Comparison of the insulin-glucose relationship in the steady state and perturbed state

A thesis submitted in partial fulfilment of the requirements of the degree of Doctor of Philosophy by

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CERTIFICATE BY SUPERVISOR

Certified that the work incorporated in the thesis titled 'Comparison of insulin-glucose relationship in the steady state and perturbed state' submitted by Manawa Diwekar-Joshi was carried out by the candidate, under my supervision. The work presented here or any part of it has not been included in any other thesis submitted previously for the award of any degree or diploma from any other university or institution.

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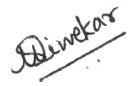

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I. Publications

Following are the publications which resulted from the work of this thesis and related projects:

1. Patil P, Lalwani P, Vidwans H, Kulkarni S, Bais D, **Diwekar-Joshi M**, Rasal M, Bhasme N, Naik M, Batwal S and Watve M. (2021). A multidimensional functional fitness score has a stronger association with type 2 diabetes than obesity parameters in cross sectional data', *PLOS ONE*, 16(2), p. e0245093. https://doi.org/10.1371/journal.pone.0245093.

2. **Diwekar-Joshi, M**., & Watve, M. (2020). Driver versus navigator causation in biology: the case of insulin and fasting glucose. *PeerJ*, *8*, e10396. https://doi.org/10.7717/peerj.10396

3. Chawla, S., Pund, A., B., V., Kulkarni, S., **Diwekar-Joshi, M**., & Watve, M. (2018). Inferring causal pathways among three or more variables from steady state correlations in a homeostatic system. *PLOS ONE*, *13*(10), e0204755. https://doi.org/10.1371/journal.pone.0204755

4. Watve, M., & **Diwekar-Joshi, M**. (2016). What to expect from an evolutionary hypothesis for a human disease: The case of type 2 diabetes. *HOMO*, *67*(5), 349–368. https://doi.org/10.1016/j.jchb.2016.07.001

5. Watve, M., Bodas, A., & **Diwekar, M**. (2014). Altered autonomic inputs as a cause of pancreatic β-cell amyloid. *Medical Hypotheses*, *82*(1), 49–53. https://doi.org/10.1016/j.mehy.2013.11.002

II. Abbreviations used in the thesis

ACCORD	Action to Control Cardiovascular Risk in Diabetes
ADA	American Diabetes Association
AMPK	AMP-Activated Protein Kinase
BATIRKO	Brown adipose tissue insulin receptor knock-out
BDNF	Brain derived neurotropic factor
BMI	Body mass index
C	Control
CI	Confidence interval
CNS	Central nervous system
CPCSEA	Committee for the purpose of control and supervision of experimental animals
CRISIS	Coronary Risk of Insulin Sensitivity in Indian Subjects
CSS	Consequential steady state
D	Diabetic
DM	Diabetes mellitus
DZX	Diazoxide
EM	Evolutionary medicine
Eq	Equation
ER	Endoplasmic reticulum
FDG-PET	Fluorodeoxyglucose-Positron emission tomography
FFA	Free fatty acid
FG	Fasting glucose
FGF	Fibroblast growth factor
FI	Fasting insulin
FIRKO	Fat insulin receptor knock-out
GDM	Gestational diabetes mellitus
GTT	Glucose tolerance test
GWAS	Genome wide association study
HbA1c	Glycated haemoglobin
HOMA	Homeostatic model assessment
IAEC	Institutional animal ethics committee

IDE	Insulin degrading enzyme
IDF	International Diabetes Federation
IFG	Impaired fasting glucose
IMTG	Intramyocellular triacylglycerol
ip	intra peritoneal
IPGTT	Intra peritoneal glucose tolerance test
IR	Insulin resistance
IRKO	Insulin receptor knock-out
KO	Knock-out
LIRKO	Liver insulin receptor knock-out
MIRKO	Muscle insulin receptor knock-out
MODY	Maturity onset diabetes of the young
mTOR	Mechanistic/mammalian target of rapamycin
n/N/No.	Sample size, number
ND	Non-diabetic
NFG	Normal fasting glucose
NHP	Newcastle Heart Project
NHP-Eur	Newcastle Heart Project-European
NHP-SA	Newcastle Heart Project-South Asian
NICE	National institute for health and clinical excellence
NPH	Neutral protamine hagedorn
OCT	Octreotide
OGTT	Oral glucose tolerance test
ON	Overnight
PAP	Predictive adaptive programming
PCOS	Poly-cystic ovarian syndrome
PEPCK	Phosphoenolpyruvate carboxykinase
PMNS	Pune Maternal Nutrition Study
PS	Perturbed state
R	Pearson's correlation coefficient
R2	R-squared
RCT	Randomized controlled trial
S6K1	Ribosomal protein S6 kinase beta-1

SD	Sprague Dawley
SE	Standard error
SGLT2	Sodium glucose co-transporter 2
SS	Steady state
STZ	Streptozotocin
T	Treated
T1D	Type 1 diabetes
T1DM	Type 1 diabetes mellitus
T2D	Type 2 diabetes
T2DM	Type 2 diabetes mellitus
TSS	Targeted steady state
UKPDS	United Kingdom Prospective Diabetes Study
VADT	Veterans Affairs Diabetes Trial
WHO	World Health Organisation
WHR	Waist to hip ratio
βIRKO	β-cell insulin receptor knock-out

III. Abstract

Altered insulin-glucose relationship is the focus of type 2 diabetes pathophysiology and treatment. Despite decades of research on the cause and mechanisms of the disorder, there are many inconsistencies and gaps in the knowledge. I have explored these inconsistencies in detail and highlighted the need to re-examine the insulin-glucose relationship. The focus of this thesis is the difference in the glucose-insulin relationship in the steady state (fasting) versus the perturbed state (post meal/post glucose load). These distinct relationships between glucose and insulin in the two states led to the question of distinct causal roles of insulin in glucose homeostasis in the two states. This distinction in inferring the causality between homeostatic variables (not only glucose and insulin, but others as well) in these two states has not been made earlier.

In homeostatic systems, causality in a steady state can be qualitatively different from that in a perturbed state. On a broader scale there is a need to differentiate driver causality from navigator causality. A driver is essential for reaching a destination but may not have any role in deciding the destination. A navigator on the other hand has a role in deciding the destination and the path but may not be able to drive the system to the destination. The failure to differentiate between types of causalities is likely to have resulted into many misinterpretations in physiology and bio-medicine.

I have used multiple approaches to address these differences in the insulin-glucose relationship in two states. With these approaches, I have critically re-examined the causal role of insulin in glucose homeostasis. The approaches used are:

1. Systematic review of literature and meta-analysis of experiments in which the insulin levels or insulin action has been altered. I have compared the effect of this alteration on the steady and perturbed state glucose with the help of four separate meta-analyses.
2. Making differential predictions from alternative homeostasis models of glucose homeostasis and testing them in human epidemiological data.

3. Differentiating between steady state and post-meal state glucose levels in streptozotocin-treated rats in primary experiments

The results of the three approaches converge on the common inference, namely the role of insulin in determining steady state glucose is different than its role in determining the perturbed state glucose. I have then delineated the causal relations between fasting glucose, fasting insulin and insulin resistance using a novel statistical method developed in our lab. Using these results, I also make a case for the concept of driver and navigator causation with the example of insulin and glucose. The different approaches lead to the conclusion that although insulin action hastens the return to a steady state after a glucose load, there is no evidence that insulin action determines the steady state level of glucose. Thus, insulin appears to be a driver but not a navigator for steady state glucose level. The insulin-glucose example suggests that we may have to carefully re-examine causal inferences from perturbation experiments and set up revised norms for experimental design for causal inference for any homeostatic systems, or for any homeostatic variables. Thus, this study could be one more piece of evidence which will stimulate research to look for factors and therapies other than insulin to control and manage type 2 diabetes.

Since I am suggesting a new relationship between insulin-glucose, rather a new role of insulin in the glucose dynamics, I also revisited the evolutionary theories behind the causes of type 2 diabetes. The new understanding of insulin-glucose relationship has many implications to the hypotheses about evolutionary origins of type 2 diabetes. It makes the case for obesity centred hypotheses substantially weak and strengthens the behaviour and reproduction-centred hypotheses. Thus, this work could be the basis for developing new behaviour-oriented therapies to control type 2 diabetes and the related complications.

IV. Synopsis

Type 2 diabetes mellitus (T2DM) is a leading (direct or indirect) cause of death. The global burden on the health systems due to T2DM has also increased drastically over the last decade. The focus of T2DM, and in turn the rationale of treatment, is the altered relationship between insulin and glucose. The role of insulin in glucose homeostasis has been studied in detail for a long time now. Despite decades on research on the cause and pathophysiology of the disorder, there are many inconsistencies and lacunae. I have explored these inconsistencies in detail and highlighted the need to re-examine the insulin-glucose relationship.

While re-examining this relationship, I focussed on the differences in the relationship between insulin-glucose in the steady state (fasting) versus the perturbed state (post meal/post glucose load). These distinct relationships between glucose and insulin in the two states led to the question of distinct causal roles of insulin in glucose homeostasis in the two states. This distinction in inferring the causality between homeostasis variables (not only glucose and insulin, but others as well) in these two states has not been made earlier. I have thus tried to shine a light on the methods or processes of inferring causal relations in bio-medicine using the example of glucose and insulin.

Inferring causality from experimental intervention or perturbation is perceived to be a more sound approach than inferring causation from cross-sectional correlation. I try to show here, that there could be logical fallacies even in interventional inference. In homeostatic systems, causality in a steady state can be qualitatively distinct from that in a perturbed state. On a broader scale, there is a need to differentiate driver causality from navigator causality. A driver is essential for reaching a particular destination but may not have any role in deciding the destination. A navigator on the other hand has a role in deciding the destination and the path but may not be able to drive the system to the destination. The failure to differentiate between these types of causalities is likely to have resulted into many misinterpretations and gaps in the understanding in physiology and bio-medicine. I have tried to illustrate this by critically re-examining a specific case of

the causal role of insulin in glucose homeostasis using multiple approaches in a comprehensive manner.

The first approach is a systematic review of literature and meta-analysis of experiments in which the insulin levels or insulin action has been altered. The effect of this alteration on the steady and perturbed state glucose is compared with the help of four separate meta-analyses. In the second approach, I make differential predictions from alternative homeostasis models of glucose homeostasis and test them in human epidemiological data. In the next approach, I differentiate between steady state and post-meal state glucose levels in streptozotocin-treated rats in primary experiments.

The results of these three approaches have converged on the common inference, namely the role of insulin in determining steady state glucose is drastically different than its role in determining the perturbed state glucose. This leads to a fundamental question of determining causality in homeostatic variables in the steady state. I will then try to delineate the causal relations between fasting glucose, fasting insulin and insulin resistance using a novel statistical method developed in our lab. Here, I also make a case for the concept of driver and navigator causation with the example of insulin and glucose. I will also give examples to try and apply this concept in a broader sense.

All these approaches converge on the inference that there is a significant difference in the insulin-glucose relationship in the steady state as compared to that in the perturbed state. Although insulin action hastens the return to a steady state after a glucose load, there is no evidence that insulin action determines the steady state level of glucose. Insulin, unlike the popular belief in medicine, appears to be a driver but not a navigator for steady state glucose level. It is quite likely therefore that the current line of clinical action in the field of type 2 diabetes has limited success largely because it is based on a misinterpretation of insulin-glucose relationship. The insulin-glucose example suggests that we may have to carefully re-examine causal inferences from perturbation experiments and set up revised norms for experimental design for causal inference for any homeostatic systems, or for any homeostatic variables. Thus, this study could be

one more piece of evidence which will stimulate research to look for factors and therapies other than insulin to control and manage type 2 diabetes.

Since I am suggesting a new relationship between insulin-glucose, rather a new role of insulin in the glucose dynamics, I also revisited the evolutionary theories behind the causes of type 2 diabetes. The new understanding of insulin-glucose relationship has many implications to the hypotheses about evolutionary origins of type 2 diabetes. It makes the case for obesity centred hypotheses substantially weak and strengthens the behaviour and reproduction-centred hypotheses. Thus, this work could be the basis for developing new behaviour-oriented therapies to control type 2 diabetes and the related complications.

Chapter 1: Introduction

1.1 Diabetes mellitus

The strongest motivation to study glucose physiology is its dysregulation. Chronically elevated levels of glucose result in diabetes mellitus (DM), popularly called diabetes. Globally, 425 million adults are estimated to have diabetes currently compared to 108 million in 1980 (IDF Atlas, 8th Edition, WHO Report on Diabetes 2016). Around 72.9 million Indians suffer from diabetes (IDF Atlas, 8th Edition). Diabetes places a large financial burden on individuals due to the cost of medications and hospitalization arising from the complications of diabetes. Around 5% to 20% of the total health expenditure of majority of the countries are spent on diabetes and related complications (IDF Atlas, 8th Edition).

1.1.1 Diagnostic criteria of diabetes

Raised blood sugar level is the diagnostic criteria for diabetes mellitus. According to the current guidelines by the American Diabetes Association (ADA), diabetes may be diagnosed based on the levels of glycated haemoglobin (HbA1c) or glucose in the blood. A person is said to be diabetic if the

1. Plasma HbA1c ≥ 6.5% OR
2. Fasting plasma glucose is ≥ 126 mg/dL (7 mmol/L) OR
3. The 2-hour post-prandial glucose is ≥ 200 mg/dL (11.1 mmol/L) in an oral glucose tolerance test (OGTT) OR
4. Random plasma glucose ≥ 200 mg/dl (11.1 mmol/dL) in a patient showing classic symptoms of hypoglycaemia or hyperglycaemia

The reasons for the chronic rise in sugar are believed to be either that the body cannot produce enough insulin (absolute insulin insufficiency) or cannot use the available insulin effectively (relative insulin insufficiency).

1.1.2 Classification of diabetes

Although diabetes is a group of complex and heterogenous symptoms, attempts have been made to classify the different types. Currently there are four main

categories (Canivell and Gomis, 2014).

Type 1 diabetes mellitus (T1DM): Approximately 5-10% of the total cases suffer from this type. It is characterised by an auto-immune destruction of β-cells which leads to near complete insulin insufficiency (Epstein, Atkinson and Maclaren, 1994). Patients with T1DM are completely dependent on insulin therapy for their survival. Although auto-immune destruction is the major cause for insulin insufficiency, some idiopathic forms of T1DM are also present. (Poretsky, 2010)

Type 2 diabetes mellitus (T2DM): This type accounts for almost 90% of the cases of diabetes. Insulin resistance and β-cell dysfunction are believed to the two hallmarks of T2DM which result into metabolic alterations. Hyperinsulinemia in the earlier stages, followed by a fasting hyperglycaemia are the typical marks of T2DM (Poretsky, 2010)

Gestational diabetes mellitus (GDM): In GDM, women with no history of diabetes develop a high blood sugar during pregnancy. It accounts for 7% of all the pregnancies and increases the risk of development of diabetes even after the delivery (Poretsky, 2010).

Other specific types: There are other types of diabetes which account for only a minor proportion of all the cases. Maturity Onset Diabetes of the Young (MODY) is a cluster of monogenic disorders in which a genetic mutation causes hyperglycaemia by affecting glucose sensing or insulin secretion, but insulin action is not affected (DeFronzo *et al.*, 2015).

1.2 Prevalent theories about pathophysiology of T2DM

The classical theory of T2DM which has been the mainstream thinking in this field was developed in the 1970s and 1980s. Considering recent evidence, many of the assumptions of this theory are challenged. Before examining the challenges, I will outline the classical line of thinking, that has been the backbone of clinical practice today.

T2DM was earlier called as the non-insulin dependent form of diabetes and T1DM was known as the insulin dependent form of diabetes. This classification

or nomenclature stems from the inherent dysfunction of insulin in T2DM as opposed to the low levels of insulin in T1DM. Insulin resistance and β-cell dysfunction are the two main causes for the relative insulin insufficiency and the pathophysiology of T2DM (DeFronzo, 1988; Kudva and Butler, 1997). The mainstream thinking that dominated the T2DM field comprises three causal steps namely obesity causes insulin resistance, β-cells compensate for insulin resistance by producing more insulin and insufficient compensation by β-cells leads to hyperglycaemia (Figure 1). Hyperglycaemia is believed to be the main cause of all the complications seen in the T2DM patients. I will discuss each of these steps in detail.

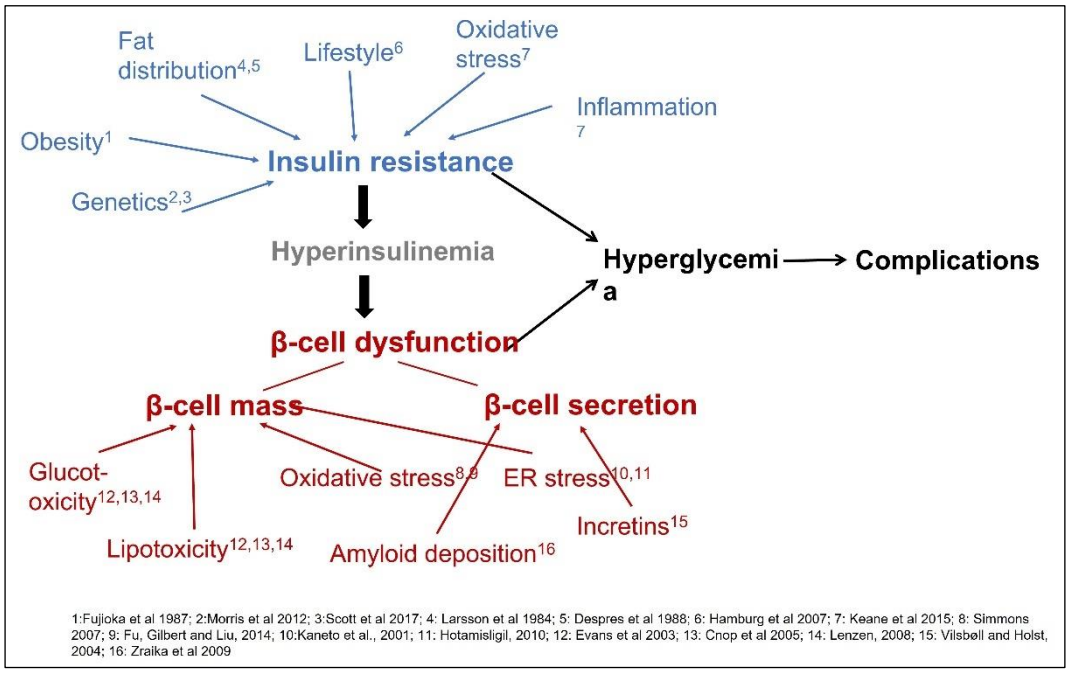


Figure 1: Infographic showing the current classical theory to explain the pathophysiology of type 2 diabetes mellitus.

1.2.1 Causes of insulin resistance

Lack or decrease of insulin action despite normal or increased production of insulin is termed as insulin resistance. It is a pathological state in which insulin-dependent cells show an inappropriate response to the insulin hormone (Samuel and Shulman, 2016). Although insulin resistance was first defined in 1966, the concept became widespread after the 1988 Banting lecture given by Reaven (Reaven, 1995). Insulin resistance is a consistent finding in T2DM and may be

present for a long time before the onset of the actual disease. A variety of risk factors are associated with insulin resistance and a myriad of molecular mechanisms have been implicated to cause insulin resistance. Obesity (Fujioka *et al.*, 1987), genetics (Morris *et al.*, 2012; Scott *et al.*, 2017), fat distribution (Larsson *et al.*, 1984; Després *et al.*, 1988), lifestyle (Hamburg *et al.*, 2007), oxidative stress and inflammation (Keane *et al.*, 2015), pain (Greisen *et al.*, 2001; Zhai *et al.*, 2016), sarcopenia (Srikanthan, Hevener and Karlamangla, 2010; Cleasby, Jamieson and Atherton, 2016), muscle soreness (Kirwan *et al.*, 1992) are some of the factors associated with insulin resistance. All these factors are not independent of each other and there are positive and negative feedback mechanisms which link these factors with insulin resistance. Insulin resistance has been studied in different tissues. Muscle, liver and adipocytes are the tissues which have been studied the most to find out their contributions to the T2DM pathophysiology.

Over the last few years, around 127 different sites in the human genome linked to propensity of obesity have been identified using GWAS studies (Alonso *et al.*, 2016; Castillo, Orlando and Garver, 2017). However the genes identified using the GWAS studies can explain only 10-15% of the variance in insulin resistance and the clinical applications of these findings are still limited (Alonso *et al*., 2016, Vidwans and Watve 2017, Watve, 2013. A more recent line of work is to study the interaction between the genetics and the environmental factors to study the propensity and progression of obesity, type 2 diabetes and other metabolic disorders. The most frequently studied environmental factors include diet and nutrition and lifestyle parameters like exercise and sleep. Epigenetic factors are now being explored as the link or the mechanism through which the genetic and environmental factors could interact to regulate the risk or propensity of metabolic disorders (Pillon *et al.*, 2021). It is however quite difficult to study the causal nature of any of this factor due to confounding effect of the other factors in the system and there are various ways in which efforts have been made to try and delineate the causal effects of such factors (Pillon *et al.*, 2021).

1.2.2 Compensatory hyperinsulinemia

According to the classical line of thinking, in the initial stages of insulin resistance, the β-cells start secreting insulin in a higher amount to compensate for the insulin resistance. Hence increased level of insulin in the plasma is also considered to be an indication of insulin resistance. This is known as compensatory hyperinsulinemia (DeFronzo *et al.*, 2015). The increased insulin secretion by the β-cells is believed to be due to an increased β-cell mass (Pick *et al.*, 1998). Another postulated reason for high levels of insulin is a change in the sensitivity of the β-cells to glucose, due to which glucose-stimulated insulin secretion increases. This is attributed to a change in the dynamics of the enzyme hexokinase (Cockburn *et al.*, 1997). As insulin resistance progresses, there is progressive degeneration of the β-cell mass as well as function due to exhaustion because of the compensatory response (DeFronzo *et al.*, 2015). Along with this 'exhaustion', the increased levels of glucose also cause decrease in the β-cell mass and/or function. Glucotoxicity and lipotoxicity are the two terms which describe this effect of glucose and lipids on the β-cell dysfunction as diabetes sets in (Poitout and Robertson, 2002).

1.2.3 Causes of β-cell dysfunction

Both β-cell dysfunction and insulin resistance are implicated as the major causes of type 2 diabetes mellitus. They are not independent of each other. The amount of insulin produced by the β-cells is a result of a variety of factors like β-cell mass, integrity of the β-cell structure and control of insulin secretion by nutrient and hormonal signals. A variety of causes have been attributed to β-cell dysfunction which manifests as reduced β-cell mass, reduced glucose stimulated insulin response and integrity of β-cell structure (Cerf 2013).

β-cell mass: The β-cell mass that is increased in the prediabetic state, decreases with advancement of T2DM. The factors implicated in β-cell death are inflammatory cytokines (Lin *et al.*, 2012; Cerf, 2013) oxidative stress (Simmons, 2007; Fu, Gilbert and Liu, 2014) endoplasmic reticulum (ER) stress (Kaneto *et al.*, 2001; Hotamisligil, 2010), autophagy (Lee, Giordano and Zhang, 2012), amyloid formation (Zraika *et al.*, 2009) and gluco-lipotoxicity (Evans *et al.*, 2003; Cnop *et al.*, 2005; Lenzen, 2008). All these factors are interlinked and enhance or

inhibit each other's actions making it difficult to segregate the exact role of each individual factor on the β-cell mass.

β-cell secretion*:* Despite the β-cell mass being optimal, the insulin levels can be reduced. There are many factors which influence the glucose-stimulated secretion of insulin from the islets which may or may not depend on obesity. Some of them are incretins (Vilsbøll and Holst, 2004), uncoupling protein-2 (Chan *et al.*, 2004), ER stress (Hasnain, Prins and McGuckin, 2016), inflammation (Fizelova *et al.*, 2017), autonomic nervous system (Campfield and Smith, 1980).

1.2.4 Fasting and post-prandial hyperglycaemia

The diagnostic criteria of T2DM are based on the blood glucose levels at two different timepoints during the day:

1. Fasting (no caloric intake for at least 8 hours): If the fasting glucose levels are above 126mg/dl, the person is said to be diabetic.
2. Post-prandial (after 2 hours following ingestion of 75g glucose load): If the post-prandial glucose levels are above 200mg/dl, the person is said to be diabetic (IDF Atlas, 8th Edition).

If either of these two readings are above the prescribed level, the person is said to be diabetic and some form of treatment regimen for control of hyperglycaemia is recommended. A more detailed description of the glucose dynamics is captured by the Oral Glucose Tolerance Test (OGTT). An OGTT records the disposal of glucose after ingestion of a 75g bolus of glucose. After a fast of 8 hours, blood is withdrawn for insulin and glucose measurement. The subject is then given a glucose bolus of 75g after which blood is drawn at intervals of about 15 to 30 minutes up to 2 hours. Both insulin and glucose levels are measured at these time points. The nature of the curve of insulin and glucose values against time represents the different patterns of glucose homeostasis and alterations in these curves are valuable tools to understand the glucose dysregulation which occurs during diabetes (Holt *et al.*, 2010; Watve, 2013). In T2DM, fasting, post-prandial or both glucose levels could be higher than normal and there are specific physiological processes associated with these two glucose levels. Additionally, a third parameter is used to monitor the blood glucose level: glycated haemoglobin

(abbreviated as HbA1c). HbA1c is used to as a measure of the previous two to three-month average blood sugar level and essentially incorporates both fasting and post-meal glucose levels. HbA1c along with fasting and post-prandial glucose levels is used to set glycaemic targets during treatments (WHO Report on diabetes, 2016)

1.2.5 Complications due to hyperglycaemia

The complications due to hyperglycaemia are multiple and have been classified into two main types: microvascular and macrovascular. The main microvascular complications include retinopathy, pathology in the renal glomerulus and peripheral neuropathies (Holt *et al.*, 2010; DeFronzo *et al.*, 2015). Macrovascular complications mainly comprise of the cardiovascular complications and diabetic foot (Holt *et al.*, 2010; Watve, 2013; DeFronzo *et al.*, 2015). Prolonged exposure to high glucose levels is believed to be the main cause of all microvascular complications UKPDS 33, 1998; UKPDS 38, 2008). Although all the cells in the body of a diabetic are exposed to high levels of glucose, only some kinds of cells show the effects of hyperglycaemia. This can be attributed to the differences in the tissue specific glucose transporters and their differences in their modulation in response to the extracellular glucose concentrations (Giardino, Edelstein and Brownlee, 1996; Holt *et al.*, 2010).

1.2.6 Treatment regimens in practice

Different strategies have been used to treat or control diabetes. Majority of these strategies aim at controlling the blood glucose levels in the hope that the complications which arise due to hyperglycaemia will be kept in check. These treatment regimens/strategies are based on the prevalent theories about the pathophysiology of diabetes. These treatment regimens can be classified into four broad categories.

a. Exercise and diet
b. Oral anti-hyperglycaemic agents
c. Insulin treatment and
d. Surgical options

I will highlight each of this category in detail below.

a. Exercise and diet

The first line of treatment for hyperglycaemia is exercise and/or modification in the diet (Kirwan, Sacks and Niewoudt, 2017). Low calorie diets which result in weight loss have been effective in treating T2DM and can result in remission in some cases as well (Lean *et al.*, 2018; Lean, 2019). Exercise improves insulin sensitivity, and this effect could be independent of the weight. It has been shown that insulin function improves without any loss in weight (Kirwan, Sacks and Niewoudt, 2017; Zanuso *et al.*, 2017). However, both diet and exercise have issues with long term compliance and are generally combined with some anti-hyperglycaemic agents in the long run.

b. Oral anti-hyperglycaemic agents

Exercise and diet aim to improve the functioning of insulin and thus reduce the blood sugar levels. Anti-hyperglycaemic agents have a similar aim, though the way in which this is achieved could be different. Anti-hyperglycaemic agents could act in one of the following ways:

i. Increase insulin production
ii. Increase insulin sensitivity of tissues
iii. Decrease endogenous glucose production
iv. Reduce/delay glucose uptake from the intestine
v. Increase the excretion of glucose from the kidneys

Based on these modes of actions, different molecules currently in use as oral antihyperglycemic agents are given in table 1.

Table 1: Brief Summary of the oral antihyperglycemic agents currently in use. Adapted from Cheng & Fantus, 2005; Inzucchi, 2002.

Name of the chemical/pharmaceutical family	**Brief mode of action**	**Efficacy in terms of reduction of HbA1c %**
Sulfonylureas	Increase insulin secretion	1.0-1.5
Non-sulfonylureas	Increase insulin secretion	0.5-1.5
Thiozolidinediones	Increase insulin sensitivity and reduce release of FFAs	1.0-1.5

Biguanides	Decrease hepatic glucose production and enhance glucose uptake by muscles	1.0-1.5
α-Glucosidase Inhibitors	Delay intestinal carbohydrate absorption	0.5-1.0
Intestinal Lipase Inhibitors	Decrease energy intake	0.3-0.9

A recent addition to the set of oral-antihyperglycemic agents is that of SGLT2 inhibitors. These molecules inhibit the sodium glucose cotransporter 2 in the kidney to reduce the reabsorption of glucose by the kidneys (Inzucchi *et al.*, 2015). Although a multitude of options of pharmaceuticals are present most of them have inherent side effects and in their efficacy reduces in the long-term (Inzucchi, 2002; Cheng and Fantus, 2005; DeFronzo, Eldor and Bdul-Ghani, 2013).

c. ***Insulin treatment***

It is estimated that 50% people suffering from T2DM require insulin injections at some timepoint in their life (Mayfield and White, 2004). Doctors recommend insulin therapy when the glucose lowering regimens stop working and the HbA1c levels are persistently above 7.5%. Insulin is generally administered by subcutaneous injections, but insulin pumps are also available which modulate the doses based on the food intake. An insulin regimen can also be given in combination with an oral-antihyperglycemic agent. The dose and frequency of insulin is adjusted by the clinician based on the fasting, post-prandial and HbA1c levels of the patients and the intended glycaemic targets of the therapy (DeFronzo *et al.*, 2015). However, there are some side effects of insulin treatment. Main side effects are:

1. Severe hypoglycaemia: The two trials Action to Control Cardiovascular Risk in Diabetes (ACCORD) and Veterans Affair Diabetes Trial (VADT) report that the incidence of severe hypoglycaemia increased after intensive glycaemic control using insulin (Duckworth *et al.*, 2009; Skyler *et al.*, 2009; Bonds *et al.*, 2010). UKPDS also shows 160% increase in hypoglycaemic events in the insulin treatment group (UKPDS 33, 1998; UKPDS 38, 2008)

2. Increased mitogenic activity: Although we focus on the glucose lowering action of insulin, it has a variety of other roles in normal physiology. One of its important functions is that of mitogenesis. Hence treatment with exogenous insulin might increase risk for certain types of cancers (Lebovitz, 2011).

d. Surgery for treatment of T2DM

One of the recent additions in the treatment regimen of T2DM is metabolic surgery. Metabolic surgery-based treatment is broadly defined as gastrointestinal modifications with an intent to reduce obesity and diabetes. Multiple randomised clinical trials have shown success in use of metabolic surgeries in the treatment of T2DM (Gloy *et al.*, 2013; Cummings and Rubino, 2018). Gloy et al 2013 report that bariatric surgery led to an increased weight loss and greater remission rates of T2D and a general improvement in the quality of life as compared to non-surgical treatment options in case of obese patients (Gloy *et al.*, 2013). However, it has also been shown that the positive effects of metabolic surgery in reducing glucose levels could be independent of the actual weight loss (Thaler and Cummings, 2009).

1.3 Problems with the current thinking about T2DM

The mainstream thinking that has dominated the field comprises of three causal steps namely obesity causes insulin resistance, β-cells compensate for insulin resistance by producing more insulin and insufficient compensation by β-cells leads to hyperglycaemia. However, one also finds several discordant notes in literature. I will now focus on the inconsistencies or the points which remain unexplained by the classical thinking about the pathophysiology of T2DM (Figure 2).

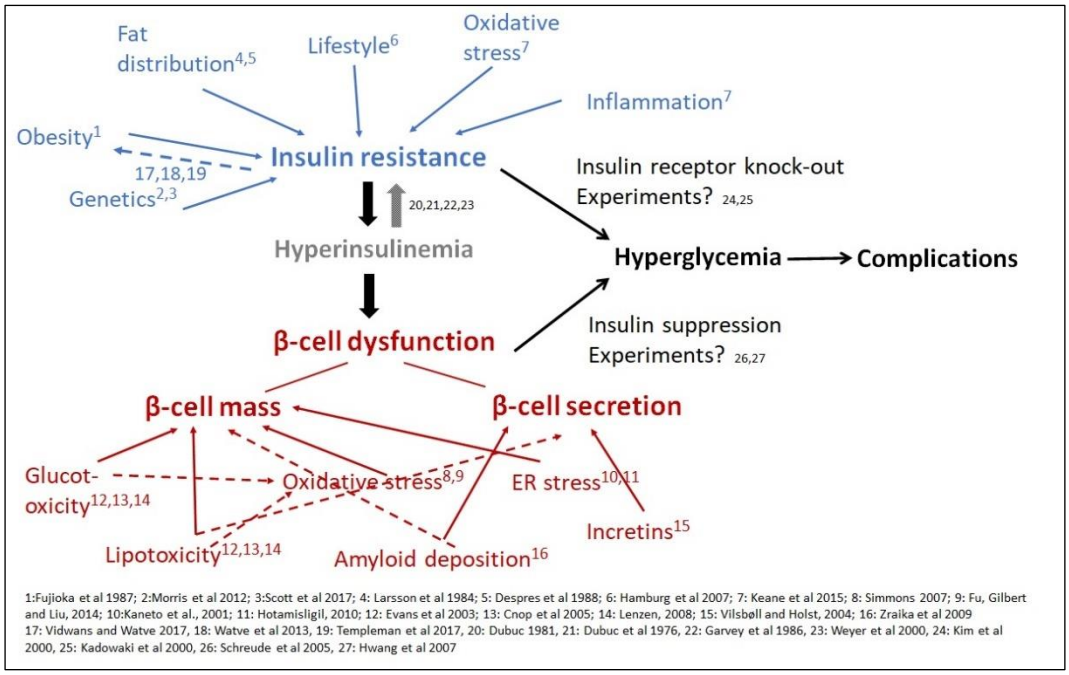


Figure 2: Infographic showing the inconsistencies and unanswered questions in the current picture explaining the pathophysiology of type 2 diabetes mellitus

1.3.1 Causes of insulin resistance

Obesity has been implicated as one of the main reasons for insulin resistance according to the mainstream thinking about T2DM (Fujioka *et al.*, 1987; Reaven, 1995; Samuel and Shulman, 2016). However, recent trend in diabetes research is questioning the arrow of causation between obesity and insulin resistance. If the statistics are seen which describe the association between obesity and insulin resistance, a weak correlation is seen between insulin resistance and obesity. Vidwans and Watve review the association between HOMA-IR and measures of obesity. They report that the mode of the R^2 values of the correlation beten HOMA-IR and obesity measures (BMI/WHR/leptin concentration) is between 0 and 0.1, and median around 0.15 which indicates that in majority of the studies obesity can explain only 10 to 15 % of variance in insulin resistance (Vidwans and Watve, 2017). Increasing numbers of non-obese, insulin resistant diabetics also point to other reasons for insulin resistance apart from obesity (Ruderman *et al.*, 1998; Conus *et al.*, 2004; Succurro *et al.*, 2008). This is true especially in India (Zheng *et al.*, 2011; George, Jacob and Fogelfeld, 2017). Secondly, not all obese people are diabetic (Succurro *et al.*, 2008; Virtue and Vidal-Puig, 2008). As I have already mentioned above, there are many causes for insulin resistance apart from obesity (Després *et al.*, 1988; Hamburg *et al.*, 2007; Keane *et al.*, 2015;

Cleasby, Jamieson and Atherton, 2016) which have not been studied in as much details in the pathophysiology of T2DM. Free fatty acids and circulating lipids are implicated in the mechanism of the cause of insulin resistance due to obesity. But the classical hypotheses such as Randle hypothesis have failed to get experimental support (An *et al.*, 2004; Cozzone *et al.*, 2006; Monetti *et al.*, 2007; Hue and Taegtmeyer, 2009). Thus, a look at other possible mechanisms for insulin resistance apart from obesity in greater details is needed

1.3.2 Hyperinsulinemia: Compensatory or primary?

The next important point in the classical theory of T2DM pathophysiology is that of compensatory hyperinsulinemia. β-cells are said to produce more insulin after insulin resistance sets in to try and keep the insulin functioning normal (Monnier, Lapinski and Colette, 2003; DeFronzo *et al.*, 2015). Certainly, there is an association between increased levels of insulin and insulin resistance, but the direction of causality is not that clear as there are only a few studies which look at the time course. In the studies which do look at the time course, it is seen that the first detectable change is a slight hypoglycaemia indicating that hyperinsulinemia sets in first (Neel, 1962; Dubuc, 1976, 1981; Le Stunff and Bougneres, 1994; Franckhauser *et al.*, 2002). The question of the order of occurrence of insulin resistance and hyperinsulinemia has been critically addressed before in a few studies (Shanik *et al.*, 2008). There are different aspects of this question which I will elaborate below pointwise.

1.3.2.1 Insulinomas

There are natural scenarios where insulin overproduction takes place or in some cases, such conditions have been induced experimentally. Insulin is produced excessively in case of pancreatic tumours. In such cases the overproduction of insulin is normally accompanied by insulin resistance and upon removal of the tumour, the insulin sensitivity increases again (Nankervis *et al.*, 1985; Pontiroli, Alberetto and Pozza, 1992; Sawicki *et al.*, 1992; Del Prato *et al.*, 1993; Leonetti *et al.*, 1993; Liu *et al.*, 2000). Thus, in case of insulin producing tumours, it is seen that hyperinsulinemia is primary whereas insulin resistance seems to respond to and compensate for the altered insulin levels.

1.3.2.2 Insulin receptor knock-outs (IRKOs)

Let us now have a look at the reverse scenario where insulin resistance is the primary event. This has been done experimentally by knocking out the insulin receptors in specific tissues. In the Muscle insulin receptor knock-out (MIRKO) mice, the insulin receptor in the muscle tissue is knocked out making it insulin resistant. Even when the muscle tissue is insulin resistant, the insulin levels in these mice remain normal (Kim *et al.*, 2000). A whole-body insulin receptor knock-out does become hyperinsulinemic (Terauchi and Kadowaki, 2002). Liver insulin receptor knock-out (LIRKO) mice do show hyperinsulinemia, but they also show hyperglycaemia. Thus, the increased insulin levels could be a result of the increased glucose levels (Kadowaki, 2000). Thus, it is difficult to conclusively say that insulin resistance results in compensatory hyperinsulinemia unless it is accompanied by an increased level of glucose in the blood. Thus, the normoglycemic hyperinsulinemic insulin resistant state cannot be explained solely using the compensatory hyperinsulinemia theory (Watve, 2013).

1.3.2.3 Hyperinsulinemia and insulin resistance: the mechanisms

To resolve the question, what comes first hyperinsulinemia or insulin resistance, a closer look is needed, at the mechanisms given in the literature for either direction of the arrow. First, I will focus on the mechanism by which insulin resistance leads to increased insulin levels which is a postulate of the classical theory of T2DM pathophysiology. The pancreas or the β-cells need to "know/measure" the insulin resistance to give a compensatory response to the insulin resistance. According to the current model, the increased insulin resistance results in hyperglycaemia which results in an increased secretion of insulin by the β-cells. Thus, the mechanism of insulin resistance to hyperinsulinemia is via blood glucose. There is no other suggested mechanism by which this takes place. However, this mechanism does not explain the hypoglycaemia which is seen in the early phases of T2DM. In the early stages, hyperinsulinemia and hypoglycaemia are present at the same time. Thus, the insulin levels in this stage are not a result of the increased blood glucose levels. But there is no other mechanism suggested for this increased insulin (Holt *et al.*, 2010; Watve, 2013; DeFronzo *et al.*, 2015).

On the other hand, there are several mechanisms and evidence which explain how increased insulin could lead to a decrease in insulin sensitivity.

1. Insulin itself can change the responsiveness of its target tissue or cell and can downregulate its receptors (Shanik *et al.*, 2008; Watve, 2013)
2. Transgenic hyperinsulinemia: Mice were transfected stably with extra copies of the insulin gene to achieve basal insulin levels which were two or four-fold higher than normal. Despite having these high insulin levels, the mice had normal weight and normal fasting glucose but higher post-feeding glucose levels. Thus, insulin resistance was a result of increased insulin levels and this was mediated by lower binding of the insulin receptor as well as hypertriglyceridemia (Marbán and Roth, 1996; Shanik *et al.*, 2008).
3. The mTOR/S6K1 pathway acts as a built-in negative feedback loop to regulate insulin action. Insulin signalling pathway activates mTOR which in turn activates S6K1 signalling downstream. This results in reduced binding of the insulin receptor (Zick, 2005). Thus, there is an automatic reduction in the insulin response in case of increased insulin signalling (Watve, 2013).
4. Amylin is synthesized, packaged, and secreted simultaneously with insulin in the β-cells. If insulin secretion increases, amylin levels also increase. It has been demonstrated by several different researchers that amylin induces insulin resistance (Molina *et al.*, 1990; Sowa *et al.*, 1990; Frontoni *et al.*, 1991; Tabata *et al.*, 1992; Ye *et al.*, 2001; Dominici *et al.*, 2014).
5. Another mechanism by which insulin induces insulin resistance is via the protein Klotho (Chen *et al.*, 2007). Insulin secretion stimulates the release of the soluble protein Klotho and it is known that Klotho induces insulin resistance (Kurosu, 2005; Bartke, 2006).
6. Increased levels of insulin induce the secretion of serotonin in the brain and a long-term increase in serotonin signalling is known to bring on insulin resistance (Fernstrom and Wurtman, 1971; Luo, Luo and Cincotta, 1999).
7. Insulin is known to induce lipogenesis (Hua *et al.*, 2016; Titchenell *et al.*, 2016) and increased levels of free fatty acids (FFAs) and triglycerides are known to induce insulin resistance (Boden, 1997; Shi *et al.*, 2006).

Multiple demonstrated mechanisms explain the causal direction from hyperinsulinemia to insulin resistance. However, there is no clear demonstrated

mechanism which explains how insulin resistance leads to compensatory hyperinsulinemia in the absence of hyperglycaemia. And if hyperinsulinemia precedes insulin resistance, what exactly is the cause of β-cell dysfunction? According to the classical thinking, the "compensation" response of β-cells is the main cause implicated in the dysfunction of the β-cells.

