are six features in this category. Features are binary in nature denoting whether a T2DM patient suffered from a given comorbidity or not.

2. Food habits: This class of features considers the food habits of T2DM patients. The features considered here are how frequently the patient took the food items: Milk or Curd, Pulses or Beans, Dark leafy vegetables, Fruits, Eggs, Fish, Chicken, Fried food and Aerated drinks. There are nine features in this category. Features are categorical and ordinal in nature having four possible values: 'Daily', 'Occasionally', 'Weekly' and 'Never'.

3. Addiction history: This class of features considers the addiction pattern of T2DM patients. There are two features in this class, both binary in nature encoding whether a patient is a Smoker or whether a patient takes Alcohol.

4. Socio-demographic features: These include features such as Sex, Age, Wealth index, Education level, Religion and Caste along with Body Mass Index (BMI) and Hemoglobin level of the patient. There are eight features in this category.

5. Living conditions: This class of features report the living conditions of the patients. The features in this class consider whether a patient lives in a household possessing refrigerator, bicycle,

motorbike, four wheeler vehicle and livestock. Also there are features denoting type of residence, household structure, frequency of household members smoking inside the house, type of cooking fuel used, source of drinking water and time to reach the nearest drinking water source. Thus, there are eleven features belonging to this category.

In the analysis, 36 features or factors were considered to investigate significant patient populations among diabetes patients. Both continuous and categorical features were present among these thirty six features. Among the categorical features there were both ordinal features and nominal features. As already mentioned, ordinal features have a sense of order among them, such as the features from the 'food habits' category whereas nominal features are categorical features with no sense of order such as sex of a patient. For our dataset the continuous features were: Age, BMI, Haemoglobin level and Time to get to drinking water source; whereas the nominal features were: Sex, Religion, Caste, Household structure, Type of place of residence, Type of cooking fuel and Source of drinking water. The rest of the features were ordinal features.

7.2.2 Machine Learning workflow

The clustering paradigm first applies UMAP on continuous, nominal and ordinal features separately. For each of these feature categories a lower dimensional embedding of the dataset is produced which were finally embedded to extract clusters using the DBSCAN algorithm, a clustering algorithm used for extracting clusters from data based on data density. One advantage of this algorithm is that one does not need to specify the number of clusters from beforehand. DBSCAN considers closely or densely located points, as clusters [240]. For UMAP, same values for the parameters nneighbours= 30 and mindistance= 0.1 were used for all the feature types. For the *continuous features* the metric measure used was *Euclidean*. For the *nominal features* the metric measure used was *Hamming*. For the *ordinal features* the metric measure used was *Canberra*. It is a weighted version of the Manhattan measure.

For the categorical and ordinal features a two dimensional representation of each data point was produced by taking into consideration the first two UMAP coordinates. For the nominal features a one dimensional representation was produced, since the data points are too scattered in this case as shown in Figure 6.2 and thus can lead to too many clusters. Thus, every data point was represented by a five dimension representation, two for each of the continuous and ordinal features and one for the nominal features. Finally, clusters were designated in the five dimensional representation using DBSCAN (eps= 1, minpoints= 200). After selecting the final clusters, they were characterized by summarizing all the 36 variables separately for each cluster. The continuous variables were summarized as their mean and the standard error of the mean. The categorical variables were summarized as their frequency distribution and the proportion of each value within each cluster.

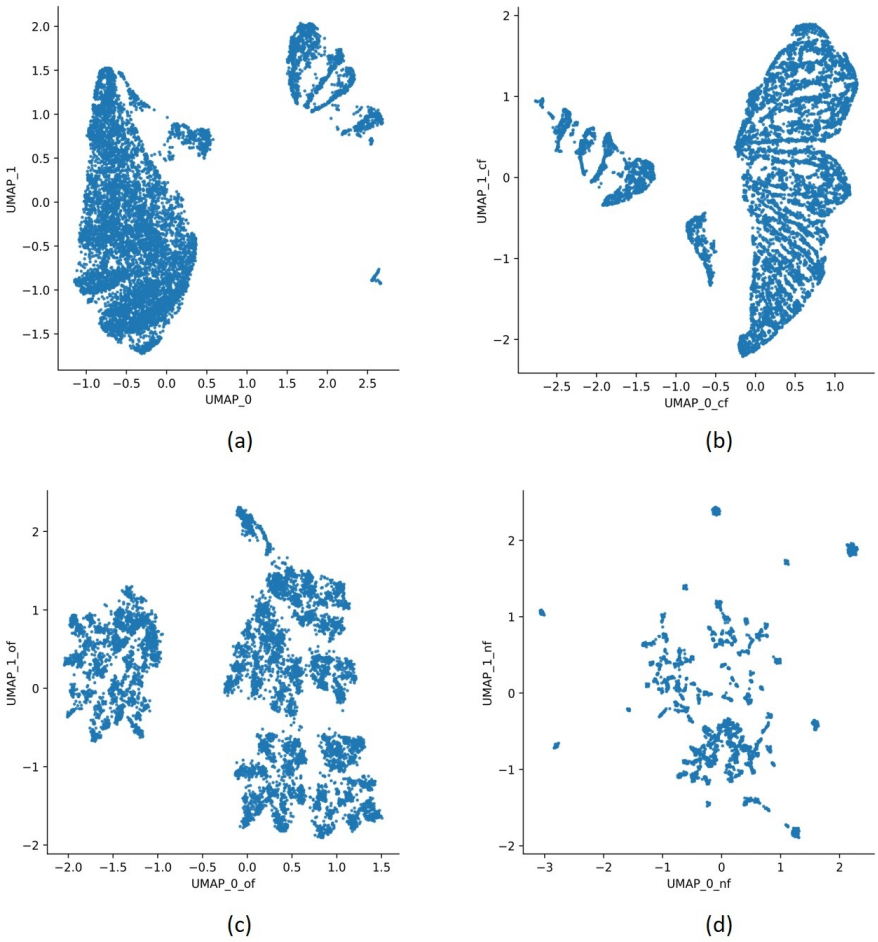
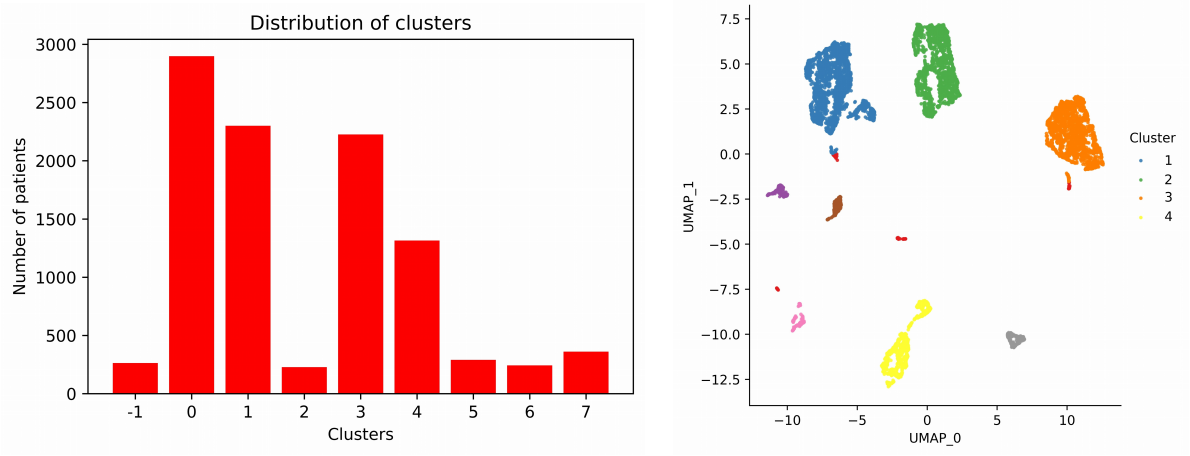


Figure 7.2: (a) Figure showing UMAP clusters for all the features with Euclidean metric (b) Figure showing UMAP clusters for continuous features with Euclidean metric (c) Figure showing UMAP clusters for ordinal features with Canberra metric (d) Figure showing UMAP clusters for nominal features with Hamming metric