1.3.3 β-cell exhaustion and insulin insufficiency leading to hyperglycaemia

According to the classical thinking about the T2DM pathophysiology, the "exhaustion" of β-cells due to overproduction of insulin during compensatory hyperinsulinemia causes β-cell dysfunction (Weir and Bonner-Weir, 2004; Holt *et al.*, 2010; DeFronzo *et al.*, 2015). In T2DM, reduction of β-cell mass is evident especially in the later stages. A 20-50% loss in the β-cell mass in diabetic subjects as compared to control subjects is demonstrated (Clark *et al.*, 1990; Porte and Kahn, 2001; Butler *et al.*, 2003). These numbers are reported using post-mortem studies of both diabetic and control subjects. Thus, a substantial portion of the β-cell mass survives even after years of diabetes. The points which show some inconsistencies and require a critical look are

i. Is exhaustion the main reason for this reduction in β-cell mass?
ii. How much reduction in insulin does this reduced β-cell mass cause?
iii. Are insulin resistance and β-cell dysfunction necessary and sufficient for hyperglycaemia?

i. There is no evidence that individual β-cells secrete more than normal amounts of insulin in a hyperinsulinemic state. Increased insulin secretion in a prediabetic state is achieved by increased β-cell number rather than increased secretion by individual cells (Hardikar *et al.*, 2015). If cells are not overworking, there is no reason to get exhausted. Chronic overuse of any organ in any body leading to its degradation or dysfunction independent of ageing is not that common. Exhaustion/overuse as a reason for degradation has been applied predominantly to pancreas and no other organ in the body (Watve, 2013). It has been sufficiently demonstrated that only hyperinsulinemia, independent of hyperglycaemia is not sufficient to cause a reduction in the β-cell mass (Kadowaki, 2000; Watve, 2013). Apart from overuse, there are many other reasons which lead to reduction in β-cell mass. They are ER stress (Kaneto *et al.*, 2001; Hotamisligil, 2010), oxidative

stress (Simmons, 2007; Fu, Gilbert and Liu, 2014), gluco-lipotoxicity (Evans *et al.*, 2003; Cnop *et al.*, 2005; Lenzen, 2008) and amyloid toxicity. The relative importance of these mechanisms is not known. At present there is no clarity on what causes the loss in number or function of β-cells.

ii. The second question which needs to be addressed, is how much the destruction of β-cells in diabetes is and how much is the insulin production reduced. The capacity of pancreas far exceeds than what is needed for normal functioning. Some experiments have demonstrated that as far as 85% of pancreas mass needs to be reduced to see hyperglycaemia (Clark *et al.*, 2001). It has also been shown that β-cells have some capacity for regeneration (Bernard *et al.*, 1998). Post-mortem studies on human diabetic subjects have shown that even in overt diabetics the β-cell destruction is not complete and about 20-50% of the β-cell mass remains even after decades of diabetes (Clark *et al.*, 2001; Deng *et al.*, 2004). Thus, reduction in β-cell number is not sufficient to explain the relative insulin insufficiency. Alternative reasoning is required to explain reduced "compensation".

iii. It needs to be examined critically whether a combination of insulin resistance and β-cell dysfunction is necessary and sufficient to cause fasting and post-prandial hyperglycaemia. This can be tested in normoglycemic hyperinsulinemic insulin resistant systems. If the insulin production in such a system is suppressed, then according to the classical thinking, sugar levels should increase. And in turn it can be said that insulin resistance and β-cell dysfunction/insulin insufficiency are enough to explain hyperglycaemia. Such experiments have been performed. It has been demonstrated in obese Zucker rats that suppression of insulin using diazoxide actually results in reduction of the fasting glucose levels due to an increase in insulin sensitivity (Schreuder *et al.*, 2005; Alemzadeh *et al.*, 2008). Insulin suppression by protein deprivation resulted in decreased levels of insulin, but this was accompanied by reduced glucose as well, indicating that reduced insulin leads to increased insulin sensitivity (Schteingart *et al.*, 1979). Another set of experiments cast a doubt on the sufficiency of insulin resistance and β-cell dysfunction to cause hyperglycaemia. These are the experiments about insulin receptor knock-outs (IRKOs). In most of these experiments, whenever the tissue

specific insulin receptors have been knocked out, the surprising result is that the sugar levels remain normal (Kadowaki, 2000; Michael *et al.*, 2000).

1.3.4 Ineffectiveness of the treatment strategies

In the earlier sections, I have shown that there are some inconsistencies in the classical thinking about T2DM which need to be addressed. Additionally, I would now like to focus on the treatment regimens in practice based on the classical insulin centric thinking. Looking at the treatment effectiveness is important because the clinicians' main aim is that of treatment of the disease despite any inconsistencies in the basic science behind it. The effectiveness of treatment of any disease or disorder can be best judged by having a look at the randomised clinical trials performed for that disease. Most of the clinical trials aim at controlling the glucose levels with the aim of preventing or delaying the complications caused due to increased sugar levels. They compare different methods of achieving this glycaemic control, based on different aspects of the physiology of glucose and insulin signalling. With increasing attempts to control the glucose levels, the glycaemic control actually becomes worse (Holman *et al.*, 2008) and only normalizing blood glucose is not enough to prevent the complications of diabetes (UKPDS 33, 1998). In the UKPDS trial it was seen that in the long term, any method of glucose control could achieve the glycaemic goal of HbA1c less than 7% only in about 25% of the subjects. This goal could be achieved by 42% subjects on insulin, 24% on sulphonylurea, 13% on metformin and 8% on dietary therapy. Thus, even if the insulin therapy shows the better results amongst the four different kinds of treatment, the success rate is less than 50% in the long run (UKPDS 33, 1998). In the UKPDS trial, intensive control of glucose using insulin therapy showed positive effects on diabetes related events, but the diabetes related mortality or all-cause mortality did not improve as compared to the other groups (Nathan, 1998).

A few other trials such as ACCORD (Skyler *et al.*, 2009; Bonds *et al.*, 2010) or NICE (The NICE-SUGAR Study Investigators, 2009) reported higher mortality in the tight glucose control group than the relaxed control group. Summing across trials currently there is no evidence that the insulin-glucose cantered treatment has a consistent beneficial effect in arresting diabetic complications. Thus, there is a

need to revisit the classical theory to come up with better or more effective treatment strategies.

1.3.5 Need to look beyond insulin?

To come up with alternative and better treatment regimens, we need to look beyond insulin. This trend has been on the rise in the past few years in both the research and the clinical community with many new drug targets and regimens in practice like the SGLT-2 receptors (Inzucchi *et al.*, 2015) and metabolic surgeries (Gloy *et al.*, 2013) as treatment options. Apart from this, there are several other molecules or pathways which affect glucose homeostasis some of which are dependent on insulin while some are independent. Whether and to what extent these molecules/pathways contribute to T2DM is not clearly known. At a very early stage of diabetes research, Claude Bernard demonstrated that damage to medulla oblongata caused severe hyperglycaemia and therefore thought that brain was the main centre for regulation of blood glucose (Bernard, 1879; Schwartz, 2005). After the discovery of insulin, the focus shifted to peripheral mechanisms and the role of brain was largely forgotten. Today the interest in the role of CNS in glucose regulation is revived considerably but it is not clear to what extent the CNS contributes to hyperglycaemia in T2DM. It is known that sympathetic stimulation increases liver glucose production whereas parasympathetic stimulation increases β-cell proliferation and insulin secretion (Nonogaki, 2000). There is evidence for a number of other molecules and metabolites being involved in the regulation of glucose which are dependent or independent of insulin like brain-derived neurotrophic factor (BDNF), which controls the hepatic glucose production independent of insulin (Meek *et al.*, 2013). Kulkarni et al 2017 describe a network model of T2DM in which they give a comprehensive list of all inter organ signals which contribute to the pathophysiology of T2DM (Kulkarni, Sharda and Watve, 2017). Their model identified close to 70 different molecules and behaviours which were altered during T2DM and could potentially serve as therapy targets (Kulkarni, Sharda and Watve, 2017). Thus, there is a growing need to look beyond insulin as a therapy in type 2 diabetes mellitus.

1.4 Why is T2DM irreversible?

There are three possible reasons why any disease or disease state is irreversible:

(a) Is there any pathophysiological mechanism that is irreversible? If insulin resistance and β-cell loss are central to T2DM, both are demonstrably reversible processes. Therefore, the classical theory does not explain why T2DM is not reversible.

(b) We do not have the technology to reverse the pathophysiological change: All types of treatment to increase insulin sensitivity, increase insulin secretion, exogenous insulin with more and more sophisticated devices have been used in the treatment. But these treatments may at the most keep hyperglycaemia in check for some time. Even this aim is not achieved consistently in the long run (UKPDS 33, 1998). So, although technology doesn't seem to have left any stone unturned, a cure for T2DM is still far from sight. The only claims for T2DM reversal are based on extreme diet and lifestyle interventions (Lean *et al.*, 2018) whose mechanism of action has not been elucidated. T2DM reversal has not been achieved by insulin sensitizing drugs and/or insulin supplementation.

(c) The third possibility is that our current interpretation/theorization of the pathophysiology of T2DM is wrong or inadequate. We have failed to identify certain mechanisms central to the pathophysiology, or the classical hypothesis is wrong. Given the inadequacy of (a) and (b), we should be open to this possibility. My aim in this thesis is to examine this possibility using multiple approaches.

1.5 Evolutionary medicine

The rationale of this thesis is to re-examine the insulin-glucose relationship with an aim to come up new basis for clinical implications. Another important aspect of disease research is to look at the cause of the disease from an evolutionary perspective. Research related to pathophysiology of any disease is based on the "how" question regarding the anatomy and physiology of the human body. Evolution addresses the "why" question. A combined understanding of how and why of a disease is likely to increase the precision and effectiveness of its treatment. Evolutionary medicine (EM) tries to explain vulnerability to a disease along with differences in vulnerabilities in the population.

EM has received serious criticism on many points. The point stated most frequently and probably the most important is that EM fails to provide insights

relevant in clinical practice (Cournoyea, 2010). The adaptationist view of EM is also criticised often. The evidence required to support adaptive argument is almost always circumstantial/ inferential and hence the argument must be considered hypothetical (Gluckman, Hanson and Spencer, 2005). Many EM theories are based on the mismatch between ancestral and the present-day environment. Claims about ancestral conditions are speculative and there are limitations in making inferences about them. Without the use of rigor for hypothesis building and efforts in making and testing differential predictions, evolutionary medicine remains at the line between science and philosophy (Cournoyea, 2010). Thus, a rigorous theoretical and empirically testable approach needs to be employed while looking at diseases from the evolutionary point of view. With this need in mind, set of criteria was developed to be expected from an evolutionary theory for any human disease and outlined an approach to evaluate alternative evolutionary hypotheses using this set (Watve and Diwekar-Joshi, 2016). After re-examining the insulin-glucose relationship, I will return to EM and examine the implications of the new understanding about insulin-glucose relationship.

1.6 Aims of the thesis

Insulin and absolute or relative insufficiency is believed to be the central cause of hyperglycaemia in T2DM. A plethora of molecules other than insulin are known to influence the glucose homeostasis (Kulkarni, Sharda and Watve, 2017). Insulin treatment to regulate the glucose levels tightly is seen to effective on a short-term basis. This treatment however fails in the long run (Holman *et al.*, 2008). There are also many points unexplained by the classical insulin centric theory. Hence, I aim to critically re-examine the role of insulin in glucose homeostasis using multiple approaches which are complementary to each other.

(i) systematic review and meta-analysis of tissue specific insulin receptor knock-out experiments,

(ii) systematic review and meta-analysis of insulin suppression and insulin enhancement experiments,

(iii) epidemiological data,

(iv) differentiating steady state and post-meal state glucose levels in streptozotocin treated rats in primary experiments, and

(v) inferring causality from steady state correlations

After revisiting the insulin-glucose relationship or re-examining the role of insulin in the glucose homeostasis, I would like to explore this from the evolutionary point of view as well.

1.7 Arrangement of the thesis

With the aims of the thesis mentioned above, and the approaches outlined in the earlier section, this is the arrangement of the thesis in brief:

Chapter 1: Introduction

Introduction to the classical theory to explain the pathophysiology of type 2 diabetes mellitus, and the discrepancies in the classical theory leading to the need of critical re-examination of the insulin-glucose relationship.

Chapter 2: Does impairment of insulin signalling affect steady state glucose? A meta-analyses approach

Using meta-analyses from experiments from published literature, I will demonstrate in this chapter that increasing or decreasing insulin or insulin action *in vivo* does not alter the steady state glucose, but it alters the perturbed state.

Chapter 3: Theoretical, mathematical, and statistical considerations

In this chapter, I will explore possible explanations for the consistent results of the meta-analyses, namely the failure of experimental insulin signal impairment to alter steady state glucose level. I will also make differential predictions from alternative homeostasis models that and test them in human epidemiological data.

Chapter 4: Does impairment of insulin signalling affect steady state glucose? The streptozotocin (STZ) model

In this chapter, I will summarise the primary experiments performed on Sprague Dawley rats. Suppression of insulin using Streptozotocin alters the steady state glucose, but not the perturbed state glucose. This concurs with the result of the meta-analyses and the statistical analyses of the epidemiological data.

Chapter 5: Fasting glucose and fasting insulin and insulin resistance: inferring causal relations

In the earlier chapters, I have established that the relationship between insulin and glucose is different in the steady state versus the perturbed state. Impairment of the insulin function/signalling does lead to a change in the glucose levels in the fasting state, but only in the post-meal or perturbed state. But this change is significant only in the perturbed/post-meal state. This leads to a broader question of finding out the causality in the steady state versus the perturbed state. Using a novel statistical and theoretical method developed in our lab, I will delineate the relationship between fasting glucose, fasting insulin and insulin resistance.
I will then highlight the role of insulin as a driver, but not a navigator in glucose regulation. The main clinical implication of this is that we need to focus on other potential navigators of glucose which could prove to be alternate or supplementary therapy targets to insulin. I also expand on this driver-navigator causation concept on a broader sense in homeostasis.

Chapter 6: Implications for evolutionary medicine

In the earlier chapters, I re-examined the insulin-glucose relationship with a view of distinguishing between the relationship in the steady state versus the perturbed state. After having gained certain insights into it, I will then examine the implications of this new understanding about insulin-glucose relationship from an evolutionary point of view.

Chapter 7: Conclusions and outlook

I will summarise the conclusions of each chapter and discuss the implications of these conclusions on current understanding of type 2 diabetes. I will also give a brief outlook about how can these lines of work be taken further.

1.8 References

Alemzadeh, R. *et al.* (2008) 'Diazoxide enhances basal metabolic rate and fat oxidation in obese Zucker rats', *Metabolism*, 57(11), pp. 1597–1607. doi: 10.1016/j.metabol.2008.06.017.

Alonso, R. *et al.* (2016) 'The Genetics of Obesity', in *Translational Cardiometabolic Genomic Medicine*. Elsevier, pp. 161–177. doi: 10.1016/B978-0-12-799961-6.00007-X.

An, J. *et al.* (2004) 'Hepatic expression of malonyl-CoA decarboxylase reverses muscle, liver and whole-animal insulin resistance', *Nature Medicine*, 10(3), pp. 268–274. doi: 10.1038/nm995.

Bartke, A. (2006) 'Long-lived Klotho mice: new insights into the roles of IGF-1 and insulin in aging', *Trends in Endocrinology & Metabolism*, 17(2), pp. 33–35. doi: 10.1016/j.tem.2006.01.002.

Bernard, C. (1879) 'Leçons de physiologie opératoire', *Librairie J.-B. Baillie et fils*, p. 650.

Bernard, C. *et al.* (1998) 'Pancreatic beta-cell regeneration after 48-h glucose infusion in mildly diabetic rats is not correlated with functional improvement', *Diabetes*, 47(7), pp. 1058–1065. doi: 10.2337/diabetes.47.7.1058.

Boden, G. (1997) 'Role of Fatty Acids in the Pathogenesis of Insulin Resistance and NIDDM', *Diabetes*, 46(1), pp. 3–10. doi: 10.2337/diab.46.1.3.

Bonds, D. E. *et al.* (2010) 'The association between symptomatic, severe hypoglycaemia and mortality in type 2 diabetes: Retrospective epidemiological analysis of the ACCORD study', *BMJ (Online)*, 340(7738), p. 137. doi: 10.1136/bmj.b4909.

Butler, A. E. *et al.* (2003) 'Beta-Cell Deficit and Increased -Cell Apoptosis in Humans With Type 2 Diabetes', *Diabetes*, 52(1), pp. 102–110. doi: 10.2337/diabetes.52.1.102.

Campfield, L. A. and Smith, F. J. (1980) 'Modulation of insulin secretion by the autonomic nervous system', *Brain Research Bulletin*, 5, pp. 103–107. doi: 10.1016/0361-9230(80)90238-5.

Canivell, S. and Gomis, R. (2014) 'Diagnosis and classification of autoimmune diabetes mellitus', *Autoimmunity Reviews*, pp. 403–407. doi: 10.1016/j.autrev.2014.01.020.

Cerf, M. E. (2013) 'B-cell Dysfunction and Insulin Resistance', *Frontiers in Endocrinology*, 4. doi: 10.3389/fendo.2013.00037.

Chan, C. B. *et al.* (2004) 'Uncoupling Protein 2 and Islet Function', *Diabetes*, 53(Supplement 1), pp. S136–S142. doi: 10.2337/diabetes.53.2007.S136.

Chen, C.-D. *et al.* (2007) 'Insulin stimulates the cleavage and release of the extracellular domain of Klotho by ADAM10 and ADAM17', *Proceedings of the National Academy of Sciences*, 104(50), pp. 19796–19801. doi: 10.1073/pnas.0709805104.

Cheng, A. Y. Y. and Fantus, I. G. (2005) 'Cheng, Fantus - 2005 - Oral antihyperglycemic therapy for type 2 diabetes mellitus', 172(2), pp. 213–226.

Clark, A. *et al.* (1990) 'Islet amyloid polypeptide in diabetic and non-diabetic Pima Indians', *Diabetologia*, 33(5), pp. 285–289. doi: 10.1007/BF00403322.

Clark, A. *et al.* (2001) 'Decreased insulin secretion in type 2 diabetes: a problem of cellular mass or function?', *Diabetes*, 50(Supplement 1), pp. S169–S171. doi: 10.2337/diabetes.50.2007.S169.

Cleasby, M. E., Jamieson, P. M. and Atherton, P. J. (2016) 'Insulin resistance and sarcopenia: mechanistic links between common co-morbidities', *Journal of Endocrinology*, 229(2), pp. R67–R81. doi: 10.1530/JOE-15-0533.

Cnop, M. *et al.* (2005) 'Mechanisms of Pancreatic -Cell Death in Type 1 and Type 2 Diabetes:

Many Differences, Few Similarities', *Diabetes*, 54(Supplement 2), pp. S97–S107. doi: 10.2337/diabetes.54.suppl_2.S97.

Cockburn, B. N. *et al.* (1997) 'Changes in Pancreatic Islet Glucokinase and Hexokinase Activities With Increasing Age, Obesity, and the Onset of Diabetes', *Diabetes*, 46(9), pp. 1434–1439. doi: 10.2337/diab.46.9.1434.

Conus, F. *et al.* (2004) 'Metabolic and behavioral characteristics of metabolically obese but normal-weight women', *Journal of Clinical Endocrinology and Metabolism*, 89(10), pp. 5013–5020. doi: 10.1210/jc.2004-0265.

Cournoyea, M. (2010) An epistemic and ethical critique of evolutionary medicine. Munk School Briefings, University of Toronto, ISSN 1715-3484.

Cozzone, D. *et al.* (2006) 'Activation of liver X receptors promotes lipid accumulation but does not alter insulin action in human skeletal muscle cells', *Diabetologia*, 49(5), pp. 990–999. doi: 10.1007/s00125-006-0140-8.

Cummings, D. E. and Rubino, F. (2018) 'Metabolic surgery for the treatment of type 2 diabetes in obese individuals LAGB Laparoscopic adjustable gastric banding NIH National Institutes of Health QALY Quality-adjusted life-year'. Diabetologia, 358280, pp. 257–264. doi: 10.1007/s00125-017-4513-y.

DeFronzo, R. A. (1988) 'The Triumvirate: -Cell, Muscle, Liver: A Collusion Responsible for NIDDM', *Diabetes*, 37(6), pp. 667–687. doi: 10.2337/diab.37.6.667.

DeFronzo, R. A. *et al.* (eds) (2015) *International Textbook of Diabetes Mellitus*. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9781118387658.

DeFronzo, R. A., Eldor, R. and Bdul-Ghani, M. A. (2013) 'Pathophysiologic approach to therapy in patients with newly diagnosed type 2 diabetes', *Diabetes Care*, 36(SUPPL.2). doi: 10.2337/dcS13-2011.

Deng, S. *et al.* (2004) 'Structural and Functional Abnormalities in the Islets Isolated From Type 2 Diabetic Subjects', *Diabetes*, 53(3), pp. 624–632. doi: 10.2337/diabetes.53.3.624.

Després, J. P. *et al.* (1988) 'Abdominal adipose tissue and serum HDL-cholesterol: association independent from obesity and serum triglyceride concentration.', *International journal of obesity*, 12(1), pp. 1–13. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2966132.

Dominici, F. P. *et al.* (2014) 'Modulation of the action of insulin by angiotensin-(1–7)', *Clinical Science*, 126(9), pp. 613–630. doi: 10.1042/cs20130333.

Dubuc, P. U. (1976) 'The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice', *Metabolism*, 25(12), pp. 1567–1574. doi: 10.1016/0026-0495(76)90109-8.

Dubuc, P. U. (1981) 'Non-Essential Role of Dietary Factors in the Development of Diabetes in ob/ob Mice', *The Journal of Nutrition*, 111(10), pp. 1742–1748. doi: 10.1093/jn/111.10.1742.

Duckworth, W. *et al.* (2009) 'Glucose Control and Vascular Complications in Veterans with Type 2 Diabetes', *New England Journal of Medicine*, 360(2), pp. 129–139. doi: 10.1056/NEJMoa0808431.

Epstein, F. H., Atkinson, M. A. and Maclaren, N. K. (1994) 'The Pathogenesis of Insulin-Dependent Diabetes Mellitus', *New England Journal of Medicine*, 331(21), pp. 1428–1436. doi: 10.1056/NEJM199411243312107.

Evans, J. L. *et al.* (2003) 'Are Oxidative Stress-Activated Signaling Pathways Mediators of Insulin Resistance and -Cell Dysfunction?', *Diabetes*, 52(1), pp. 1–8. doi: 10.2337/diabetes.52.1.1.

Fernstrom, J. D. and Wurtman, R. J. (1971) 'Brain Serotonin Content: Increase Following Ingestion of Carbohydrate Diet', *Science*, 174(4013), pp. 1023–1025. doi: 10.1126/science.174.4013.1023.

Fizelova, M. *et al.* (2017) 'Differential Associations of Inflammatory Markers With Insulin Sensitivity and Secretion: The Prospective METSIM Study', *The Journal of Clinical Endocrinology & Metabolism*, 102(9), pp. 3600–3609. doi: 10.1210/jc.2017-01057.

Franckhauser, S. *et al.* (2002) 'Increased Fatty Acid Re-esterification by PEPCK Overexpression in Adipose Tissue Leads to Obesity Without Insulin Resistance', *Diabetes*, 51(3), pp. 624–630. doi: 10.2337/diabetes.51.3.624.

Frontoni, S. *et al.* (1991) 'In Vivo Insulin Resistance Induced by Amylin Primarily Through Inhibition of Insulin-Stimulated Glycogen Synthesis in Skeletal Muscle', *Diabetes*, 40(5), pp. 568–573. doi: 10.2337/diab.40.5.568.

Fu, Z., Gilbert, E. R. and Liu, D. (2014) 'Regulation of insulin synthesis and secretion and pancreatic beta-cell dysfuntion in diabetes', *Curr Diabetes Rev.*, 9(1), pp. 25–53.

Fujioka, S. *et al.* (1987) 'Contribution of intra-abdominal fat accumulation to the impairment of glucose and lipid metabolism in human obesity.', *Metabolism: clinical and experimental*, 36(1), pp. 54–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3796297.

Garvey, W. T., Olefsky, J. M. and Marshall, S. (1986) 'Insulin Induces Progressive Insulin Resistance in Cultured Rat Adipocytes: Sequential Effects at Receptor and Multiple Postreceptor Sites', *Diabetes*, 35(3), pp. 258–267. doi: 10.2337/diab.35.3.258.

George, A. M., Jacob, A. G. and Fogelfeld, L. (2017) 'Lean diabetes mellitus: An emerging entity in the era of obesity', *World Journal of Diabetes*, 6(4), p. 613. doi: 10.4239/wjd.v6.i4.613.

Giardino, I., Edelstein, D. and Brownlee, M. (1996) 'BCL-2 expression or antioxidants prevent hyperglycemia-induced formation of intracellular advanced glycation endproducts in bovine endothelial cells.', *Journal of Clinical Investigation*, 97(6), pp. 1422–1428. doi: 10.1172/JCI118563.

Gloy, V. L. *et al.* (2013) 'Bariatric surgery versus non-surgical treatment for obesity: a systematic review and meta-analysis of randomised controlled trials', *Bmj*, 347(oct22 1), pp. f5934–f5934. doi: 10.1136/bmj.f5934.

Gluckman, P. D., Hanson, M. A. and Spencer, H. G. (2005) 'Predictive adaptive responses and human evolution', *Trends in Ecology & Evolution*, 20(10), pp. 527–533. doi: 10.1016/j.tree.2005.08.001.

Greisen, J. *et al.* (2001) 'Acute pain induces insulin resistance in humans', *Anesthesiology*, 95(3), pp. 578–584. doi: 10.1097/00000542-200109000-00007.

Hamburg, N. M. *et al.* (2007) 'Physical Inactivity Rapidly Induces Insulin Resistance and Microvascular Dysfunction in Healthy Volunteers', *Arteriosclerosis, Thrombosis, and Vascular Biology*, 27(12), pp. 2650–2656. doi: 10.1161/ATVBAHA.107.153288.

Hardikar, A. A. *et al.* (2015) 'Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation', *Cell Metabolism*, 22(2), pp. 312–319. doi: 10.1016/j.cmet.2015.06.008.

Hasnain, S. Z., Prins, J. B. and McGuckin, M. A. (2016) 'Oxidative and endoplasmic reticulum stress in β-cell dysfunction in diabetes', *Journal of Molecular Endocrinology*, 56(2), pp. R33–R54. doi: 10.1530/JME-15-0232.

Holman, R. R. *et al.* (2008) '10-Year Follow-up of Intensive Glucose Control in Type 2 Diabetes', *New England Journal of Medicine*, 359(15), pp. 1577–1589. doi: 10.1056/NEJMoa0806470.

Holt, R. I. G. *et al.* (eds) (2010) *Textbook of Diabetes*. Oxford, UK: Wiley-Blackwell. doi: 10.1002/9781444324808.

Hotamisligil, G. S. (2010) 'Endoplasmic Reticulum Stress and the Inflammatory Basis of Metabolic Disease', *Cell*, 140(6), pp. 900–917. doi: 10.1016/j.cell.2010.02.034.

Hua, Z. G. *et al.* (2016) 'Glucose and Insulin Stimulate Lipogenesis in Porcine Adipocytes: Dissimilar and Identical Regulation Pathway for Key Transcription Factors', *Molecules and Cells*, 39(11), pp. 797–806. doi: 10.14348/molcells.2016.0144.

Hue, L. and Taegtmeyer, H. (2009) 'The Randle cycle revisited: a new head for an old hat', *American Journal of Physiology-Endocrinology and Metabolism*, 297(3), pp. E578–E591. doi: 10.1152/ajpendo.00093.2009.

Hwang, D. Y. *et al.* (2007) 'Significant change in insulin production, glucose tolerance and ER stress signaling in transgenic mice coexpressing insulin-siRNA and human IDE', *International Journal of Molecular Medicine*, 19(1), pp. 65–73. doi: 10.3892/ijmm.19.1.65.

IDF, International Diabetes Federation Atlas, 8th Edition. (2017) ISBN 978-2-930229-87-4 https://www.diabetesatlas.org/

Inzucchi, S. E. (2002) 'Oral antihyperglycemic therapy for type 2 diabetes: Scientific review', *Journal of the American Medical Association*, 287(3), pp. 360–372. doi: 10.1001/jama.287.3.360.

Inzucchi, S. E. *et al.* (2015) 'SGLT-2 inhibitors and cardiovascular risk: Proposed pathways and review of ongoing outcome trials', *Diabetes and Vascular Disease Research*, 12(2), pp. 90–100. doi: 10.1177/1479164114559852.

Kadowaki, T. (2000) 'Insights into insulin resistance and type 2 diabetes from knockout mouse models', *Journal of Clinical Investigation*, 106(4), pp. 459–465. doi: 10.1172/JCI10830.

Kaneto, H. *et al.* (2001) 'Activation of the Hexosamine Pathway Leads to Deterioration of Pancreatic β-Cell Function through the Induction of Oxidative Stress', *Journal of Biological Chemistry*, 276(33), pp. 31099–31104. doi: 10.1074/jbc.M104115200.

Keane, K. N. *et al.* (2015) 'Molecular Events Linking Oxidative Stress and Inflammation to Insulin Resistance and β -Cell Dysfunction', *Oxidative Medicine and Cellular Longevity*, 2015, pp. 1–15. doi: 10.1155/2015/181643.

Kim, J. K. *et al.* (2000) 'Redistribution of substrates to adipose tissue promotes obesity in mice with selective insulin resistance in muscle', *Journal of Clinical Investigation*, 105(12), pp. 1791–1797. doi: 10.1172/JCI8305.

Kirwan, J. ., Sacks, J. and Niewoudt, S. (2017) 'The essential role of exercise in the management of type 2 diabetes', *Cleveland Clinic Journal of Medicine*, 84(7 suppl 1), pp. S15–S21. doi: 10.3949/ccjm.84.s1.03.

Kirwan, J. P. *et al.* (1992) 'Eccentric exercise induces transient insulin resistance in healthy individuals', *Journal of Applied Physiology*, 72(6), pp. 2197–2202. doi: 10.1152/jappl.1992.72.6.2197.

Kudva, Y. C. and Butler, P. C. (1997) 'Insulin Secretion in Type II Diabetes Mellitus', in *Clinical Research in Diabetes and Obesity*. Totowa, NJ: Humana Press, pp. 119–136. doi: 10.1007/978-1-4757-3906-0_7.

Kulkarni, S., Sharda, S. and Watve, M. (2017) 'Bi-stability in type 2 diabetes mellitus multi-organ signalling network', *PLOS ONE*. Edited by C. Cras-Méneur, 12(8), p. e0181536. doi: 10.1371/journal.pone.0181536.

Kurosu, H. (2005) 'Suppression of Aging in Mice by the Hormone Klotho', *Science*, 309(5742),

pp. 1829–1833. doi: 10.1126/science.1112766.

Larsson, B. *et al.* (1984) 'Abdominal adipose tissue distribution, obesity, and risk of cardiovascular disease and death: 13 year follow up of participants in the study of men born in 1913.', *BMJ*, 288(6428), pp. 1401–1404. doi: 10.1136/bmj.288.6428.1401.

Lean, M. E. *et al.* (2018) 'Primary care-led weight management for remission of type 2 diabetes (DiRECT): an open-label, cluster-randomised trial', *The Lancet*, 391(10120), pp. 541–551. doi: 10.1016/S0140-6736(17)33102-1.

Lean, M. E. J. (2019) 'Low-calorie diets in the management of type 2 diabetes mellitus', *Nature Reviews Endocrinology*. Springer US, 15(5), pp. 251–252. doi: 10.1038/s41574-019-0186-6.

Lebovitz, H. E. (2011) 'Insulin: Potential negative consequences of early routine use in patients with type 2 diabetes', *Diabetes Care*, 34(SUPPL. 2). doi: 10.2337/dc11-s225.

Lee, J., Giordano, S. and Zhang, J. (2012) 'Autophagy, mitochondria and oxidative stress: cross-talk and redox signalling', *Biochemical Journal*, 441(2), pp. 523–540. doi: 10.1042/BJ20111451.

Lenzen, S. (2008) 'Oxidative stress: the vulnerable β-cell', *Biochemical Society Transactions*, 36(3), pp. 343–347. doi: 10.1042/BST0360343.

Leonetti, F. *et al.* (1993) 'Absence of clinically overt atherosclerotic vascular disease and adverse changes in cardiovascular risk factors in 70 patients with insulinoma', *Journal of Endocrinological Investigation*, 16(11), pp. 875–880. doi: 10.1007/BF03348949.

Lin, C.-Y. *et al.* (2012) 'Flavonoids protect pancreatic beta-cells from cytokines mediated apoptosis through the activation of PI3-kinase pathway', *Cytokine*, 59(1), pp. 65–71. doi: 10.1016/j.cyto.2012.04.011.

Liu, J. *et al.* (2000) 'The Intracellular Mechanism of Insulin Resistance in Pancreatic Cancer Patients 1', *The Journal of Clinical Endocrinology & Metabolism*, 85(3), pp. 1232–1238. doi: 10.1210/jcem.85.3.6400.

Luo, S., Luo, J. and Cincotta, A. H. (1999) 'Chronic Ventromedial Hypothalamic Infusion of Norepinephrine and Serotonin Promotes Insulin Resistance and Glucose Intolerance', *Neuroendocrinology*, 70(6), pp. 460–465. doi: 10.1159/000054508.

Marbán, S. L. and Roth, J. (1996) 'Transgenic hyperinsulinemia: A mouse model of insulin resistance and glucose intolerance without obesity', in *Lessons from Animal Diabetes VI*. Boston, MA: Birkhäuser Boston, pp. 201–224. doi: 10.1007/978-1-4612-4112-6_13.

Mayfield, J. A. and White, R. D. (2004) 'Insulin therapy for type 2 diabetes: rescue, augmentation, and replacement of beta-cell function.', *American family physician*, 70(3), pp. 489–500. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15317436.

Meek, T. H. *et al.* (2013) 'BDNF Action in the Brain Attenuates Diabetic Hyperglycemia via Insulin-Independent Inhibition of Hepatic Glucose Production', *Diabetes*, 62(5), pp. 1512–1518. doi: 10.2337/db12-0837.

Michael, M. D. *et al.* (2000) 'Loss of Insulin Signaling in Hepatocytes Leads to Severe Insulin Resistance and Progressive Hepatic Dysfunction', *Molecular Cell*, 6(1), pp. 87–97. doi: 10.1016/S1097-2765(05)00015-8.

Molina, J. M. *et al.* (1990) 'Induction of Insulin Resistance In Vivo by Amylin and Calcitonin Gene-Related Peptide', *Diabetes*, 39(2), pp. 260–265. doi: 10.2337/diab.39.2.260.

Monetti, M. *et al.* (2007) 'Dissociation of Hepatic Steatosis and Insulin Resistance in Mice Overexpressing DGAT in the Liver', *Cell Metabolism*, 6(1), pp. 69–78. doi: 10.1016/j.cmet.2007.05.005.

Monnier, L., Lapinski, H. and Colette, C. (2003) 'Contributions of fasting and postprandial glucose to overall hyperglycemia of type 2 diabetic patients', *Diabetes Care*, 26(3), pp. 881–883.

Morris, A. P. *et al.* (2012) 'Large-scale association analysis provides insights into the genetic architecture and pathophysiology of type 2 diabetes.', *Nature genetics*, 44(9), pp. 981–90. doi: 10.1038/ng.2383.

Nankervis, A. *et al.* (1985) 'Hyperinsulinaemia and insulin insensitivity: studies in subjects with insulinoma', *Diabetologia*, 28(7), pp. 427–431. doi: 10.1007/BF00280885.
Nathan, D. M. (1998) 'Some answers, more controversy, from UKPDS', *The Lancet*, 352(9131), pp. 832–833. doi: 10.1016/S0140-6736(98)22937-0.

Neel, J. V (1962) 'Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"?', *American journal of human genetics*, 14, pp. 353–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13937884.

Nonogaki, K. (2000) 'New insights into sympathetic regulation of glucose and fat metabolism', *Diabetologia*, 43(5), pp. 533–549. doi: 10.1007/s001250051341.

Pick, A. *et al.* (1998) 'Role of apoptosis in failure of beta-cell mass compensation for insulin resistance and beta-cell defects in the male Zucker diabetic fatty rat', *Diabetes*, 47(3), pp. 358–364. doi: 10.2337/diabetes.47.3.358.

Pillon, N. J. *et al.* (2021) 'Metabolic consequences of obesity and type 2 diabetes: Balancing genes and environment for personalized care', *Cell*, 184(6), pp. 1530–1544. doi: 10.1016/j.cell.2021.02.012

Poitout, V. and Robertson, R. P. (2002) 'Minireview: Secondary β-Cell Failure in Type 2 Diabetes—A Convergence of Glucotoxicity and Lipotoxicity', *Endocrinology*, 143(2), pp. 339–342. doi: 10.1210/endo.143.2.8623.

Pontiroli, A. E., Alberetto, M. and Pozza, G. (1992) 'Patients with insulinoma show insulin resistance in the absence of arterial hypertension', *Diabetologia*, 35(3), pp. 294–295. doi: 10.1007/BF00400934.

Poretsky, L. (ed.) (2010) *Principles of Diabetes Mellitus*. Boston, MA: Springer US. doi: 10.1007/978-0-387-09841-8.

Porte, D. and Kahn, S. E. (2001) 'Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms', *Diabetes*, 50(Supplement 1), pp. S160–S163. doi: 10.2337/diabetes.50.2007.S160.

Del Prato, S. *et al.* (1993) 'Mechanisms of fasting hypoglycemia and concomitant insulin resistance in insulinoma patients', *Metabolism*, 42(1), pp. 24–29. doi: 10.1016/0026-0495(93)90167-M.

Reaven, G. M. (1995) 'Pathophysiology of insulin resistance in human disease', *Physiological Reviews*, 75(3), pp. 473–486. doi: 10.1152/physrev.1995.75.3.473.

Ruderman, N. *et al.* (1998) 'The metabolically obese, normal-weight individual revisited', *Diabetes*, 47(5), pp. 699–713. doi: 10.2337/diabetes.47.5.699.

Samuel, V. T. and Shulman, G. I. (2016) 'The pathogenesis of insulin resistance: Integrating signaling pathways and substrate flux', *Journal of Clinical Investigation*, 126(1), pp. 12–22. doi: 10.1172/JCI77812.

Sawicki, P. T. *et al.* (1992) 'Normal blood pressure in patients with insulinoma despite hyperinsulinemia and insulin resistance.', *Journal of the American Society of Nephrology : JASN*, 3(4 Suppl), pp. S64-8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1457762.

Schreuder, T. *et al.* (2005) 'Diazoxide-mediated insulin suppression in obese men: a dose-response

study*', *Diabetes, Obesity and Metabolism*, 7(3), pp. 239–245. doi: 10.1111/j.1463-1326.2004.00449.x.

Schteingart, D. E. *et al.* (1979) 'Suppression of Insulin Secretion by Protein Deprivation in Obesity', in, pp. 125–132. doi: 10.1007/978-1-4615-9110-8_19.

Schwartz, M. W. (2005) 'Diabetes, Obesity, and the Brain', *Science*, 307(5708), pp. 375–379. doi: 10.1126/science.1104344.

Scott, R. A. *et al.* (2017) 'An Expanded Genome-Wide Association Study of Type 2 Diabetes in Europeans', *Diabetes*, 66(11), pp. 2888–2902. doi: 10.2337/db16-1253.

Shanik, M. H. *et al.* (2008) 'Insulin Resistance and Hyperinsulinemia: Is hyperinsulinemia the cart or the horse?', *Diabetes Care*, 31(Supplement 2), pp. S262–S268. doi: 10.2337/dc08-s264.

Shi, H. *et al.* (2006) 'TLR4 links innate immunity and fatty acid–induced insulin resistance', *Journal of Clinical Investigation*, 116(11), pp. 3015–3025. doi: 10.1172/JCI28898.

Simmons, R. A. (2007) 'Role of metabolic programming in the pathogenesis of β-cell failure in postnatal life', *Reviews in Endocrine and Metabolic Disorders*, 8(2), pp. 95–104. doi: 10.1007/s11154-007-9045-1.

Skyler, J. S. *et al.* (2009) 'Intensive glycemic control and the prevention of cardiovascular events: Implications of the ACCORD, ADVANCE, and VA diabetes trials', *Diabetes Care*, 32(1), pp. 187–192. doi: 10.2337/dc08-9026.

Sowa, R. *et al.* (1990) 'Islet amyloid polypeptide amide causes peripheral insulin resistance in vivo in dogs', *Diabetologia*, 33(2), pp. 118–120. doi: 10.1007/BF00401051.

Srikanthan, P., Hevener, A. L. and Karlamangla, A. S. (2010) 'Sarcopenia Exacerbates Obesity-Associated Insulin Resistance and Dysglycemia: Findings from the National Health and Nutrition Examination Survey III', *PLoS ONE*. Edited by C. P. Earnest, 5(5), p. e10805. doi: 10.1371/journal.pone.0010805.

Study, I. T. N.-S. (2009) 'Intensive versus Conventional Glucose Control in Critically Ill Patients', *New England Journal of Medicine*, 360(13), pp. 1283–1297. doi: 10.1056/NEJMoa0810625.

Le Stunff, C. and Bougneres, P. (1994) 'Early Changes in Postprandial Insulin Secretion, Not in Insulin Sensitivity, Characterize Juvenile Obesity', *Diabetes*, 43(5), pp. 696–702. doi: 10.2337/diab.43.5.696.

Succurro, E. *et al.* (2008) 'Insulin Secretion in Metabolically Obese, but Normal Weight, and in Metabolically Healthy but Obese Individuals', *Obesity*, 16(8), pp. 1881–1886. doi: 10.1038/oby.2008.308.

Tabata, H. *et al.* (1992) 'Islet amyloid polypeptide (IAPP/amylin) causes insulin resistance in perfused rat hindlimb muscle.', *Diabetes research and clinical practice*, 15(1), pp. 57–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1541236.

Terauchi, Y. and Kadowaki, T. (2002) 'Insights into Molecular Pathogenesis of Type 2 Diabetes from Knockout Mouse Models.', *Endocrine Journal*, 49(3), pp. 247–263. doi: 10.1507/endocrj.49.247.

Templeman, N. M. *et al.* (2017) 'A causal role for hyperinsulinemia in obesity', *Journal of Endocrinology*, 232(3), pp. R173–R183. doi: 10.1530/JOE-16-0449.

Thaler, J. P. and Cummings, D. E. (2009) 'Hormonal and Metabolic Mechanisms of Diabetes Remission after Gastrointestinal Surgery', *Endocrinology*, 150(6), pp. 2518–2525. doi: 10.1210/en.2009-0367.

Titchenell, P. M. *et al.* (2016) 'Direct Hepatocyte Insulin Signaling Is Required for Lipogenesis but Is Dispensable for the Suppression of Glucose Production', *Cell Metabolism*, 23(6), pp. 1154–1166. doi: 10.1016/j.cmet.2016.04.022.

UK Prospective Diabetes Study Group (1998) 'Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)', *The Lancet*, 352(9131), pp. 837–853. doi: 10.1016/S0140-6736(98)07019-6.

UK Prospective Diabetes Study Group (2008) 'Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes (UKPDS 38). UK Prospective Diabetes Study Group.', *BMJ (Clinical research ed.)*, 317(7160), pp. 703–13. doi: 10.1007/s10291-013-0357-1.

Vidwans, H. B. and Watve, M. G. (2017) 'How much variance in insulin resistance is explained by obesity?', *Journal of Insulin Resistance*, 2(1), p. 7. doi: 10.4102/jir.v2i1.22.

Vilsbøll, T. and Holst, J. J. (2004) 'Incretins, insulin secretion and Type 2 diabetes mellitus', *Diabetologia*, 47(3), pp. 357–366. doi: 10.1007/s00125-004-1342-6.

Virtue, S. and Vidal-Puig, A. (2008) 'It's not how fat you are, it's what you do with it that counts', *PLoS Biology*, 6(9), pp. 1819–1823. doi: 10.1371/journal.pbio.0060237.

Watve, M. (2013) *Doves, Diplomats, and Diabetes*. New York, NY: Springer New York. doi: 10.1007/978-1-4614-4409-1. https://www.who.int/publications/i/item/9789241565257

Watve, M. and Diwekar-Joshi, M. (2016) 'What to expect from an evolutionary hypothesis for a human disease: The case of type 2 diabetes', *HOMO*, 67(5), pp. 349–368. doi: 10.1016/j.jchb.2016.07.001.

Weir, G. C. and Bonner-Weir, S. (2004) 'Five Stages of Evolving Beta-Cell Dysfunction During Progression to Diabetes', *Diabetes*, 53(Supplement 3), pp. S16–S21. doi: 10.2337/diabetes.53.suppl_3.S16.

Weyer, C. *et al.* (2000) 'A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia', *Diabetes*, 49(12), pp. 2094–2101. doi: 10.2337/diabetes.49.12.2094.

WHO Report on Diabetes. World Health Organiszation, Global Report on Diabetes. (2016). ISBN 978 92 4 156525 7.

Ye, J.-M. *et al.* (2001) 'Evidence that amylin stimulates lipolysis in vivo: a possible mediator of induced insulin resistance', *American Journal of Physiology-Endocrinology and Metabolism*, 280(4), pp. E562–E569. doi: 10.1152/ajpendo.2001.280.4.E562.

Zanuso, S. *et al.* (2017) 'Exercise in type 2 diabetes: genetic, metabolic and neuromuscular adaptations. A review of the evidence', *British Journal of Sports Medicine*, 51(21), pp. 1533–1538. doi: 10.1136/bjsports-2016-096724.

Zhai, X. *et al.* (2016) 'A Correlative Relationship Between Chronic Pain and Insulin Resistance in Zucker Fatty Rats: Role of Downregulation of Insulin Receptors', *The Journal of Pain*, 17(4), pp. 404–413. doi: 10.1016/j.jpain.2015.12.003.

Zheng, W. *et al.* (2011) 'Association between Body-Mass Index and Risk of Death in More Than 1 Million Asians', *New England Journal of Medicine*, 364(8), pp. 719–729. doi: 10.1056/NEJMoa1010679.

Zick, Y. (2005) 'Ser/Thr Phosphorylation of IRS Proteins: A Molecular Basis for Insulin Resistance', *Science Signaling*, 2005(268), pp. pe4–pe4. doi: 10.1126/stke.2682005pe4.

Zraika, S. *et al.* (2009) 'Oxidative stress is induced by islet amyloid formation and time-dependently mediates amyloid-induced β-cell apoptosis', *Diabetologia*, 52(4), pp. 626–635. doi: 10.1007/s00125-008-1255-x.

Chapter 2: Does impairment of insulin signalling affect steady state glucose? A meta-analyses approach

2.1 Introduction

2.1.1: A brief history of insulin: Why is insulin considered to be the central and practically the only mechanism of glucose regulation?

Claud Bernard demonstrated that damage to medulla oblongata results in hyperglycaemia (Bernard, 1879). This was one of the first and major breakthroughs in understanding the regulation of glucose. The second major advancement was the demonstration by von Mering and Minkowski that removal of the pancreas resulted in hyperglycaemia (Mering and Minkowski, 1890) and further that pancreatic extracts resulted in lowering of plasma glucose. The active chemical from pancreas was eventually purified and called as insulin (Karamitsos, 2011). The discovery and success of insulin in treating diabetes was astounding. Hence insulin became the modal molecule in glucose homeostasis and the role of brain and other mechanisms were practically forgotten. The prevalent type of diabetes then was what we would label as T1DM today which is characterised by an almost complete destruction of pancreatic β-cells. For T2DM like patients, diet therapy was the main mode of treatment and insulin was administered for patients who could not follow the diet or had severe hyperglycaemia (Maria Rotella, Pala and Mannucci, 2013) The insulin in use initially was short acting and of bovine or porcine origin, and the compliance to it was also poor, which resulted in a lot of fluctuations in the glucose. In 1950, the Neutral Protamine Hagedorn (NPH) insulin was developed and instantly became the favoured treatment for diabetes. The first oral anti hyperglycaemic agent Tolbutamide (a sulfonylurea) was used for the first time in 1957. For the first 34 years, insulin was the drug of choice regardless of the type of diabetes (Maria Rotella, Pala and Mannucci, 2013).

The distinction between type 1 and 2 developed over the next five decades along with the realization that insulin levels may be normal or even raised in T2DM and that a substantial proportion of β-cells survives lifelong (Clark *et al.,* 1990; Porte and Kahn, 2001; Butler *et al.*, 2003). However, by now the research about glucose homeostasis was so insulin-centric, that the inability of normal or raised levels of

insulin to keep plasma glucose normal was labelled as “insulin resistance”. This was done without looking at alternative possibilities and the concept got wide uncritical acceptance. Although insulin resistance as a phenomenon is well established and its molecular mechanisms elucidated with substantial details, the question whether altered insulin signalling is solely responsible for fasting hyperglycaemia of T2DM or other insulin independent mechanisms play a significant role is not clearly answered.

There are many evidences which lead us to re-examine the role of insulin in glucose regulation in relation to T2DM (Corkey, 2012; Pories and Dohm, 2012; Watve, 2013). Exogenous insulin and other insulin-centred lines of treatment provide short term glucose control but have failed to reduce complications and all-cause mortality in T2DM (Meinert *et al.*, 1970; UK Prospective Diabetes Study Group, 1998a, 1998b, 1998c; King, Peacock and Donnelly, 2001; ACCORD, 2008). In the long run the normalization of glucose also gets difficult in majority of cases (UK Prospective Diabetes Study Group, 1998a, 1998b). A number of mechanisms are known to influence glucose dynamics, partially or completely independent of insulin signalling, including autonomic signals (Nonogaki, 2000; Schwartz, 2005), glucocorticoids (Goldstein *et al.*, 1993; Gathercole and Stewart, 2010; Di Dalmazi *et al.*, 2012; Kuo *et al.*, 2015), insulin independent glucose transporters (Carruthers *et al.*, 2009) and certain other hormones and growth factors (Clemmons, 2004; Jansen *et al.*, 2006; Messmer-Blust *et al.*, 2012; Suh *et al.*, 2014). Analysis of multi-organ signalling network models have also raised doubts about the central role of insulin and insulin resistance in T2D (Kulkarni, Sharda and Watve, 2017).

2.1.2 Why is it necessary to differentiate between steady state effects and perturbed state effects?

The diagnosis of glucose is done based on the two different glucose readings taken either at the fasting state or after a meal/post-prandial state (IDF Diabetes Atlas, 8th Edition, 2017). The fasting state is generally accepted to be a steady state for glucose concentration for several reasons. In a given healthy individual the fasting glucose levels are stable in time (Lerner and Porte, 1972; Halter *et al.*, 1985). The post-prandial peak of glucose and insulin returns to the fasting level

within a few hours and remains stable over a long time. The fasting state is considered and modelled as a steady state by the homeostasis model of assessment (HOMA) which is a widely used model based on negative feedback loops that give rise to a steady state dynamics for glucose and insulin (Turner *et al.*, 1979; Matthews *et al.*, 1985). The steady state predictions arising from the model were tested against the fasting glucose values in normal and diabetic subjects (Turner *et al.*, 1979; Matthews *et al.*, 1985). The negative feedback loops are assumed to work through insulin and therefore insulin is taken as a determinant of steady state glucose level. The fasting glucose levels of non-diabetic people are generally between 70-110mg/dl and the glucose levels begin to rise as early as 10 minutes after a meal and come back to this level after around 2 hours after the meal (Enzo Bonora *et al.*, 2001; DeFronzo *et al.*, 2015).

The post-meal/post-prandial glucose is also the non-steady state glucose or the perturbed state glucose. The peak value of glucose attained after any meal is determined by a variety of factors like the timing, composition, and amount of the meal. In a healthy person, the post-prandial glucose levels do not cross 140mg/dl and return to the pre-meal levels in about 2-3 hours. In T2DM, the time at which the peak glucose level is attained, as well as the magnitude of this peak is altered (Enzo Bonora *et al.*, 2001; DeFronzo *et al.*, 2015).

The individual or differential contributions of fasting and post-meal glucose to that of HbA1c have been studied. Data from different cohorts of T1DM and T2DM patients have been studied and the overall conclusion is that the fasting glucose levels are better correlated with HbA1c as compared to the post-meal levels (Enzo Bonora *et al.*, 2001). People have recently begun to study the individual contributions of fasting versus post-meal glucose on the hyperglycaemia related complications of diabetes. Although most clinical trials have glycaemic aims determined by the HbA1c levels or the fasting glucose levels, only in the case of gestational diabetes the post-prandial glucose levels are targeted specifically with the aim of reducing the complications. (Enzo Bonora *et al.*, 2001). Monneir et al have studied the relative contributions of fasting and post-prandial glucose to the overall hyperglycaemia in T2DM patients who were on anti-hyperglycaemic treatments other than insulin and acarbose (Monnier,

Lapinski and Colette, 2003). Their results indicate that there is a gradient in the relative contributions of the fasting and post-prandial glucose levels when the patients shift from moderate to high hyperglycaemia. The changes or deviations in the post-prandial glucose levels contribute predominantly in subjects with moderate T2DM whereas the relative contribution of fasting glucose levels increases as the diabetes aggravates (Monnier, Lapinski and Colette, 2003). Thus, there are differences in the patterns of the fasting and post prandial hyperglycaemia in T2DM patients. The relative contribution of insulin to these two different levels of glucose also could be different and different physiological mechanisms could be playing a role in controlling these two levels of glucose. We would like to study the difference in the glucose and insulin relationship in these two different states to shed some more light on the causal factors of hyperglycaemia.

2.1.3 The approach taken in this chapter

We aim to re-examine the glucose and insulin relationship in steady state versus the perturbed state. To examine the relationship between two variables (glucose and insulin in this case) we searched the literature for four different scenarios:

1. Continuous increase in the glucose levels and its effect on insulin level
2. Continuous increase in the insulin levels/insulin action and its effect on glucose level
3. Continuous decrease in the glucose levels and its effect on insulin levels
4. Continuous decrease in the insulin levels/insulin action and its effect on glucose levels

A plethora of such experiments already exists in literature. These experiments had different aims and did not explore the differences in the steady state and perturbed state relationships in glucose and insulin. So, we aimed to re-examine the existing data with a new aim. I will discuss all the four scenarios in details below.

1. Continuous increase in the glucose levels and its effect on insulin levels

Jetton et al (Jetton *et al.*, 2008) infused intra venous glucose (20% glucose w/v) continuously for 4 days in rats. Insulin levels increased significantly at days 1 and 2 after the infusion but came back to normal by day 3 and 4 even as the infusion continued. Infusion with a higher concentration of glucose (up to 35%) also yielded similar results (Steil *et al.*, 2001). This demonstrates that whenever there

is an external input perturbation of the glucose level, there an altered insulin level in response. But if the glucose input is maintained constant and allowed to reach a steady state, insulin returns to the pre-perturbation level. This line of experiments appears to be limited in literature.

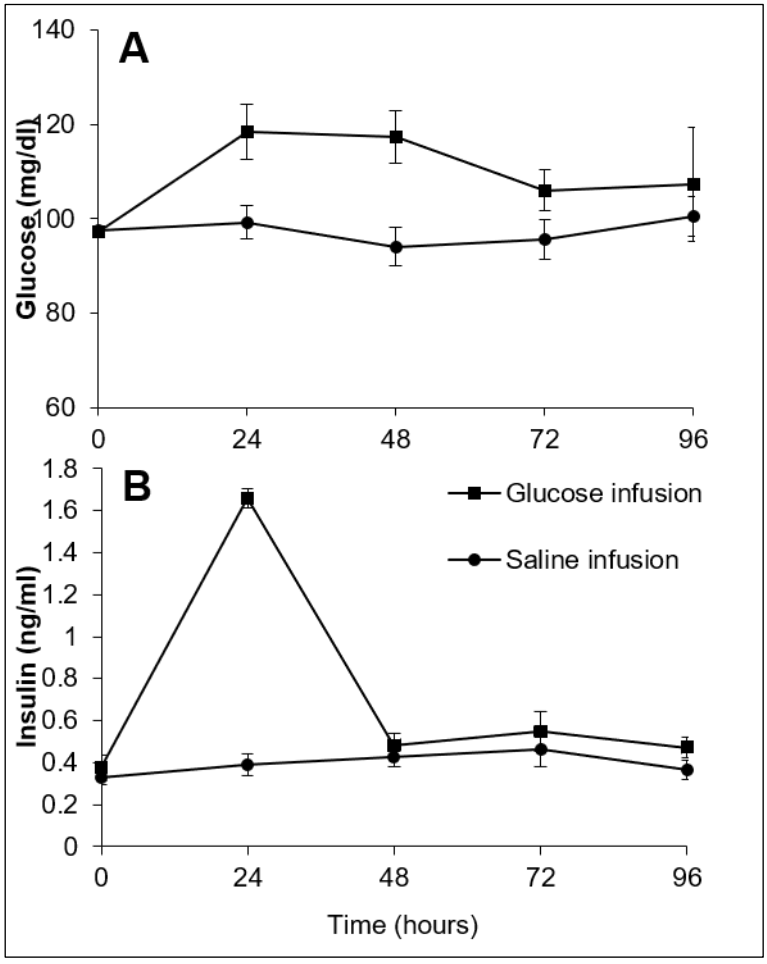


Figure 1: Effects of chronically increased glucose on the steady state: Plasma glucose (A) and insulin (B) levels of 20% continuous glucose-infused rats. A: Glucose levels were elevated at day 1 and 2 after infusion but decreased and returned close to normal on day 3 and remained so through further infusion. B: A 4-fold increase in plasma insulin was seen at day 1 in the glucose-infusion group but were normalized after day 2; n=12 (saline control) and n=16 (20% Glucose Infusion) *p<0.001. Figure reproduced from data by (Jetton *et al.*, 2008).

2. ***Continuous increase in the insulin levels/insulin action and its effect on glucose levels***

Studies show insulin infusion experiments on a short time frame. Since we wanted to explore the effect of a sustained insulin increase, the model we chose was that of insulin degrading enzyme knockouts or inhibitors. An interplay between insulin secretion and insulin breakdown maintains the level of insulin in plasma. Plasma

insulin has a half-life of 4 to 9 minutes (Tomasi *et al.*, 1967; Hulse, Ralat and Wei-Jen, 2009). Insulin is degraded predominantly by the insulin degrading enzyme (IDE) (Shen *et al.*, 2006; Hulse, Ralat and Wei-Jen, 2009). Inhibition of IDE has been considered as a therapeutic option for type 2 diabetes (Costes and Butler, 2014; Maianti *et al.*, 2014). We performed a systematic literature review to find out experiments performed in which IDE was inhibited and an oral glucose tolerance test (OGTT) performed. We wanted to see the differential effect of increase in insulin levels (due to the inhibition of IDE) on the steady state and perturbed state glucose. OGTT, by design, shows the steady state (fasting) and perturbed state (post glucose loading) of glucose and insulin. The methods and results used to analyse the differential effect of insulin increase on glucose levels has been explained in detail in the methods and results section of this chapter below.

3. Continuous decrease in the glucose levels and its effect on insulin level

Even though this is a theoretically possible scenario, this has not been performed in human/animal models because sustained hypoglycaemia is often fatal. I did not find experiments that show the effect of sustained lower glucose level on insulin dynamics.

4. ***Continuous decrease in the insulin levels/insulin action and its effect on glucose level***

This is the most relevant scenario physiologically as insulin levels and/or action reduce in T1D as well as in T2D to some extent. The absolute insulin levels reduce in T1D whereas the insulin action and/or insulin levels are reduced in T2D. This chapter explores this scenario in detail with the help of three meta-analyses (please refer to the methods and results section below). We chose three different approaches used in the literature to supress insulin/insulin action.

(i) Insulin receptor knock-outs (IRKO): Tissue specific knockouts of the insulin receptor (Kitamura, Kahn and Accili, 2003) which result in downregulation of the insulin action. The absolute levels of insulin may or may not be affected. These data exclusively consist of experiments in rodent models.

(ii) Diazoxide (DZX): Diazoxide is a potassium channel activator which causes reduction in β-cell insulin secretion by keeping the cells in a

hyperpolarized state by opening the channel (Panten *et al.*, 1989). It has been used as a drug to modulate insulin secretion for research and therapeutic purposes (Doyle, 2003).

(iii) Octreotide (OCT): Octreotide is a somatostatin analogue which inhibits the secretion of insulin and growth hormone. It has been used to reduce insulin secretion *in vitro* and *in vivo* (Lamberts *et al.*, 1996).

Streptozotocin (STZ) is a commonly used treatment in rodent models for destroying β-cells and thereby preventing insulin production. But I have not considered STZ in this chapter since it will be dealt with in greater details using primary experiments in the next chapter.

2.2 Methods

2.2.1 Meta-analyses: Search strategy

We performed four separate meta-analyses to see the differential effect of insulin increase or suppression on the steady state (fasting) and perturbed state (post glucose bolus) glucose. The four meta-analyses were as follows:

1. Insulin degrading enzyme
2. Insulin receptor knockouts
3. Insulin suppression by diazoxide
4. Insulin suppression by octreotide

The details of all the meta-analyses are given in the table 1 below and the Prisma flowcharts can be found in the Appendix. Inhibition of the IDE was chosen as the model of insulin increase (Scenario 2). After the systematic search and screening, the data from 6 different studies was used for the meta-analysis. There are different ways of suppressing insulin or insulin action (scenario 4) and we chose three of them in human or rodent models. The three methods were (i) insulin receptor knock-outs (ii) diazoxide (iii) octreotide. Details of the methods of the meta-analyses are given in table 1. After the primary and secondary screening, 16, 8 and 14 papers were used for data extraction and analyses respectively for the three methods. All the meta-analyses were registered on the PROSPERO database and the details can be found in table 1.

Table 1: Details of the meta-analyses used to study the action of insulin modulation on steady state and perturbed state glucose.

Meta-analysis → Task performed ↓	**Insulin degrading enzyme (IDE) inhibition/knockout (Scenario 2)**	**Insulin receptor knock-outs (IRKOs) (Scenario 4)**	**Suppression of insulin using Diazoxide (DZX) (Scenario 4)**	**Suppression of insulin using Octreotide (OCT) (Scenario 4)**
Key word used for the first search on the Pubmed data-base	"insulin degrading enzyme"	"insulin receptor knockout"	"diazoxide and diabetes"; "insulin suppression"	"octreotide and diabetes"; "insulin suppression"
Number of hits in the first search	1179	78	1043	1202
Inclusion criteria for primary screening	Study showing experiments with IDE inhibition and OGTT	Study showing experiments with IRKOs and OGTT	Study showing stable insulin suppression using diazoxide and an OGTT after insulin suppression.	Study showing stable insulin suppression using octreotide and an OGTT after insulin suppression.
Number of papers shortlisted after primary screening	33	36	239	289
Inclusion criteria for secondary screening	Study showing experiments with IDE inhibition, included fasting and post glucose bolus readings of control and IDE inhibition	Study showing similar methods of making the IRKO; included fasting and post glucose bolus readings of the control and IRKO	Study showing similarities in the concentration of DZX used; and included fasting and post glucose bolus readings of the control and DZX subjects	Study showing similarities in the concentration of OCT used; and included fasting and post glucose bolus readings of the control and OCT subjects

Papers shortlisted after secondary screening; used for data **extraction**	6	16	6 (human studies) 2 (rodent studies)	14 (human studies)
Studies used in the final meta-analysis (rodent model)	(Farris *et al.*, 2003; Abdul-Hay *et al.*, 2011; Maianti *et al.*, 2014; Deprez-Poulain *et al.*, 2015; Durham *et al.*, 2015; Villa-Pérez *et al.*, 2018)	(Brüning *et al.*, 1998; Lauro *et al.*, 1998; Wojtaszewski *et al.*, 1999; Mauvais-Jarvis *et al.*, 2000; Dodson Michael *et al.*, 2000; Guerra *et al.*, 2001; Blüher *et al.*, 2002; Otani, 2003; Cohen *et al.*, 2004; Okada *et al.*, 2007; Ealey *et al.*, 2008; Escribano *et al.*, 2009; Kawamori *et al.*, 2009; Haas *et al.*, 2012; Softic *et al.*, 2016; Sakaguchi *et al.*, 2017)	(Leahy, Bumbalo and Chen, 1994; Matsuda *et al.*, 2002)	None
Studies used in the final meta-analyses (human studies)	None	None	(Wigand and Blackard, 1979; Schreuder *et al.*, 2005; Due *et al.*, 2007; van Boekel *et al.*, 2008; Ramanathan, Arbeláez and Cryer, 2011; Brauner *et al.*, 2016)	(Williams *et al.*, 1986, 1988; Davies *et al.*, 1986; Johnston *et al.*, 1986; Candrina, Gussago and Giustina, 1988; Giustina *et al.*, 1991; Piaditis *et al.*, 1996; Savage, Mohamed-Ali and Williams, 1998; Marfella, Nappo and Angelis,

				2000; Ronchi *et al.*, 2002; Parkinson *et al.*, 2002; Lustig *et al.*, 2003; Breckenridge *et al.*, 2007; Madsen *et al.*, 2011)
PROSPERO ID	CRD42019140619	CRD42019132379	CRD42020141688	CRD42020141464

2.2.2 Data extraction from the shortlisted papers

The glucose and insulin values were extracted from the shortlisted papers using a freeware called WebPlot digitizer (https://automeris.io/WebPlotDigitizer). The reliability of the WebPlotDigitier has been studied by using comparative methods (Burda *et al.*, 2017; Drevon, Fursa and Malcolm, 2017). The software has been used previously for the extraction of data for meta analyses (Koren *et al.*, 2019; Neale *et al.*, 2019), just like we have used it. The values were extracted from graphs, tables, or texts.

2.2.3 Statistics used in the meta-analyses:

The data from the OGTT was extracted from all the shortlisted papers for the three meta-analyses. The steady state and perturbed state values of glucose (with the 95% confidence intervals) of the controls and insulin modification were extracted from the papers. The glucose values of the treated (T) group (insulin modification) were compared with that of the untreated/control (C) group. The number of T>C and T<C were counted using the absolute values of the means of the glucose of the treated and the control as well as the 95% CI of the means. In case of the 95% CI comparison, T >C if the lower 95% CI of the T was greater than the upper 95%CI of C. Similarly, T<C if the upper 95%CI of T was less than the lower 95% CI of C. The frequency of T>C and T<C was compared using a chi-square test. We chose this non-parametric approach to analyse the meta-analyses data since the pooling of data was done across studies where the age, weight class of animals, day/time of the reading differed. Use of non-parametric tests for meta-analysis has been recommended as it does not assume homogeneity of conditions or when you are not sure about the normality of the distributions (Hedges and Olkin, 1984; Kitchen, 2009)

2.3 Results

2.3.1 Effect of increase in insulin on steady state and perturbed state glucose: Meta-analysis of inhibition of IDE

The model of choice for a sustained increase in insulin levels is by knocking out or inhibiting the insulin degrading enzyme (IDE). We shortlisted 6 papers which performed chemical inhibition or knock-out of the IDE which had a total of 18 studies. All 18 studies have the fasting (time 0) and post glucose bolus time 120

minutes readings, but not all of them have the intermittent readings. We compared the glucose values of the treated (T) which was IDE inhibition/KO and control (C) at every time point (table 3). The number of T>C and T<C were counted using the absolute values of the means of the glucose of the treated and the control as well as the 95% CI of the means. In case of the 95% CI comparison, T >C if the lower 95% CI of the T was greater than the upper 95%CI of C. Similarly, T<C if the upper 95%CI of T was less than the lower 95% CI of C. The frequency of T>C and T<C was compared using a chi-square test. In case of IDE suppression, the expectation is that the glucose values of the treated should be smaller than that of the control as a decrease in IDE results in the increase in insulin values. However, we see no significant decrease in the treated as compared to the control. The treated and the control show very similar glucose levels in the fasting state and the difference is not significant in any of the later time points either (Table 3 and figure 2). The actual curve of the OGTT of the treated and the control also show a remarkably similar trend except one experiment. In the study (Maianti *et al.*, 2014) the diet induced obese mice when treated with the chemical inhibitor of the IDE, show a completely different trend as compared to the control or the lean mice treated with the inhibitor. The post-bolus glucose values in this case do not return to the fasting values after 120 minutes. This is also very surprising, since IDE inhibition is expected to increase the insulin levels, and in turn the glucose levels should reduce (figure 3).

Table 2: Details of the 6 studies used in the IDE inhibition meta-analysis. All studies were carried out on mouse models.

Sr. No.	Reference	Method used to inhibit IDE	Fasting duration before the GTT	Glucose concentration/ mode of glucose infusion used in GTT	Sample size
1	Villa Perez et al 2018	Liver specific IDE knock-out	16 hours	2g/kg dextrose given i.p.	n= 9 to 13 for each group
2	Deprez-Poulain et al 2018	Inhibition of catalytic site of IDE using the inhibitor BDM44768	6 hours	1.5g/kg glucose for IPGTT and 2 or 3g/kg glucose for OGTT	n= 4 to 7 for each group
3	Durham et al 2015	Inhibition of IDE using an N-terminal exosite (NTE)	Overnight	2g/kg dextrose given orally	n=6 for each group
4	Maianti et al 2014	Inhibition of IDE using a non-catalytic site binding inhibitor	14 hours	1.5g/kg glucose for IPGTT and 3g/kg glucose for OGTT	n=5 to 7 for each group
5	Abdul Hay et al 2010	IDE-KO created by Cre-lox recombination	6 to 9 hours	1g/kg dextrose given i.p.	n=10 to 12 for each group
6	Farris et al 2003	IDE-/- mice created by gene trapping method	Overnight	2g/kg dextrose given i.p.	n=6 (IDE-/-) n=4 (Control)

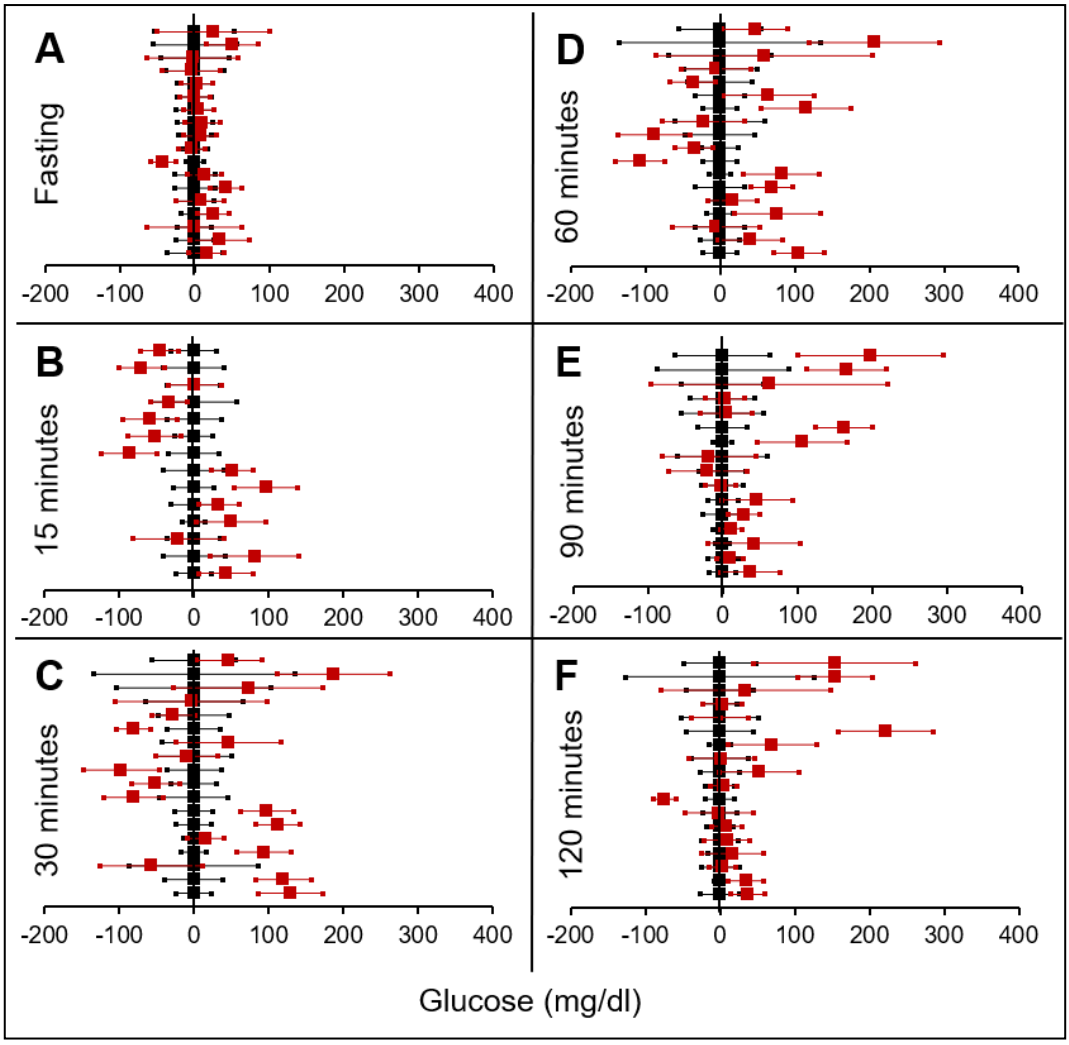


Figure 2: Glucose levels for control (black squares) and IDE-inhibition (red squares) models at steady and perturbed state. X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and IDE-inhibition were compared using an OGTT. IDE-inhibition glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points (B to F) post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

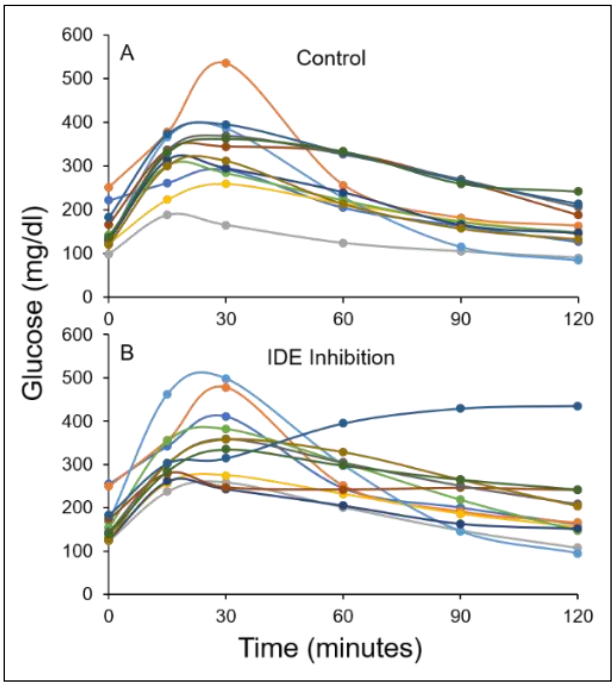


Figure 3: GTT curves of all Control (A) and IDE inhibition (B) models from all the studies from the meta-analysis. Each curve represents the glucose values from the GTT of each study. The SEs for the control curves ranged from 2 to 12% and that of the IDE inhibition ranged from 3 to 21%.

Table 3: Comparison between steady state (fasting) and perturbed state (post glucose load) of control (C) and IDE suppression/treated (T).

Time point	**Total studies**	**T>C**	**T<C**	***p* using chi square**	**T > C individually significant based on 95% CI**	**T < C individually significant based on 95% CI**
Fasting	18	12	6	0.157	0	1
15 min	14	7	7	0.999	3	5
30 min	18	10	8	0.637	5	2
60 min	18	11	7	0.346	5	1
90 min	16	13	3	0.012*	4	0
120 min	18	15	2	0.002*	2	1

2.3.2 Effect of decrease in insulin signalling on steady state and perturbed state glucose: Meta-analysis of insulin receptor knock-out

We use four different tissue specific knockouts for the analysis. Liver insulin receptor knockout (LIRKO), Muscle insulin receptor knockout (MIRKO), fat/adipose insulin receptor knockout (FIRKO) and β-cell insulin receptor knockout (βIRKO). We shortlisted 16 papers (table 4) which had IRKOs in rodent models with a total of 46 studies. All studies included the glucose readings at time

0 (fasting) and at time 120 minutes. The intermittent time point glucose values were not present in all the studies. A generalized trend in the total picture collated over all the IRKOs seen in the meta-analysis is that along the GTT curve, a significantly higher glucose level is seen in the knock-outs/treated as compared to the controls (figure 4). This trend is seen particularly and consistently at 30, 60 and 120 minutes (figure 4). However, the fasting glucose level is not significantly different in the meta-analysis (figure 4). In some studies, fasting glucose is significantly greater in the knock-outs than the controls, however in some other studies it is significantly lower as well. In 29 out of 46 studies there is no significant difference between the knock-outs/treated and control in the fasting state (Table 2). This trend was seen consistently in MIRKO (figure 6), LIRKO (figure 7) and βIRKO (figure 8). Only in FIRKO (figure 5) there were more studies showing higher fasting glucose in the knock-outs than in the controls (Guerra *et al.*, 2001; Softic *et al.*, 2016; Sakaguchi *et al.*, 2017), but when compared using the non-parametric chi-square test, this trend was not significant. In FIRKO, the 30, 60 and 120 minute glucose was not significantly different in the knock-outs than the controls. The inconsistencies in the FIRKO fasting glucose levels could be a result of the differences in the duration of fasting used for the GTTs. Although the glucose levels in the control are lower than the FIRKO/BATIRKO in the fasting conditions, there could be possible reasons for that. For example, in the study Sakaguchi et al., 2017, fasting for the GTT was carried out only for 6 hours as against 16 hours/overnight in other studies. Secondly, one of the knock-outs in this study is a double knock-out of insulin receptor and the insulin-like growth factor 1 receptor which could be a possible reason for the higher glucose levels in the fasting condition. In case of the Guerra et al., 2001; Softic et al., 2016, the animals have been fasted overnight for the GTT. The tests have been performed on FIRKO and WT of different ages. The impairment of glucose tolerance increases with age, though this also is not seen consistently across all the studies. In the case of Blüher et al. (2002), the fasting duration for the GTT is 16 h, highest in all the studies and in this case the treated glucose levels in the fasting condition are equal to or lower than that of the controls. In the LIRKO knock-outs in none of the studies the fasting sugar is significantly higher than the controls. This contradicts the classical belief that

liver insulin resistance is mainly responsible for fasting hyperglycaemia in T2DM (Johnson *et al.*, 1972; Bock *et al.*, 2007).

Table 4: Details of the 16 studies used in the IRKO meta-analysis

Sr. No.	Reference	Type of IRKO	Method used to make the knock-out	Fasting duration before the GTT	Glucose concentration/ mode of glucose infusion used in GTT	Sample size
1	Sakaguchi et al 2017	inducible-DKO IR and IGF-IR, BATIRKO	Cre-lox lines	6 hours	2g/kg dextrose given orally	Control n=13, IRKO, n=12
2	Softic et al 2016	FIRKO (12 weeks old)	Cre-lox lines	Overnight	Random fed	n=12 to 30 for each group
		FIRKO (52 weeks old male mice)				n=5 to 6 for each group
3	Haas et al 2012	LIRKO	Cre-lox lines	Overnight	1g/kg dextrose given i.p.	n=3 to 5 for each group
4	Kawamori et al 2009	αIRKO (2,5, 12-month-old mice) fed/fasted	Cre-lox lines	16 hours	Random fed	n=6 to 8 for each group
		αIRKO (2,5 month old mice) GTT		16 hours	1g/kg dextrose given i.p.	n=3 to 12 for each group
5	Escribano et al 2009	inducible LIRKO	Cre-lox lines	16 hours	2g/kg dextrose i.p.	n=10 to 20 for each group
6	Ealey et al 2008	MIRKO	Cre-lox lines	Overnight	2g/kg dextrose i.p.	n=7 to 13 for each group

7	Okada et al 2007	βIRKO, LIRKO and βIRKO-LIRKO (4-5 weeks old male mice)	Cre-lox lines	Overnight	2g/kg dextrose i.p.	n=8 for each group
		βIRKO (20 weeks old, male mice; chow and HFD)				n=9 to 16 for each group
8	Cohen et al 2004	LIRKO (2 month old mice)	Cre-lox lines	16 hours	2g/kg dextrose i.p.	n=17 for control n=25 for LIRKO
9	Otani et al 2004	βIRKO-Non-diabetic (ND)	Cre-loxP system	4 hours	2g/kg dextrose i.p.	n= 35 for control, n=28 for βIRKO(ND)
		βIRKO-Diabetic (D)				n=10 for βIRKO(D)
10	Blueher et al 2002	FIRKO (2 month and 10 month old mice)	Cre-loxP system	16 hours	2g/kg dextrose i.p.	n=8 for each group
11	Guerra et al 2001	BATIRKO (3, 6 and 9 month old male and female mice)	Cre-loxP system	Overnight	2g/kg dextrose i.p.	n=10 to 20 for each group
12	Lauro et al 1998	Insulin receptor (Ins R) and Ins R K1030 mutatnt	Cre-loxP system,	Overnight	2g/kg dextrose i.p.	n=8 for each group
13	Mauvais-Jarvis et al 2000	MIRKO, βIRKO and βIRKO-MIRKO (2 and 6 month old mice	Cre-loxP system	Overnight	2g/kg dextrose i.p.	n=28 to 32 for each group
14	Micheal et al 2000	LIRKO (2 and 6 month old mice)	Cre-loxP system	16 hours	2g/kg dextrose i.p.	n=8 for each group

15	Wojtaszewski et al 1999	MIRKO	Cre-loxP system	Overnight	2g/kg dextrose i.p.	n= 7 to 8 for each group
16	Bruening et al 1998	MIRKO	Cre-loxP system	Overnight	2g/kg dextrose i.p.	n=8 for each group

Table 5: Comparison between steady state (fasting) and perturbed state (post glucose load) glucose between control (C) and treated/IRKOs (T).

Time point	Total studies	T>C	T<C	*p* using chi square	T > C individually significant based on 95% CI	T < C individually significant based on 95% CI
ALL IRKOs						
Fasting	46	25	20	0.454	13	4
15 min	14	7	7	0.999	4	2
30 min	40	36	4	<0.0001*	22	1
60 min	40	36	4	<0.0001*	24	1
120 min	46	37	9	<0.0001*	24	2
FIRKO						
Fasting	12	9	3	0.083	9	1
15 min	3	1	2	0.566	1	1
30 min	9	7	2	0.095	6	1
60 min	9	7	2	0.095	5	1
120 min	12	9	3	0.83	7	2
MIRKO						
Fasting	10	3	7	0.205	0	2
15 min	6	3	3	0.999	1	0
30 min	10	9	1	0.011*	3	0
60 min	10	9	1	0.011*	3	0
120 min	10	6	4	0.527	3	0
LIRKO						
Fasting	9	4	5	0.739	0	0

15 min	1	1	0	N.A.	0	0
30 min	9	9	0	.003*	6	0
60 min	9	9	0	.003*	7	0
90 min	9	9	0	.003*	5	0
120 min	9	7	2	0.094	4	0
βIRKO						
Fasting	8	6	2	0.157	4	0
15 min	2	2	0	0.157	2	0
30 min	8	7	1	0.033*	6	0
60 min	8	7	1	0.033*	7	0
90 min	4	4	0	0.046*	4	0
120 min	8	8	0	0.0046*	7	0

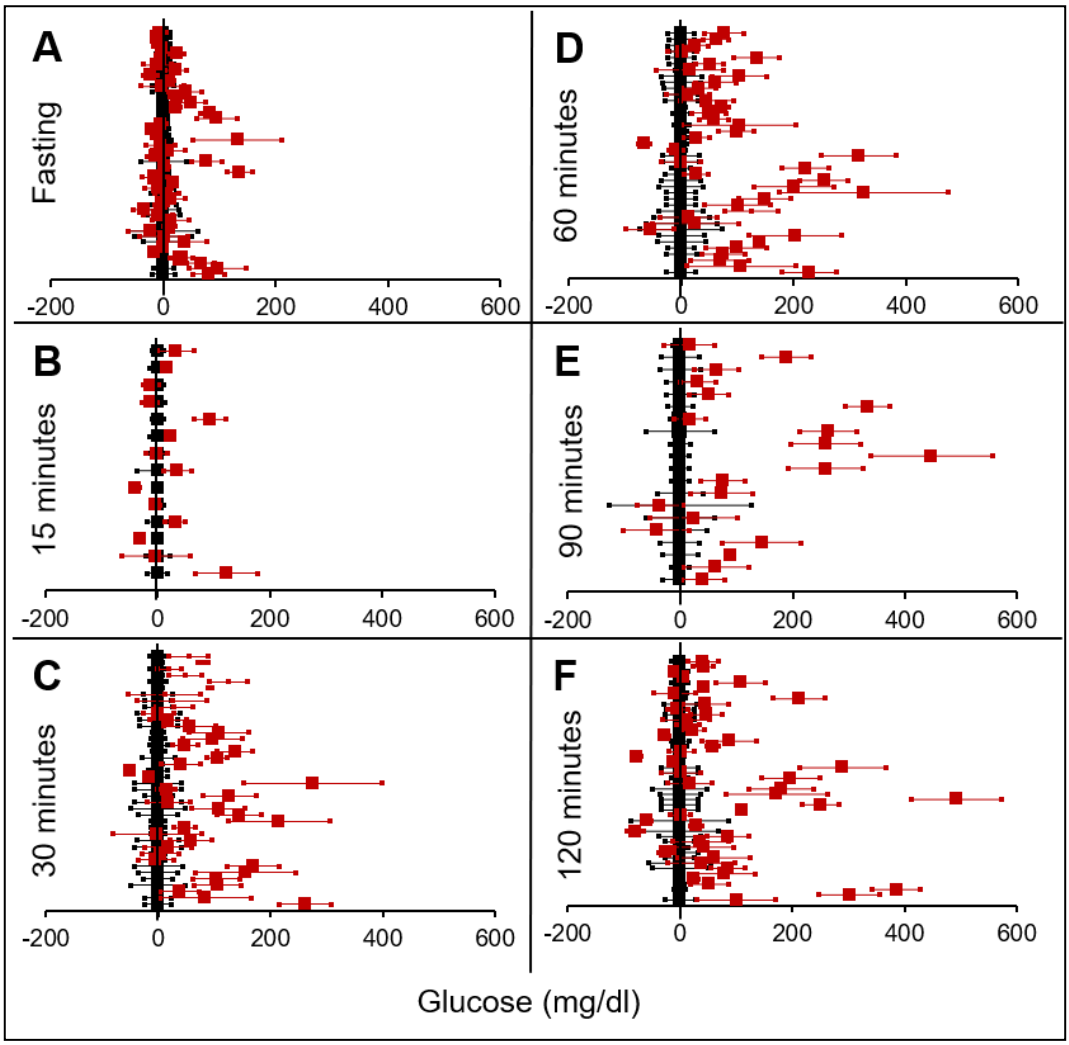


Figure 4: Glucose levels for control (black squares) and IRKO (red squares) at steady state and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and IRKO were compared using an OGTT. IRKO glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

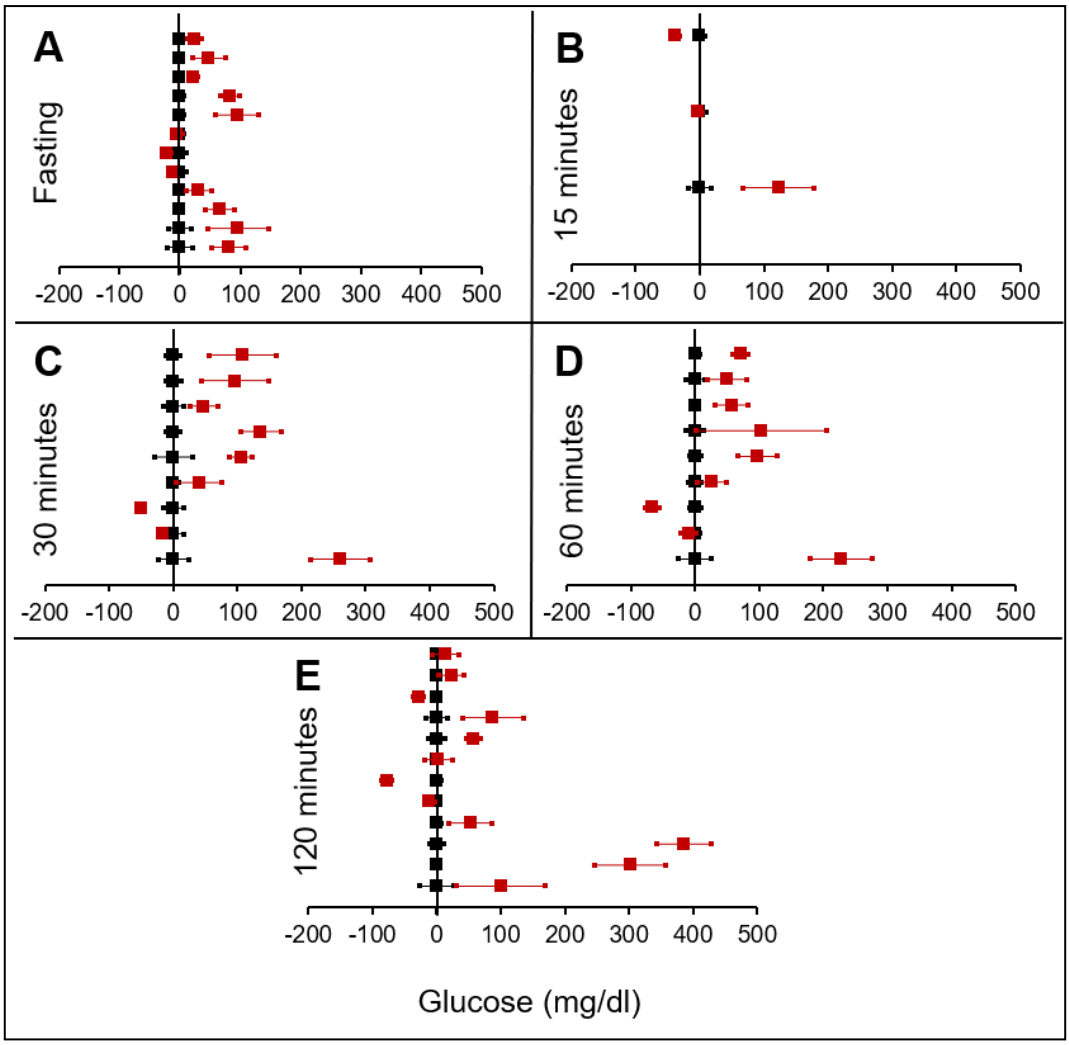


Figure 5: Glucose levels for control (black squares) and FIRKO (red squares) at steady state and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and FIRKO were compared using an OGTT. FIRKO glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes and (E) 120 minutes.