7.3 Results

7.3.1 Identification of four significant clusters within the T2DM population

Using the clustering paradigm, seven subpopulations were detected among the patients with 261 patients considered as outliers. The distribution of clusters is shown in Figure 7.3a. A UMAP was further performed on the five dimensional reduced representation of the data to visualize the clusters detected by DBSCAN. For this the data points were labelled using the DBSCAN clustering labels and were color coded in the UMAP representation of the five dimensional reduced data as shown in Figure 7.3b. This provides validation to the fact the clustering done by DBSCAN makes sense. Four significant patient subpopulations containing 2898, 2301, 2226 and 1315 data points were finally obtained which were characterized next.



(a) (b)

Figure 7.3: (a) Distribution of clusters detected by DBSCAN on the five dimensional reduced representation of the data (b) UMAP clusters for five dimensional reduced representation of the data annotated by the DBSCAN generated clusters

7.3.2 Non-obese T2DM clusters with lower average age

Age and obesity are the most important risk factors for T2DM. However, there was a heterogeneity in both these variables across all the clusters. Interestingly, the mean Age and BMI both were lower in Cluster 2 (Age: 38.3 ± 0.19 years, BMI: 23.9 ± 0.1) and Cluster 4 (Age: 37.9 ± 0.26 years, BMI: 23.6 ± 0.13) compared to Cluster 1 (Age: 41.3 ± 0.14 years, BMI: 26.7 ± 0.09) and Cluster 3 (Age: 39.9 ± 0.18 years, BMI: 26±0.11). However distribution of males and females has been found to be similar across all the clusters. Thus the unsupervised clustering workflow identifies non-obese T2DM clusters present in the T2DM population.

7.3.3 Non-obese T2DM clusters majorly belong to rural residence

Proportion of rural residents was high in Cluster 2 (69.4% were Rural residents) and Cluster 4 (72.02% were Rural residents) compared to the other clusters (31.3% in Cluster 1 and 49.19% in Cluster 3). Surprisingly, only 4.3% people in Cluster 2 and 8.37% in Cluster 4 belonged to the richest quintile of the Wealth Index category whereas 64.04% in Cluster 1 and 54.9% in Cluster 3 belonged to the same.

Table 7.1: Detailed cluster-specific analysis for all numerical and categorical variables

(continued in the next page)

Identified clusters		Cluster 1	Cluster 2	Cluster 3	Cluster 4
Cluster Size (N)		2898	2301	2226	1315
Cont. Variables (Mean ± SE)					
Age (yrs)		41.3 ± 0.14	38.3 ± 0.19	39.9 ± 0.18	37.9 ± 0.26
Body Mass Index (kg/m2)		26.7 ± 0.09	23.9 ± 0.1	26 ± 0.11	23.6 ± 0.13
Haemoglobin (gm/dl)		12.5 ± 0.04	12.3 ± 0.04	12.1 ± 0.04	12.3 ± 0.06
Time to Water Source (min)		0.1 ± 0.01	0.02 ± 0.01	0.09 ± 0.01	18.6 ± 0.39
Cat.Variables	Value for cat. variables				
Sex	Male	558 (19.25)	457 (19.86)	270 (12.13)	323 (24.56)
	Female	2340 (80.75)	1844 (80.14)	1956 (87.87)	992 (75.44)

Table 7.1: Detailed cluster-specific analysis for all numerical and categorical variables

(continued in the next page)