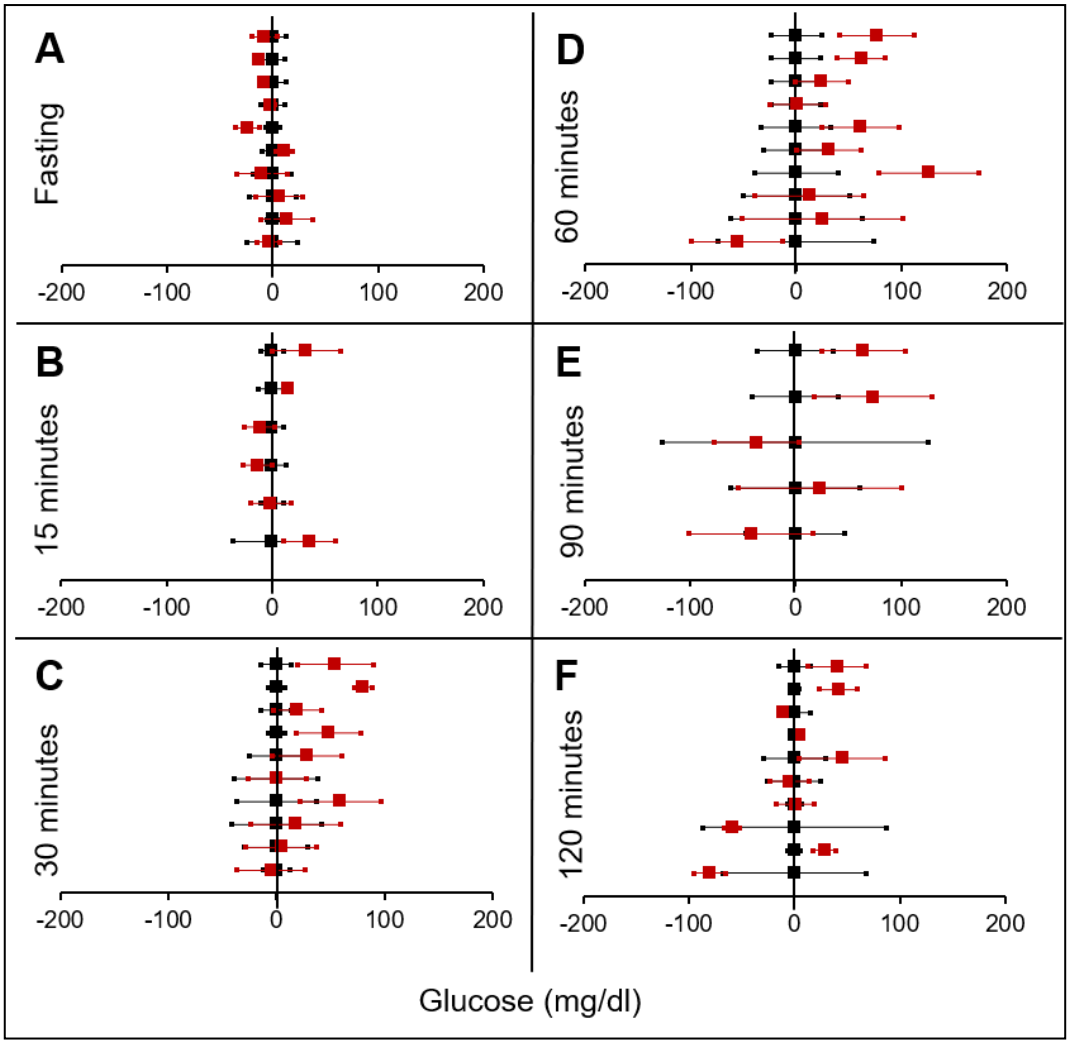


Figure 6: Glucose levels for control (black squares) and MIRKO (red squares) at steady state and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and MIRKO were compared using an OGTT. MIRKO glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

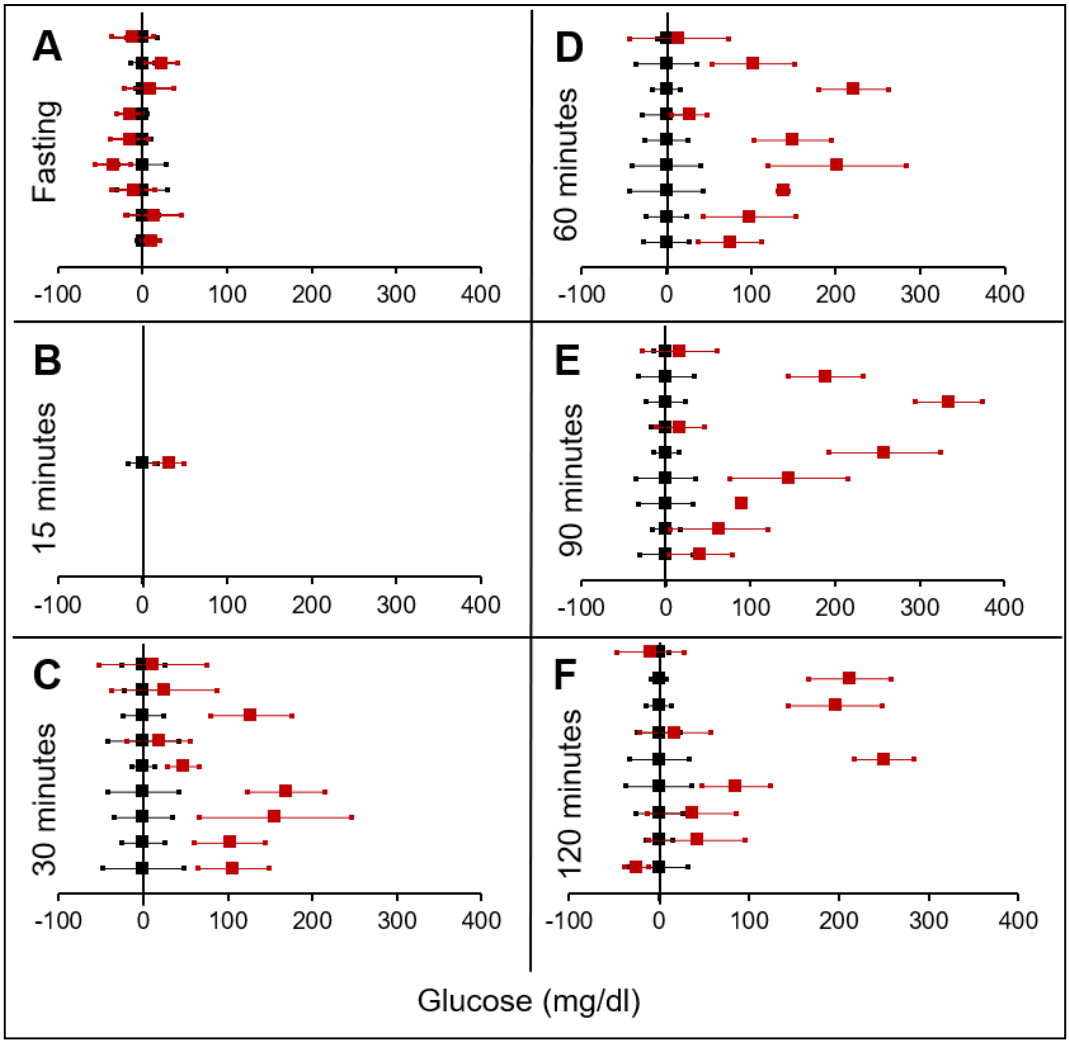


Figure 7: Glucose levels for control (black squares) and LIRKO (red squares) at steady state and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and LIRKO were compared using an OGTT. LIRKO glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

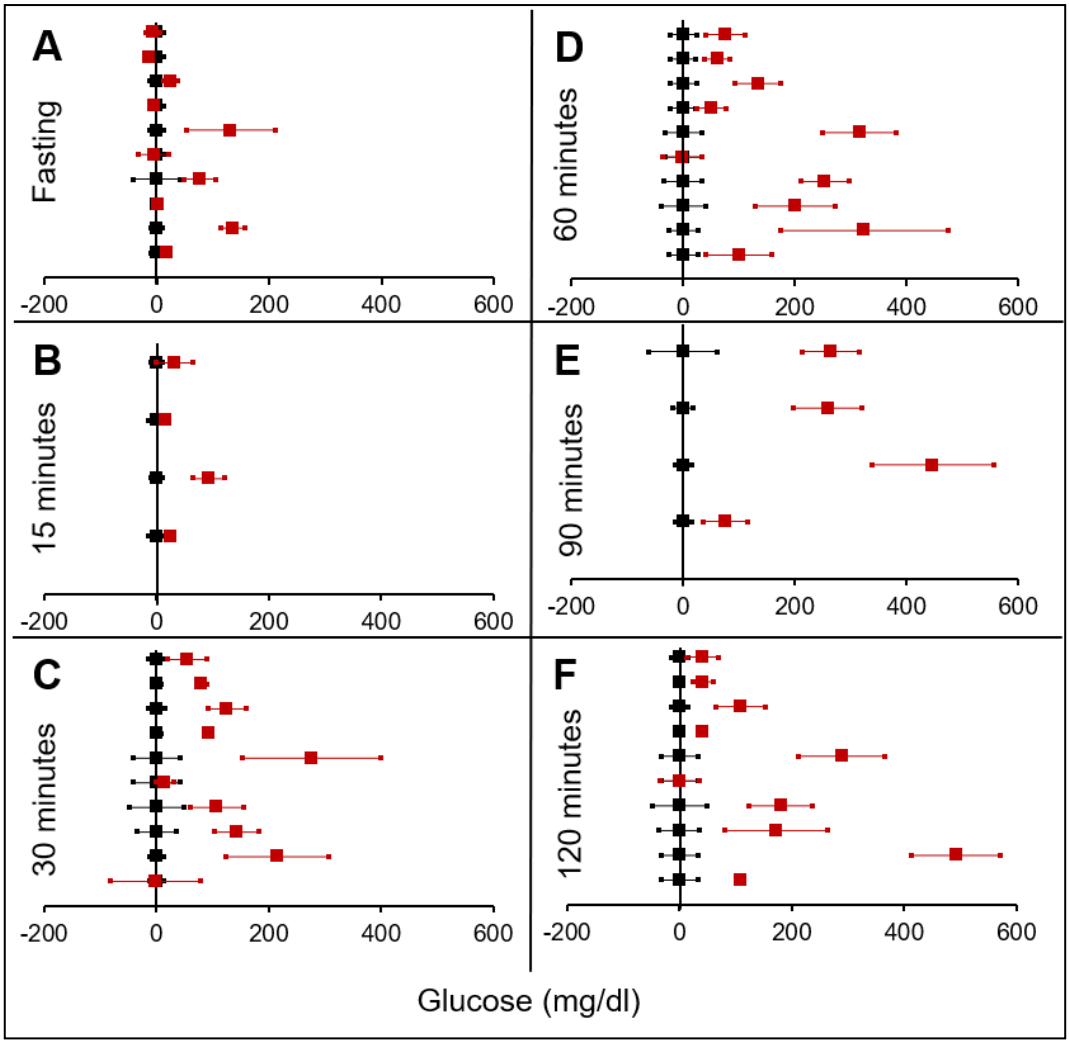


Figure 8: Glucose levels for control (black squares) and βIRKO (red squares) at steady state and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and βIRKO were compared using an OGTT. βIRKO glucose levels are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

2.3.3 Effect of decrease in insulin on steady state and perturbed state glucose

2.3.3.1 Meta-analysis of inhibition of insulin using Diazoxide and Octreotide

After studying the effects of increase in glucose, increase in insulin, decrease in insulin action on steady state glucose, we wanted to study the effect of decrease in the absolute levels of insulin on steady state glucose levels. A variety of methods have been used to supress the insulin in vivo. These studies have predominantly been done on rodents. A few molecules however have been used on human subjects as well. Diazoxide and Octreotide have been used in human subjects as

well to suppress insulin in cases of pancreatic or pituitary tumors which result in the over expression of insulin and/or growth hormone Doyle 2003; Lamberts et al. 1996; Panten et al. 1989). We performed two separate meta-analyses to study the effect of insulin suppression on steady and perturbed state glucose values (table 1).

Meta-analysis for treatment with Diazoxide

We shortlisted 8 papers for insulin suppression with DZX out of which 6 papers included human studies while 2 papers included studies on rodent models (table1). The 8 papers had 16 separate groups in which control and treated glucose values could be compared. The expectation is that on suppression of insulin, the glucose levels should be elevated. The fasting glucose levels in the control and the treated group however did not differ significantly (figure 9 and table 6). At the time point of 30 minutes, there was a significant increase in the glucose levels of the treated versus the control (table 6).

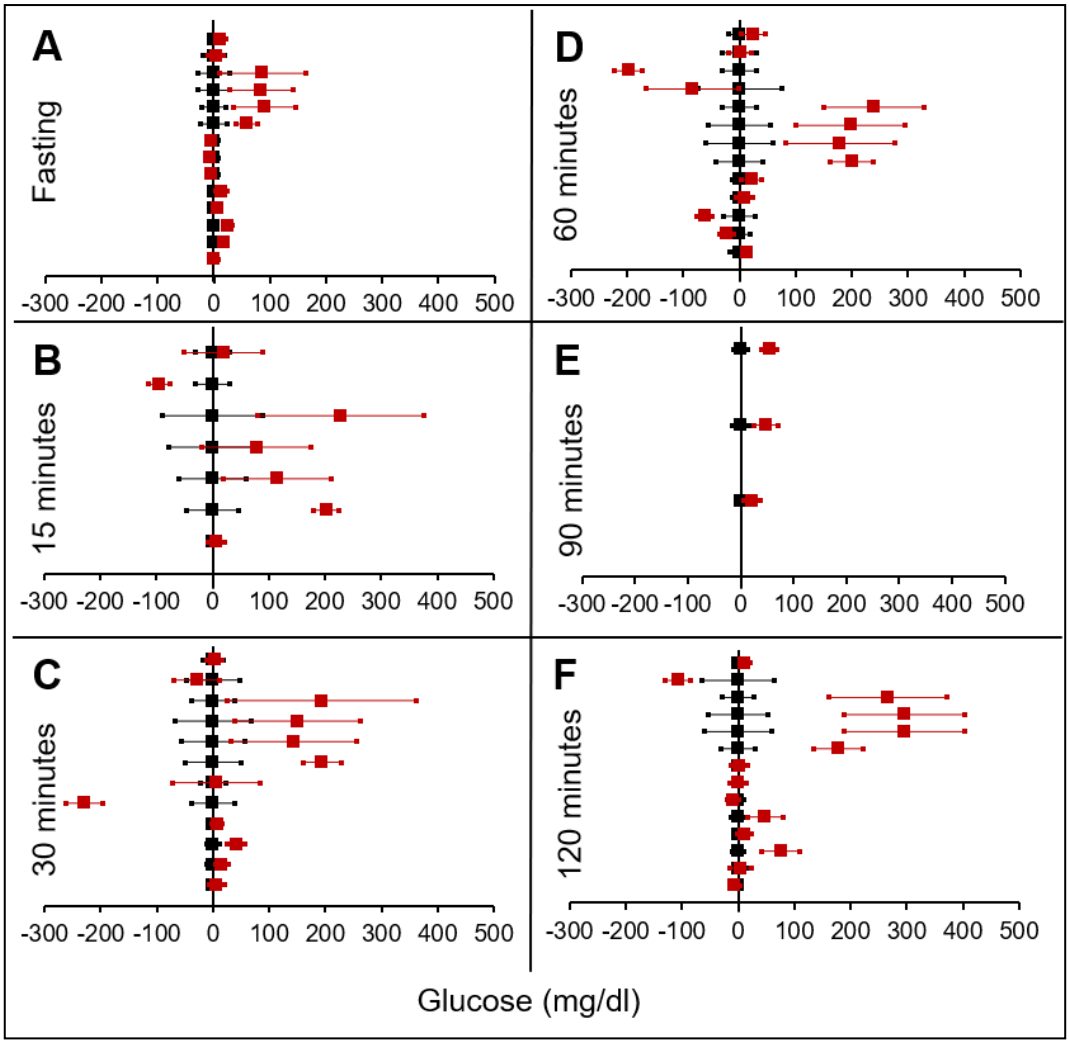


Figure 9: Glucose levels for control (black squares) and treatment with DZX (red squares) models at steady and perturbed state. The X-axis represents the glucose level and the Y-axis represents experiments from different studies from the shortlisted papers in which control and treated were compared using an OGTT. Glucose levels after treatment with DZX are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 15 minutes (C) 30 minutes (D) 60 minutes (E) 90 minutes and (F) 120 minutes.

Table 6: Comparison between steady state (fasting) and perturbed state (post glucose load) of control (C) and insulin suppression or treated (T).

Time	Total studies	T>C		T<C	*p* using chi square	T > C individually significant based on 95% CI	T < C individually significant based on 95% CI
Diazoxide treatment							
Fasting	14	10		3	0.052	0	5
15 min	7	6		1	0.059	1	1
30 min	12	10		2	0.021*	2	1
60 min	13	9		4	0.166	4	2
90 min	3	3		0	0.083	2	0
120 min	14	10		3	0.052	6	1
Octreotide treatment							
Fasting	15	6		7	0.781	0	0
30 min	14	4		10	0.108	0	2
60 min	14	4		10	0.108	2	1
90 min	13	5		8	0.405	1	0
120 min	15	7		8	0.797	1	1

Meta-analysis for treatment with Octreotide:

We shortlisted 14 papers for the meta-analysis of Octreotide treatment for insulin suppression. All the papers included human studies and there were 15 separate groups where the control and treated glucose values could be compared at different time points. Octreotide is a known somatostatin analogue and it is known to suppress the insulin and/or growth hormone secretion (Lamberts *et al.*, 1996). Therefore, t is expected that the glucose levels should increase on treatment with Octreotide. There was no significant difference between the treated and control at any time point (figure 10 and table 6).

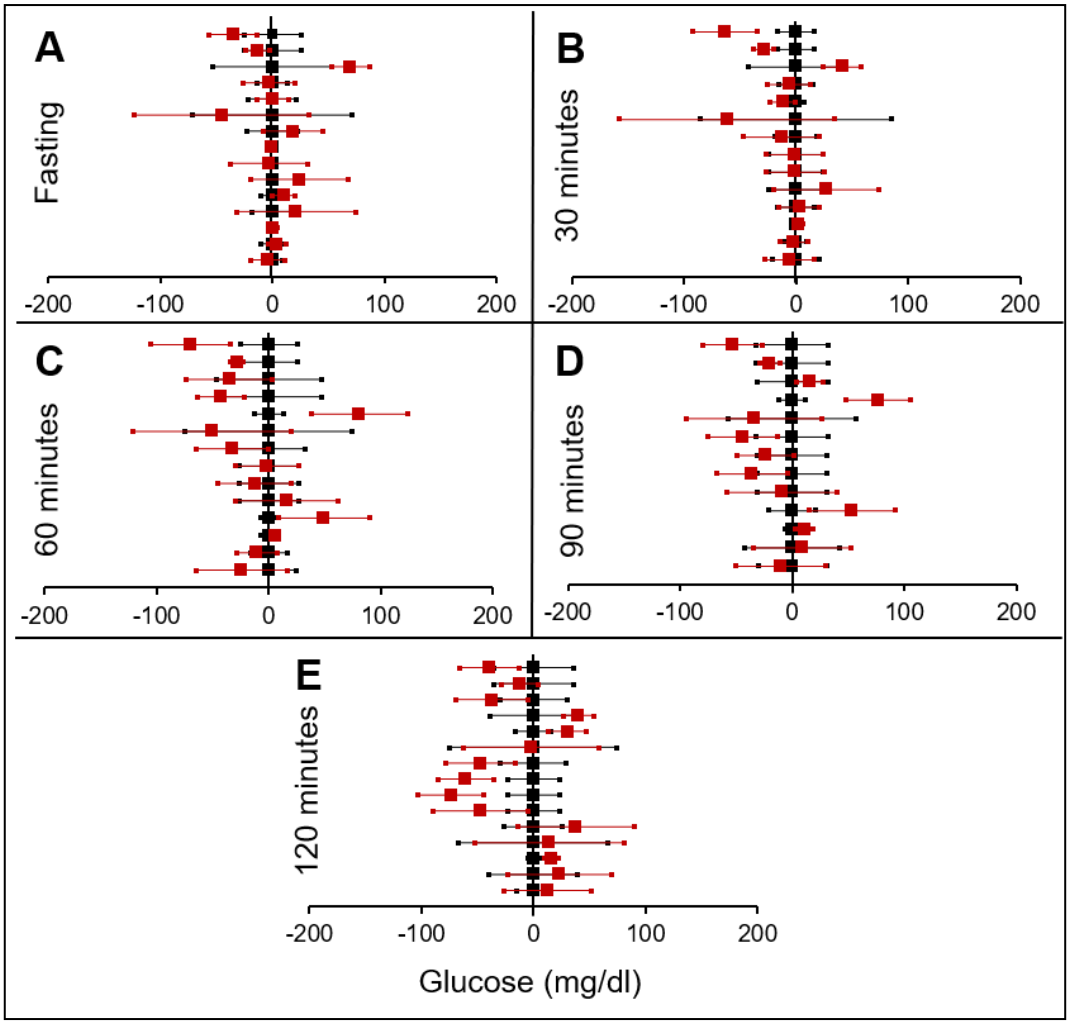


Figure 10: Glucose levels for control (black squares) and treatment with OCT (red squares) models at steady and perturbed state. The X-axis represents the glucose levels and the Y-axis represents experiments from different studies from the shortlisted papers in which control and treated were compared using an OGTT. Glucose levels after treatment with OCT are normalized to that of the control and the difference is expressed with ± 95% CI. Glucose levels of control are expressed as 0 ± 95% CI. Steady state is represented by the fasting glucose (A) and the perturbed state is represented by different time points post glucose load when the readings are taken: (B) 30 minutes (C) 60 minutes (D) 90 minutes and (E) 120 minutes.

2.3.3.2 Suppression of insulin by protein deprivation

Another method for insulin suppression is dietary protein deprivation. This method has been performed in experimentally in rodents. This also led to a decrease in plasma insulin levels; however fasting glucose levels did not increase (Schteingart *et al.*, 1979).

2.3.3.3 Suppression of insulin by siRNA

Transgenic mice for insulin-siRNA along with IDE overexpression, showed decreased levels of insulin. Again, the fasting glucose levels remained normal while there was a change in glucose tolerance curve (figure 11) (Hwang *et al.*, 2007)

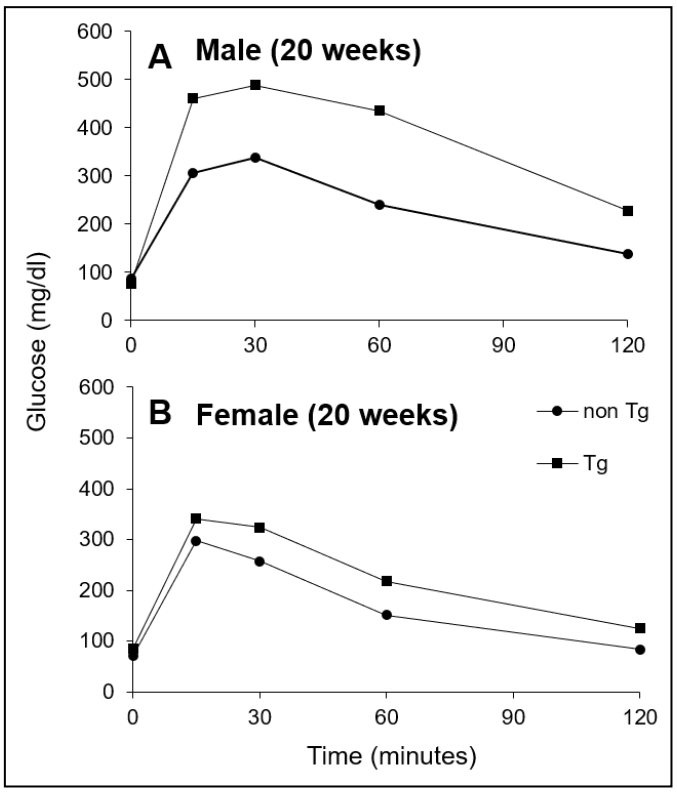


Figure 11: Fasting glucose levels in both the siRNA treated and untreated group remain unaltered in male (A) and female (B) mice. 15 minutes after the glucose injection, the treated mice show higher glucose levels relative to the untreated mice and this effect is seen throughout till 120 minutes. Figure reproduced without permission from data by (Hwang *et al.*, 2007).

2.3.3.4 Suppression of insulin by partial gene ablation:

In rodents, there are two insulin genes *Ins1* and *Ins2* (Duvillie *et al.*, 1997). A double knock-out of both the genes results in death, but ablation of either of the genes does not alter the glucose tolerance significantly suggesting redundancy (Mehran *et al.*, 2012). There are studies in which one gene is completely knocked out and the other one is a heterozygote (Mehran *et al.*, 2012; Templeman, Clee and Johnson, 2015; Dionne *et al.*, 2016; Templeman *et al.*, 2017; Page *et al.*, 2019). Reduced insulin gene dosage did not consistently result into fasting hyperglycaemia in these studies although it offered protection against some of the effects of hyperinsulinemia.

2.4 Conclusion

Using systematic literature review and meta-analyses approach we have tried to see the effect of change in one variable (either glucose or insulin) on the steady state and perturbed levels of the other (insulin or glucose respectively). Increase in glucose can result in an immediate increase in the insulin level, however sustained increased in glucose input results in the insulin levels coming back to normal as shown by Jetton et al 2008 and Steil et al 2001.

When the insulin levels are increased in a sustained manner, the expectation according to the classical theories of glucose homeostasis are that glucose levels will go down due to the increased insulin level. This however is not seen as evident by the insulin degrading enzyme meta-analysis. This meta-analysis focussed on studies in which the IDE was knocked down/inhibited in a sustained manner. The results of this meta-analysis indicate that the glucose levels in the fasting or the steady state remain unaltered. At all the time points during the OGTT, the number of experiments in which the glucose of the treated (IDE inhibited) exceeds that of control are more than those in which the glucose of treated is less than that of the control. This is quite contrary to what is expected. The difference between the glucose levels of the treated and control are different only in the perturbed state (90 or 120 minutes after ingestion of glucose in the GTT) but not in the fasting/steady state (table 2).

The focus of this study however was to see the effect of reduced insulin levels or reduced insulin action on the steady state and perturbed state glucose levels since this scenario is the most relevant to T2DM. This was analysed using three different meta-analyses. The first meta-analysis focused on the knockouts of tissue specific insulin receptors in rodent models. The glucose levels at steady state and perturbed state of the treated (IRKO) and the wild-type/control were compared here. This meta-analysis clearly focussed on the tissue specific knockouts of the insulin receptors. It can be seen from the analysis that irrespective of the specific tissue in which the insulin receptor was knocked out, the perturbed state glucose (post-feeding) is affected but not the steady state (fasting), with the fat insulin receptor knockouts being the only exception.

Different fasting intervals have been used across the studies compared. A wide range of 4 to 16 hours has been used, which could be a possible problem in comparing fasting glucose across different studies. No study clearly reported how much time is required to reach a steady state in a knock-out. In 10 studies, which had the fasting time was reported as 16 hours, none had fasting sugar significantly different for controls. In the 13 experiments in which the fasting glucose was high, the fasting duration was either between 4 to 12 hours or it was not precisely reported. Therefore, it is possible that in some of the experiments, glucose steady state was not yet achieved at the time point defined as fasting. This bias increases the probability that higher fasting glucose is reported for the knock-outs. However, since we do not see a significant difference in the meta-analysis, the inference that IRKO does not alter fasting glucose is unlikely to be a result of the bias. In fact, any possible correction to the bias might nullify the apparent residual difference. Therefore, despite some inconsistency across studies, a robust generalization is that IRKOs have significantly increased plasma glucose over controls at 30 to 120 minutes post-glucose load, but they do not appear to affect steady state fasting glucose.

The next two meta-analyses focussed on looking at the effect of insulin suppression on the glucose levels in the steady state and the perturbed state. Individually, each meta-analysis consisted of studies in which insulin was suppressed using diazoxide and octreotide, respectively. In both the meta-analyses, the expectation according to the prevalent theory is that since insulin is suppressed, the glucose levels should increase in the treated animals/subjects. However, it is seen that there is no significant effect of the suppression of insulin, especially on the fasting or steady state glucose. In case of the perturbed state/post-meal glucose as well, there is no significant change in the glucose level despite the treatment with diazoxide or octreotide.

Even though IRKO and DZX/OCT act to reduce insulin action or insulin levels, there is a fundamental difference in the way they work. IRKO or insulin receptor knock-outs are made by knocking out the insulin receptor using Cre-lox recombination whereas DZX and OCT are chemicals which act in different ways to reduce insulin action. In case of DZX and OCT, these are chemical molecules

which reduce the insulin action, but they do not do it in a specific manner. Whereas in case of IRKO, the action of the insulin receptor and in turn the downstream signalling of insulin is reduced to a greater extent. This difference in the mode of action could explain the results that in IRKO the plasma glucose levels at 30 to 120 minutes post-load glucose are higher without affecting the steady state values as compared to that in DZX and OCT. The overall insulin/insulin action lowering effect of DZX and OCT is lower than that of IRKO. These could be the possible explanations from this observation.

Thus, alteration in the insulin levels/insulin signalling has a significant and consistent effect on the perturbed state glucose level but there is no consistent effect on the steady state glucose level across studies.

2.5 References:

Abdul-Ghani, M. A., Tripathy, D. and DeFronzo, R. A. (2006) 'Contributions of beta-cell dysfunction and insulin resistance to the pathogenesis of impaired glucose tolerance and impaired fasting glucose.', *Diabetes care*, 29(5), pp. 1130–9. doi: 10.2337/diacare.2951130.

Abdul-Hay, S. O. *et al.* (2011) 'Deletion of insulin-degrading enzyme elicits antipodal, age-dependent effects on glucose and insulin tolerance.', *PloS one*, 6(6), p. e20818. doi: 10.1371/journal.pone.0020818.

ACCORD (2008) 'Effects of Intensive Glucose Lowering in Type 2 Diabetes', *New England Journal of Medicine*, 358(24), pp. 2545–2559. doi: 10.1056/NEJMoa0802743.

Bernard, C. (1879) 'Leçons de physiologie opératoire', *Librairie J.-B. Baillie et fils*, p. 650.

Blüher, M. *et al.* (2002) 'Adipose tissue selective insulin receptor knockout protects against obesity and obesity-related glucose intolerance', *Developmental Cell*. doi: 10.1016/S1534-5807(02)00199-5.

Bock, G. *et al.* (2007) 'Contribution of Hepatic and Extrahepatic Insulin Resistance to the Pathogenesis of Impaired Fasting Glucose: Role of Increased Rates of Gluconeogenesis', *Diabetes*, 56(6), pp. 1703–1711. doi: 10.2337/db06-1776.

van Boekel, G. *et al.* (2008) 'Weight loss in obese men by caloric restriction and high-dose diazoxide-mediated insulin suppression', *Diabetes, Obesity and Metabolism*, 10(12), pp. 1195–1203. doi: 10.1111/j.1463-1326.2008.00878.x.

Brauner, R. *et al.* (2016) 'Diazoxide in children with obesity after hypothalamic-pituitary lesions: A randomized, placebo-controlled trial', *Journal of Clinical Endocrinology and Metabolism*, 101(12), pp. 4825–4833. doi: 10.1210/jc.2016-2126.

Breckenridge, S. M. *et al.* (2007) 'Glucagon, in concert with insulin, supports the postabsorptive plasma glucose concentration in humans', *Diabetes*, 56(10), pp. 2442–2448. doi: 10.2337/db07-0751.

Brüning, J. C. *et al.* (1998) 'A muscle-specific insulin receptor knockout exhibits features of the metabolic syndrome of NIDDM without altering glucose tolerance', *Molecular Cell*. doi: 10.1016/S1097-2765(00)80155-0.

Burda, B. U. *et al.* (2017) 'Estimating data from figures with a Web-based program: Considerations for a systematic review', *Research Synthesis Methods*, 8(3), pp. 258–262. doi: 10.1002/jrsm.1232.

Butler, A. E. *et al.* (2003) '-Cell Deficit and Increased -Cell Apoptosis in Humans With Type 2 Diabetes', *Diabetes*, 52(1), pp. 102–110. doi: 10.2337/diabetes.52.1.102.

Candrina, R., Gussago, A. and Giustina, G. (1988) 'Effect of a new long-acting somatostatin analogue (SMS 201–995) on glycemic and hormonal response to a mixed meal in acromegalic patients', *Journal of Endocrinological Investigation*, 11(1), pp. 21–26. doi: 10.1007/BF03350089.

Carruthers, A. *et al.* (2009) 'Will the original glucose transporter isoform please stand up!', *American Journal of Physiology-Endocrinology and Metabolism*, 297(4), pp. E836–E848. doi: 10.1152/ajpendo.00496.2009.

Clark, A. *et al.* (1990) 'Islet amyloid polypeptide in diabetic and non-diabetic Pima Indians', *Diabetologia*, 33(5), pp. 285–289. doi: 10.1007/BF00403322.

Clemmons, D. R. (2004) 'Role of Insulin-Like Growth Factor Iin Maintaining Normal Glucose Homeostasis', *Hormone Research in Paediatrics*, 62(1), pp. 77–82. doi: 10.1159/000080763.

Cohen, S. E. *et al.* (2004) 'Effects of insulin-sensitising agents in mice with hepatic insulin resistance', *Diabetologia*. doi: 10.1007/s00125-003-1320-4.

Corkey, B. E. (2012) 'Banting Lecture 2011: Hyperinsulinemia: Cause or Consequence?', *Diabetes*, 61(1), pp. 4–13. doi: 10.2337/db11-1483.

Costes, S. and Butler, P. C. (2014) 'Insulin-Degrading Enzyme Inhibition, a Novel Therapy for Type 2 Diabetes?', *Cell Metabolism*, 20(2), pp. 201–203. doi: 10.1016/j.cmet.2014.07.016.

Di Dalmazi, G. *et al.* (2012) 'Glucocorticoids and Type 2 Diabetes: From Physiology to Pathology', *Journal of Nutrition and Metabolism*, 2012, pp. 1–9. doi: 10.1155/2012/525093.

Davies, R. R. *et al.* (1986) 'Effects of somatostatin analogue SMS 201-995 in non-insulin-dependent diabetes.', *Clinical endocrinology*, 25(6), pp. 739–747.

DeFronzo, R. A. *et al.* (eds) (2015) *International Textbook of Diabetes Mellitus*. Chichester, UK: John Wiley & Sons, Ltd. doi: 10.1002/9781118387658.

Deprez-Poulain, R. *et al.* (2015) 'Catalytic site inhibition of insulin-degrading enzyme by a small molecule induces glucose intolerance in mice', *Nature Communications*, 6. doi: 10.1038/ncomms9250.

Dionne, D. A. *et al.* (2016) 'in Mice With Genetically Reduced Insulin', 157(July), pp. 2724–2734. doi: 10.1210/en.2016-1102.

Dodson Michael, M. *et al.* (2000) 'Loss of Insulin Signaling in Hepatocytes Leads to Severe Insulin Resistance and Progressive Hepatic Dysfunction', *Molecular Cell*, 6, pp. 87–97.

Doyle, M. E. (2003) 'Pharmacological Agents That Directly Modulate Insulin Secretion', *Pharmacological Reviews*, 55(1), pp. 105–131. doi: 10.1124/pr.55.1.7.

Drevon, D., Fursa, S. R. and Malcolm, A. L. (2017) 'Intercoder Reliability and Validity of WebPlotDigitizer in Extracting Graphed Data', *Behavior Modification*, 41(2), pp. 323–339. doi: 10.1177/0145445516673998.

Dubuc, P. U. (1981) 'Non-essential role of dietary factors in the development of diabetes in ob/ob mice.', *The Journal of nutrition*, 111(10), pp. 1742–8. doi: 10.1093/jn/111.10.1742.

Due, A. *et al.* (2007) 'No effect of inhibition of insulin secretion by diazoxide on weight loss in hyperinsulinaemic obese subjects during an 8-week weight-loss diet', *Diabetes, Obesity and Metabolism*, 9(4), pp. 566–574. doi: 10.1111/j.1463-1326.2006.00645.x.

Durham, T. B. *et al.* (2015) 'Dual exosite-binding inhibitors of insulin-degrading enzyme challenge its role as the primary mediator of insulin clearance in vivo', *Journal of Biological Chemistry*, 290(33), pp. 20044–20059. doi: 10.1074/jbc.M115.638205.

Duvillie, B. *et al.* (1997) 'Phenotypic alterations in insulin-deficient mutant mice', *Proceedings of the National Academy of Sciences*, 94(10), pp. 5137–5140. doi: 10.1073/pnas.94.10.5137.

Ealey, K. N. *et al.* (2008) 'Reduced susceptibility of muscle-specific insulin receptor knockout mice to colon carcinogenesis', *Am J Physiol Gastrointest Liver Physiol*, 294(3), pp. G679-86. doi: 10.1152/ajpgi.00526.2007.

Enzo Bonora, MD, P. F. L. *et al.* (2001) 'Postprandial Blood Glucose', *Diabetes Care*, 24(4), pp. 775–778. doi: 10.2337/diacare.24.4.775.

Escribano, O. *et al.* (2009) 'Role of a Liver-Pancreas Endocrine Axis Through Insulin Receptor A Isoform', *Diabetes*, 58(April), pp. 820–828. doi: 10.2337/db08-0551.

Farris, W. *et al.* (2003) 'Insulin-degrading enzyme regulates the levels of insulin, amyloid beta-

protein, and the beta-amyloid precursor protein intracellular domain in vivo.', *Proceedings of the National Academy of Sciences of the United States of America*, 100(7), pp. 4162–7. doi: 10.1073/pnas.0230450100.

Gathercole, L. L. and Stewart, P. M. (2010) 'Targeting the pre-receptor metabolism of cortisol as a novel therapy in obesity and diabetes', *The Journal of Steroid Biochemistry and Molecular Biology*, 122(1–3), pp. 21–27. doi: 10.1016/j.jsbmb.2010.03.060.

Giustina, A. *et al.* (1991) 'Low-dose octreotide is able to cause a maximal inhibition of the glycemic responses to a mixed meal in obese type 2 diabetic patients treated with insulin', *Diabetes Research and Clinical Practice*, 14(1), pp. 47–54. doi: 10.1016/0168-8227(91)90052-F.

Goldstein, R. E. *et al.* (1993) 'Effects of chronic elevation in plasma cortisol on hepatic carbohydrate metabolism', *American Journal of Physiology-Endocrinology and Metabolism*, 264(1), pp. E119–E127. doi: 10.1152/ajpendo.1993.264.1.E119.

Guerra, C. *et al.* (2001) 'Brown adipose tissue – specific insulin receptor knockout shows diabetic phenotype without insulin resistance', *The Journal of Clinical Investigation*, 108(8), pp. 1205–1213. doi: 10.1172/JCI200113103.Introduction.

Haas, J. T. *et al.* (2012) 'Hepatic Insulin Signaling Is Required for Obesity-Dependent Expression of SREBP-1c mRNA but Not for Feeding-Dependent Expression', *Cell Metabolism*, 15(6), pp. 873–884. doi: 10.1016/j.cmet.2012.05.002.

Halter, J. B. *et al.* (1985) 'Glucose regulation in non-insulin-dependent diabetes mellitus. Interaction between pancreatic islets and the liver.', *The American journal of medicine*, 79(2B), pp. 6–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2863979.

Hedges, L. V. and Olkin, I. (1984) 'Nonparametric estimators of effect size in meta-analysis.', *Psychological Bulletin*, 96(3), pp. 573–580. doi: 10.1037/0033-2909.96.3.573.

Hulse, R. E., Ralat, L. a and Wei-Jen, T. (2009) 'Structure, function, and regulation of insulin-degrading enzyme.', *Vitamins and hormones*, 80(08), pp. 635–648. doi: 10.1016/S0083-6729(08)00622-5.

Hwang, D. *et al.* (2007) 'Significant change in insulin production, glucose tolerance and ER stress signaling in transgenic mice coexpressing insulin-siRNA and human IDE', *International Journal of Molecular Medicine*. doi: 10.3892/ijmm.19.1.65.

IDF Diabetes Atlas, 8th Edition'. Brussels, Belgium: International Diabetes Federation, 2017. Available at: http://www.diabetesatlas.org.

Jansen, C. *et al.* (2006) 'Does Epidermal Growth Factor Participate in the Regulation of Glucose, Insulin and Glucagon Levels?', *European Surgical Research*, 38(4), pp. 377–384. doi: 10.1159/000094533.

Jetton, T. L. *et al.* (2008) 'Enhanced β-cell mass without increased proliferation following chronic mild glucose infusion', *American Journal of Physiology-Endocrinology and Metabolism*, 294(4), pp. E679–E687. doi: 10.1152/ajpendo.00569.2007.

Johnson, M. E. *et al.* (1972) 'The regulation of gluconeogenesis in isolated rat liver cells by glucagon, insulin, dibutyryl cyclic adenosine monophosphate, and fatty acids.', *The Journal of biological chemistry*, 247(10), pp. 3229–35. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4337509.

Johnston, D. G. *et al.* (1986) 'Effects of SMS 201-995 on intermediary metabolism and endocrine status in normal and diabetic humans', *The American Journal of Medicine*, 81(6 SUPPL. 2), pp. 88–93. doi: 10.1016/0002-9343(86)90589-9.

Karamitsos, D. T. (2011) 'The story of insulin discovery', *Diabetes Research and Clinical*

Practice, 93, pp. S2–S8. doi: 10.1016/S0168-8227(11)70007-9.

Kawamori, D. *et al.* (2009) 'Insulin Signaling in α Cells Modulates Glucagon Secretion In Vivo', *Cell Metabolism*, 9(4), pp. 350–361. doi: 10.1016/j.cmet.2009.02.007.

King, P., Peacock, I. and Donnelly, R. (2001) 'The UK Prospective Diabetes Study (UKPDS): clinical and therapeutic implications for type 2 diabetes', *British Journal of Clinical Pharmacology*, 48(5), pp. 643–648. doi: 10.1046/j.1365-2125.1999.00092.x.
Kitamura, T., Kahn, C. R. and Accili, D. (2003) 'Insulin Receptor Knockout Mice', *Annual Review of Physiology*, 65(1), pp. 313–332. doi: 10.1146/annurev.physiol.65.092101.142540.

Kitchen, C. M. R. (2009) 'Nonparametric vs Parametric Tests of Location in Biomedical Research', *American Journal of Ophthalmology*, 147(4), pp. 571–572. doi: 10.1016/j.ajo.2008.06.031.

Koren, L. *et al.* (2019) 'Towards the validation of endogenous steroid testing in wildlife hair', *Journal of Applied Ecology*. Edited by C. Bieber, 56(3), pp. 547–561. doi: 10.1111/1365-2664.13306.

Kulkarni, S., Sharda, S. and Watve, M. (2017) 'Bi-stability in type 2 diabetes mellitus multi-organ signalling network', *PLOS ONE*. Edited by C. Cras-Méneur, 12(8), p. e0181536. doi: 10.1371/journal.pone.0181536.

Kuo, T. *et al.* (2015) 'Regulation of Glucose Homeostasis by Glucocorticoids', in, pp. 99–126. doi: 10.1007/978-1-4939-2895-8_5.

Lamberts, S. W. J. *et al.* (1996) 'Octreotide', *New England Journal of Medicine*. Edited by A. J. J. Wood, 334(4), pp. 246–254. doi: 10.1056/NEJM199601253340408.

Lauro, D. *et al.* (1998) 'Impaired glucose tolerance in mice with a targeted impairment of insulin action in muscle and adipose tissue.', *Nature genetics*, 20(3), pp. 294–298. doi: 10.1038/3112.

Leahy, J. L., Bumbalo, L. M. and Chen, C. (1994) 'Diazoxide causes recovery of beta-cell glucose responsiveness in 90% pancreatectomized diabetic rats.', *Diabetes*, 43(2), pp. 173–9. doi: 10.1046/j.1464-5491.1999.00150.x.

Lerner, R. L. and Porte, D. (1972) 'Acute and steady state insulin responses to glucose in nonobese diabetic subjects', *Journal of Clinical Investigation*, 51(7), pp. 1624–1631. doi: 10.1172/JCI106963.

Lustig, R. H. *et al.* (2003) 'Octreotide therapy of pediatric hypothalamic obesity: A double-blind, placebo-controlled trial', *Journal of Clinical Endocrinology and Metabolism*, 88(6), pp. 2586–2592. doi: 10.1210/jc.2002-030003.