Identified clusters		Cluster 1	Cluster 2	Cluster 3	Cluster 4
History of Asthma	No	2737 (94.44)	2064 (89.7)	1999 (89.8)	1121 (85.25)
	Yes	161 (5.56)	237 (10.3)	227 (10.2)	194 (14.75)
History of Thyroid Disorder	No	2636 (90.96)	2135 (92.79)	1992 (89.49)	1196 (90.95)
	Yes	262 (9.04)	166 (7.21)	234 (10.51)	119 (9.05)
History of Heart Disease	No	2729 (94.17)	2107 (91.57)	1996 (89.67)	1174 (89.28)
	Yes	169 (5.83)	194 (8.43)	230 (10.33)	141 (10.72)
History of Cancer	No	2876 (99.24)	2272 (98.74)	2161 (97.08)	1246 (94.75)
	Yes	22 (0.76)	29 (1.26)	65 (2.92)	69 (5.25)
Ever suffered from TB	No	2890 (99.72)	2287 (99.39)	2218 (99.64)	1305 (99.24)
	Yes	8 (0.28)	14 (0.61)	8 (0.36)	10 (0.76)
Milk/Curd intake freq	Never	201 (6.94)	183 (7.95)	110 (4.94)	123 (9.35)
	Weekly	461 (15.91)	551 (23.95)	293 (13.16)	405 (30.8)
	Occasionally	611 (21.08)	669 (29.07)	447 (20.08)	291 (22.13)
	Daily	1625 (56.07)	898 (39.03)	1376 (61.81)	496 (37.72)
Pulses/Beans intake freq	Never	13 (0.45)	17 (0.74)	18 (0.81)	9 (0.68)
	Weekly	255 (8.8)	248 (10.78)	152 (6.83)	198 (15.06)
	Occasionally	1263 (43.58)	937 (40.72)	936 (42.05)	574 (43.65)
	Daily	1367 (47.17)	1099 (47.76)	1120 (50.31)	534 (40.61)
Green vegetables intake freq	Never	7 (0.24)	12 (0.52)	10 (0.45)	9 (0.68)
	Weekly	324 (11.18)	259 (11.26)	279 (12.53)	142 (10.8)
	Occasionally	1000 (34.51)	796 (34.59)	792 (35.58)	483 (36.73)
	Daily	1567 (54.07)	1234 (53.63)	1145 (51.44)	681 (51.79)
Fruit intake freq	Never	50 (1.73)	65 (2.82)	74 (3.32)	41 (3.12)
	Weekly	897 (30.95)	1148 (49.89)	872 (39.17)	750 (57.03)
	Occasionally	1203 (41.51)	818 (35.55)	810 (36.39)	386 (29.35)
	Daily	748 (25.81)	270 (11.73)	470 (21.11)	138 (10.49)
Egg intake freq	Never	97 (3.35)	85 (3.69)	1983 (89.08)	41 (3.12)
	Weekly	1005 (34.68)	963 (41.85)	153 (6.87)	520 (39.54)
	Occasionally	1537 (53.04)	1100 (47.81)	80 (3.59)	678 (51.56)
	Daily	259 (8.94)	153 (6.65)	10 (0.45)	76 (5.78)
Fish intake freq	Never	222 (7.66)	106 (4.61)	2162 (97.12)	83 (6.31)
	Weekly	994 (34.3)	1006 (43.72)	35 (1.57)	593 (45.1)
	Occasionally	1210 (41.75)	987 (42.89)	20 (0.9)	563 (42.81)
	Daily	472 (16.29)	202 (8.78)	9 (0.4)	76 (5.78)
Chicken/Meat intake freq	Never	53 (1.83)	58 (2.52)	2175 (97.71)	33 (2.51)
	Weekly	1274 (43.96)	1150 (49.98)	32 (1.44)	640 (48.67)
	Occasionally	1475 (50.9)	1032 (44.85)	18 (0.81)	612 (46.54)
	Daily	96 (3.31)	61 (2.65)	1 (0.04)	30 (2.28)
Fried food intake freq	Never	179 (6.18)	161 (7)	276 (12.4)	95 (7.22)
	Weekly	1275 (44)	988 (42.94)	1114 (50.04)	631 (47.98)
	Occasionally	1071 (36.96)	849 (36.9)	715 (32.12)	408 (31.03)
	Daily	373 (12.87)	303 (13.17)	121 (5.44)	181 (13.76)
Aerated drink intake freq	Never	512 (17.67)	475 (20.64)	409 (18.37)	262 (19.92)
	Weekly	1579 (54.49)	1258 (54.67)	1200 (53.91)	744 (56.58)
	Occasionally	597 (20.6)	449 (19.51)	497 (22.33)	236 (17.95)
	Daily	210 (7.25)	119 (5.17)	120 (5.39)	73 (5.55)
Alcoholic	No	2627 (90.65)	2027 (88.09)	2171 (97.53)	1127 (85.7)
	Yes	271 (9.35)	274 (11.91)	55 (2.47)	188 (14.3)
Smoker	No	2770 (95.58)	2192 (95.26)	2197 (98.7)	1234 (93.84)
	Yes	128 (4.42)	109 (4.74)	29 (1.3)	81 (6.16)
Indoor Smoking freq	Never	1849 (63.8)	1138 (49.46)	1429 (64.2)	690 (52.47)
	Weekly	222 (7.66)	264 (11.47)	176 (7.91)	129 (9.81)
	Less than monthly	72 (2.48)	72 (3.13)	71 (3.19)	33 (2.51)
	Monthly	78 (2.69)	72 (3.13)	68 (3.05)	36 (2.74)
	Daily	677 (23.36)	755 (32.81)	482 (21.65)	427 (32.47)
Residence	Urban	1991 (68.7)	704 (30.6)	1131 (50.81)	368 (27.98)
	Rural	907 (31.3)	1597 (69.4)	1095 (49.19)	947 (72.02)
Wealth Index	Poorest	1 (0.03)	287 (12.47)	82 (3.68)	301 (22.89)
	Poorer	8 (0.28)	519 (22.56)	154 (6.92)	285 (21.67)
	Middle	151 (5.21)	698 (30.33)	245 (11.01)	339 (25.78)
	Richer	882 (30.43)	698 (30.33)	523 (23.5)	280 (21.29)
	Richest	1856 (64.04)	99 (4.3)	1222 (54.9)	110 (8.37)
Highest Education level	No education	388 (13.39)	758 (32.94)	416 (18.69)	472 (35.89)
	Primary level	347 (11.97)	373 (16.21)	303 (13.61)	240 (18.25)
	Secondary level	1641 (56.63)	1006 (43.72)	1106 (49.69)	530 (40.3)
	Higher level	522 (18.01)	164 (7.13)	401 (18.01)	73 (5.55)

Table 7.1: Detailed cluster-specific analysis for all numerical and categorical variables

Identified clusters		Cluster 1	Cluster 2	Cluster 3	Cluster 4
Religion	Hindu	1822 (62.87)	1544 (67.1)	1947 (87.47)	975 (74.14)
	Muslim	627 (21.64)	472 (20.51)	46 (2.07)	210 (15.97)
	Christian	313 (10.8)	210 (9.13)	13 (0.58)	97 (7.38)
	Others	136 (4.69)	75 (3.26)	220 (9.88)	33 (2.51)
Caste/Tribe	OBC	1331 (45.93)	871 (37.85)	805 (36.16)	472 (35.89)
	SC	384 (13.25)	517 (22.47)	328 (14.73)	343 (26.08)
	ST	303 (10.46)	385 (16.73)	86 (3.86)	258 (19.62)
	General	880 (30.37)	528 (22.95)	1007 (45.24)	242 (18.4)
Blood Pressure	No	1594 (55)	1443 (62.71)	1281 (57.55)	849 (64.56)
	Yes	1304 (45)	858 (37.29)	945 (42.45)	466 (35.44)
Possess Refrigerator	No	131 (4.52)	2296 (99.78)	762 (34.23)	989 (75.21)
	Yes	2767 (95.48)	5 (0.22)	1464 (65.77)	326 (24.79)
Possess Bicycle	No	1503 (51.86)	1055 (45.85)	1013 (45.51)	617 (46.92)
	Yes	1395 (48.14)	1246 (54.15)	1213 (54.49)	698 (53.08)
Possess Motorbike	No	825 (28.47)	1590 (69.1)	734 (32.97)	884 (67.22)
	Yes	2073 (71.53)	711 (30.9)	1492 (67.03)	431 (32.78)
Possess Car/Truck	No	2217 (76.5)	2226 (96.74)	1840 (82.66)	1273 (96.81)
	Yes	681 (23.5)	75 (3.26)	386 (17.34)	42 (3.19)
Cooking Fuel used	Other	1 (0.03)	4 (0.17)	0 (0)	1 (0.08)
	Plant based	354 (12.22)	1018 (44.24)	437 (19.63)	723 (54.98)
	Livestock based	47 (1.62)	297 (12.91)	211 (9.48)	104 (7.91)
	Gas/Oil	2460 (84.89)	965 (41.94)	1562 (70.17)	476 (36.2)
	Electricity	36 (1.24)	17 (0.74)	16 (0.72)	11 (0.84)
Household Structure	Non-nuclear	1310 (45.2)	1016 (44.15)	1120 (50.31)	564 (42.89)
	Nuclear	1588 (54.8)	1285 (55.85)	1106 (49.69)	751 (57.11)
Possess Livestock	No	2226 (76.81)	1155 (50.2)	1474 (66.22)	646 (49.13)
	Yes	672 (23.19)	1146 (49.8)	752 (33.78)	669 (50.87)
Drinking Water Source	Unprotrected sources	76 (2.62)	146 (6.35)	44 (1.98)	204 (15.51)
	Protected sources	739 (25.5)	998 (43.37)	686 (30.82)	522 (39.7)
	Community service	1991 (68.7)	1112 (48.33)	1448 (65.05)	508 (38.63)
	Bottled water	86 (2.97)	43 (1.87)	46 (2.07)	77 (5.86)
	Other	6 (0.21)	2 (0.09)	2 (0.09)	4 (0.3)