Madsen, M. *et al.* (2011) 'Cotreatment with pegvisomant and a Somatostatin Analog (SA) in SA-responsive acromegalic patients', *Journal of Clinical Endocrinology and Metabolism*, 96(8), pp. 2405–2413. doi: 10.1210/jc.2011-0654.

Maianti, J. P. *et al.* (2014) 'Anti-diabetic activity of insulin-degrading enzyme inhibitors mediated by multiple hormones', *Nature*. Nature Publishing Group, 511(7507), pp. 94–98. doi: 10.1038/nature13297.

Marfella, R., Nappo, F. and Angelis, L. De (2000) 'Hemodynamic effects of acute hyperglycemia in type 2 diabetic patients', *Diabetes Care*, 23(5), pp. 658–63. Available at: http://care.diabetesjournals.org/content/23/5/658.short.

Maria Rotella, C., Pala, L. and Mannucci, E. (2013) 'Role of Insulin in the Type 2 Diabetes Therapy: Past, Present and Future', *International Journal of Endocrinology and Metabolism*, 11(3). doi: 10.5812/ijem.7551.

Matsuda, M. *et al.* (2002) 'Rescue of beta-cell exhaustion by diazoxide after the development of diabetes mellitus in rats with streptozotocin-induced diabetes', *European Journal of Pharmacology*, 453(1), pp. 141–148. doi: 10.1016/S0014-2999(02)02389-0.

Matthews, D. R. *et al.* (1985) 'Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28(7), pp. 412–419. doi: 10.1007/BF00280883.

Mauvais-Jarvis, F. *et al.* (2000) 'A model to explore the interaction between muscle insulin resistance and beta-cell dysfunction in the development of type 2 diabetes', *Diabetes*. doi: 10.2337/diabetes.49.12.2126.

Mehran, A. E. *et al.* (2012) 'Article Hyperinsulinemia Drives Diet-Induced Obesity Independently of Brain Insulin Production', *Cell Metabolism*. Elsevier Inc., 16(6), pp. 723–737. doi: 10.1016/j.cmet.2012.10.019.

Meinert, C. L. *et al.* (1970) 'A study of the effects of hypoglycemic agents on vascular complications in patients with adult-onset diabetes. II. Mortality results.', *Diabetes*, 19, p. Suppl:789-830. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4926376.

Mering, J. von J. and Minkowski, O. (1890) 'Diabetes mellitus nach Pankreasexstirpation', *Vogel, Leipzig*, p. 28.

Messmer-Blust, A. F. *et al.* (2012) 'RTEF-1 Attenuates Blood Glucose Levels by Regulating Insulin-Like Growth Factor Binding Protein-1 in the Endothelium', *Circulation Research*, 111(8), pp. 991–1001. doi: 10.1161/CIRCRESAHA.112.268110.

Monnier, L., Lapinski, H. and Colette, C. (2003) 'Contributions of fasting and postprandial glucose to overall hyperglycemia of type 2 diabetic patients', *Diabetes Care*, 26(3), pp. 881–883.

Neale, R. E. *et al.* (2019) 'The effect of sunscreen on vitamin D: a review', *British Journal of Dermatology*, 181(5), pp. 907–915. doi: 10.1111/bjd.17980.

Nonogaki, K. (2000) 'New insights into sympathetic regulation of glucose and fat metabolism', *Diabetologia*, 43(5), pp. 533–549. doi: 10.1007/s001250051341.

Okada, T. *et al.* (2007) 'Insulin receptors in beta-cells are critical for islet compensatory growth response to insulin resistance.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(21), pp. 8977–82. doi: 10.1073/pnas.0608703104.

Otani, K. (2003) 'Reduced -cell mass and altered glucose sensing impair insulin-secretory function in IRKO mice', *AJP: Endocrinology and Metabolism*. doi: 10.1152/ajpendo.00533.2001.

Page, M. M. *et al.* (2019) 'Reducing insulin via conditional partial gene ablation in adults reverses diet-induced weight gain'. doi: 10.1096/fj.201700518R.

Panten, U. *et al.* (1989) 'Control of insulin secretion by sulfonylureas, meglitinide and diazoxide in relation to their binding to the sulfonylurea receptor in pancreatic islets', *Biochemical Pharmacology*, 38(8), pp. 1217–1229. doi: 10.1016/0006-2952(89)90327-4.

Parkinson, C. *et al.* (2002) 'A comparison of the effects of pegvisomant and octreotide on glucose, insulin, gastrin, cholecystokinin, and pancreatic polypeptide responses to oral glucose and a standard mixed meal', *Journal of Clinical Endocrinology and Metabolism*, 87(4), pp. 1797–1804. doi: 10.1210/jcem.87.4.8432.

Piaditis, G. P. *et al.* (1996) 'The effect of sequential administration of octreotide alone and octreotide/growth hormone simultaneously on buserelin stimulated ovarian steroid secretion in women with polycystic ovary syndrome', *Clinical Endocrinology*, 45(5), pp. 595–604. doi: 10.1046/j.1365-2265.1996.00854.x.

Pories, W. J. and Dohm, G. L. (2012) 'Diabetes: Have We Got It All Wrong?: Hyperinsulinism as the culprit: surgery provides the evidence', *Diabetes Care*, 35(12), pp. 2438–2442. doi: 10.2337/dc12-0684.

Porte, D. and Kahn, S. E. (2001) 'Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms', *Diabetes*, 50(Supplement 1), pp. S160–S163. doi: 10.2337/diabetes.50.2007.S160.

Ramanathan, R. P., Arbeláez, A. M. and Cryer, P. E. (2011) 'Partial inhibition of insulin secretion results in glucose intolerance but not hyperglucagonemia', *Diabetes*, 60(4), pp. 1324–1328. doi: 10.2337/db10-1586.

Ronchi, C. *et al.* (2002) 'Effects of two different somatostatin analogs on glucose tolerance in acromegaly', *Journal of Endocrinological Investigation*, 25(6), pp. 502–507. doi: 10.1007/BF03345491.

Sakaguchi, M. *et al.* (2017) 'Adipocyte Dynamics and Reversible Metabolic Syndrome in Mice with an Inducible Adipocyte-Specific Deletion of the Insulin Receptor', *Cell Metabolism*, 25(2), pp. 448–462. doi: 10.1016/j.cmet.2016.12.008.

Savage, M. W., Mohamed-Ali, V. and Williams, G. (1998) 'Suppression of post-glucose hyperinsulinaemia does not affect blood pressure in either normotensive or hypertensive subjects.', *Clinical science (London, England : 1979)*, 94(6), pp. 609–614.

Schreuder, T. *et al.* (2005) 'Diazoxide-mediated insulin suppression in obese men: a dose-response study.', *Diabetes, obesity & metabolism*, 7(3), pp. 239–45. doi: 10.1111/j.1463-1326.2004.00449.x.

Schteingart, D. E. *et al.* (1979) 'Suppression of insulin secretion by protein deprivation in obesity', *Metabolism*, 28(9), pp. 943–949. doi: 10.1016/0026-0495(79)90095-7.

Schwartz, M. W. (2005) 'Diabetes, Obesity, and the Brain', *Science*, 307(5708), pp. 375–379. doi: 10.1126/science.1104344.

Shen, Y. *et al.* (2006) 'Structures of human insulin-degrading enzyme reveal a new substrate recognition mechanism', *Nature*, 443(7113), pp. 870–874. doi: 10.1038/nature05143.

Softic, S. *et al.* (2016) 'Lipodystrophy due to adipose tissue-specific insulin receptor knockout results in progressive NAFLD', *Diabetes*, 65(8), pp. 2187–2200. doi: 10.2337/db16-0213.

Steil, G. M. *et al.* (2001) 'Adaptation of β-cell mass to substrate oversupply: enhanced function with normal gene expression', *American Journal of Physiology-Endocrinology and Metabolism*, 280(5), pp. E788–E796. doi: 10.1152/ajpendo.2001.280.5.E788.

Suh, J. M. *et al.* (2014) 'Endocrinization of FGF1 produces a neomorphic and potent insulin sensitizer', *Nature*, 513(7518), pp. 436–439. doi: 10.1038/nature13540.

Templeman, N. M. *et al.* (2017) 'Sensitivity in Old Mice and Extends Lifespan Reduced Circulating Insulin Enhances Insulin Sensitivity in Old Mice and Extends Lifespan', *CellReports*. ElsevierCompany., 20(2), pp. 451–463. doi: 10.1016/j.celrep.2017.06.048.

Templeman, N. M., Clee, S. M. and Johnson, J. D. (2015) 'Suppression of hyperinsulinaemia in growing female mice provides long-term protection against obesity', pp. 2392–2402. doi: 10.1007/s00125-015-3676-7.

Tomasi, T. *et al.* (1967) 'Insulin Half-Life in Normal and Diabetic Subjects.', *Experimental Biology and Medicine*, 126(1), pp. 315–317. doi: 10.3181/00379727-126-43434.

Turner, R. C. *et al.* (1979) 'Insulin deficiency and insulin resistance interaction in diabetes: Estimation of their relative contribution by feedback analysis from basal plasma insulin and glucose concentrations', *Metabolism*, 28(11), pp. 1086–1096. doi: 10.1016/0026-0495(79)90146-

X.

UK Prospective Diabetes Study Group (1998a) ‘Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)’, *The Lancet*, 352(9131), pp. 837–853. doi: 10.1016/S0140-6736(98)07019-6.

UK Prospective Diabetes Study Group (1998b) ‘Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38’, *BMJ*, 317(7160), pp. 703–713. doi: 10.1136/bmj.317.7160.703.

UK Prospective Diabetes Study Group (1998c) ‘United Kingdom Prospective Diabetes Study 24: A 6-Year, Randomized, Controlled Trial Comparing Sulfonylurea, Insulin, and Metformin Therapy in Patients with Newly Diagnosed Type 2 Diabetes That Could Not Be Controlled with Diet Therapy’, *Annals of Internal Medicine*, 128(3), p. 165. doi: 10.7326/0003-4819-128-3-199802010-00001.

Villa-Pérez, P. *et al.* (2018) ‘Liver-specific ablation of insulin-degrading enzyme causes hepatic insulin resistance and glucose intolerance, without affecting insulin clearance in mice’, *Metabolism*. The Authors, 88, pp. 1–11. doi: 10.1016/J.METABOL.2018.08.001.

Watve, M. (2013) *Doves, Diplomats, and Diabetes*. New York, NY: Springer New York. doi: 10.1007/978-1-4614-4409-1.

Wigand, J. P. and Blackard, W. G. (1979) ‘Downregulation of insulin receptors in obese man’, *Diabetes*, 28(4), pp. 287–291. doi: 10.2337/diab.28.4.287.

Williams, G. *et al.* (1986) ‘Postprandial effects of SMS 201-995 on gut hormones and glucose tolerance’, *Scandinavian Journal of Gastroenterology*, 21(S119), pp. 73–83. doi: 10.3109/00365528609087434.

Williams, G. *et al.* (1988) ‘Postprandial glycaemic effects of a long-acting somatostatin analogue (octreotide) in non-insulin dependent diabetes mellitus’, *Hormone and Metabolic Research*, 20(3), pp. 168–170. doi: 10.1055/s-2007-1010784.

Wojtaszewski, J. F. P. *et al.* (1999) ‘Exercise modulates postreceptor insulin signaling and glucose transport in muscle-specific insulin receptor knockout mice’, *Journal of Clinical Investigation*. doi: 10.1172/JCI7961.

Chapter 3: Theoretical, mathematical, and statistical considerations

3.1 Introduction

In the earlier chapter I have established the differences in the effect of insulin on the steady state and perturbed state glucose using meta-analyses. In this chapter I would like to focus on the theoretical foundations of the insulin-glucose relationship. The unexpected yet consistent result of all the meta-analyses is that impairment of the insulin signal fails to alter steady state glucose level. The possible explanations for this result will be explored in this chapter. Simultaneously, differential predictions from alternative models of glucose homeostasis will also be made that can be tested in human epidemiological data. These predictions will then be tested using epidemiological data to examine whether the relationship between glucose and insulin in fasting/steady state is the same as that in the post-meal/perturbed state. The work described in this chapter has been published as parts of two papers Chawla et al 2018 and Diwekar-Joshi and Watve 2020.

Mathematical models of glucose homeostasis assume the fasting steady state to be a balance between glucose consumption and liver glucose production. These models predict that the regression and correlation parameters between insulin and glucose would be similar although the range of values will be different. We test the actual correlation and regression parameters in three individual data sets which include glucose and insulin values from fasting and post-glucose load from normal and diabetic human subjects.

3.2 Methods and Results

3.2.1 Models for glucose homeostasis

Fasting glucose is used interchangeably as steady state glucose for several reasons. In a healthy person, the fasting blood glucose levels are stable in time (Lerner and Porte, 1972; Halter *et al.*, 1985). After a perturbation like intake of food, both insulin and glucose go up and then return to the fasting level in a few hours and then remain stable. The most widely used model to explain the function of insulin, the HOMA, uses the fasting state of glucose and insulin as steady states

and suggests methods to calculate insulin resistance based on that (Turner *et al.*, 1979; Matthews *et al.*, 1985). Insulin is taken as a determining factor of the steady state glucose levels and the action of insulin is assumed to be through negative feedback loops. Many models of glucose homeostasis work on this assumption. There are a few non-steady state models as well (Palumbo *et al.*, 2013).

As we have seen from chapter 2, the relationship between glucose and insulin is significantly different in the steady state and the perturbed state and hence we can ask a critical question in glucose homeostasis: Is the steady state/fasting glucose level a consequential result of the balance between glucose production and glucose utilization rates (consequential steady state CSS) or whether there is a target glucose level that is maintained by sensing and correcting any changes in it (targeted steady state TSS).

I will use the level in a water tank as an analogy to demonstrate the difference between the two (figure 1). If a water tank has an inlet tap filling in water at a constant rate and has an outlet at the bottom through which water goes out proportionate to the pressure of the water column, a steady state is reached invariably. The steady state level is decided by the rate of water flowing in and the size of the outlet. This is a CSS which will change with any change in the size/capacity of the inlet or outlet. In contrast to CSS, in a TSS there is a desired or set or targeted water level. Sensors are placed above and below the desired level such that when the level goes below the lower sensor the input is switched on or its rate increased and/or output switched off or its rate decreased.

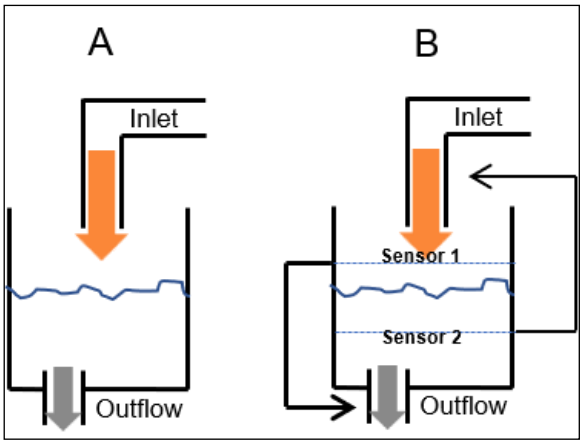


Figure 1: Consequential steady state (A) and Targeted steady state (B) models of homeostasis shown with a tank water level analogy. In CSS, a change in size of inlet or outlet, analogous to insulin sensitivity can change the steady state level. In a TSS model, a change in the inlet or outlet will alter the time required to reach a steady state but will not change the steady state level itself.

If we compare the water tank with glucose homeostasis, we can see that in a fasting state, hepatic glucose production is analogous to the input to the tank and uptake of glucose by the tissues is analogous to the size of the outlet. Both these processes are a function of insulin signalling. Most mathematical models of glucose regulation assume CSS (Tomasi *et al.*, 1967; Turner *et al.*, 1979; Matthews *et al.*, 1985; Bergman, 1989, 2005; Makroglou, Li and Kuang, 2006, Palumbo *et al.*, 2013).

We would like to examine critically which model: CSS or TSS describes glucose homeostasis more aptly. If TSS model is more relevant, insulin resistance and relative insulin deficiency will not lead to a changed steady state in the glucose. The time required for reaching the steady state after perturbation might change, even though the steady state level per se might now. If CSS model is relevant, insulin resistance or reduced insulin levels are likely to change fasting glucose levels. As seen in chapter 2, the meta-analyses show that IRKOs or insulin suppression experiments do not alter fasting steady state. This lack of change of steady state and the delay in reaching the steady state indicates that TSS is more relevant as a model to describe glucose homeostasis.

For the TSS model to work, there must be mechanisms of sensing any departure from the ‘targeted’ steady state. Such mechanisms are not known in peripheral system, but glucose sensing neurons are certainly known to be present in the

brain. Therefore, if TSS is a more appropriate model, the CNS mechanisms are likely to be central to glucose homeostasis, particularly in determining the steady state levels; whereas insulin signalling would determine the rate at which a steady state is reached after perturbation.

3.2.2 Making testable predictions from the models

We now try to make testable predictions of TSS and CSS models. We first look at the situation in a healthy individual with normal glucose regulation.

3.2.2.1 Exercise and glucose regulation

Increased glucose utilization would decrease fasting or steady state glucose levels by the CSS model but not by the TSS model. We can test this prediction. Experiments on human subjects have shown that prolonged exercise does not reduce plasma glucose, in fact it might increase (Coggan, 1991). To match with experimental data, CSS models of glucose dynamics during exercise need to include additional terms which involve central mechanisms. Possible central mechanisms include parasympathetic and sympathetic control of hepatic glucose production in response to exercise (Roy and Parker, 2006). This CSS model is closer to a TSS model. If TSS model describes glucose homeostasis more appropriately, reduced insulin signalling is not expected to change steady state glucose but only alter the time course to reach a steady state.

3.2.2.2 Attaining an hyperinsulinemic normoglycemic state

The CSS and TSS models have differences in the mechanism of attaining a hyperinsulinemic normoglycemic prediabetic state. By the classical CSS based pathway, the primary event is the obesity induced insulin resistance. Insulin resistance causes reduced glucose uptake, and the increased blood glucose triggers a compensatory insulin response. This increased insulin response or hyperinsulinemia compensates for insulin resistance and thus the fasting glucose levels remain normal. Chawla et al 2018 have given an in-depth analysis of the model. They matched the predictions of this model with empirical data to refute this model (Chawla *et al.*, 2018). This refutation is not surprising as one can intuitively see that once the increased insulin levels normalize glucose, there is no reason why insulin levels continue to remain high. Hence, a steady state with

hyperinsulinemia and normal glucose levels is impossible by the CSS model but it does exist in prediabetic subjects.

Testable prediction of CSS model

If a "compensatory" insulin response is mediated by glucose, one would expect a positive correlation between fasting glucose (FG) and fasting insulin (FI) and no correlation between insulin resistance and β cell responsiveness.

Testable prediction of TSS model

On the other hand, a compensatory response is possible in either way by the TSS model. Primary insulin resistance may increase the glucose levels transiently. When the glucose sensing mechanisms detect a change, a compensatory response comes into effect, which will remain operational due to hysteresis unless glucose levels reach the lower margin of normal level. By this mechanism, a hyperinsulinemic normoglycemic state is possible. Alternatively, primary hyperinsulinemia (Garvey, Olefsky and Marshall, 1986; Weyer *et al.*, 2000; Shanik *et al.*, 2008; Corkey, 2012) can also be compensated by increased insulin resistance by hitting the lower level of sensing which would trigger compensatory insulin resistance. Even in this case a hyperinsulinemic normoglycemic state is possible. Both glucose sensing neurons and neuronal regulation of insulin release and liver glucose production are well known.

In the compensatory response mediated by TSS pathways there need not be a correlation between fasting insulin and fasting glucose, but insulin resistance and β-cell response would be correlated.

3.2.2.3 Testable predictions from a generalized CSS model

A variety of models are present in literature to try and explain glucose dynamics. We use a generalized minimal CSS model using the following assumptions.

1. The blood glucose level *G* increases by two processes (i) absorption from gut and (ii) glucose production by the liver.
2. Gut absorption *Gt* to be independent of standing blood glucose as well as insulin

3. Liver glucose production has a maximum rate *L* which has two feedback inhibitors namely direct feedback inhibition by glucose and that by standing insulin which depends upon the insulin sensitivity of liver.
4. Glucose is cleared from blood via two mechanisms (i) insulin independent and (ii) insulin dependent.
5. The blood insulin *I* is a balance between insulin release by pancreatic β-cells, the rate being a function of plasma glucose and a rate of insulin degradation which is directly proportional to standing plasma insulin level.
6. We assume all relationships to be linear and use the model framework of Chawla et al 2018 (Chawla *et al.*, 2018).

The equations:

$$\frac{dG}{dt} = Gt + L - K_1.G - I_{SENS}.K_2.I. \qquad \text{Equation 1}$$

$$\frac{dI}{dt} = K_3.G - d.I \qquad \text{Equation. 2}$$

Where:

G: Plasma glucose

Gt: Gut glucose

L: Maximum rate of liver glucose production

I: Plasma insulin

K_1: Rate constant for glucose uptake by tissues as well as direct feedback inhibition of liver glucose production

K_2: Rate constant for insulin mediated inhibition of liver glucose production as well as insulin mediated glucose uptake,

I_{SENS}: Insulin sensitivity which is assumed to be unity normally and decreases with insulin resistance.

K_3: Rate constant for glucose stimulated insulin secretion.

d: Rate of insulin clearance

We use simulations with normally distributed errors to study how the correlation between plasma glucose and insulin is affected by the parameters as well as by the standard deviation of errors. We use the errors additively or multiplicatively. For

simulations using additive errors, we add normally distributed error terms e_1 and e_2 to both the equations.

$$\frac{dG}{dt} = Gt + L - K_1.G - I_{SENS}.K_2.I. + e_1 \qquad \text{Equation. 3}$$

$$\frac{dI}{dt} = K_3.G - d.I + e_2 \qquad \text{Equation. 4}$$

For simulations using multiplicative error, we give normal distributions to K_1, K_2, K_3 and I_{SENS}. Realistic ranges for the parameters are taken from (Chawla *et al.*, 2018). Simulations show that if we use an additive error model, and if the parameters of glucose insulin relationship are the same, the regression-correlation parameters for the insulin-glucose relationship are not significantly different during fasting/steady state ($Gt=0$) and at any time post-meal ($Gt> 0$). The only difference is in the range of glucose and insulin distribution (figure 2)

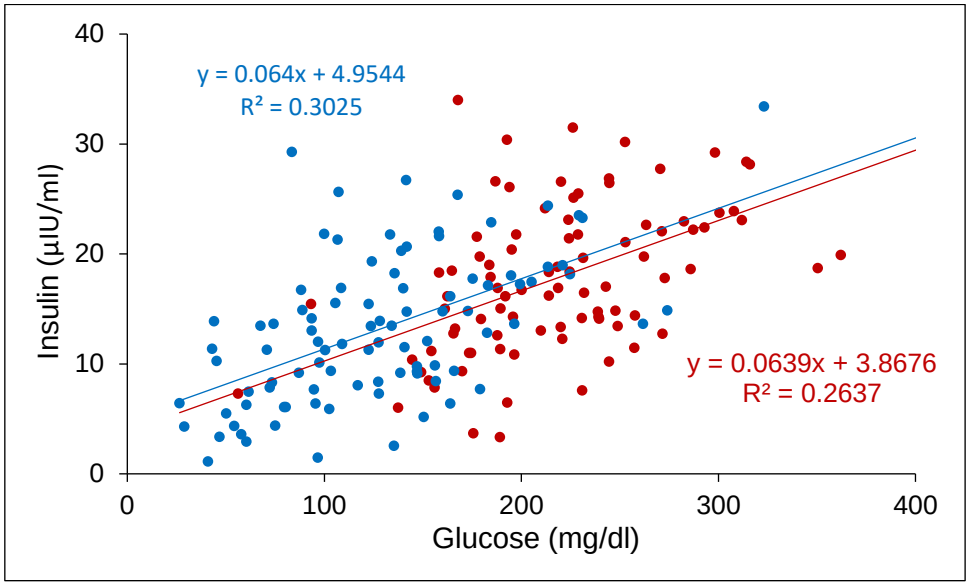


Figure 2: The glucose insulin scatter in a fasting steady state (blue circles) and in a post-meal arbitrary but constant time interval (red circles) in an additive error model. A sample result is shown in which K_1=0.1, K_2=0.9, I_{SENS} is randomized between 0.1 and 1 and K_3=0.015 and d=0.15. The error standard deviations are 15 and 1 respectively.

In simulations with multiplicative errors, the post-meal insulin-glucose correlation was weaker than the fasting steady state correlation (figure 3). This is the likely result of the errors increasing in proportion to larger values of glucose and insulin, and due to the addition of the variable (gut absorption) to the model.

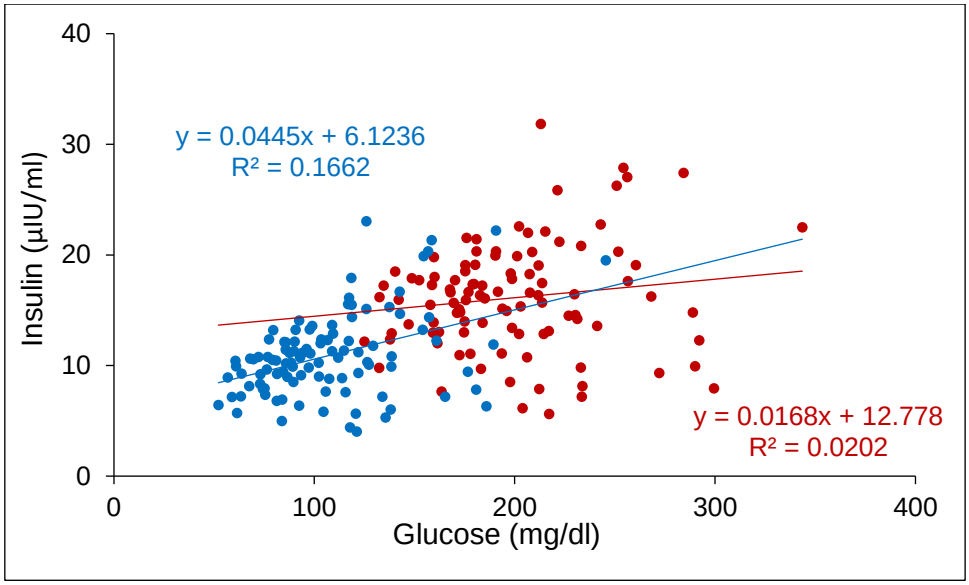


Figure 3: The glucose insulin scatter in a fasting steady state (red squares) and at a post-meal arbitrary but constant time point (blue diamonds) in a multiplicative error model. A sample result is shown in which the mean (standard deviations) of the parameters were K_1=0.1 (0.02), K_2=0.9 (0.5), I_{SENS} is randomized between 0.1 and 1 K_3=0.0015 (0.0002) and d =0.15 (0.005). In all the simulations the correlation coefficient and regression slopes of the post-meal scatters were less than or equal to the corresponding fasting parameters. This contrasts the epidemiological patterns in which the fasting correlations are substantially weaker than the post-meal correlations.

Over a wide range of parameters, the slopes of lines and correlation coefficients changed. The generalization that R^2 as well as the regression slope of the fasting scatter was less than or equal to that of the post-meal condition remained constant and robust. In some parameter space, that is when the variability in insulin response was substantially greater than the variability in insulin resistance, the correlation between glucose and insulin was negative. But again, the fasting correlation was equal or stronger than the post-meal correlation.

Thus, a generalization can be made that if the model parameters remain the same, the glucose insulin correlation in steady state is stronger or equal to the post-meal correlation. Logically and intuitively sound, this generalization is unlikely to be specific to any form of equations based on the assumptions of the CSS class of models.

Testable predictions

According to the CSS model, the R^2 and the slope of regression between glucose and insulin for post-meal (perturbed) scatters are weaker or equal than that for the fasting (steady state).

With a TSS model there should not be a correlation in fasting glucose (FG) and fasting insulin (FI). On impairment of insulin signalling, the time required to attain a steady state can be substantially longer, overnight fasting may not ensure a steady state in all individuals. Fasting hyperglycaemia in T2DM can have two alternative (but not mutually exclusive) causes. Either it represents the failure to reach a steady state in the specified fasting period, or it is because of mechanisms other than reduced insulin action. In population data, if some individuals have reached a steady state but a few others haven't we would expect a correlation in FG and FI but significantly weaker than the post-meal correlation.

3.2.3 Data sets used to test the predictions

The three data sets used here come from two different studies: (i) Coronary Risk of Insulin Sensitivity in Indian Subjects (CRISIS) study, Pune, India (Yajnik *et al.*, 2007) and (ii) Newcastle Heart Project (NHP), UK (Bhopal et al., 1999). Data from the NHP is divided into two groups as the subjects belong to different ethnicities namely European white and south Asian. We have analysed the two groups separately, since there are differences in the tendency to develop metabolic syndrome depending on the ethnicity (Bhopal, 2013; Gujral et al., 2013). Hence testing of prediction of the models with the data has been done independently for the three data sets. All the studies are population surveys that include non-diabetic (fasting glucose values less than 110mg/dl) and diabetic individuals (fasting glucose values above 110 mg/dl) subjects. The clinical history, morphometric parameters, glucose, and insulin during fasting and oral glucose tolerance test (OGTT) of the subjects were recorded. In the analysis, we included only the non-diabetic groups in which the homeostatic mechanism can be assumed to be intact and therefore any hypothesis about it can be tested. Most of the individuals in the diabetic group would be under different drug regime affecting insulin-glucose dynamics in different ways and therefore we exclude that group for the analysis.

3.2.4 Statistics

Linear regression and correlation were used to compare the insulin-glucose relationship in steady state (fasting) versus perturbed state (post glucose load) in the three data sets. The regression and correlation between the derived parameters of HOMA-IR and HOMA-β were also used to test the predictions of the models.

3.2.5 Results

In all the three data sets there was weak (R^2 range 0.017 to 0.057) yet significant correlation between fasting glucose (FG) and fasting insulin (FI). Contrary to the prediction of the CSS model, the post-meal insulin-glucose correlation was stronger (R^2 range 0.28 to 0.34) and the slope of the regression much steeper than in the fasting state. The correlation between HOMA-IR and HOMA-β is strong (R^2 range 0.20 to 0.83) as predicted by the TSS model and not by the CSS model. The HOMA-IR HOMA-β correlation, as well as the difference between the regression correlation parameters between fasting and post-meal data were compatible with predictions of the TSS model. However, although weak, there is significant correlation between FG and FI unlike what may be expected by a steady state TSS model. This incompatibility is not sufficient to falsify the TSS model as the failure of a small proportion of individuals to reach a steady state at overnight fasting could explain the weak correlation. It is also likely that the assumption of fasting may not be true for the entire sample. Even if a small number of individuals do not comply with the overnight fasting instructions, a positive correlation could be seen. This possibility is extremely difficult to exclude in human data.

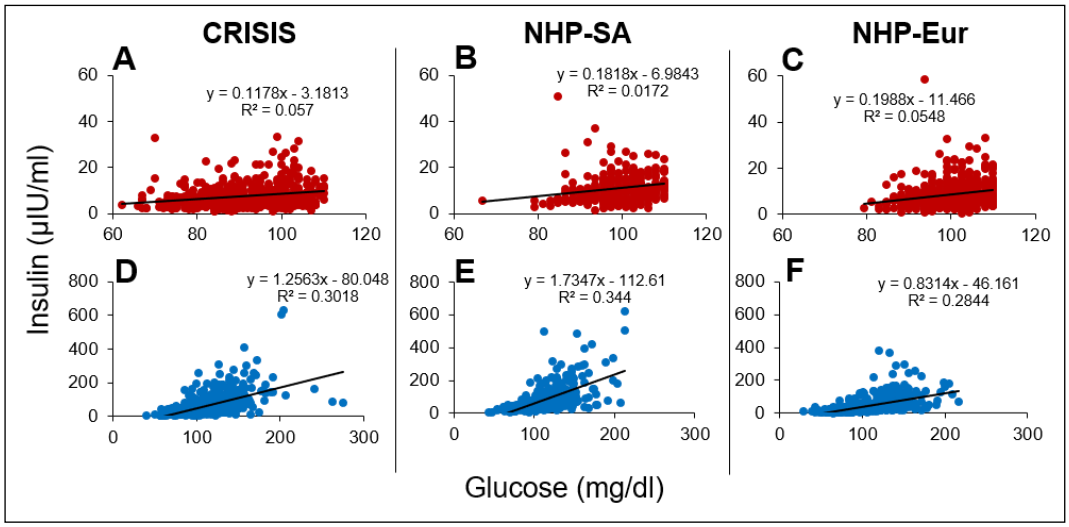


Figure 4: The Fasting Glucose-Fasting Insulin (A to C), Post-meal Glucose-Post-meal Insulin (D to F) scatter plots in non-diabetic subjects. The FG-FI correlation is weak as compared to post-meal correlation.

Table 1: Correlation and regression parameters for the insulin-glucose relationship in steady and perturbed states in the normal and diabetic subjects

	Steady state (fasting)			Perturbed state (2 hours post glucose bolus)		
Parameter → Data set ↓	**R-squared (variance explained)**	**p value**	**Slope (95% CI bounds)**	**R-squared (variance explained)**	**p value**	**Slope (95% CI bounds)**
Normal fasting glucose (NFG) subjects						
CRISIS (N=522)	0.0570 (5.7%)	<0.0001*	0.1178 (0.0765 to 0.1591)	0.3018 (30.18%)	<0.0001*	1.2563 (1.0917 to 1.4209)
NHP-South Asian (N=310)	0.0172 (1.72%)	0.021*	0.1818 (0.0279 to 0.3356)	0.344 (34.4%)	<0.0001*	1.7347 (1.4661 to 2.0033)
NHP-European (N=574)	0.0548 (5.48%)	<0.0001*	0.1988 (0.131 to 0.2666)	0.2844 (28.44%)	<0.0001*	0.8314 (0.7231 to 0.9397)
Impaired fasting glucose (IFG) subjects						
CRISIS (N=42)	0.007 (0.7 %)	0.940	0.012 (-0.035 to 0.059)	0.002 (0.2%)	0.836	0.033 (-0.214 to 0.280)
NHP-South Asian (N=143)	0.0001 (0.01%)	0.907	0.002 (-0.032 to 0.036)	0.015 (1.5%)	0.285	-0.09 (-0.211 to 0.031)
NHP-European (N=174)	0.0000(0%)	0.937	-0.001 (-0.033 to 0.031)	0.039 (3.9%)	0.009*	0.172 (0.043 to 0.3)
NFG and IFG combined						
CRISIS (N=564)	0.044 (4.4%)	<0.0001*	0.056 (0.034 to 0.078)	0.124 (12.4%)	<0.0001*	0.515 (0.402 to 0.629)
NHP-South Asian (N=453)	0.016 (1.6%)	0.006*	0.041 (0.012 to 0.070)	0.025 (2.5%)	0.0007*	0.183 (0.078 to 0.288)
NHP-European (N=748)	0.022 (2.2%)	0.00004*	0.045 (0.024 to 0.066)	0.150 (15%)	<0.0001*	0.413 (0.342 to 0.484)

The insulin-glucose correlation pattern is substantially altered in the IGF data (table 1). However, most individuals in the IGF group were under treatment, the drug regime of every individual being widely different. The drugs are likely to produce substantial noise in the data. Therefore, any inferences from this group are unlikely to be useful in testing the CSS versus TSS predictions.

The support of TSS model over CSS model in epidemiological data is important because it accounts for the failure of impairment of insulin signalling to alter fasting glucose but alter the nature of the glucose tolerance curve and delay the return to the steady state level.

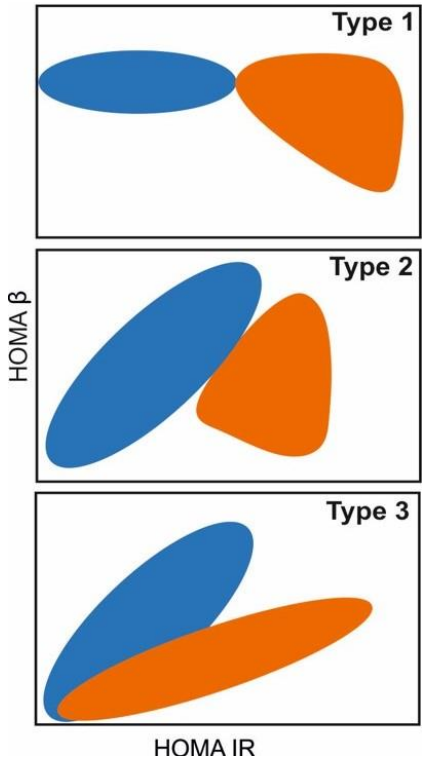


Figure 5: HOMA-IR and HOMA-β relationship based on alternative pathophysiological models The blue scatter area represents the NFG group and the orange scatter area represents the IFG group.

In a different approach, the HOMA-IR, HOMA-β scatter can be used to test alternative pathophysiological models (figure 5).

(i) In the classical view, obesity induced insulin resistance is primary, and the compensatory insulin response is mediated by glucose stimulated insulin response. However, the β-cell responsiveness is not assumed to increase. If this is true, and if we assume that HOMA-IR is a faithful measure of insulin resistance

and HOMA- β a faithful measure of β-cell response, then in the NFG group, there should be no correlation between HOMA-IR and HOMA β. The IFG group should lie to the right of the NFG group since high insulin resistance is the primary and necessary condition for IFG. The IFG range may extend downwards on the HOMA β axis as in type 1 pattern of figure 5.

(ii) If, on the other hand, we assume that β-cell response also increases in response to insulin resistance, then we expect a positive correlation between HOMA-IR, HOMA β in the NFG group. In this case the IFG group should still lie only at higher values of HOMA-IR compared to the NFG group but lower on the HOMA β axis as in type 2 pattern of figure 5

(iii) Alternatively, we may assume that the fasting sugar is independent of insulin action and therefore HOMA-IR and HOMA β do not really reflect on insulin action and β-cell response, respectively. In that case we expect that HOMA-IR, HOMA β will be positively correlated simply because they have a common numerator term in their calculation. If glucose increases independent of insulin, HOMA-IR will increase and HOMA β decrease simply because of the formula for calculating the two indices. In such a case IFG would lie to the right and lower position as compared to NFG throughout the NFG range, as depicted in type 3 patters of figure 5. Here it is not necessary that IFG is seen only at high values of HOMA-IR.

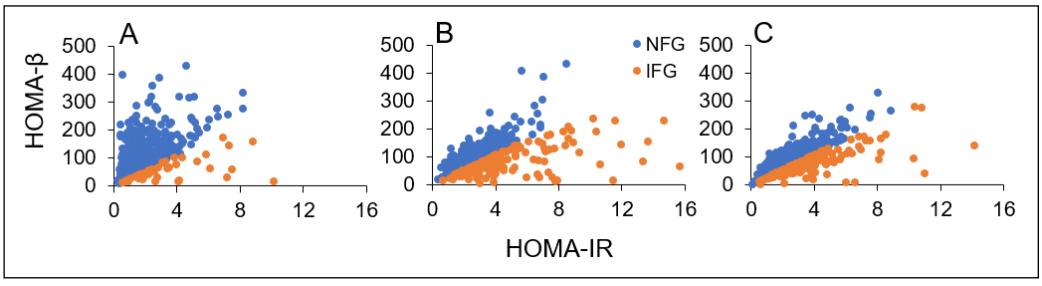


Figure 6: HOMA-IR-HOMA-β scatters in the three data sets. A: CRISIS, B: NHP-SA and C: NHP-Eur.

Using the same three data sources as above, we see that in all the three the type 3 pattern is followed by the distributions of NFG and IFG in the HOMA-IR, HOMA β scatter (figure 6). This pattern further supports the hypothesis that change in fasting sugar is independent of insulin and insulin resistance and HOMA-IR,

HOMA β do not really represent insulin resistance and β cell responsivity as classically assumed (Chawla et al 2018).

3.3 Conclusion

In short from multiple predictions, the classical CSS based model fails to get its predictions supported by human epidemiological data. The data are more compatible with the alternative hypothesis that fasting glucose is not decided by insulin action. Glucose regulation follows a TSS model, where the target is set by mechanisms independent of insulin.

3.4 References

Bergman, R. N. (1989) 'Toward Physiological Understanding of Glucose Tolerance: Minimal-Model Approach', *Diabetes*, 38(12), pp. 1512–1527. doi: 10.2337/diab.38.12.1512.

Bergman, R. N. (2005) 'Minimal Model: Perspective from 2005', *Hormone Research in Paediatrics*, 64(3), pp. 8–15. doi: 10.1159/000089312.

Bhopal, R. *et al.* (1999) 'Heterogeneity of coronary heart disease risk factors in Indian, Pakistani, Bangladeshi, and European origin populations: cross sectional study', *BMJ*, 319(7204), pp. 215–220. doi: 10.1136/bmj.319.7204.215.

Bhopal, R. S. (2013) 'A four-stage model explaining the higher risk of Type 2 diabetes mellitus in South Asians compared with European populations', *Diabetic Medicine*, 30(1), pp. 35–42. doi: 10.1111/dme.12016.

Chawla, S. *et al.* (2018) 'Inferring causal pathways among three or more variables from steady state correlations in a homeostatic system', *PLOS ONE*. Edited by M. Ruscica, 13(10), p. e0204755. doi: 10.1371/journal.pone.0204755.

Coggan, A. R. (1991) 'Plasma Glucose Metabolism During Exercise in Humans', *Sports Medicine*, 11(2), pp. 102–124. doi: 10.2165/00007256-199111020-00003.

Corkey, B. E. (2012) 'Banting Lecture 2011: Hyperinsulinemia: Cause or Consequence?', *Diabetes*, 61(1), pp. 4–13. doi: 10.2337/db11-1483.

Diwekar-Joshi, M., & Watve, M. (2020). Driver versus navigator causation in biology: the case of insulin and fasting glucose. *PeerJ*, *8*, e10396. https://doi.org/10.7717/peerj.10396

Garvey, W. T., Olefsky, J. M. and Marshall, S. (1986) 'Insulin Induces Progressive Insulin Resistance in Cultured Rat Adipocytes: Sequential Effects at Receptor and Multiple Postreceptor Sites', *Diabetes*, 35(3), pp. 258–267. doi: 10.2337/diab.35.3.258.

Gujral, U. P. *et al.* (2013) 'Type 2 diabetes in South Asians: similarities and differences with white Caucasian and other populations', *Annals of the New York Academy of Sciences*, 1281(1), pp. 51–63. doi: 10.1111/j.1749-6632.2012.06838.x.

Halter, J. B. *et al.* (1985) 'Glucose regulation in non-insulin-dependent diabetes mellitus. Interaction between pancreatic islets and the liver.', *The American journal of medicine*, 79(2B), pp. 6–12. Available at: http://www.ncbi.nlm.nih.gov/pubmed/2863979.

Lerner, R. L. and Porte, D. (1972) 'Acute and steady state insulin responses to glucose in nonobese diabetic subjects', *Journal of Clinical Investigation*, 51(7), pp. 1624–1631. doi: 10.1172/JCI106963.

Makroglou, A., Li, J. and Kuang, Y. (2006) 'Mathematical models and software tools for the insulin-glucose regulatory system and diabetes: an overview', *Applied Numerical Mathematics*, 56(3–4), pp. 559–573. doi: 10.1016/j.apnum.2005.04.023.

Matthews, D. R. *et al.* (1985) 'Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28(7), pp. 412–419. doi: 10.1007/BF00280883.

Palumbo, P. *et al.* (2013) 'Mathematical modeling of the glucose–insulin system: A review', *Mathematical Biosciences*, 244(2), pp. 69–81. doi: 10.1016/j.mbs.2013.05.006.

Roy, A. and Parker, R. S. (2006) 'DYNAMIC MODELING OF EXERCISE EFFECTS ON PLASMA GLUCOSE AND INSULIN LEVELS', *IFAC Proceedings Volumes*, 39(2), pp. 509–514. doi: 10.3182/20060402-4-BR-2902.00509.

Shanik, M. H. *et al.* (2008) 'Insulin Resistance and Hyperinsulinemia: Is hyperinsulinemia the cart or the horse?', *Diabetes Care*, 31(Supplement 2), pp. S262–S268. doi: 10.2337/dc08-s264.

Tomasi, T. *et al.* (1967) 'Insulin Half-Life in Normal and Diabetic Subjects.', *Experimental Biology and Medicine*, 126(1), pp. 315–317. doi: 10.3181/00379727-126-43434.