7.3.4 Non-obese T2DM clusters comprises of people from economically disadvantaged strata having a lower quality of life

Analysis reveal several other factors supporting the fact that T2DM sub-populations from Cluster 2 and Cluster 4 have a considerably lower quality of life.

It is observed that only 0.22% and 24.79% of patients belonging to Cluster 2 and Cluster 4 respectively possess a refrigerator compared to 95.48% and 65.77% of patients belonging to Cluster 1 and Cluster 3 respectively (Table 7.1). Only 30.9% and 32.78% of patients belonging to Cluster 2 and Cluster 4 respectively possess a motorbike compared to 71.53% and 67.03% of patients belonging to Cluster 1 and Cluster 3 respectively. Only 3.26% and 3.19% of patients belonging to

Cluster 2 and Cluster 4 respectively possess a car/truck compared to 23.5% and 17.34% of patients belonging to Cluster 1 and Cluster 3 respectively. 44.24% and 54.98% of patients belonging to Cluster 2 and Cluster 4 respectively, use plant based cooking fuel, which is relatively cheap, compared to 12.22% and 19.63% of patients belonging to Cluster 1 and Cluster 3 respectively. Moreover, only 41.94% and 36.2% of patients belonging to Cluster 2 and Cluster 4 respectively use Gas/Oil based cooking fuel, which is relatively expensive, compared to 84.89% and 70.17% of patients belonging to Cluster 1 and Cluster 3 respectively. 6.35 % and 15.51% of patients belonging to Cluster 2 and Cluster 4 respectively, drink water from unprotected sources, compared to 2.62% and 1.98% of patients belonging to Cluster 1 and Cluster 3 respectively.

7.3.5 Obese T2DM cluster with a strict non-vegetarian dietary pattern

Intake of non-vegetarian foods is invariably low in Cluster 3. Around 90% of the population in Cluster 3 had no intake of Egg (89.08%), fish (97.12%), chicken or meat (97.71%) whereas only less than 10% of the population in all the other 3 clusters had no intake of these non-vegetarian foods (Table 1). Though the Cluster 3 population had the highest daily intake of milk/curd (61.81%) and pulses/beans (50.31%) compared to the other clusters, other clusters also had almost similar proportion of people taking milk/curd and pulses/beans daily. Intake of other foods like dark leafy vegetables, fruits, fried foods and aerated drinks showed similar distribution across all the clusters.