Turner, R. C. *et al.* (1979) 'Insulin deficiency and insulin resistance interaction in diabetes: Estimation of their relative contribution by feedback analysis from basal plasma insulin and glucose concentrations', *Metabolism*, 28(11), pp. 1086–1096. doi: 10.1016/0026-0495(79)90146-X.

Weyer, C. *et al.* (2000) 'A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia', *Diabetes*, 49(12), pp. 2094–2101. doi: 10.2337/diabetes.49.12.2094.

Yajnik, C. S. *et al.* (2007) 'Adiposity, inflammation and hyperglycaemia in rural and urban Indian men: Coronary Risk of Insulin Sensitivity in Indian Subjects (CRISIS) Study', *Diabetologia*, 51(1), pp. 39–46. doi: 10.1007/s00125-007-0847-1.

Chapter 4: Does impairment of insulin signalling affect steady state glucose? The streptozotocin (STZ) model

4.1 Introduction

Streptozotocin (STZ) induced β-cell destruction is a popular model of rodent hyperglycaemia (Szkudelski, 2001; Akbarzadeh *et al.*, 2007; Zhang *et al.*, 2008). STZ is believed to act by specifically destroying the insulin producing β-cells of the pancreatic islets (Szkudelski, 2001). The STZ model has been considered a strong support to the idea that disruption of insulin signalling is the sole or main cause of glucose dysregulation. Therefore, it is necessary to examine the strength of this evidence, vis a vis alternative possible interpretation. A low dose of STZ that destroys a substantial population of β-cells but does not lead to total destruction of their population is often perceived as a model for T2DM whereas a high dose of STZ that destroys the β-cell population almost entirely is perceived as a model of T1DM (Gajdosík *et al.*, 1999; Zhang *et al.*, 2008). We searched literature to look for studies that carefully differentiated between steady state glucose from post-load glucose in STZ models but surprisingly did not find any studies that make this distinction clearly. Therefore, we could not perform meta-analysis like the other glucose and insulin modulation methods described in the chapter 2.

There are certain other paradoxes associated with STZ experiments too. By the classical belief, STZ destroys the β-cells and this is said to be the reason for long term hyperglycaemia in the treated animals. However, some studies show that β-cell population regenerates quite rapidly in the STZ treated animals (Wang, Bouwens and Klöppel, 1996; Movassat and Portha, 1999). In general, the β-cells have good reproductive capacity of regeneration as well as neogenesis (Levine and Itkin-Ansari, 2008; Porat *et al.*, 2011). The regenerated cells have also been shown to stain normally for insulin content (Cano *et al.*, 2008). However, despite revival of the β-cells with normal insulin content, hyperglycaemia continues. This paradox has not been addressed satisfactorily, and it raises the possibility that the long-lasting hyperglycaemia after STZ treatment is not because of destruction of

β-cells alone but due to some other effect of the STZ treatment underappreciated so far.

The critical questions asked in this study were:

(i) What are the insulin levels in STZ animals showing hyperglycaemia? Does STZ lead to absence or significantly reduced levels of insulin and does that adequately explain the hyperglycaemia observed?

(ii) Does STZ treatment affect both steady state and perturbed state glucose in a similar way? If reduced insulin response to glucose is the main cause of hyperglycaemia, then the increase in fasting and post load glucose is expected to be similar as predicted by the model in the last chapter. The regression-correlation between glucose and insulin in the fasting versus post-meal state should have similar parameters despite a different range. If they are affected in significantly different ways, additional or alternative explanation is required.

(iii) Is the effect of STZ treatment on SS and PS glucose levels explained by inadequate insulin? If yes, how much variance is glucose in explained by insulin within and between treatments?

(iv) Since some experiments show that the β-cells can regenerate after STZ treatment (Wang, Bouwens and Klöppel, 1996; Movassat and Portha, 1999), we wanted test whether the hyperglycaemic effect is reduced by giving more time for regeneration of cells.

To answer these questions, we designed and conducted experiments to differentially study the steady state and perturbed state glucose levels in rats treated with STZ. We performed two different experiments to see the differential effect of STZ on the SS and PS glucose. In the first experiment we tested the effect of STZ treatment over a period of 12 days starting at day 4. However, to consider the possibility that β cell regeneration may have been completed before day 4, in the second experiment we also tried to see the effect of the STZ treatment at days 2, 4 and 6. Additionally, we monitored the dynamics of plasma glucose post-feeding to see the time taken to reach a steady state. The work done described in this chapter has been published as a part of this paper (Diwekar-Joshi and Watve, 2020).

4.2 Experimental methods

4.2.1 Animal model and conditions

Approval by the Ethics Committee

The experiments performed on Sprague Dawley (SD) rats were approved by the Institutional Animal Ethics Committee at IISER, Pune (Protocol Number IISER/IAEC/2016-02/006). Refer to the Appendix for the approval certificate. This committee is constituted by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Govt. of India.

Housing of the animals

The SD rats were housed at a temperature of 23±2°C with a 12-hour light/dark cycle and standard rat chow (Altromin rat/mice maintenance diet) and water available *ad libitum*. The bedding of the cages was changed every three days till the rats were of a suitable weight for the experiment. The bedding was changed daily after injection of STZ for the duration of the experiment. There were no extra measures taken for the enrichment of the animals.

Euthanasia

At the end of the of glucose and insulin readings for the duration of the experiments (either 6 days or 12 days), the animals were euthanized with an intra-peritoneal (IP) injection of thiopentone (100-120mg/kg body weight).

4.2.2 STZ treatment for insulin suppression

Male, SD rats weighing 180-200grams were injected intra-peritoneal (IP) with STZ at a dose of either 50mg/kg or 70mg/kg body weight. The STZ was dissolved in Citrate Buffer (Citric Acid: 0.1M and Sodium Citrate: 0.1M). Injection of citrate buffer (CB) alone was used as control.

4.2.3 Steady state and perturbed state glucose and insulin in a 12 day follow up

Figure 1 gives the schematic representation of the experimental procedure followed. Three days after the STZ injection, the rats were fasted for 16 hours; after which fasting glucose was measured. The rats were then given 40 grams of Standard chow. Three hours after the food was given, the post-feeding glucose was measured. The food was weighed again after five hours and removed to start the 16-hour fasting for the next day. This protocol was repeated for 12 days and

body weight, food weight and glucose readings were taken daily. 12 animals per group were used for this experiment.

Sampling for glucose estimation:

Fasting and 3 hours post feeding glucose was measured using a handheld Accu-Chek Glucometer in this experiment. This measurement required about 0.5-1µl of blood which was withdrawn using the tail prick method. Since the volume of blood required for this test was small, this sampling was carried out every day. Hence, we had the fasting and post-feeding glucose readings for all the animals on all the 12 days (figure 1)

Sampling for insulin estimation:

On the days four, 8 and 12, 500µl to 1ml blood was drawn from the retro-orbital sinus from the rats for the measurement of insulin using isoflurane for anaesthesia. (figure 1). Insulin was measured Rat-Insulin ELISA kit from Thermo-Fischer according to the manufacturer's instructions. To minimize the stress of repeated blood collection, blood was not drawn at the fasting and post feeding time-points from the same animal. Thus, half the number of rats were anaesthetised, and blood was drawn from them at the fasting time point on the days four, 8 and 12 and the remaining at the post-feeding time point. So, we had half the number (6 animals per condition) of insulin readings for the fasting and post-feeding condition on the days four, 8 and 12 as compared to the glucose readings from all the animals (12 per condition) for all the 12 days.

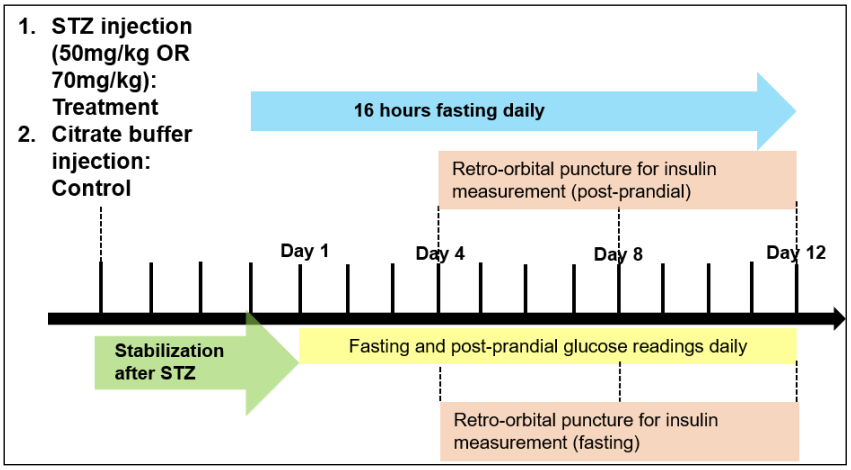


Figure 1: Schematic representation of the experimental protocol followed to test the effect of insulin suppression on steady and perturbed state of glucose.

4.2.4 Time required to reach a steady state

According to the published literature, fasting durations ranged from four hours to 20 hours. We selected 16 hours as the duration of fasting based on review of literature (Reed *et al.*, 2000; Zhang *et al.*, 2008; Nowland, Hugunin and Rogers, 2011; Arindkar *et al.*, 2012). In another experiment, we wanted to test if the assumption that fasting glucose is steady state glucose is correct, i.e., whether a steady state is reached in 16 hours. The food was removed from the STZ and Control animals after overnight *ad libitum* availability and glucose readings were taken after 3 hours, 6 hours, 9 hours, 12 hours and 16 hours. After a recovery of three days, glucose levels were measured directly at 16 hours after removing the food. 8 STZ treated animals and 10 Control animals were used for this experiment.

4.2.5 Short term effect of STZ on steady and perturbed state of glucose

We also performed one more experiment in which we tried to have a look at the short term or immediate effect of STZ suppression on glucose and insulin. In the earlier experiment (figure 1) we started measuring the effect of STZ on glucose on the fourth day after STZ injection since by a commonly followed norm, a three-day stabilisation period after the injection is given before starting fasting protocol. The first insulin measurement was done on the fourth day after starting the fasting protocol. Along with the long-term or delayed effects of STZ, we also wanted to see the short-term effects on the levels of steady state glucose and insulin after the STZ treatment. In this experiment, we started the fasting protocol on the second day after the STZ treatments and took the readings on day four and day six as well. The fasting duration, method of feeding and weighing of food was identical to the first experiment. Figure 2 shows the timeline and protocol for this experiment.

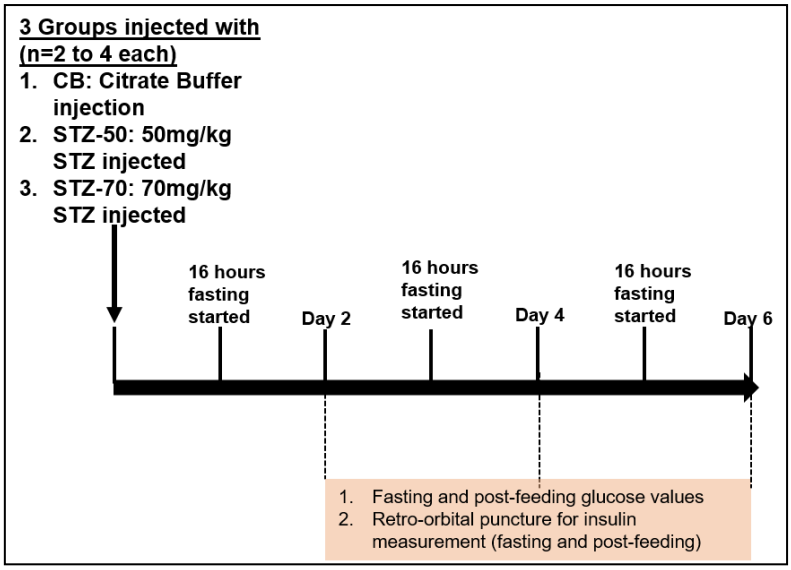


Figure 2: Schematic representation of the experimental protocol followed to test the short-term effect of insulin suppression on steady and perturbed state of glucose.

4.2.6 Statistical analysis

Glucose and insulin levels of the control and the STZ treated rats were compared using a non-parametric Mann-Whitney U test. Since individual responses to STZ treatment are highly variable and the resultant distribution is likely to be skewed with the possibility of outliers, non-parametric statistics was considered more appropriate for comparison. In addition to these tests, the correlation between glucose and insulin in the steady state (fasting) and perturbed state (post-feeding) was also compared using the Pearson's correlation coefficient (r), but was also backed by non-parametric Spearman's ranked correlations. For some of the questions, such as whether insulin and glucose in the same animal at a given time are correlated, data from both experiments and the three treatment groups was analysed separately as well as after pooling.

4.3 Results

4.3.1 Effect of STZ treatment on body weight and food intake

In all the three groups, the body weight increased over the span of 12 days (figure 3). There was no significant difference in the body weights of the three groups to begin with and the increase in the body weight in each group across 12 days was also not significant, although there was a weak but consistent trend of lower body weights in the STZ treated animals. It has been reported in literature that STZ treatment results in an increased food intake (Hebden *et al.*, 1986; Gelling *et al.*,

2004). We also measured the food intake of the three groups across the 12 days of the experiment (figure 4). There is no significant difference in the morning (3 hours after taking the fasting glucose reading)/afternoon (5 hours after taking the post-feeding glucose reading) or the total (morning + afternoon) food intake of the three groups.

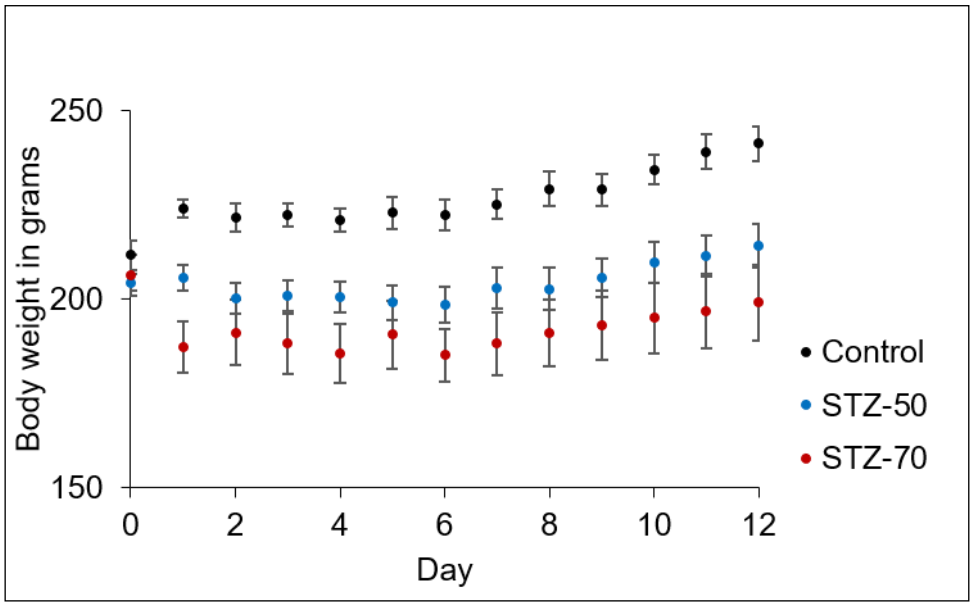


Figure 3: Effect of STZ treatment on the body weight of the SD rats. Results expressed as the mean ± SD for each group. The sample sizes are as follows for the three groups: N=14 for Control, N=12 for STZ-50 and N=9 for STZ-70.

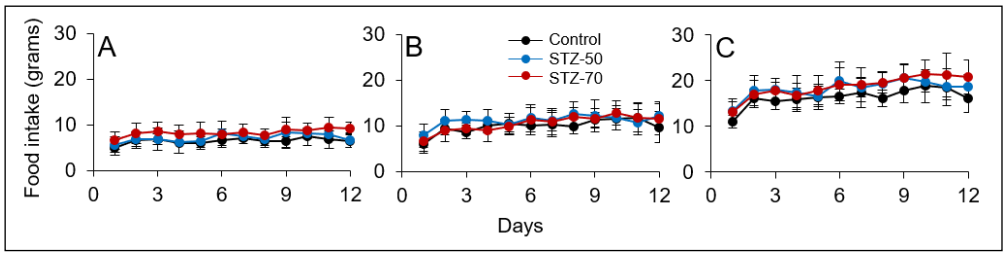


Figure 4: Effect of STZ treatment on A: Morning food intake, B: Afternoon food intake and C: Total food intake of the SD rats. Results expressed as mean ± SD for each group. The sample sizes are as follows for the three groups: N=14 for Control, N=12 for STZ-50 and N=9 for STZ-70.

4.3.2 Differential effect of STZ injection on fasting and post-feeding glucose levels

We used the STZ treatment to induce hyperglycaemia in SD rats. We used two different doses of STZ namely 50mg/kg and 70mg/kg. These doses were decided based on the review of literature and had been used earlier to induce a T2D-like

hyperglycaemia in rats (Gajdosík *et al.*, 1999; Freitas *et al.*, 2015). We performed three different experiments on the STZ treated SD rats to answer different questions. The starting condition of the animals, the mode of injection of the STZ and the measurement of glucose levels after the injection was however carried out in similar manner. This enabled us to pool the data from all the experiments in the context of certain questions. The pattern seen in the pooled data are that an STZ dose of 70mg/dl or even 50mg/dl resulted in an increased post-feeding glucose (> 200mg/dl) in almost all rats. The effect of the STZ treatment on the fasting glucose levels of the rats was not the same as that on the post-feeding levels. If we look at the distribution of the glucose levels in the STZ treated rats (figure 3), we see that the mode of the distribution in case of the fasting glucose values is within the normal range for treatments with 50 and 70mg/kg body weight whereas the mode for post-feeding is in the higher than normal range (>200mg/dl). As expected, the mode for the 70mg/kg STZ treatment is higher than that of 50mg/kg treatment.

Also, the distribution appears to be bi-modal for the fasting glucose values. The fasting glucose of a significant proportion of STZ rats remained normal (<140mg/dl) in spite of having high post-feeding levels of glucose (table 1). In case of treatment with 50mg/kg STZ, 17 out of 23 rats had normal fasting glucose and increased post-feeding glucose levels and only 6 out of the 23 (26.09%) had increased fasting glucose levels. In 70 mg/Kg treatment there was a greater proportion of rats with IFG 9 out of 22 (40%). Still in both groups majority of animals had normal fasting glucose but increased post feeding glucose. Figure 4 shows the scatters of the post-feeding glucose levels against the fasting glucose levels of the STZ treated rats. Almost all of the STZ treated rats have high post-feeding glucose, but the fasting glucose levels of the STZ-treated rats fall into two distinct groups with an apparent gap in between (figure 3 and 4).

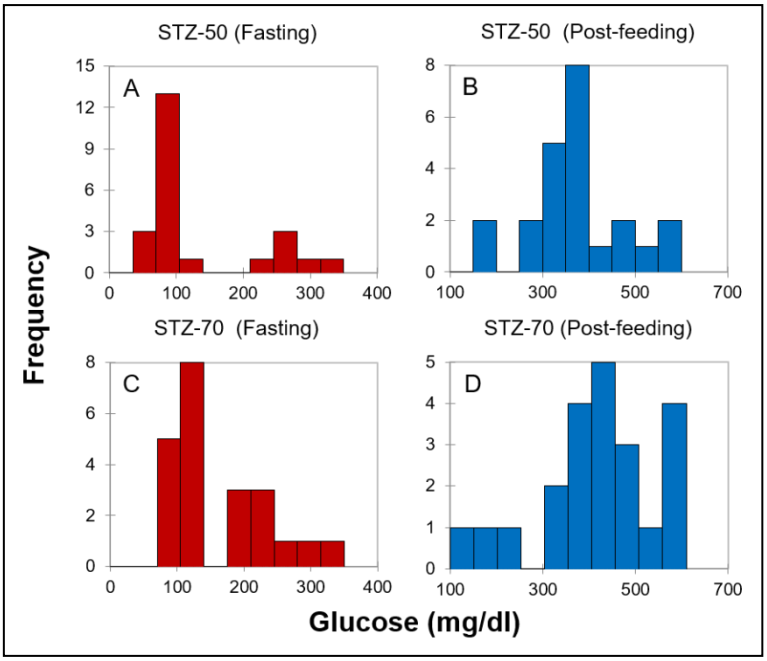


Figure 5: Distribution of the glucose values at fasting and post-feeding conditions after STZ treatment. (A) and (B) represent treatment with 50mg/kg body weight of STZ (n=23)at the fasting and post-feeding time-points respectively whereas (C) and (D) represent treatment with 70mg/kg (n=22) fasting and post-feeding time-points respectively.

Table 1: Effect of the STZ dose on the fasting and post-feeding glucose values of SD rats.

Category	**STZ 50mg/kg body weight**	**STZ 70mg/kg body weight**
High fasting glucose/High post-feeding glucose	6 (26.09%)	9(40.91%)
Normal-fasting glucose/High post-feeding glucose	17 (73.91%)	13(59.09%)
Total sample size (n)	23	22

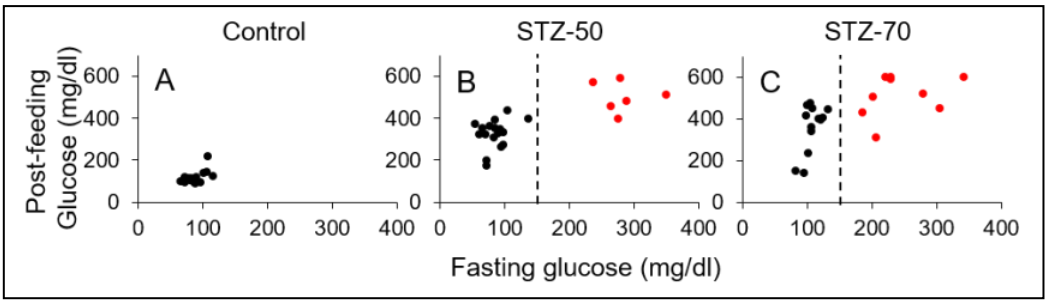


Figure 6: Post-feeding glucose versus fasting glucose of SD rats treated with (A) Control injected with only citrate buffer (B) 50mg/kg body weight of STZ and (C) 70mg/kg body weight of STZ. Black circles represent the rats with normal fasting glucose and orange circles represent the rats with high fasting glucose.

In short, pooled across experiments, the effect of STZ treatment in both the doses was very clearly and consistently seen in the post feeding glucose levels, but the

effect on fasting glucose was highly variable across individuals and a substantial fraction of individuals did not show fasting glucose levels higher than normal.

4.3.3 Fasting and post-feeding glucose in 12-day follow up after insulin suppression by STZ treatment

To see the consistency of glucose dysregulation over time in the same individual, we followed up marked individuals for 12 days. The three groups were as earlier i.e.

(i) Control: Intra-peritoneal injection of only Citrate Buffer

(ii) STZ-50: Intra-peritoneal injection of STZ at a dose of 50mg/kg body weight of the rat

(iii) STZ-70: Intra-peritoneal injection of STZ at a dose of 70mg/kg body weight of the rat

After a stabilization period of three days after the STZ treatment, the glucose in 16 hour fasting and post-feeding conditions was monitored over a period of 12 days. The duration of fasting was chosen as 16 hours based on earlier studies (Nowland, Hugunin and Rogers, 2011). In the rodent studies shortlisted in the IRKO meta-analysis, we have seen that the 30% of the studies that have used 16 hours as the fasting duration were least likely to show increased fasting glucose. We performed pair wise Mann Whitney U tests between Control and STZ-50 glucose values at the fasting and post-feeding time points. Similar procedure was followed for that of the STZ-70 group as well. Table 2 and figure 7 shows that the difference in the glucose levels between the treated and control was highly significant during the post-feeding time point for both STZ-50 and 70 on all the 12 days. In the case of STZ-50 treatment, the difference between fasting sugar in the control and treated was not significant on any of the 12 days. In 4 of the 12 days the mean in the STZ-50 group was lower than the control group. This means that the failure to get significance was not due to inadequate sample size alone. In the case of the higher dose of STZ-70, it was seen that the fasting glucose was significantly higher on 6 out of the 12 days. However, unlike STZ-50, the mean and median glucose was always higher than the control.

Table 2: p-values of the pair wise Mann-Whitney U tests between the glucose levels of control and treated rats. * represent the $p < 0.05$.

Time point →	Fasting		Post-feeding	
Difference between → Day ↓	Control (N=14) and STZ-50 (N=12)	Control (N=14) and STZ-70 (N=9)	Control (N=14) and STZ-50 (N=12)	Control (N=14) and STZ-70 (N=9)
1	0.2187	0.00022*	0.0088*	0.00105*
2	0.69654	0.09492	0.0012*	0.00094*
3	0.22628	0.06724	0.00044*	0.00024*
4	0.30302	0.101	0.00084*	0.0006*
5	0.35238	0.0278*	0.00168*	0.00075*
6	0.93624	0.03486*	0.00032*	0.00016*
7	0.5552	0.23014	0.00062*	0.00075*
8	0.3843	0.0278*	0.00056*	0.00017*
9	0.1543	0.07186	0.0007*	0.00117*
10	0.50286	0.07186	0.00142*	0.0018*
11	0.32708	0.01684*	0.00014*	0.00059*
12	0.15854	0.03236*	0.00132*	0.00025*

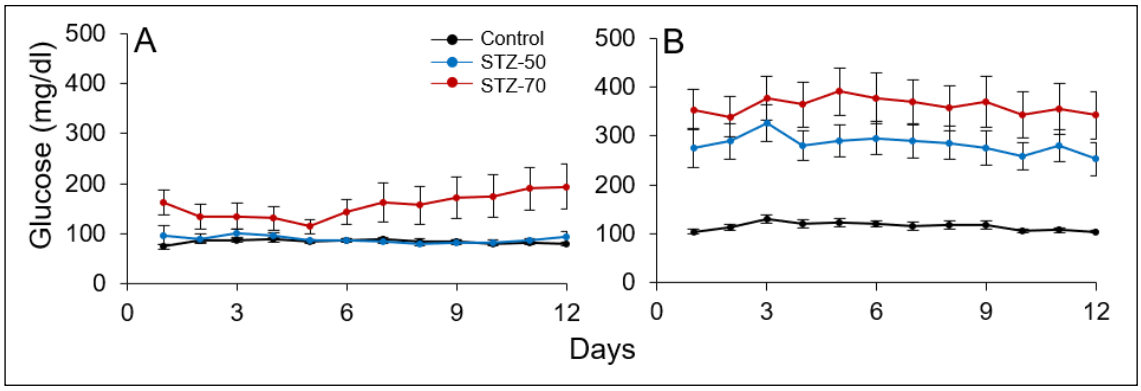


Figure 7: Effect of the STZ treatment on the (A) Fasting and (B) Post-feeding glucose levels in Control, STZ-50 and STZ-70 SD rats. The glucose values are mean ± SE for each group with the sample sizes for the three groups: N=14 for Control, N=12 for STZ-50 and N=9 for STZ-70.

However, if we look at the fasting glucose of the same animal over the 12 days, we see that with only one exception, no individual consistently showed a higher-than-normal fasting glucose on all the days even in the STZ-70 group. In contrast, the animals that showed higher post feeding glucose, remained higher than normal consistently on all days.

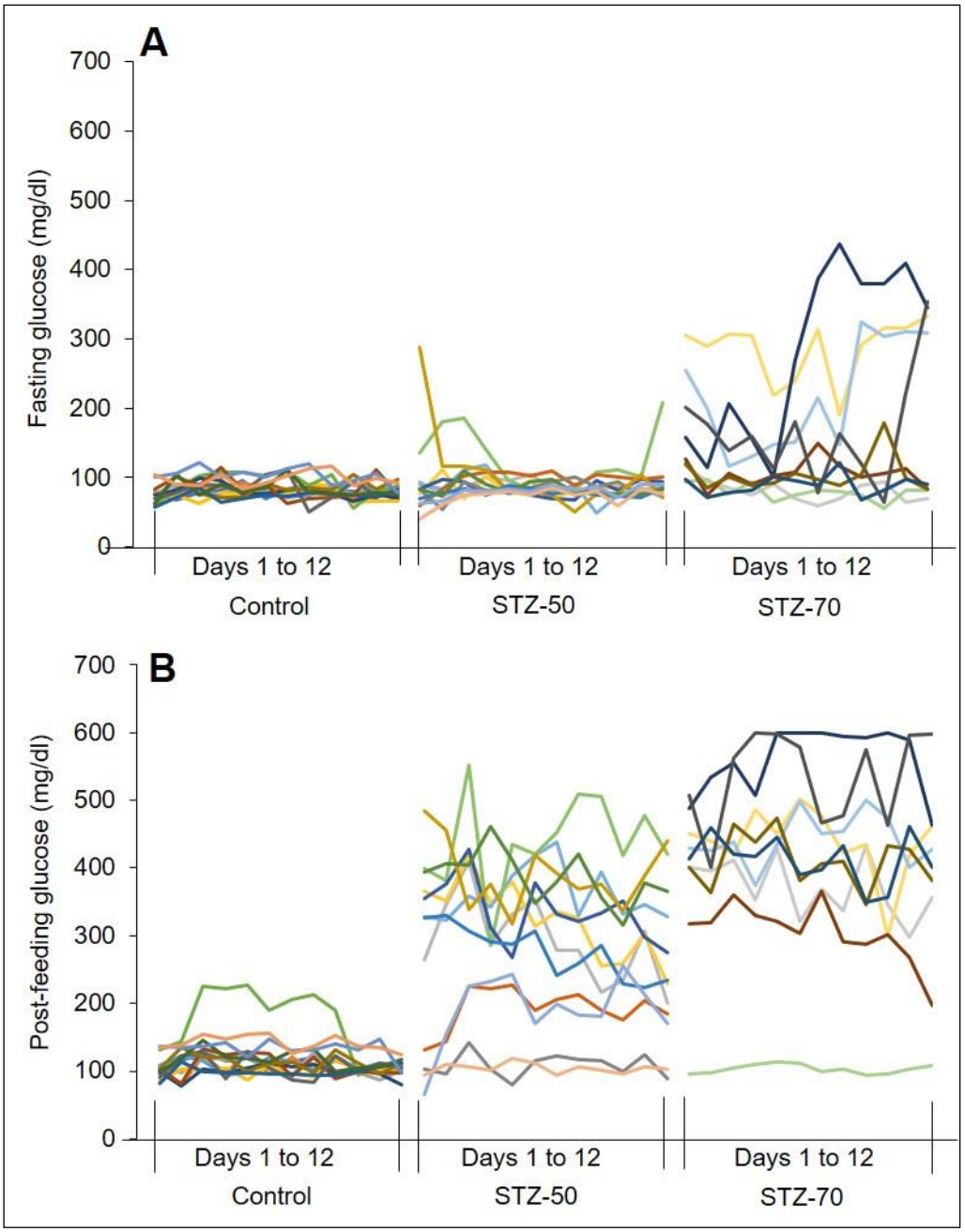


Figure 8: Fasting(A) and post-feeding (B) glucose of each individual animal over the span of 12 days.

The 12 day follow up experiments reveals that the effect of STZ is consistent in the expected direction on the post feeding glucose level but is inconsistent and highly variable across individuals as well as across time for the same individual. The glucose levels do not show any time trend over the 12 days tested. Since β cell population is known to recover by regeneration, we would expect a gradual progress towards normalcy of glucose levels, which is not seen over the 12 days. Since the hyperglycemia following STZ treatment is assumed to be due to lack of

insulin producing cells and thereby absolute deficiency of insulin, we estimated insulin on selected samples as follows.

4.3.4 Fasting and post-feeding insulin after STZ treatment

We measured the insulin levels of the control and treated rats at the two time points fasting and post-feeding just as we did in case of the glucose measurement. However, insulin measurement needs at least 500µl of blood and we need to anesthetize the animal to obtain this blood. The retro-orbital sinus was chosen as the site of blood withdrawal. We did not draw blood at the fasting and post-feeding time-point from the same animal as the anaesthesia given during the blood withdrawal for the fasting time-point would have influenced the food intake/behaviour as well as metabolism on the post-feeding time-point. So, we used half the rats in each group for blood withdrawal at the fasting time-point and half at the post-feeding time point. Additionally, we could not draw blood for insulin on all 12 days like that for glucose because of the effects of the anaesthesia. So, the blood withdrawal was done on the days 4, 8 and 12 of the experiment. The blood withdrawn was separated immediately into serum and the serum was stored at -80°C. Insulin levels were measured using the Rat-insulin ELISA kit (Thermo-Fisher) as per the manufacturer's instructions. The samples were run in duplicates and the samples where the single values differed more than 10% from the mean were not used for further analysis. We analysed the difference between the insulin levels of the control and treated at the fasting and post-feeding time-points. Treatment with neither of the doses of STZ showed a significant change in the insulin levels in the fasting state, but the post feeding insulin levels of STZ animals was lower than the controls (figure 9).

STZ treatment at both doses increased the variance between fasting insulin levels of individuals but the means were not significantly reduced as compared to the controls. For STZ-50, the mean fasting insulin was actually greater than the control, although not significantly. This means that the failure to detect significance was not due to small sample size alone. The direction of change is also not as expected. In the post-feeding data, however, the mean levels of insulin were consistently lower than the control. Particularly in STZ 70 post-meal insulin was clearly deficient as compared to control on all the three sampling days.

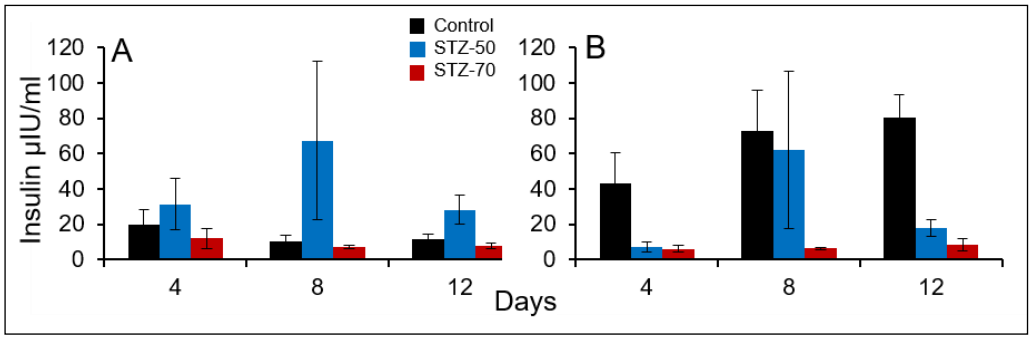


Figure 9: Effect of STZ treatment on the insulin levels of SD rats. Insulin levels were measured at fasting (A) and 3 hours post-feeding (B) on the days 4,8 and 12 of the 12 day follow-up experiment. The insulin values are mean ± SE for each group with the sample size of 3 to 6 for each group.

It is quite likely that the failure to get significant difference between groups is a result of high within group variance, particularly within the STZ treated groups. So, we pooled the readings of the three groups (days 4,8 and 12) and used scatter plots to see whether and to what extent variance in insulin explains the variance in glucose. (figure 10).

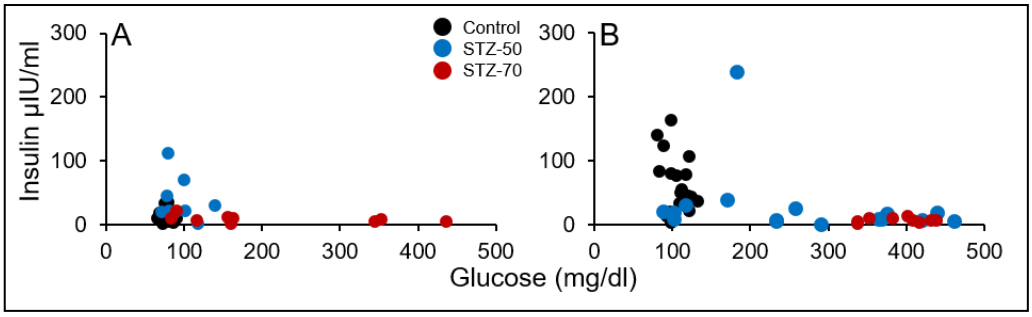


Figure 10: Glucose and insulin scatter in the 12-day follow up experiment. (A) Correlation in the fasting condition and (B) Correlation in the post-feeding condition. See parameters in table 3.

Table 3: Coefficients of determination (R-squared) and correlation coefficients (*r*) of the insulin-glucose scatters for the 12 day follow up experiment. The correlations within control and treatment groups are consistently negative, although not significant. The lack of significance may be because of small sample size. However, the variance in glucose explained by insulin is always small. Pooled over all treatments, in the fasting condition insulin-glucose correlation is not significant and the variance in glucose explained by insulin is only 4.6%. However, post feeding the correlation was significant with 48.5 % variance in glucose being explained by insulin.

Condition →	**Fasting**		**Post-feeding**	
Group ↓	**R-squared**	***r***	**R-squared**	***r***
Control	0.01254	-0.11198, n=14 p=0.703	0.13788	-0.37133, n=18 p=0.13
STZ-50	0.06961	-0.26384, n=9 p=0.494	0.056697	-0.23811, n=16 p=0.374
STZ-70	0.216469	-0.46526, n=9 p=0.207	0.005724	0.07566, n=9 p=0.8479
Control+STZ-50+STZ-70	0.046	- 0.214, n=32 p = 0.239	0.235	-0.485, n=43 p = 0.001

If the difference in glucose levels were mainly caused by the difference in insulin, we would have expected a negative correlation between glucose and insulin in the data pooled over the three treatment groups. We see such a significant correlation for the post-feeding levels but not for the fasting levels. This is because there is a large overlap in the insulin levels of the control and STZ treated groups in fasting condition. Only two animals in the control group have higher fasting insulin than the STZ groups. This means it is not true that after destruction of the β cells by STZ, insulin is not produced at all. Although we did not histologically test the regeneration of β cells, fasting insulin appears to have normalized indicating adequate level of regeneration. Nevertheless, the insulin response to feeding appears to be still deficient in the STZ groups.

4.3.5 Effect of the duration of fasting

In the meta-analyses reported in the earlier chapter we have seen that the duration of fasting used in rodent experiments was highly variable ranging from 4 hours to up to 24 hours. Most studies reported using 16 hours as the duration of fasting before conducting an oral glucose tolerance test. This variability poses a problem as the exact time when the steady state is reached is not known. We performed an experiment to see how much time was required to reach a steady state of glucose after removal of food. We removed the food from the STZ and Control animals

after overnight ad libitum availability and measured the glucose after 3 hours, 6 hours, 9 hours, 12 hours and16 hours after food removal. After a recovery of three days, we repeated the experiment but measured glucose directly at 16 hours after removing the food. Nine STZ-50 animals and 10 Control animals were used for this experiment.

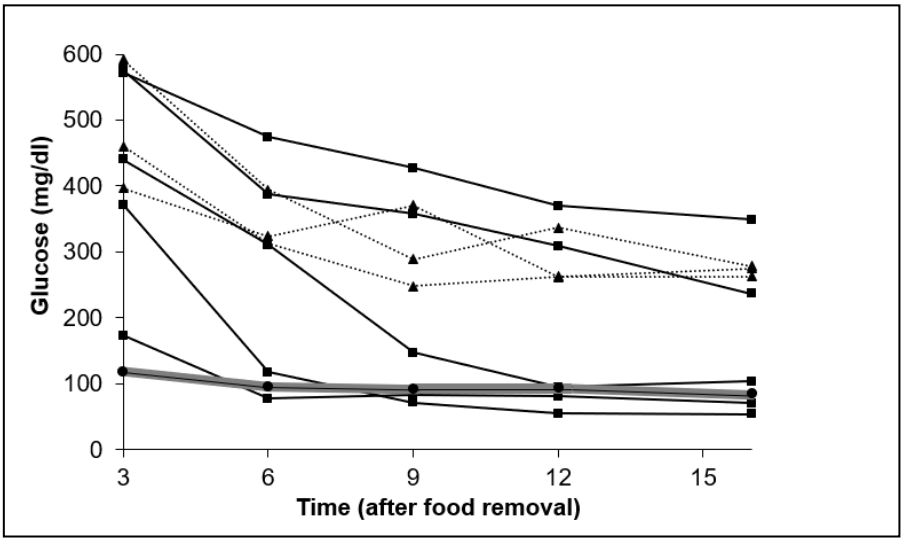


Figure 11: Time course of glucose on 16 hours fasting. The grey band represents the upper and lower bounds of 95%CI of the control group with the mean glucose values represented by filled circles. Filled squares represent individuals from the STZ group that showed a monotonic decrease in glucose levels. In three animals the glucose levels reduced at or below the control levels and in two others they showed a continued monotonic decrease but did not reach the normal level in 16 hours. Filled triangles with dotted lines represent the individual time courses of the three STZ treated rats which showed some indications of stabilizing at a steady state above the normal.

A close look at the time course of fasting in the two groups revealed that in 4 out of 9 STZ animals the glucose levels reached the normal range but with substantial delay as compared to control animals (figure 11). Animals that showed delay in returning to steady state did not show a higher steady state glucose level. In two more animals the levels did not reach the normal range till 16 hours, but a monotonic decrease continued throughout the period, indicating that their blood glucose may not have reached a steady state in 16 hours. Only in 3 animals the 16-hour glucose was higher than the control range with some indications of stabilizing at a higher level.

It is possible that the stress of repeated sampling might have caused some metabolic disturbance leading to a higher glucose level in some of the animals. When after a gap of few days the same animals were subject to a similar

experiment but were sampled only once after 16 hours, there glucose levels were lower than the repeated sampling experiment (figure 12).

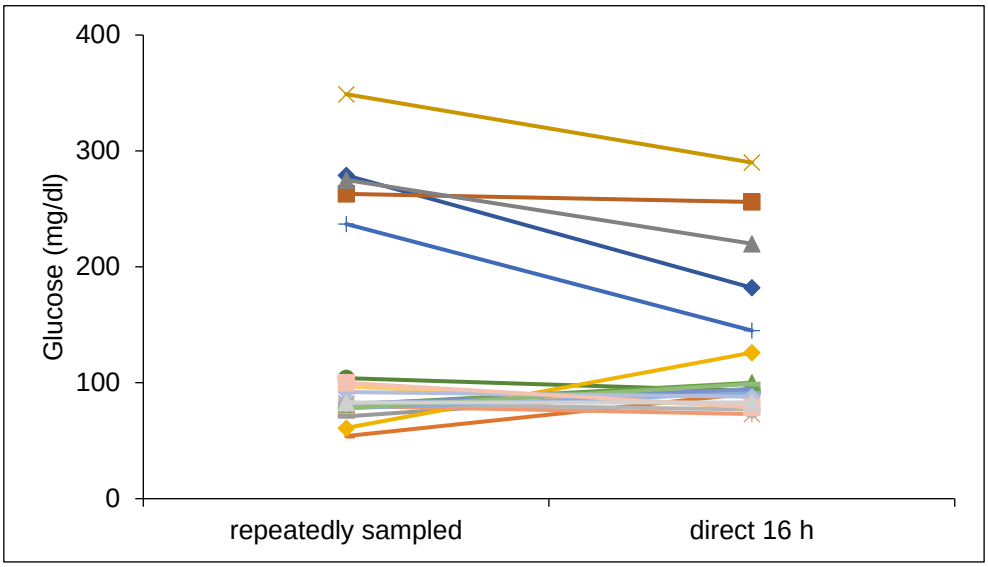


Figure 12: 16-hour glucose in the repeated sampling experiment versus single sampling after 16 hours. Most animals show a lower level if sampled only once.

This experiment showed that there is certainly a variable lag in reaching the steady state in the STZ treated animals. The delay in returning to steady state seems to be independent of the steady state level. Although 3 animals appeared to have reached a higher steady state, in this experiment, we have seen earlier in the 12 day follow up experiment that no single animal showed consistently higher fasting glucose on all the 12 days (figure 8). Therefore, although a delay can certainly be inferred with confidence, evidence is not sufficient to conclude that STZ treated animals show a consistent higher steady state glucose.

4.3.6 The 2 to 6 day experiment

The effect of STZ treatment on the fasting or steady state glucose and the post-feeding or perturbed state glucose was apparent in the 12 day follow up experiment. In the 12 day follow up experiment performed earlier; we started the fasting protocol after a three-day stabilising period after giving the STZ injection. We wanted to check if the β-cells actually recovered even before this three day period and that is the reason why we see the normal levels of the fasting glucose levels. In short, we wanted to see if we can capture the effect of β-cell regeneration in a shorter time frame. The design of this experiment was similar to

that of the first experiment except that the fasting protocol was started immediately one day after the STZ treatment (figure 2). The fasting protocol was followed and the glucose and insulin were measured on the days two, four and six after the STZ injections. In this set up, the fasting and post-feeding insulin was measured on the same animal, taking the risk of subjecting the animal to a greater stress, but in tern one source of variability was reduced.

Effect on glucose levels

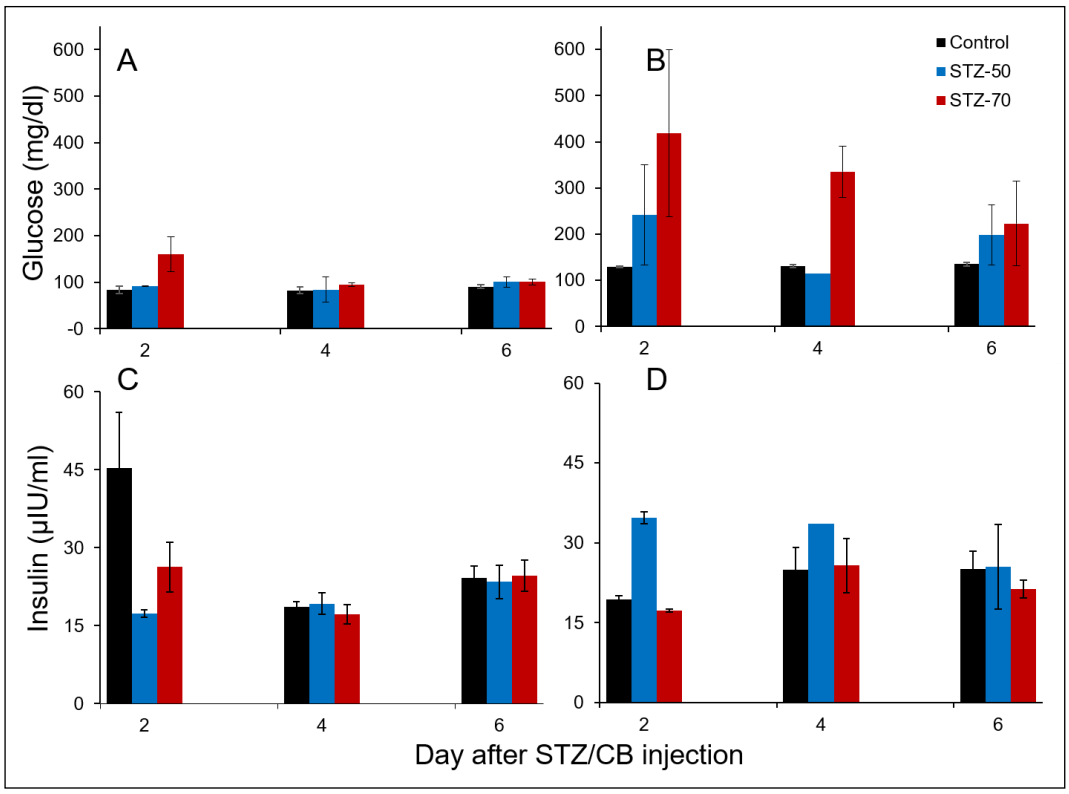


Figure 13: Time course of glucose and insulin on 2, 4 and 6 days of STZ treatment. A. Fasting glucose B. Post feeding glucose C. Fasting insulin and D. Post feeding insulin. There is a weak trend of decreasing glucose and improvement in insulin between day 2 and day 6, which is compatible with the expectation of improving glucose control by regeneration of β cells. Except for post-meal glucose, in STZ-70, the trends are not significant.

Table 4: p-values of the pair wise Mann-Whitney U tests between the glucose levels of the control and treated rats. * represent the p-values < 0.05. Sample size for each group is between 3 to 10.

Time point →	**Fasting**		**Post-feeding**	
Difference between → Day ↓	**Control and STZ-50**	**Control and STZ-70**	**Control and STZ-50**	**Control and STZ-70**
2	0.62414	0.14156	0.01878*	0.0784
4	0.9442	0.3757	0.0932	0.133-
6	0.5926	0.30942	0.5927	0.7702

The fasting glucose did not differ from the control on any of the days (table 4). But we see a weak time trend in this experiment (figure 13) in the post-meal glucose which was significantly greater than control on day 2 for STZ-50 and marginally significant for STZ-70. On days 4 and 6 the post-meal glucose was higher than the control, but the difference was no more significant. This trend is compatible with the expectation of regeneration of β cells in 3-4 days after STZ

treatment (Movassat & Portha, 1999; Wang et al., 1996) but the trend is too weak to conclude either way. On the other hand, we did not observe an increase in fasting or post feeding insulin levels during this time. Therefore, these data do not clearly reflect on the possibility of β cell regeneration between 2 to 6 days.
In this experiment, in data pooled over the three days (figure 14), the insulin levels of the STZ treated animals overlapped considerably with those of the control animals. Although some animals in STZ 50 and 70 group showed high post feeding glucose, their insulin levels were not significantly lower than controls.

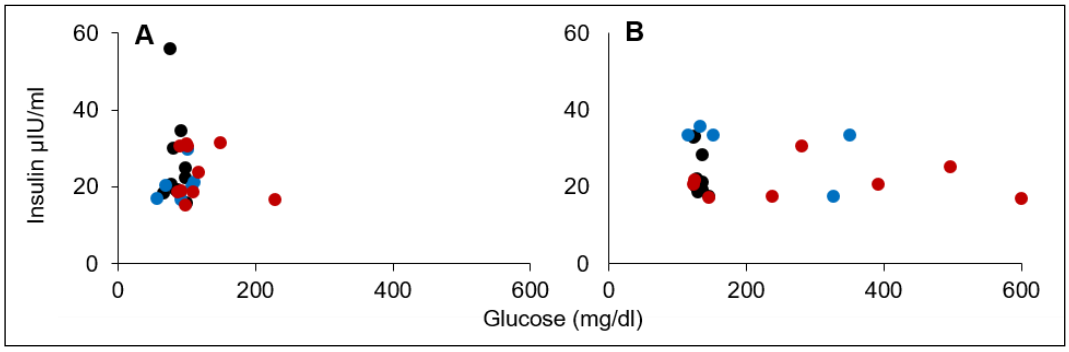


Figure 14: Insulin glucose scatters pooled across days two, four and six for Control (black circles), STZ-50 (blue circles) and STZ-70 (red circles) in the fasting (A) and post-feeding (B) conditions.

4.3.7 Insulin-glucose correlation in data pooled over all experiments:

Individual animals show substantial variability in the effects of STZ. However, if the main mechanism behind STZ induced hyperglycaemia is β-cell damage and thereby insulin suppression, we would expect a negative correlation between glucose and insulin across the board. We pooled the data from the 12 day follow up and the 2,4,6 day follow up experiments. In the control as well as two treatment groups the correlations are consistently negative although not all are individually significant. When pooled across the board, fasting glucose and insulin were not significantly correlated with each other, but post-feeding glucose and insulin were significantly negatively correlated. Overall, the variance in glucose explained by insulin was small.

Table 5: Coefficient of determination (R-squared) values for the correlation between glucose and insulin of the control and treated rats in the pooled data from short-term and long-term experiments.

Condition → **Group ↓**	**Fasting**		**Post-feeding**	
	R-squared	***r***	**R-squared**	***r***
Control	0.001912	0.0437, n=24 p=0.8393	0.32173*	-0.5672, n=27 p=0.002
STZ-50	0.014023	-0.1184, n=16 p=0.6633	0.05418	-0.23276, n=21 p=0.31
STZ-70	0.291223*	-0.540, n=19 p=0.0172	0.08915	-0.2986, n=17 p=0.245
Control+STZ-50+STZ-70	0.048	-0.219, n=59 p=0.096	0.152	-0.39, n=65 p=0.0013

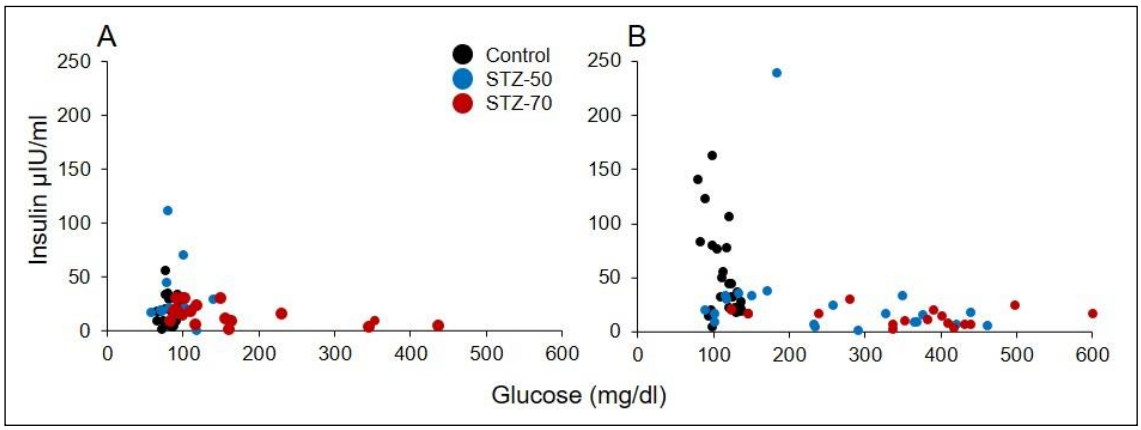


Figure 15: Insulin glucose scatters pooled across long-term and short-term experiments. STZ-50 (blue circles) and STZ-70 (red circles) in the fasting (A) and post-feeding (B) conditions.

4.4 Discussion

Streptozotocin (STZ) is a widely used model of rodent diabetes and the mainstream belief is that it acts by specifically destroying insulin producing β cell population. It was believed earlier that the β-cell population, once destroyed, does not regenerate. Regeneration capacity of β-cells having been demonstrated clearly after STZ treatment; a paradox arose. The prior belief that the STZ treated animals show lifelong insulin deficiency and that explains their persistent hyperglycaemia is seriously under doubt.

STZ has been shown to induce certain other physiological changes in the body along with β-cell destruction. Particularly relevant is the down-regulation of growth factors such as FGF21 by STZ (Omar *et al.*, 2014; Kim *et al.*, 2015) and reduction in glut-1 expression in the brain (Chapter 12, Watve, 2013). The claim

that intra cerebroventricular single injection of FGF1 normalizes sugar levels in STZ model of diabetes, along with other rodent diabetes models (Scarlett *et al.*, 2016) has raised many new possibilities. On this background, it is necessary to reexamine the classical belief that STZ acts by at least partly irreversible destruction of β cells alone.

In this context our experiments do not clearly falsify the β-cell centered hypothesis of STZ action but certainly expose some anomalies and raises important questions. In the earlier chapter, we demonstrated that if the insulin signalling machinery is impaired by some method, the change of this impairment is consistently evident on the post-feeding glucose levels but not on the fasting glucose levels. We have now demonstrated even in the STZ model, that change in fasting glucose and change in post feeding glucose do not show the same patterns. The treatment with STZ affects the fasting and post-feeding levels of glucose in a different manner. Firstly, we see that after an injection of STZ, most the rats still continue to show normal levels of fasting glucose even though they show high levels of the post-feeding glucose (figure 3 and table 1). This effect is expectedly more profound in STZ-50 as compared to STZ-70. Though even in STZ-70, a significant number of rats continue to show normal fasting glucose levels. Further, the animals that showed higher fasting glucose on a given day did not necessarily show the same on all days, which post feeding glucose was more consistently high.

By an insulin resistance-based CSS model of glucose homeostasis that we saw in the last chapter, if the insulin-glucose relationship parameters in the fasting and post feeding state are the same, the insulin-glucose correlation and regression should show comparable parameters. This we did not see in human epidemiological data, and we do not see it in the STZ experiment as well. It would be interesting to see whether the alternative actions of STZ such as its effect on growth factor signalling and glut-1 expression can explain the patterns inadequately explained by the β-cell destruction alone. But this exploration is beyond the scope of this thesis.

4.5 References

Akbarzadeh, A. *et al.* (2007) 'Induction of diabetes by Streptozotocin in rats', *Indian Journal of Clinical Biochemistry*, 22(2), pp. 60–64. doi: 10.1007/BF02913315.

Arindkar, S. *et al.* (2012) 'The Effect of Fasting on Haematology Serum Biochemistry Parameters on STZ Induced CD1 Mice and Diabetic db/db Mice', *Journal of Drug Metabolism & Toxicology*, 3(6). doi: 10.4172/2157-7609.1000137.

Cano, D. A. *et al.* (2008) 'Regulated -Cell Regeneration in the Adult Mouse Pancreas', *Diabetes*, 57(4), pp. 958–966. doi: 10.2337/db07-0913.

Diwekar-Joshi, M. and Watve, M. (2020) 'Driver versus navigator causation in biology: the case of insulin and fasting glucose', *PeerJ*, 8, p. e10396. doi: 10.7717/peerj.10396.

Freitas, S. C. F. *et al.* (2015) 'Effect of aerobic exercise training on regional blood flow and vascular resistance in diabetic rats', *Diabetology & Metabolic Syndrome*, 7(1), p. 115. doi: 10.1186/s13098-015-0109-1.

Gajdosík, A. *et al.* (1999) 'Streptozotocin-induced experimental diabetes in male Wistar rats.', *General physiology and biophysics*, 18 Spec No, pp. 54–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10703720.

Gelling, R. W. *et al.* (2004) 'Effect of Uncontrolled Diabetes on Plasma Ghrelin Concentrations and Ghrelin-Induced Feeding', *Endocrinology*, 145(10), pp. 4575–4582. doi: 10.1210/en.2004-0605.

Hebden, R. A. *et al.* (1986) 'The influence of streptozotocin-induced diabetes mellitus on fluid and electrolyte handling in rats', *Clinical Science*, 70(1), pp. 111–117. doi: 10.1042/cs0700111.

Kim, J.-H. *et al.* (2015) 'Fibroblast growth factor 21 analogue LY2405319 lowers blood glucose in streptozotocin-induced insulin-deficient diabetic mice by restoring brown adipose tissue function', *Diabetes, Obesity and Metabolism*, 17(2), pp. 161–169. doi: 10.1111/dom.12408.

Levine, F. and Itkin-Ansari, P. (2008) 'β-cell regeneration: Neogenesis, replication or both?', *Journal of Molecular Medicine*, 86(3), pp. 247–258. doi: 10.1007/s00109-007-0259-1.

Movassat, J. and Portha, B. (1999) 'Beta-cell growth in the neonatal Goto-Kakisaki rat and regeneration after treatment with streptozotocin at birth', *Diabetologia*, 42(9), pp. 1098–1106. doi: 10.1007/s001250051277.

Nowland, M. H., Hugunin, K. M. S. and Rogers, K. L. (2011) 'Effects of short-term fasting in male Sprague-Dawley rats', *Comparative Medicine*, 61(2), pp. 138–144.

Omar, B. A. *et al.* (2014) 'Fibroblast Growth Factor 21 (FGF21) and Glucagon-Like Peptide 1 Contribute to Diabetes Resistance in Glucagon Receptor-Deficient Mice', *Diabetes*, 63(1), pp. 101–110. doi: 10.2337/db13-0710.

Porat, S. *et al.* (2011) 'Control of Pancreatic β Cell Regeneration by Glucose Metabolism', *Cell Metabolism*, 13(4), pp. 440–449. doi: 10.1016/j.cmet.2011.02.012.

Reed, M. J. *et al.* (2000) 'A new rat model of type 2 diabetes: The fat-fed, streptozotocin-treated rat', *Metabolism: Clinical and Experimental*, 49(11), pp. 1390–1394. doi: 10.1053/meta.2000.17721.

Scarlett, J. M. *et al.* (2016) 'Central injection of fibroblast growth factor 1 induces sustained remission of diabetic hyperglycemia in rodents', *Nature Medicine*, 22(7), pp. 800–806. doi: 10.1038/nm.4101.

Szkudelski, T. (2001) 'The mechanism of alloxan and streptozotocin action in B cells of the rat

pancreas.', *Physiological research*, 50(6), pp. 537–46. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11829314.

Wang, R. N., Bouwens, L. and Kl�ppel, G. (1996) 'Beta-cell growth in adolescent and adult rats treated with streptozotocin during the neonatal period', *Diabetologia*, 39(5), pp. 548–557. doi: 10.1007/BF00403301.

Watve, M. (2013) *Doves, Diplomats, and Diabetes*. New York, NY: Springer New York. doi: 10.1007/978-1-4614-4409-1.

Zhang, M. *et al.* (2008) 'The Characterization of High-Fat Diet and Multiple Low-Dose Streptozotocin Induced Type 2 Diabetes Rat Model', *Experimental Diabetes Research*, 2008, pp. 1–9. doi: 10.1155/2008/704045.

Chapter 5: Fasting glucose and fasting insulin and insulin resistance: inferring causal relations

5.1 Introduction

In the chapters 2, 3 and 4 we have established that the relationship between insulin and glucose is different in the steady state versus the perturbed state. Impairment of the insulin function/signalling does lead to a change in the glucose levels. But this change is significant only in the perturbed/post-meal state. The three approaches used in the earlier chapters consistently show that impairment of insulin signalling does not affect the steady state represented by the fasting glucose levels. This leads to a broader question of finding out the causality in the steady state versus the perturbed state. To begin with, inferring causality in biology and medicine is a difficult area with philosophical as well as methodological issues. Causality can be inferred using correlations in longitudinal/times series data or cross-sectional data. There have been many debated instances of inferring causality from correlations in biomedicine (Baker, 2008; Gerber and Offit, 2009; Ratzan, 2010) There have been many attempts to develop sound methods to address questions of causal inference from correlation data which include Hill criteria (Hill, 1965), path analysis (Niles, 1923; Wright, 1934, 1960; Meehl and Waller, 2002), the use of instrumental variables (Greenland, 2000), Granger causality (Granger, 1969), Rubin causal model (Rosenbaum and Rubin, 1983), or additive noise models (Peters, Janzing and Scholkopf, 2011).

In a massive team effort, our lab developed a novel set of methods to infer causal pathways reliably from cross-sectional correlations, provided there were three or more inter-correlated variables measured over the same individuals in a population. The team developed a set of general predictions and showed that different causal pathways make different predictions along these paths, using which hypothetical causal pathways can be rejected or accepted. I will elaborate on this in the section 5.1.

In experimental biology causal inferences are made from perturbation experiments. As a general principle, if a perturbation in X changes Y, X is said to

have a causal effect on Y. However, this is not as straightforward as it appears and there are many logical traps in such inferences. I will elaborate on this in the section 5.2.

We are fortunate that we have both types of data to infer causal relationships between glucose and insulin and I will discuss both in this chapter to see whether they converge on the inferences. This work has been published partly in the paper by Chawla et al 2018 from our lab and partly in the paper Diwekar-Joshi and Watve, 2020.

5.2 Inferring causality from steady state correlations: a novel approach

In the team effort from our lab, we established certain novel methods to infer causal pathways from steady state correlations (Chawla et al 2018). To state in brief, these methods use the following lines of pathway predictions.

If A, B and C are the three inter-correlated variables, the causal linkages between the three can be inferred based on,

1. Whether r^2_{AC} can be estimated from the product of $r^2_{AB}$and r^2_{BC}. For some pathways $r^2{}_{AC} = r^2{}_{AB.}r^2{}_{BC}$ for some pathways $r^2{}_{AC} < r^2{}_{AB.}r^2{}_{BC}$ and $r^2{}_{AC} > r^2{}_{AB.}r^2{}_{BC}$ for other pathways.

2. Whether slope M_{ca}can be estimated from the product of the slopes $M_{ba}$and M_{cb}, the three possibilities here being like those mentioned in 1.

3. Whether the residuals of the regression of *B* on *A* (E_{ba}) are correlated with those of *C* on *B*(E_{cb}): The errors or residuals in a regression are assumed to be random independent errors. However, if there are loops, convergent or confounding elements in a pathway, $E_{ba}$and E_{cb} do not remain independent. Based on the nature of dependence between $E_{ba}$and E_{cb}, presence of, and possible nature of the loops and convergence can be inferred.

4. a. Whether correction for *A* improves or reduces the correlation of *B* with *C*, i.e. whether $r^2_{E_{ba}C}$ is greater or lesser than r^2_{BC}.

b. Whether the extent to which $r^2_{E_{ba}C}$is greater or lesser than r^2_{BC}can be predicted by r^2_{AB}.

5.2.1 Applying the method to the example of fasting glucose, fasting insulin and insulin resistance

After developing rigorous statistical methods for hypothetical pathway testing, we used these methods to analyse the relationship between steady state plasma insulin, plasma glucose and insulin resistance. My role in this teamwork was in applying the methods developed by my colleagues to the epidemiological data from the sources described in chapter 3. Based on the novel principles and methods, the classical genesis of an insulin resistant, hyperinsulinemic normoglycemic state was examined and it got rejected by the analysis. The details of this work are published (Chawla *et al.*, 2018). I will only include a small part of this analysis here.

5.2.1.1 The possible pathways

We can use FG, FI and HOMA-IR as the three variables and test the classical pathway(s) against a null model in which FG and FI do not affect each other and HOMA-IR is only a derived variable.

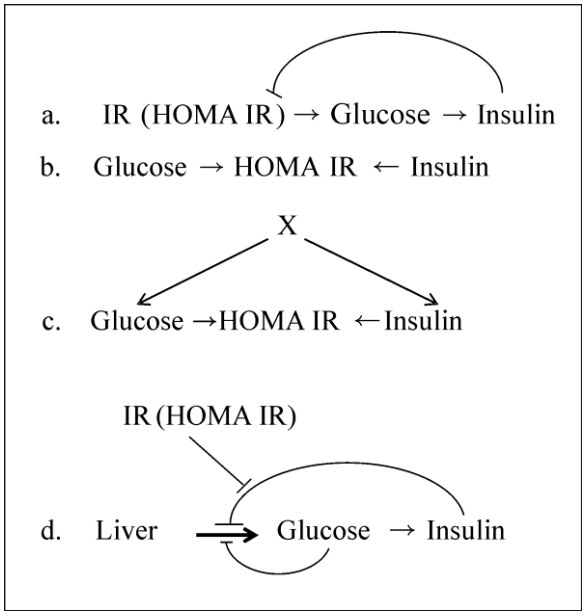


Figure 1: Possible pathways between insulin resistance, FG and FI: a) A simplified single feedback pathway that approximates the negative feedback pathway. b) A null model assuming FG and FI to be independent and HOMA-IR, a derived construct. c) An improvised null model with an external causal factor influencing FG and FI. d) The classically perceived pathway with dual feedback from glucose and insulin. (Already published in Chawla et al 2018)

We compared the regression-correlational predictions of the following possible alternative pathways. The classical pathway is depicted in figure 1a in which insulin resistance is primary, it reduces glucose disposal rate thereby increases FG. The rise in FG induces a rise in insulin. The insulin in turn suppresses glucose level. Pathway 1b assumes that FG and FI are independent of each other and HOMA-IR is only a derived variable. This can be considered as a null hypothesis to test 1a. In fact, for testing the classical pathway, a null hypothesis should have been necessary but has not been considered so far. Pathway 1c is a refinement of 1b in which FG and FI can be affected by something else such as autonomic signals and HOMA-IR is only a derived variable. In both 1b and 1c, HOMA-IR does not represent insulin resistance. In 1d, liver glucose production is the focal variable which is under dual feedback control from glucose and insulin and the feedback from insulin is affected by insulin resistance.

5.2.1.2 Testing these pathways analytically and using simulations

Since from experimental literature we have estimates of realistic parameters in these pathways, we can use them analytically or in simulations to test alternative causal pathways. The parameters taken from literature are as follows.

Table 1: Real life values of the parameters used in the simulations. These values have been obtained from the estimates published in literature.

Symbol	Description of the parameter	Estimated mean value used (units)
d	Rate constant for insulin degradation	0.15/min
k_3	Rate constant for glucose-stimulated insulin secretion	0.08µIU/mg/min
L	liver glucose production independent of insulin	20 mg/dl/min
k_1	Rate constant for glucose feedback	0.5
k_2	Rate constant for insulin feedback	2

***d* (Insulin degradation rate):**

The half-life of insulin has been determined experimentally in various model systems ranging from isolated cells to humans. To calculate the rate constant for insulin degradation, we used the half-life of insulin estimated as 5 to 6 minutes (Tomasi *et al.*, 1966; Matthews *et al.*, 1985).

***k_3* (Rate constant for glucose-stimulated insulin secretion):**

The value for this parameter was calculated based on experiments on isolated human pancreatic islets which show glucose-stimulated insulin secretion (Marchetti *et al.*, 1994; Chirieac *et al.*, 2000; Westerlund and Bergsten, 2001). Insulin secretion was measured in this study after exposing the islet cells to different concentrations of glucose. The mean value of 0.08 was used for this parameter (Marchetti *et al.*, 1994). Similar studies have also been performed in rat islets (Westerlund and Bergsten, 2001) and in vivo as well (Chirieac *et al.*, 2000). It is known that in humans, insulin secretion is stimulated by glucose above a threshold estimated to be 63mg/dl. Therefore, the index HOMA-β has a denominator as glucose concentration above the threshold.

L (Rate constant for liver glucose production independent of insulin):

The net hepatic glucose production has been measured using a variety of tracer techniques and is reported to be around 10-15mg/dl (Shulman *et al.*, 1990; Rothman *et al.*, 1991). Absence of insulin signalling, such as during extreme hypoglycaemia results in a 25-30% increase in hepatic glucose production (Chu *et al.*, 1997; Moore, Connolly and Cherrington, 1998). Hence the value of L used in the simulations was 20mg/dl.

Insulin sensitivity:

Since no genuine measure of insulin sensitivity independent of insulin and glucose measurement is available, we assume the normal healthy insulin sensitivity to be unity. At the normal level of insulin sensitivity, the reduction in liver glucose production mentioned above is brought about by a fasting insulin level of 5 to 10 μIU. The rate constant K_2 for insulin feedback can be calculated from this to range between 0.5 and 1. Given these estimates the constant for glucose feedback K_1 needed to give the normal fasting value of insulin was calculated as 0.15 to 0.2.

Tissue glucose uptake:

To countercheck our estimates of K_1 and K_2, we calculated the normal glucose uptake by tissues based on these parameters, which ranges between 15 to 20 mg/dl/min. Fludeoxyglucose (FDG)-Positron emission tomography (PET) scanning has been used to measure the glucose uptake in specific muscles. Based

on these results, the average whole body muscle glucose uptake is close to 18 mg/dl (Nuutila *et al.*, 2000; Hällsten *et al.*, 2002; Fujimoto *et al.*, 2003). These estimates match well.

Thus, we can use a set of parameters that closely approximate the real-life values. However, it needs to be realized that many of the inferences drawn from the prediction signatures are independent of the actual parameters used. Therefore, even if some of the parameter estimates used are biased or unrealistic, it is not a serious threat to the conclusions.

We use the equations in chapter 3 again here and test the predicted regression-correlation parameters against epidemiological data from the same sources as in chapter 3.

5.2.1.3 Testing the predictions against data

We tested the predictions from the pathways shown in the figure 1 against the human data. The details of the datasets have already been given in Chapter 3. The same datasets were used for this section as well. Assuming classical pathway and faithful indices: The following predictions of the classical pathway depicted in figure 1d are testable using the following approaches:

Approach 1:

1. ***HOMA-IR, FG and FI should be positively correlated to each other.*** This prediction is true in all the data sets. The correlations between FG and FI however are weak. In terms of the variance explained (range 1.7 to 5.7 %) FG and FI are poorly related (Chapter 3). The glucose homeostasis model expects a positive correlation between FG and FI. It is important to note this, since in the classical thinking, a prediabetic state is characterized by increased insulin but normal glucose levels. If the compensatory insulin response is mediated through glucose, it is impossible to have a raised FI without a proportionate rise in FG. In the pathway predictions, a positive correlation between FG and FI is expected independent of the feedback loop. However, the classical thinking tries to explain a hyperinsulinemic normoglycemic state achieved through this pathway. The poor correlation between FG and FI, and a large coefficient of variation in FI compared to FG indicates that a normoglycemic hyperinsulinemic state may indeed be achieved, but whether the classical pathway offers a sound explanation for this state

is the question. In an insulin resistant state, the level of FI seems to increase by about 10-fold the normal. However, the difference between the lower and upper limit of glucose in a pre-diabetic state is less than 1.5-fold. To achieve a tenfold increase in the effect resulting from a 1.5 fold increase in the causal variable, the slope needs to be of the order of 7 to 8. However, in the data, the regression slope ranges between 0.11 and 0.19 (Table 1, chapter 3). Therefore, the variance in FI is unlikely to be caused by variance in glucose following insulin resistance. Therefore, we need to conclude that most of the variation in FI appears to be random error (or some due to some factor other than glucose) and not a compensatory rise in response to FG. From equations 3 and 4 of chapter 3, steady state FG and FI can be derived as

$$FG = \frac{d.(L + e_1) - I_{SENS}.K_2.e_2}{K_1.d + I_{SENS}.K_2.K_3}$$

$$FI = \frac{K_3.FG + e_2}{d}$$

2. Based on the steady state equations, the slope of the regression of FI on FG should be K_3/d. Empirical estimates for values of K_3 and d are available from literature (table 1) and hence this prediction can be tested. The empirical estimates are K_3 = 0.08 µIU.mg/min and d = 0.15/min respectively, and thereby the expected slope is 0.533. In all data sets, regression slopes are significantly smaller than the slopes predicted from the empirical estimates 0.11 to 0.19. Thus, apart from a mismatch between the slope required to cause the observed variation in FI and actual slopes, the slopes expected from the empirical estimates of parameters and those obtained in regression also do not match. The latter mismatch by itself may not be sufficient to reject the pathway since a large measurement error in the X variable, i.e.,FG can lead to underestimation of regression slope, but this explanation implies that a substantial part of variation in glucose is independent of insulin resistance, and is akin to random error with respect to the hypothetical causal pathway.

3. ***HOMA-β in our assumption represents K_3.*** However, K_3 is a constant in our model, and although it may have some variability in the population, it is uncorrelated with the three variables of concern. Therefore, HOMA-β should show no significant correlation with FG, FI and HOMA-IR. However, in all the data sets HOMA-β is significantly positively correlated with FI, but negatively correlated

with FG and positively correlated with HOMA-IR. This is substantial mismatch with the classical model.

4. ***In a negative feedback pathway*** $r_{AC}^2 > r_{AB}^2 . r_{BC}^2$**.** Qualitatively this inequality is true for HOMA-IR, FG and FI in the data. However, simulations show that there is overfitting of the inequality. r_{AC}^2 in all the datasets and are substantially higher than the distribution obtained in the simulations (figure 2). The correlation between FI and HOMA-IR is far greater than that predicted by the simulations, leading to an overfitting rejection.

Thus, if we assume the two HOMA indices to faithfully represent insulin resistance and β-cell response respectively, then classical pathway needs to be rejected owing to mismatches with many of its predictions.

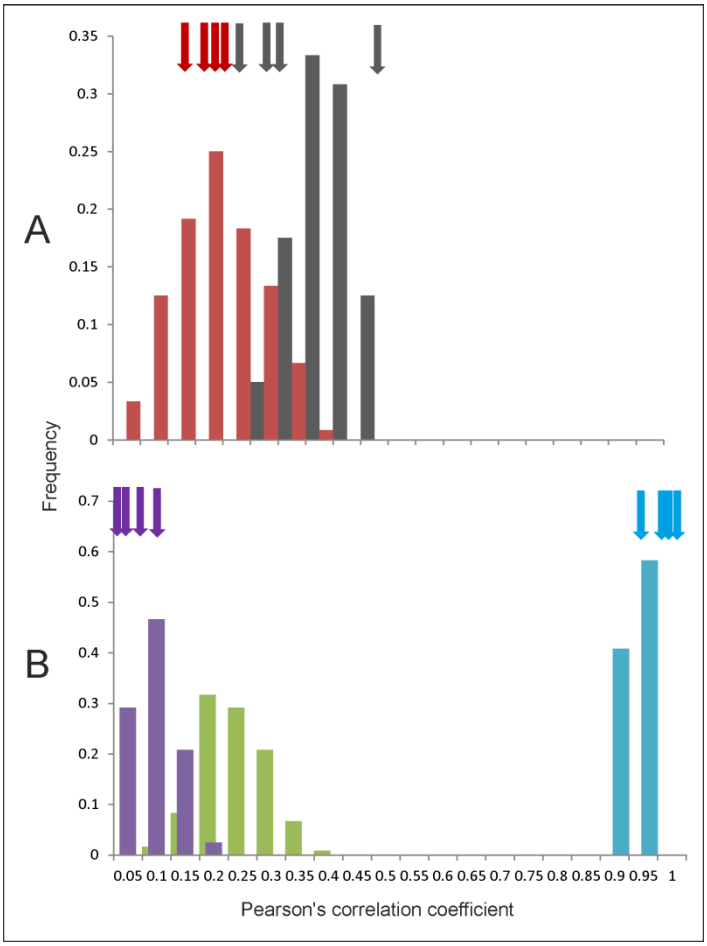


Figure 2: Frequency distribution of correlation coefficients in simulations of the classical pathway leading to prediabetic state: Bars represent the distribution of Pearson's correlations obtained in 10000 runs of simulations. The arrows indicate Pearson's correlations in the datasets of empirical data. The distribution generated by simulations matches well with the real-life correlations for true IR-FG (grey bars and arrows), FG-FI (red bars and arrows), and the product of the two (purple bars and arrows). The correlation between true IR and FI is greater than the product as predicted by the pathway (green bars, we do not have empirical estimates of these correlations) but the correlation between HOMA-IR and FI (blue bars and arrows) is substantially greater than the predicted leading

to an overfitting rejection. This indicates that either HOMA-IR as currently calculated is substantially different from true insulin resistance or the pathway get rejected based on this prediction.

Approach 2: Effects of deriving HOMA-IR and HOMA-β from FG and FI:

HOMA-IR and HOMA-β are not measured independently but are derived from FG and FI measurements. Some correlations will hence follow from the derivations themselves. The overfitting anomaly seen above could be explained as an artefact of the calculation of HOMA-IR. However, other anomalies do remain unexplained. We assume here that the classical pathway is true and therefore, FI is a linear function of FG. If FI is represented as $m.FG + e$, HOMA-IR will be correlated to FG^2. Similarly, HOMA-β should be represented as $m.FG/(FG - 63)+e$. Under normal physiological range, FG > 63 and therefore HOMA-β is a decreasing function of FG. As a result, both FI and HOMA-IR should be negatively correlated to HOMA-β. Simulations of the pathway results in a negative correlation between HOMA-IR and HOMA-β till the errors are small to moderate. These expectations do not match the empirical data, in which FI and HOMA-IR have significant positive correlations with HOMA-β. Thus, accepting the classical pathway with some allowance for artefacts coming out of the derived variables is not sufficient to explain the empirical correlations.

Approach 3:

Testing the predictions of the null model: If FG and FI are independent of each other and have some variance around a mean, HOMA-IR is expected to be positively correlated with both since it is a product of the two. FI should be positively correlated with HOMA-β, but FG should be negatively correlated with HOMA-β. In the HOMA-IR- HOMA-β relationship, FI is in the numerator of both. FG is in the numerator of HOMA-IR but in the denominator of HOMA-β. Nevertheless, since the coefficient of variation of FI is substantially greater than that of FG, FI is expected to dominate the relationship and result in a positive correlation between HOMA-IR and HOMA- β. All these predictions are observed in the data. The mismatch of the null model with the data is that it assumes FG and FI to be independent and uncorrelated. In the three sets of data, there is a significant but weak correlation between the two. The r^2 ranges from 0.017 to 0.057, and thus not more than 6 % of variance is explained by the relationship.

We need to examine now to what extent HOMA-IR faithfully represents the true insulin resistance because if it does, the classical pathway certainly gets rejected. This can be examined in the simulations since the true insulin resistance is an input variable and HOMA-IR can be calculated as an outcome of the simulations. We see that HOMA-IR is correlated well with true insulin resistance when both e_1 and e_2 are close to zero (figure 3). As the errors increase, the correlation becomes weaker. In the data, we do not have access to e_1 and e_2 but since the FG-FI correlation also becomes weaker with e_2, we can look at how HOMA-IR represents true insulin resistance at different levels of FG-FI correlation. It can be seen that as FG-FI correlation becomes weak, HOMA-IR correlation with the true insulin resistance also becomes weak (figure 3), but this relationship is affected by e_1. When e_1 is close to zero, i.e. almost all the variation in FG is explained by variation in true insulin resistance, even at low FG-FI correlation, HOMA-IR represents true insulin resistance fairly well, their correlation ranging between 0.58 and 0.7.

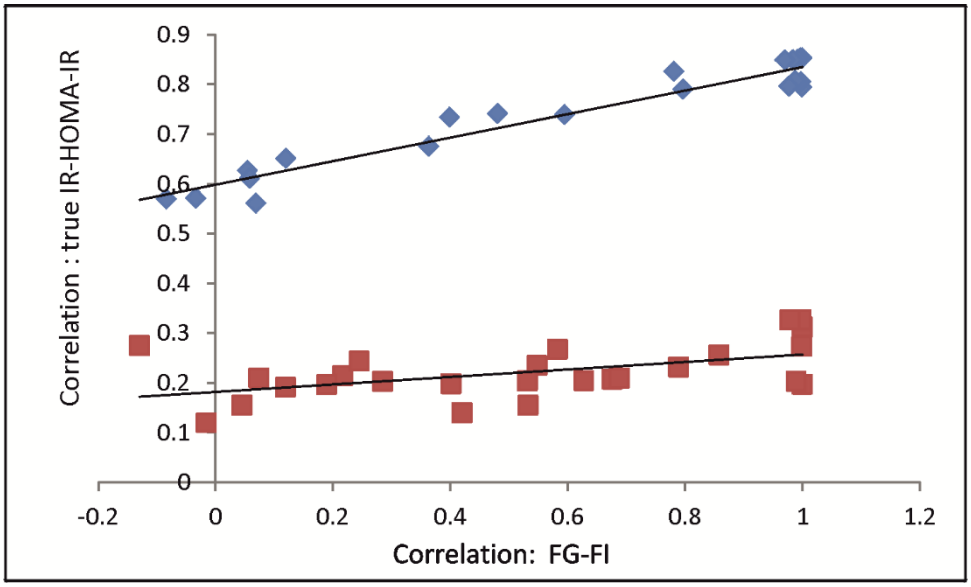


Figure 3: The reliability of HOMA-IR as an index of true insulin resistance: The pathway simulations were carried out at a standard deviation of e_1=1 (blue dots) and 10 (red dots).

The FG-FI correlation weakens with increase in e_2 which also affects the correlation between true IR and HOMA-IR. HOMA-IR is a reliable indicator of insulin resistance when e_1 is small, but at large e_1 it is a poor indicator as suggested by a weak correlation with true insulin resistance.

On the other hand, if we assume e_1 to be large i.e. most of the variation in FG is due to random error or effects independent of insulin action, HOMA-IR is poorly correlated with true insulin resistance, the correlation coefficient declining to 0.2. Thus, if we assume that the variance in FG is mainly caused by insulin resistance, then we must reject the classical pathway leading to hyperinsulinemia. Alternatively, it is likely that the classical pathway is true, but HOMA-IR does not represent true insulin resistance and that most of the variation in FG is not caused by insulin resistance. The substantially lower than expected slope of the FG-FI regression suggests large random errors in FG making the second interpretation more likely. In any case the classical pathway and the faithfulness of HOMA indices cannot be simultaneously true, and we must reject at least one of them.

Results of the alternative approaches to analyse the classical pathway and the null model converge on the inference that the null model is rejected only based on a weak but significant correlation between FG and FI. But the weak correlation in FG and FI is not adequately explained by the classical pathway owing to multiple mismatches and rejection of many of its predictions. The pathway rejection may be partially saved by saying that HOMA-IR and HOMA- β are not good indicators of insulin resistance and β-cell response and that we do not have access to true insulin resistance to test the predictions. However, the FG-FI regression slope also has a large mismatch with expectations derived from the variance in FI as well as from empirical estimates of K_3 and d. Therefore, it seems more likely that FG and FI are related by causes other than the classical pathway, and HOMA-IR and HOMA- β are derived artificial constructs that do not represent any real-life phenomena.

There are several real-life interpretations of the pathway in figure 1c. Autonomic inputs from the nervous system are known to affect both insulin secretion and liver glucose production, which might be represented by the common cause arrows of figure 1c. Alternatively, a small error in data collection can also result in the observed FG-FI correlation. The fasting sampling is done by instructing the subjects to have no food or drink after the last evening meal. However, if even a small proportion of subjects happen to consume bed tea an hour or two before sampling, their glucose as well as insulin levels could be slightly elevated simultaneously. This can result in a weak positive correlation between FG and FI in the data. Since

the fasting state is based on the honesty of the subjects and there is no independent monitoring, this source of error cannot be ignored. Thus, there are more than one possible reason for external factors causing a weak correlation between FG and FI, and the correlation is not sufficient to support the classical pathway in the presence of multiple other mismatches.

5.3 Inferring causality from interventional experiments

In classical experimental physiology, interventions or perturbations are believed to be reliable indicators of causation and there is little debate about it. If the experimenter perturbs A and finds a significant effect on B after following all fundamental principles of experimental design, the change in A is inferred to be causal to the change in B. However, there are many subtleties in drawing a causal inference from experimental interventions that have not yet attracted sufficient philosophical as well as methodological attention among experimental biologists. One such thinking trap is that in homeostatic systems the nature of causality in a perturbed state can be qualitatively different from that in equilibrium or steady state and the failure to distinguish between the two may have substantially misled biomedical research.

5.3.1 The role of growth rates in Lotka-Volterra competition models

A well worked out theoretical model that can be used to distinguish clearly between perturbed and steady state causation is the Lotka-Volterra (LV) competition model. This model describes the dynamics for interspecific competition (Gotelli, 2008). The growth of two interacting populations is modelled using logistic equations for both the populations. The changes in populations depend on the individual growth rates and the carrying capacities of the two populations (equations 1 and 2). Additional parameters are the competition coefficients α and β which represent the effects of the two species on each other. Thus, the carrying capacities K_1, K_2 and the competition coefficients α and β determine the equilibrium population (equations 3 and 4) (Gotelli, 2008).

$$\frac{dN_1}{dt} = r_1.N_1\left(\frac{K_1-N_1-\alpha.N_2}{K_1}\right) \qquad \text{Equation 1}$$

$$\frac{dN_2}{dt} = r_2.N_2\left(\frac{K_2-N_2-\beta.N_1}{K_2}\right) \qquad \text{Equation 2}$$

$$\breve{N}_1 = \frac{K_1 - \alpha.K_2}{1 - \alpha.\beta}$$ Equation 3

$$\breve{N}_2 = \frac{K_2 - \beta.K_1}{1 - \alpha.\beta}$$ Equation 4

where,

$N_1$and N_2 are the population sizes of the two competing species respectively, $\breve{N}_1$ and $\breve{N}_2$ representing steady state populations.

r_1 and r_2 are the growth rates

K_1 and K_2 are the carrying capacities

α is the competition coefficient which shows the effect of population 2 on population 1

β is the competition coefficient which shows the effect of population 1 on population 2

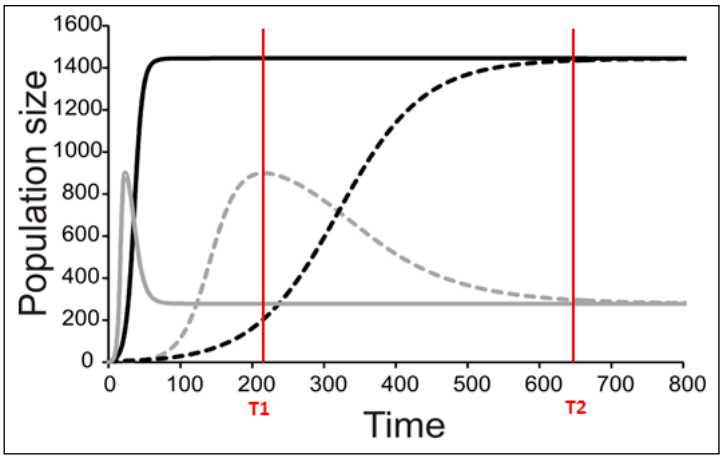


Figure 4: Simulated population dynamics of two competing species A (black lines) and B (grey lines) at different growth rates. Solid lines represent high growth rates reaching the equilibrium faster whereas dotted lines represent slow growth rates reaching the equilibrium more slowly. In both cases species A has slower growth rate but greater carrying capacity than species B. At time T1 the populations are in proportion to their growth rates but at T2, approaching equilibrium, growth rates become increasingly irrelevant in determining population sizes. Thus, growth rates are important determinants of population size in a perturbed state but not at a steady state.