7.4 Discussion

T2DM was identified as a homogeneous disease with Insulin Resistance followed by β-cell dysfunction being the underlying pathology. However recent studies have explored and found T2DM to be a heterogeneous entity with the relative contribution of Insulin Resistance and β-cell dysfunction to differ across T2DM clusters [241]. These studies were performed on clinical and

biochemical data with variables having uniform data types. On the other hand, our clustering approach takes into account the diverse data types obtained from an epidemiological dataset and discovers clusters among the T2DM population. Interestingly, two of the four clusters obtained in our study belonged to the non- obese T2DM phenotype where the mean BMI was below 25. These two non-obese clusters also had lower mean age compared to the other clusters. Both these non-obese clusters had larger proportion of rural residents and lower proportion of people belonging to the highest wealth quintile concluding to the fact that a large majority of T2DM people from rural India have lower BMI and are younger in age. The T2DM patient subpopulation belonging to these clusters have a relatively lower quality of life judging by analysis the lifestyle pattern based features. The non-obese phenotype of T2DM has been increasingly reported over the last two decades raising concern about the uniqueness of its underlying pathophysiology with a greater contribution of β-cell dysfunction compared to Insulin Resistance [13,29,163]. This non-obese T2DM phenotype has been found among Asians and studies depicting and investigating its similarities and differences has been in place. Studies have concluded T2DM to occur among the Asians at a lower BMI cut-off and also at a younger age [242,243]. This finding of two non-obese clusters with lower mean age provides confirmation to this.

Though non-obese T2DM is being considered as a unique phenotype, epidemiological studies for identifying high-risk population groups still remain undone. This is especially important for many Asian countries where over half of the T2DM population is of non-obese phenotype. This analysis, reporting an increased presence of Rural residents in both the non-obese T2DM clusters, calls for a modification in BMI and Age cut-off for T2DM screening among rural residents. However identification of risk factors for T2DM specific to the rural population needs to be done. Representation of people from the highest wealth quintile was much lower in both the non-obese T2DM clusters. T2DM is a multi-factorial disease requiring strict compliance to lifestyle modification, proper diet and antidiabetic therapy. Non-obese T2DM clusters with reduced

representation from the highest wealth quintile suggests the possibility of an unequal access to care for non-obese T2DM people thereby generating the need of a more equitable healthcare policy in terms of prevention and therapy.

On the other hand, both the obese T2DM clusters had higher age and more urban residents. The proportion of people from the highest wealth quintile was higher in both the obese clusters. Interestingly one of the obese clusters (Cluster 3) had invariably low intake of non-vegetarian foods (egg, fish, chicken and meat) pointing out to the fact this T2DM cluster comprised of non-vegetarian people mainly. Dietary requirements in diagnosed T2DM patients involves reduced amount of carbohydrates and fats with increased amount of protein-rich foods [243]. Animal products, being rich sources of dietary protein, need to be included in the diet. One of the obese T2DM clusters with a strict non-vegetarian dietary pattern suggests the need to design a proper dietary guidelines for this group.

7.5 Summary of the work

Existence of a significant non-obese T2DM patient sub-population belonging to younger age group and having larger proportions of rural residents raises with a lower quality of life, indicate the need of a different screening criteria for T2DM among rural Indian residents. The obese T2DM cluster with around 90% of people sticking to the non-vegetarian diet calls for the need of dietary guidelines for T2DM patients having a non-vegetarian dietary pattern.

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ABSTRACT

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Title of the thesis: Pathophysiological Characterization of Non-obese Type 2 Diabetes Mellitus

Background: Increasing recognition of Non-obese Type 2 Diabetes Mellitus (T2DM) as a distinct phenotype calls for investigation of its pathophysiology with identification of risk factors specific to this group. Over half of the diabetic population in India belong to the non-obese category with Body Mass Index (BMI) below 25.

Methodology: Insulin Resistance (IR) and pancreatic ß-cell function was investigated in T2DM subjects and healthy controls in a community based cohort (N=1057). Dynamic Insulin Response and Incretin Response during Oral Glucose Tolerance Test (OGTT) was investigated in non-obese and obese T2DM subjects (N=39) in a hospital cohort. Another hospital cohort of patients (N=20) undergoing bariatric surgery was followed up to study the association of incretin with glycaemic deregulation. Two of the largest epidemiological datasets of India; Clinical, Anthropometric and Biochemical Survey (CAB, N=616681) and National Family Health Survey-4 (NFHS-4, N=610498) were analysed using machine learning to identify risk factors for T2DM limited to the non-obese group. Finally unsupervised clustering was applied to identify T2DM sub-populations within the T2DM population.

Results: Compensatory hyperinsulinaemia, the hallmark for IR, was absent in non-obese T2DM. Both IR and ß-cell function was significantly lower in non-obese T2DM group compared to the obese T2DM. Non-obese T2DM subjects had reduced insulin response than obese T2DM subjects during OGTT though incretin response was comparable in both the groups. On follow-up, obese patients undergoing bariatric surgery had no change in Dipeptidyl Peptidase-4 in spite of glycaemic control. Machine learning analysis revealed males to be at higher T2DM risk only in the non-obese BMI range. Also the impact of ß-cell function was greater on T2DM risk for non-obese males. Four T2DM clusters were identified within the T2DM population in which two were characterized by normal BMI, younger age and rural residence.

Conclusion: Non-obese T2DM is characterised by impaired compensatory hyperinsulinaemia and reduced insulin response with comparable DPP4 & Incretin levels. Machine Learning Analysis on NFHS-4, CAB Survey and the community health cohort reveal Non-obese Indian males to be at higher T2DM risk with greater impact of ß-cell dysfunction. Non-obese T2DM population exists in the India who are younger in age and mainly belong to rural residence.

List of publications

Journal Publications

1. **Sarkar J**, Nargis T, Tantia O, Ghosh S, Chakrabarti P*. Increased Plasma Dipeptidyl Peptidase-4 (DPP4) Activity Is an Obesity-Independent Parameter for Glycemic Deregulation in Type 2 Diabetes Patients. *Frontiers of Endocrinology (Lausanne)*. 2019;10:505. Published 2019 Jul 25. doi:10.3389/fendo.2019.00505 [Co-first author]
2. **Sarkar J***, Maity SK, Sen A, Nargis T, Ray D, Chakrabarti P*. Impaired compensatory hyperinsulinemia among nonobese type 2 diabetes patients: a cross-sectional study. *Therapeutic Advances in Endocrinology and Metabolism*. 2019;10:2042018819889024. Published 2019 Dec 2. doi:10.1177/2042018819889024 [Co-corresponding author]
3. Subham Basu, Mahesh Barad, Dipika Yadav, Arijit Nandy, Bidisha Mukherjee, **Jit Sarkar**, Partha Chakrabarti, Satinath Mukhopadhyay, Debabrata Biswas*. DBC1, p300, HDAC3, and Siah1 coordinately regulate ELL stability and function for expression of its target genes. *Proceedings of the National Academy of Sciences* Mar 2020, 117 (12) 6509-6520; DOI: 10.1073/pnas.1912375117
4. **Sarkar J**, Maity SK, Ghosh P, Mohan V, Chakrabarti P*. Machine Learning Analysis on NFHS-4, CAB Survey and a Community Health Cohort reveal Non-obese Indian males to be at higher T2DM risk with greater impact of ß-cell dysfunction. [Under Review]
5. Bej S, **Sarkar J***, Biswas S, Mitra P, Chakrabarti P, Wolkenhauer O*. Identification and Epidemiological Characterization of Non-obese Type 2 Diabetic Sub-populations in the NFHS-4 Study using an Unsupervised Machine Learning Approach. [Under Review] [Co-first author & Co-corresponding author]
6. Ruchi Supekar, **Jit Sarkar**, Partha Chakrabarti, Subhajit Biswas*. Cross-sectional study revealed that a quarter of Kolkata city-dwellers may have silent infection (OBI) with genotype D2 Hepatitis B virus S protein mutants. [Under Review]

Conference/Symposium Publications

1. **Jit Sarkar**, Partha Chakrabarti. A web-based platform for Comprehensive clinical services in Rural India. EMBO Symposium on Big Data in Biomedicine. Published 2018.
2. **Jit Sarkar**, Sujay Krishna Maity, Pramit Ghosh, Viswanathan Mohan, Partha Chakrabarti. Non-obese Indian males with higher T2DM risk display predominant β-cell dysfunction: Corroborative findings from NFHS-4, CAB Survey and a Community Health Cohort. 6th Annual Conference of Association of Clinical Chemistry and Lab Medicine Practitioners. Published 2020.