If, in an experiment, we start at a non-equilibrium state, and observe at time T1 (figure 4), the standing populations would be inferred as a function of intrinsic growth rates r_1 and r_2. But if observed at time T2, the inference would be different. The intrinsic growth rates of both the competing populations do not determine the equilibrium populations of the two species. The magnitude of r determines the time taken by the population to reach the equilibrium or steady state (figure 4). The role of growth rates in population dynamics is well

recognized and is demonstrable in a perturbed state but it needs to be realized that it has no role in determining the steady state populations. Nevertheless, existence of non-zero positive growth rates is essential for attaining the equilibrium or returning to it if perturbed. If either or both the growth rates are made zero, the system will never attain back a stable equilibrium coexistence. Thus, the two growth rates are causal for attaining equilibrium, but they have no causal role in deciding the position of the equilibrium point. Thus, we need to distinguish between the driver cause and the navigator cause. Driver causality is a process that takes a homoeostatic system to an equilibrium point but may not have any role in deciding the attributes of the equilibrium. Navigator causality refers to the processes that determine the location of the equilibrium point and lead the driver there, but in the absence of the driver, may not be able to take the system to the steady state.

5.3.2 Driver and navigator in a homeostatic system

For a homeostatic system, the distinction between perturbed state and steady state causality is practically equivalent to driver and navigator causality. However, the driver-navigator distinction can be applied, in principle, to non-homeostatic systems as well and therefore is a broader concept.

This has relevance to experimental physiology. If knocking out a certain gene, protein or function disables homeostatic control, it does not provide us any clue as to whether it has a driver or navigator function. The experiment does not necessarily demonstrate that the gene, protein or function determines the steady state levels of the controlled variable. Since distinction between driver and navigator causality has not been explicitly made in experimental physiology, currently there are no norms or methods to resolve between the two types of causations. We use this distinction below to re-examine the role of insulin in glucose homeostasis and show that the failure to distinguish between driver and navigator causality has led to a fundamentally flawed understanding of glucose homeostasis and type 2 diabetes.

5.3.3 Why is insulin believed to regulate fasting blood sugar: a burden of history?

After the classical demonstration by Claud Bernard that damage to medulla oblongata causes hyperglycaemia (Bernard, 1879), the second major breakthrough was the demonstration by von Mering and Minkowski that pancreatectomy resulted in hyperglycaemia (Mering and Minkowski, 1890) and further that pancreatic extracts resulted in lowering of plasma glucose. The active principle eventually purified became known as insulin (Karamitsos, 2011). The discovery and success of insulin in treating diabetes was so overwhelming that insulin became the key molecule in glucose homeostasis and the role of brain and other mechanisms were practically forgotten. It should be noted that the prevalent type of diabetes then was what we would call type 1 diabetes (T1D) today in which there is almost complete destruction of pancreatic β-cells. The distinction between type 1 and 2 developed gradually over the next five decades along with the realization that insulin levels may be normal or raised in type 2 diabetes (T2D) and that a substantial population of β-cells survives lifelong (Clark *et al.*, 1990; Porte and Kahn, 2001; Butler *et al.*, 2003). However, by now the thinking about glucose homeostasis was so insulin-cantered, that the inability of normal or raised levels of insulin to keep plasma glucose normal was labelled as "insulin resistance" (Reaven, 1988) without adequately examining and eliminating alternative possibilities and the concept got wide uncritical acceptance. Although insulin receptor and downstream functions are known to be highly variable at the cellular level, the question whether altered insulin signalling is solely or mainly responsible for fasting hyperglycaemia of T2D, or other insulin independent mechanisms play a significant role is not clearly answered.

5.3.4 Do we need to look beyond insulin?

There are multiple reasons to doubt and re-examine the role of insulin in glucose regulation in relation to T2D (Corkey, 2012; Pories and Dohm, 2012; Watve, 2013). Exogenous insulin and other insulin-centered lines of treatment have largely failed to reduce diabetic complications and mortality in T2D although short term glucose lowering may be achieved (Meinert *et al.*, 1970; UKPDS 24; UKPDS 33; UKPDS 38; King, Peacock and Donnelly, 2001; ACCORD, 2008). In the long run even the glucose normalization goal is not achieved in majority of

cases (UKPDS 33; UKPDS 38). A number of mechanisms are known to influence glucose dynamics, partially or completely independent of insulin signalling, including autonomic signals (Nonogaki, 2000; Schwartz, 2005), glucocorticoids (Goldstein *et al.*, 1993; Gathercole and Stewart, 2010; Di Dalmazi *et al.*, 2012; Kuo *et al.*, 2015), insulin independent glucose transporters (Carruthers *et al.*, 2009) and certain other hormones and growth factors (Clemmons, 2004; Jansen *et al.*, 2006; Messmer-Blust *et al.*, 2012; Suh *et al.*, 2014). Analysis of multi-organ signalling network models have also raised doubts about the central role of insulin and insulin resistance in T2D (Kulkarni, Sharda and Watve, 2017).

The definitions as well as clinical measures of insulin resistance are such that the effects of all other mechanisms are accounted for under the name of "insulin resistance". For example, the HOMA-IR index is calculated as a product of fasting glucose and fasting insulin (Turner *et al.*, 1979; Matthews *et al.*, 1985). The belief that this product reflects insulin resistance is necessarily based on the assumption that insulin signalling alone quantitatively determines glucose level in a fasting steady state. The assumption has seldom been critically examined. If any other mechanisms are contributing to impaired fasting glucose, they will be included in the HOMA-IR index going by the way it is calculated and would be labelled as insulin resistance. This amounts to a circular logic. Insulin resistance is hypothesized to be responsible for the failure of insulin to control fasting glucose, and insulin resistance is measured as the inability of insulin to control fasting glucose. This makes the insulin resistance concept unfalsifiable from clinical data. We therefore need alternative and multiple approaches to test the concept.

5.3.5 A new view at the insulin-glucose relationship

In chapters 2,3 and 4 we examined the long-held belief that altered insulin signalling is responsible for deciding fasting glucose level using multiple approaches.

(1) Systematic review of experiments involving tissue specific insulin receptor knock-outs (IRKOs)

(2) Systematic review of experiments to chronically raise or lower insulin levels

(3) Primary experiments on streptozotocin (STZ) induced hyperglycaemia in rats, that differentiate between steady (fasting) and perturbed (post-feeding) state

(4) Examining the insulin resistance hypothesis for being mathematically possible and theoretically sound

(5) Analysis of insulin-glucose relationship in steady state versus post-meal perturbed state in human epidemiological data for testing the predictions of mathematical models.

The first three approaches have the advantage of using specific molecular interventions where the target is precisely known. For the analyses we chose mechanisms of insulin level/action modification which have been used extensively and have been reproduced by multiple labs world over. The possible disadvantage is that they are mostly animal experiments and doubts are expressed about whether the results are directly relevant to humans (Akhtar, 2015; Ali et al., 2018; Bracken, 2009). However, some of the experiments reported are human and they converge with the inferences of the animal experiments. In the last two approaches, human epidemiological data are used in which the experimental molecular precision is not expected, but we test certain specific predictions of the insulin resistance hypotheses using novel analytical approaches and examine whether they converge on similar inferences. The convergence of human and animal data is important to reach robust conclusions.

The experimental approaches examined in chapters 2, and 4 fail to support the classical belief about glucose insulin relationship. The insulin receptor knock-out experiments and insulin suppression or enhancement experiments converge to show that alteration in insulin levels or insulin sensitivity does not change the steady state glucose levels. Evidence that it changes the shape of the glucose curve after food intake or glucose loading is more convincing in spite of some inconsistency across different experiments. Typically return to the steady state is delayed by impaired insulin signalling but the steady state glucose level remains unchanged. Convergence of experiments using other means of causing specific alterations in insulin action strengthens the inference.

Several mathematical models attempt to capture the dynamics of glucose homeostasis. A good model should be able to explain all the empirical results summed up here. The inability of insulin receptor knockouts, insulin suppression and insulin enhancement experiments to alter steady state glucose levels, the difference in the regression correlation parameters between insulin and glucose in the steady versus perturbed state, the extremely weak correlation between fasting

glucose and fasting insulin, but very strong correlation between HOMA-IR and HOMA-β, the hyperinsulinemic-normoglycemic prediabetic state and the phenomenon of impaired glucose tolerance but normal fasting glucose. Reviewing models of glucose homeostasis is beyond the scope of this paper, but we outline here what a good model of glucose homeostasis needs to explain. In our observation, all existing models explain only some of the empirical findings. We suggest here that this inability is because of a common baseline assumption of all models that insulin signalling determines the glucose level in the fasting as well as post feeding conditions. It should be possible to construct such a model, if we realize that insulin affects glucose only in the post feeding but not in fasting conditions.

It is difficult to defend the classical assumptions about insulin-glucose relationship against the multiple convergent lines of evidence. Although results of these experiments have been there in the published literature for about two decades, these results were mostly explained away giving different excuses for different sets of experiments. The possible lines of defence would include difference between homeostatic mechanisms in rodents and humans or the possibility of non-linear nature of insulin-glucose relationship. The evidence reviewed here comes from rodents as well as humans and the glucose insulin scatters do not show any clear indication of non-linearity. Further it would be prudent to avoid making inferences based on dietary or other complex interventions since they can have multiple mechanisms of action. Specific genetic or molecular interventions are more revealing with respect to the underlying mechanisms since we can be more confident about their specificity of action. Therefore, our inference that insulin action does not influence fasting glucose levels is the most straightforward and parsimonious inference. Any other explanations will have to be supported by giving evidence for the assumptions made in those explanations.

The failure of experimental alteration in insulin signalling to alter steady state glucose raises two distinct possibilities about fasting hyperglycaemia in T2D. One is that fasting hyperglycaemia in T2D is a result of processes independent of insulin signalling such as autonomic signalling or other insulin independent mechanisms. The sympathetic tone is known to be altered in metabolic syndrome (Thorp and Schlaich, 2015) and increased sensitivity of liver to sympathetic signal

is likely to be mainly responsible to fasting hyperglycaemia (Bruce *et al.*, 1992). The other possibility is that with impaired insulin signalling overnight fasting is not sufficient to reach a steady state, therefore fasting hyperglycaemia in T2D is a non-steady state phenomenon in type 2 diabetes. The considerably weaker but still significant correlation between glucose and insulin in fasting as compared to post glucose load data suggests that both the factors are likely to be operational differentially in different individuals.

In either case certain fundamental concepts in our understanding of T2D need to be revised. First, the definition and measurement of insulin resistance using steady state glucose and insulin levels needs to be questioned. Most commonly used indices of insulin resistance are based on the assumption that insulin signalling decides the fasting steady state glucose levels, although non-equilibrium methods of assessing insulin resistance have been described (Patarrão *et al.*, 2012). In the classical view other mechanisms of glucose regulation are assumed to be absent or non-significant. If increased sympathetic signalling increases liver glucose production, HOMA-IR will still account it as "insulin resistance". The same is true about insulin resistance measured by hyperinsulinemic euglycemic clamp. The way insulin resistance is measured at the clinical level eliminates the chance of separately accounting for other mechanisms of glucose regulation. Even when experiments show that certain agents affect glucose dynamics independent of insulin action, they are typically labelled as "insulin sensitizing" agents (Hossain *et al.*, 2018). As a result, the belief that insulin is the only mechanism of glucose regulation relevant to T2D is artificially strengthened. There is a subtle circularity in the working definition of insulin resistance. Insulin resistance is blamed for the failure of normal or elevated levels of insulin to regulate glucose. To test this hypothesis, we should have an independent definition and measure of insulin resistance. Only then we can test whether and to what extent insulin resistance can alter glucose dynamics. However, clinically insulin resistance is measured by the inability of insulin to regulate glucose. Such a measure cannot be used to test the hypothesis that insulin resistance leads to the failure of insulin to regulate glucose. The unfalsifiability of the insulin resistance hypothesis arising out of this circularity has halted any attempts towards realistic assessments of the true causes of fasting hyperglycaemia in type 2 diabetes. In the molecular approach to induce insulin resistance, we have an independent definition and causality for insulin

resistance and therefore such experiments are free from circularity of definition. The results of such experiments reviewed here are therefore more revealing and reliable. Since all of them converge to show that altering insulin signalling does not alter steady state glucose levels, the insulin resistance and inadequate compensation hypothesis for steady state hyperglycaemia stands clearly rejected. The question can be turned upside down to examine whether steady state glucose level determines steady state insulin. If glucose is infused with a constant rate over a long time, insulin levels will come back to the baseline levels if glucose is not a determinant of fasting insulin. If it is, then insulin levels will stabilize at a new heightened steady state level. Jetton et al. (2008) infused intra venous glucose (20% glucose w/v) continuously for 4 days in rats (figure 5). Both glucose and insulin levels increased significantly after the infusion. However, later both glucose and insulin levels came back to normal even as the infusion continued. Increase in the concentration of the infused glucose (up to 35%) also yielded similar results (Steil *et al.*, 2001). Thus, immediately on perturbation, glucose affected insulin levels, however after allowing sufficient time to regain steady state, the infused glucose had no significant effect on insulin levels. This demonstrates that even glucose does not hold a causal relationship with insulin in a steady state whereas glucose level perturbation is certainly known to stimulate insulin response.

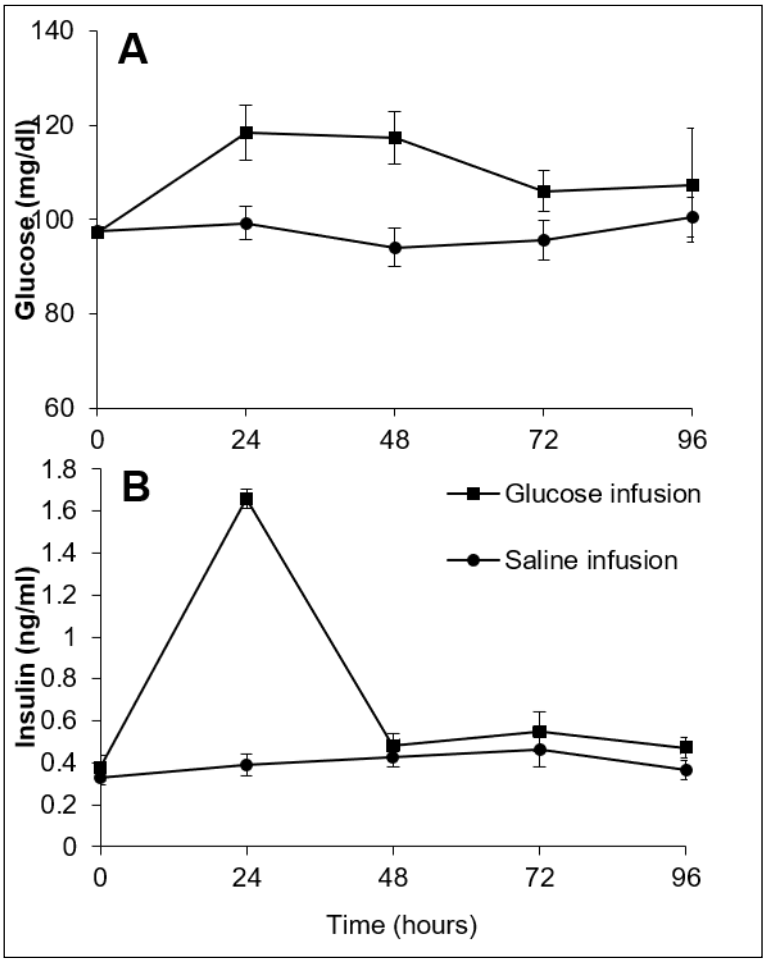


Figure 5: Insulin and glucose levels after a sustained infusion of 20%w/v glucose for 4 days. Figure reproduced from Jetton et al 2008 without permission.

The interpretation of this phenomenon needs to be done at a broader philosophical level. We point out here with specific reference to homeostatic systems that the nature of causality in a perturbed state can be qualitatively different from causality in steady state. There is a simple analogue to perturbed state versus steady state causality in one of the basic models of mathematical biology. In the classical model of logistic growth, the intrinsic growth rate *r* decides the rate at which a population can change when away from the carrying capacity *K* (Gotelli, 2008). However, the carrying capacity itself may be independent of the growth rate. A non-zero positive *r* is required to reach the steady state at *K* but *r* does not determine the steady state level. It is a function of *K* alone. Reducing *r* leads to delay in achieving a steady state but the steady state remains at the same position. The evidence reviewed here indicates that insulin action is analogous to *r* of logistic model. It is required to reach a steady state, but it does not determine the location of the steady state.

The inability to distinguish between steady state causality and perturbed state causality may have substantially misled biomedical research at times, T2D certainly being an important example. This poses an important philosophical as well as methodological problem in experimental physiology. Many systems in physiology have homeostatic steady states and we use experimental approaches to reveal them. However, most experiments are perturbation experiments, and we may be making the mistake of applying the demonstrated perturbed state causality to understand steady state systems. The apparent paradox can be resolved only by carefully designing and interpreting experiments. If a perturbation is momentary or transient, the results obtained would certainly reflect perturbed state causality but may not reflect steady state causality. On the other hand, sustained perturbations held constant for sufficiently long to allow the system to regain a steady state are necessary to establish steady state causality. If upon sustainably altering a causal factor the effect variable returns to the same steady state, it reflects only perturbed state and not steady state causality. If, on the other hand, sustained alteration in the causal factor results into an altered steady state, it indicates steady state causality.

Viewed from a slightly different and more generalized angle that goes beyond homeostatic systems, we can differentiate between two types of causalities. In driver causality the causal factor is necessary to reach a destination but does not decide the destination. In navigator causality the causal factor is crucial in determining the destination but may not be sufficient to take the system there. The evidence reviewed above indicates that insulin is a driver but not a navigator of glucose homeostasis. A non-zero level of insulin is required for reaching a homeostatic steady state. In type 1 diabetes, the almost complete absence of insulin prevents glucose homeostasis. In type 2 diabetes there are non-zero insulin levels and therefore, a steady state is possible, but insulin itself plays little role in deciding the steady state glucose level. It is more likely that neuronal and other hormonal-metabolic factors affect the steady state glucose in T2D.

Certain kinds of experimental interventions are unable to distinguish between driver versus navigator causality. Knocking out a driver or a navigator will disable the journey to the destination. Therefore, complete knockout of a cause may not distinguish between driver and navigator causality. On the other hand, experiments quantitatively altering the level of the causal factor while keeping it

non-zero and observing the effect for sufficiently long duration, can help us differentiate between drivers and navigators. A sub-normal driver will delay the time to destination but will not change the destination. On the other hand, changing the navigator may or may not alter the time, but will alter the position of the destination. The history of insulin research is that early experiments such as total pancreatectomy demonstrated the necessary role of insulin in glucose homeostasis but the distinction between driver or navigator causality was not even conceptually perceived. So, it was assumed that insulin does both the roles. Now in the presence of multiple experiments showing the precise role of insulin, we need to revive our concepts of causality. At a broader scale the insulin example warrants care in making inferences in experimental physiology, in the absence of which our understanding of the physiology of homeostatic systems can be seriously flawed.

5.4 References

ACCORD (2008) 'Effects of Intensive Glucose Lowering in Type 2 Diabetes', *New England Journal of Medicine*, 358(24), pp. 2545–2559. doi: 10.1056/NEJMoa0802743.

Akhtar, A. (2015) 'The Flaws and Human Harms of Animal Experimentation', *Cambridge Quarterly of Healthcare Ethics*, 24(04), pp. 407–419. doi: 10.1017/S0963180115000079.

Ali, Z., Chandrasekera, P. C. and Pippin, J. J. (2018) 'Animal research for type 2 diabetes mellitus, its limited translation for clinical benefit, and the way forward.', *Alternatives to laboratory animals : ATLA*, 46(1), pp. 13–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/29553794.
Baker, J. P. (2008) 'Mercury, Vaccines, and Autism', *American Journal of Public Health*, 98(2), pp. 244–253. doi: 10.2105/AJPH.2007.113159.

Bernard, C. (1879) 'Leçons de physiologie opératoire', *Librairie J.-B. Baillie et fils*, p. 650.

Bracken, M. B. (2009) 'Why animal studies are often poor predictors of human reactions to exposure', *Journal of the Royal Society of Medicine*, 102(3), pp. 120–122. doi: 10.1258/jrsm.2008.08k033.

Bruce, D. G. *et al.* (1992) 'The effects of sympathetic nervous system activation and psychological stress on glucose metabolism and blood pressure in subjects with Type 2 (non-insulin-dependent) diabetes mellitus', *Diabetologia*, 35(9), pp. 835–843. doi: 10.1007/BF00399929.

Butler, A. E. *et al.* (2003) '-Cell Deficit and Increased -Cell Apoptosis in Humans With Type 2 Diabetes', *Diabetes*, 52(1), pp. 102–110. doi: 10.2337/diabetes.52.1.102.

Carruthers, A. *et al.* (2009) 'Will the original glucose transporter isoform please stand up!', *American Journal of Physiology-Endocrinology and Metabolism*, 297(4), pp. E836–E848. doi: 10.1152/ajpendo.00496.2009.

Chawla, S. *et al.* (2018) 'Inferring causal pathways among three or more variables from steady state correlations in a homeostatic system', *PLOS ONE*. Edited by M. Ruscica, 13(10), p. e0204755. doi: 10.1371/journal.pone.0204755.

Chirieac, D. V *et al.* (2000) 'Glucose-Stimulated Insulin Secretion Suppresses Hepatic Triglyceride-Rich Lipoprotein and Apo B Production In Vivo', *Diabetes*, 49(5), p. A281.

Chu, C. A. *et al.* (1997) 'Comparison of the direct and indirect effects of epinephrine on hepatic glucose production.', *J Clin Invest*, 99, pp. 1044–1056.

Clark, A. *et al.* (1990) 'Islet amyloid polypeptide in diabetic and non-diabetic Pima Indians', *Diabetologia*, 33(5), pp. 285–289. doi: 10.1007/BF00403322.

Clemmons, D. R. (2004) 'Role of Insulin-Like Growth Factor Iin Maintaining Normal Glucose Homeostasis', *Hormone Research in Paediatrics*, 62(1), pp. 77–82. doi: 10.1159/000080763.

Corkey, B. E. (2012) 'Banting Lecture 2011: Hyperinsulinemia: Cause or Consequence?', *Diabetes*, 61(1), pp. 4–13. doi: 10.2337/db11-1483.

Di Dalmazi, G. *et al.* (2012) 'Glucocorticoids and Type 2 Diabetes: From Physiology to Pathology', *Journal of Nutrition and Metabolism*, 2012, pp. 1–9. doi: 10.1155/2012/525093.

Diwekar-Joshi, M. and Watve, M. (2020) 'Driver versus navigator causation in biology: the case of insulin and fasting glucose', *PeerJ*, 8, p. e10396. doi: 10.7717/peerj.10396.

Fujimoto, T. *et al.* (2003) 'Skeletal muscle glucose uptake response to exercise in trained and untrained men', *Medicine and Science in Sports and Exercise*, 35(5), pp. 777–783. doi: 10.1249/01.MSS.0000065070.49295.C0.

Gathercole, L. L. and Stewart, P. M. (2010) 'Targeting the pre-receptor metabolism of cortisol as a novel therapy in obesity and diabetes', *The Journal of Steroid Biochemistry and Molecular Biology*, 122(1–3), pp. 21–27. doi: 10.1016/j.jsbmb.2010.03.060.

Gerber, J. S. and Offit, P. A. (2009) 'Vaccines and Autism: A Tale of Shifting Hypotheses', *Clinical Infectious Diseases*, 48(4), pp. 456–461. doi: 10.1086/596476.

Goldstein, R. E. *et al.* (1993) 'Effects of chronic elevation in plasma cortisol on hepatic carbohydrate metabolism', *American Journal of Physiology-Endocrinology and Metabolism*, 264(1), pp. E119–E127. doi: 10.1152/ajpendo.1993.264.1.E119.

Gotelli, N. J. (2008) *A Primer of Ecology*.

Granger, C. W. J. (1969) 'Investigating Causal Relations by Econometric Models and Cross-spectral Methods', *Econometrica*, 37(3), p. 424. doi: 10.2307/1912791.

Greenland, S. (2000) 'An introduction to instrumental variables for epidemiologists', *International Journal of Epidemiology*, 29(4), pp. 722–729. doi: 10.1093/ije/29.4.722.

Hällsten, K. *et al.* (2002) 'Rosiglitazone but not metformin enhances insulin- and exercise-stimulated skeletal muscle glucose uptake in patients with newly diagnosed type 2 diabetes', *Diabetes*, 51(12), pp. 3479–3485.

Hill, A. B. (1965) 'The Environment and Disease: Association or Causation?', *Proceedings of the Royal Society of Medicine*, 58(5), pp. 295–300.

Hossain, Z. *et al.* (2018) 'Discovery of pancreastatin inhibitor PSTi8 for the treatment of insulin resistance and diabetes: Studies in rodent models of diabetes mellitus', *Scientific Reports*. Springer US, 8(1), pp. 1–13. doi: 10.1038/s41598-018-27018-8.

Jansen, C. *et al.* (2006) 'Does Epidermal Growth Factor Participate in the Regulation of Glucose, Insulin and Glucagon Levels?', *European Surgical Research*, 38(4), pp. 377–384. doi: 10.1159/000094533.

Jetton, T. L. *et al.* (2008) 'Enhanced β-cell mass without increased proliferation following chronic mild glucose infusion', *American Journal of Physiology-Endocrinology and Metabolism*, 294(4), pp. E679–E687. doi: 10.1152/ajpendo.00569.2007.

Karamitsos, D. T. (2011) 'The story of insulin discovery', *Diabetes Research and Clinical Practice*, 93, pp. S2–S8. doi: 10.1016/S0168-8227(11)70007-9.

King, P., Peacock, I. and Donnelly, R. (2001) 'The UK Prospective Diabetes Study (UKPDS): clinical and therapeutic implications for type 2 diabetes', *British Journal of Clinical Pharmacology*, 48(5), pp. 643–648. doi: 10.1046/j.1365-2125.1999.00092.x.

Kulkarni, S., Sharda, S. and Watve, M. (2017) 'Bi-stability in type 2 diabetes mellitus multi-organ signalling network', *PLOS ONE*. Edited by C. Cras-Méneur, 12(8), p. e0181536. doi: 10.1371/journal.pone.0181536.

Kuo, T. *et al.* (2015) 'Regulation of Glucose Homeostasis by Glucocorticoids', in, pp. 99–126. doi: 10.1007/978-1-4939-2895-8_5.

Marchetti, P. *et al.* (1994) 'Pulsatile insulin secretion from isolated human pancreatic islets.', *Diabetes*, 43(6), pp. 827–30.

Matthews, D. R. *et al.* (1985) 'Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28(7), pp. 412–419. doi: 10.1007/BF00280883.

Meehl, P. E. and Waller, N. G. (2002) 'The path analysis controversy: a new statistical approach to

strong appraisal of verisimilitude.', *Psychological methods*, 7(3), pp. 283–300. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12243300.

Meinert, C. L. *et al.* (1970) 'A study of the effects of hypoglycemic agents on vascular complications in patients with adult-onset diabetes. II. Mortality results.', *Diabetes*, 19, p. Suppl:789-830. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4926376.

Mering, J. von J. and Minkowski, O. (1890) 'Diabetes mellitus nach Pankreasexstirpation', *Vogel, Leipzig*, p. 28.

Messmer-Blust, A. F. *et al.* (2012) 'RTEF-1 Attenuates Blood Glucose Levels by Regulating Insulin-Like Growth Factor Binding Protein-1 in the Endothelium', *Circulation Research*, 111(8), pp. 991–1001. doi: 10.1161/CIRCRESAHA.112.268110.

Moore, M. C., Connolly, C. C. and Cherrington, a D. (1998) 'Autoregulation of hepatic glucose production.', *European journal of endocrinology*, 138(3), pp. 240–248.

Niles, H. E. (1923) 'The Method of Path Coefficients an Answer to Wright.', *Genetics*, 8(3), pp. 256–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17246012.

Nonogaki, K. (2000) 'New insights into sympathetic regulation of glucose and fat metabolism', *Diabetologia*, 43(5), pp. 533–549. doi: 10.1007/s001250051341.

Nuutila, P. *et al.* (2000) 'Enhanced stimulation of glucose uptake by insulin increases exercise-stimulated glucose uptake in skeletal muscle in humans: studies using [15O]O2, [15O]H2O, [18F]fluoro-deoxy-glucose, and positron emission tomography.', *Diabetes*, 49(7), pp. 1084–91.

Patarrão, R. S. *et al.* (2012) 'Postprandial but not fasting insulin resistance is an early identifier of dysmetabolism in overweight subjects', *Canadian Journal of Physiology and Pharmacology*, 90(7), pp. 923–931. doi: 10.1139/y2012-086.

Peters, J., Janzing, D. and Scholkopf, B. (2011) 'Causal Inference on Discrete Data Using Additive Noise Models', *IEEE Transactions on Pattern Analysis and Machine Intelligence*, 33(12), pp. 2436–2450. doi: 10.1109/TPAMI.2011.71.

Pories, W. J. and Dohm, G. L. (2012) 'Diabetes: Have We Got It All Wrong?: Hyperinsulinism as the culprit: surgery provides the evidence', *Diabetes Care*, 35(12), pp. 2438–2442. doi: 10.2337/dc12-0684.

Porte, D. and Kahn, S. E. (2001) 'Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms', *Diabetes*, 50(Supplement 1), pp. S160–S163. doi: 10.2337/diabetes.50.2007.S160.

Ratzan, S. C. (2010) 'Setting the Record Straight: Vaccines, Autism, and the Lancet', *Journal of Health Communication*, 15(3), pp. 237–239. doi: 10.1080/10810731003780714.

Reaven, G. M. (1988) 'Role of Insulin Resistance in Human Disease', *Diabetes*, 37(12), pp. 1595–1607. doi: 10.2337/diab.37.12.1595.

Rosenbaum, P. R. and Rubin, D. B. (1983) 'The central role of the propensity score in observational studies for causal effects', *Biometrika*, 70(1), pp. 41–55. doi: 10.1093/biomet/70.1.41.

Rothman, D. L. *et al.* (1991) 'Quantitation of hepatic glycogenolysis and gluconeogenesis in fasting humans with 13C NMR.', *Science (New York, N.Y.)*, 254(5031), pp. 573–6.

Schwartz, M. W. (2005) 'Diabetes, Obesity, and the Brain', *Science*, 307(5708), pp. 375–379. doi: 10.1126/science.1104344.

Shulman, G. I. *et al.* (1990) 'Quantitation of muscle glycogen synthesis in normal subjects and subjects with non-insulin-dependent diabetes by 13C nuclear magnetic resonance spectroscopy.',

The New England journal of medicine, 322(4), pp. 223–8. doi: 10.1056/NEJM199001253220403.

Steil, G. M. *et al.* (2001) 'Adaptation of β-cell mass to substrate oversupply: enhanced function with normal gene expression', *American Journal of Physiology-Endocrinology and Metabolism*, 280(5), pp. E788–E796. doi: 10.1152/ajpendo.2001.280.5.E788.

Suh, J. M. *et al.* (2014) 'Endocrinization of FGF1 produces a neomorphic and potent insulin sensitizer', *Nature*, 513(7518), pp. 436–439. doi: 10.1038/nature13540.

Thorp, A. A. and Schlaich, M. P. (2015) 'Relevance of Sympathetic Nervous System Activation in Obesity and Metabolic Syndrome', *Journal of Diabetes Research*, 2015, pp. 1–11. doi: 10.1155/2015/341583.

Tomasi, T. *et al.* (1966) 'Insulin half-life in normal and diabetic subjects.', *Revue de neuropsychiatrie infantile et d'hygiene mentale de l'enfance*, 14(12), pp. 315–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5988016.

Turner, R. C. *et al.* (1979) 'Insulin deficiency and insulin resistance interaction in diabetes: Estimation of their relative contribution by feedback analysis from basal plasma insulin and glucose concentrations', *Metabolism*, 28(11), pp. 1086–1096. doi: 10.1016/0026-0495(79)90146-X.

UK Prospective Diabetes Study Group (1998a) 'Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33)', *The Lancet*, 352(9131), pp. 837–853. doi: 10.1016/S0140-6736(98)07019-6.

UK Prospective Diabetes Study Group (1998b) 'Tight blood pressure control and risk of macrovascular and microvascular complications in type 2 diabetes: UKPDS 38', *BMJ*, 317(7160), pp. 703–713. doi: 10.1136/bmj.317.7160.703.

UK Prospective Diabetes Study Group (1998c) 'United Kingdom Prospective Diabetes Study 24: A 6-Year, Randomized, Controlled Trial Comparing Sulfonylurea, Insulin, and Metformin Therapy in Patients with Newly Diagnosed Type 2 Diabetes That Could Not Be Controlled with Diet Therapy', *Annals of Internal Medicine*, 128(3), p. 165. doi: 10.7326/0003-4819-128-3-199802010-00001.

Watve, M. (2013) *Doves, Diplomats, and Diabetes*. New York, NY: Springer New York. doi: 10.1007/978-1-4614-4409-1.

Westerlund, J. and Bergsten, P. (2001) 'Glucose Metabolism and Pulsatile Insulin Release From Isolated Islets', *Diabetes*, 50(8), pp. 1785–1790. doi: 10.2337/diabetes.50.8.1785.

Wright, S. (1934) 'The Method of Path Coefficients', *The Annals of Mathematical Statistics*, 5(3), pp. 161–215. doi: 10.1214/aoms/1177732676.

Wright, S. (1960) 'Path Coefficients and Path Regressions: Alternative or Complementary Concepts?', *Biometrics*, 16(2), p. 189. doi: 10.2307/2527551.

Chapter 6: Implications for evolutionary medicine

6.1 Introduction

The laboratory environment and the research group in which I worked are primarily evolutionary biologists, evolutionary medicine (EM) being one of the major interests. My seniors and colleagues published several papers, articles, and books on the evolutionary aspects of type 2 diabetes. However, during this work it was realized that the current perception of pathophysiology of T2DM has a few fundamental problems. Unless we have a clearer picture of the underlying physiology and the disease, the evolutionary interpretations will remain weak and unsupported. This realization led to fundamental work in the pathophysiology, namely the fundamental relationship between glucose and insulin.

In the earlier chapters, I re-examined the insulin-glucose relationship with a view of distinguishing between the relationship in the steady state versus the perturbed state. Now having obtained certain insights into it, it is natural to return to EM and examine the implications of the new understanding about insulin-glucose relationship. Some part of this following work is published as a paper on the evolutionary origins of type 2 diabetes (Watve and Diwekar-Joshi, 2016).

6.2 Evolutionary medicine

Evolutionary medicine has a long history, but there are disputes about its implications in clinical practice (Cournoyea, 2010). Evolutionary hypotheses about human anatomy such as skeletal effects of bipedalism, trade-offs associated with a large brain theorised for a long time. Physiological phenomena like the fight or flight response were described in the 1930s (Cannon, 1934) and quickly became widely accepted. However, attempts to organize evolutionary thinking in physiology and medicine do not date back to more than a few decades (Williams and Nesse, 1991; Nesse, 2001).

The foundation of medicine is based on the “how” question regarding the anatomy and physiology of the human body. Evolution addresses the “why” question. A combined understanding of how and why of a disease is likely to boost the

precision and effectiveness of its treatment. EM tries to explain vulnerability to a disease along with differences in vulnerabilities in the population. In the past EM has given us following conceptual insights in infectious diseases: evolution of virulence (Read, 1994) and evolution of antibiotic resistance (Nesse, 2001; Read and Huijben, 2009). The contribution if EM to non-infectious diseases is peripheral and its clinical implications debated.

EM has received serious criticism on several grounds. The most stated and the most important is that EM fails to provide insights relevant in clinical practice (Cournoyea, 2010). The adaptationist view of EM is also a focus of criticism. The evidence for any adaptive argument is almost always circumstantial/ inferential and hence the argument must be considered hypothetical (Gluckman, Hanson and Spencer, 2005). Many EM theories are based on the mismatch between ancestral and the current environment. Claims about ancestral conditions are speculative and there are limitations in visualizing them. Without adequate use of rigorous norms for hypothesis building and adequate efforts in making and testing differential predictions, evolutionary medicine remains at the blurred line between science and philosophy (Cournoyea, 2010). The kind of scientific rigor seen in evolutionary literature in the fields of social behaviour, sexual selection or molecular phylogeny for example, needs to be employed in EM.

We developed a set of criteria to be expected from an evolutionary theory for any human disease and outlined an approach to evaluate alternative evolutionary hypotheses using this set (Watve and Diwekar-Joshi, 2016). Such a relative evaluation approach is only occasionally used in EM (Corbett and Morin-Papunen, 2013). The analysis in the earlier chapters have refuted the classical theory of glucose regulation by insulin (more specifically in the steady state) which has implications for the evolutionary hypotheses for T2DM which we will discuss here.

6.3 Brief history of evolutionary theories about T2DM

6.3.1 Thrifty gene hypothesis

James Neel tried to come up with an evolutionary explanation for the diabetic genotype (Neel, 1962). He postulated that diabetes was a single recessive or

incompletely recessive Mendelian trait. Neel looked at the frequency of occurrence of diabetes and asked the question if it was a genetic disorder comparable to thalassemia or haemophilia. But diabetes was more frequent and increasing at a high rate. This led him to seek an adaptive or 'advantage driven' explanation for diabetes. By this time, the association between obesity and diabetes was well established. Neel postulated that "the overweight individual of 40 or 50 with mild diabetes is not so much diabetic because he is obese as he is obese because he is of a particular (diabetic) genotype". (What he refers to as "mild diabetes" is today's type 2 diabetes.) He stated that a "thrifty gene" was responsible for storage of fat in nutrient rich or "feast" conditions and allowed reutilization during starvation or "famine" conditions. Neel defined thriftiness as "being extremely efficient in intake and/or utilization of food" (Neel, 1962). Neel had a clear hypothesis for the mechanism of thrift which was based on the finding that prediabetics have higher levels of insulin. Since insulin is known to be lipogenic (Moustaïd, Jones and Taylor, 1996) high levels of insulin would lead to higher fat storage. Thus, in Neel's view rise in insulin levels was primary and obesity a secondary effect of that. Since the concept of insulin resistance was yet to be widely known then, he believed that an "anti-insulin activity" balanced the hyperinsulinemia. In due course of time excessive anti-insulin activity resulted into diabetes.

Although Neel is commonly credited as the father of thrifty gene hypothesis, Neel's concept of thrifty gene was substantially different than the currently prevalent thrifty gene concept. During 1970s and 80s the centre of thinking shifted to insulin resistance which turned the thinking upside down. Now obesity was thought to be primary which increased insulin resistance and hyperinsulinemia was a compensatory response (Matthews *et al.*, 1985; Turner *et al.*, 2007). In this view, Neel's idea of hyperinsulinemia as the mechanism of thrift was destroyed. But this was not replaced by any clearly spelt out alternative mechanism. Neel believed obesity to be a by-product of a diabetic tendency whereas the current view projects obesity as causal to insulin resistance and in-turn diabetes.

6.3.2 Thrifty phenotype hypothesis

Although Neel's thrifty gene hypothesis was largely accepted, no such "thrifty gene" was discovered. On the other hand, Hales and Barker (1991) demonstrated the association between reduced growth in early life with impaired glucose tolerance/T2DM and related disorders in later life (Hales *et al.*, 1991). This led to the concept of 'thrifty phenotype' (Hales *et al.*, 1991; Hales and Barker, 1992; Barker, 1998; Drake and Walker, 2004). This statistical association between impaired uterine development and late life metabolic disorders has been interpreted in two ways namely development constraints and developmental programming.

In the developmental constraints view, unfavourable conditions during development lead to lifetime deficiencies for example in size and function of β-cells (Barker, 1998). In the alternative view of developmental programming, under-nourishing conditions during development are taken as cues to predict life-time nutritional limitations and the body is said to be programmed for a thrifty metabolism in anticipation (Gluckman, Hanson and Spencer, 2005).

6.3.3 Criticism of the thrifty phenotype hypothesis

The concepts of thrifty genotype and thrifty phenotype have been reviewed elaborately in recent times (Wells, 2003; Speakman, 2008; Baig *et al.*, 2011; Watve, 2013). Some of the critics of thrift have admired Neel's vision (Watve, 2013). Neel's speculations were ahead of his time and had far-reaching vision Although the concept of insulin resistance was not yet common, Neel perceived it and called it 'anti-insulin activity'. Contrary to present day mainstream thinking, Neel perceived that hyperinsulinemia precedes the "anti-insulin activity". He also clearly rejected the notion of 'β-cell exhaustion' being the reason for β-cell dysfunction (Neel, 1962). I will now discuss the main grounds on which the thrifty gene and thrifty phenotype hypotheses are criticized are as follows.

1. ***Questioning ancestral feast and famine***

Fluctuations in food availability due to famines is the fundamental assumption of the thrift hypotheses. We know little about ancestral human conditions and whatever evidence we have, does not support the frequent famine assumption

(Speakman, 2008). Chronic starvation was more predominant after the beginning of agriculture as compared to the hunter-gatherer stage (Sahlins, 1974). If the history of major famines began after the emergence of agriculture, whether this period is sufficient for the evolution of thrift is questionable. Some communities adopted agriculture very recently (or not yet) but they also showed high propensity to obesity, T2DM and hypertension on adopting modern urban lifestyle (O'Dea, 1991).

2. Can it explain population variability in proneness to obesity and T2DM?

If thrift was adaptive, why isn't everybody thrifty? Human populations have large variation in the propensity to become diabetic or obese. Neel believed that polymorphism exists because of heterozygote advantage to a thrifty allele. This argument was based on the Mendelian inheritance of diabetes which was believed at that time. Neither Mendelian inheritance nor heterozygote advantage for a thrifty allele was proven. But Neel was aware that the variability needs an explanation. The later versions of thrift have not always cared to explain the variability. If thrift was adaptive to everyone, the observed variance is unlikely to arise from stable polymorphism. Transient polymorphism is likely if the selection is recent but there is no clarity about the time course of selection for thrifty gene. Genome wide association studies (GWAS) have identified a large number of loci and mutants associated with obesity. However, they together explain only about 2-5% of population variance in obesity parameters (Mutch and Clément, 2006; McCarthy and Zeggini, 2009; Li *et al.*, 2010; Kilpeläinen *et al.*, 2011) (Rankinen *et al.*, 2006; Scott *et al.*, 2007; Sladek *et al.*, 2007; Thorleifsson *et al.*, 2009).
If many genes determine the propensity to obesity or diabetes with each one having a small effect, independent segregation of alleles at each locus will give an averaging effect reducing population variance. Therefore, the population variability observed is unlikely to be genetic. The failure to detect any locus with a large population effect undermines any genetic theory and one needs to rely more on phenotypic causes of population variability. The thrifty phenotype hypothesis explains the variance at a phenotypic level depending upon early developmental history. The evidence that early life history affects life-time metabolism and health is strong and consistent across studies but whether it is sufficient to account for population variability has not been examined. Although

there is a strong correlation between low birth weight and the likelihood of obesity and T2DM in later ages, majority of adults with T2DM are not born with a lower birth weight (McCance *et al.*, 1994; Boyko, 2000). Thus, the diabetic tendency explained by the thrifty phenotype appears to be limited.

3. Do obese individuals survive starvation/famine better?

If the thrifty hypotheses are true, obese people are expected to have a better chance of survival during famines or low nutrient periods. Evidence suggests that there are no significant differences in the survival of lean or obese individuals during famines (Speakman, 2008). Moreover, there is an association between obesity and reduction in fecundity (Gesink Law, Maclehose and Longnecker, 2006; Sallmen *et al.*, 2006; Ramlau-Hansen *et al.*, 2007) (Yilmaz *et al.*, 2009). Thus, the advantage of being obese must be higher than the reproductive cost it incurs, and this has not yet been shown quantitatively. Evidence for thrifty programming is equally weak. In baboons, offspring born small performed worse in a subsequent famine contrary to the thrifty programming hypothesis (Johnson, 1987; Virgin and Sapolsky, 1997; Lea *et al.*, 2015). The same pattern is also visible in human data (Hayward, Rickard and Lummaa, 2013).

4. Human physiology does not compare with animals evolved for feast and famine

Migratory birds or hibernating animals accumulate large amounts of fat before migration or hibernation. Both the processes of fat accumulation as well as breakdown are highly efficient in these animals due to fine-tuning of metabolic pathways (Weber, 2009; Guglielmo, 2010). Humans are not well-adapted to utilization of fat during starvation. On facing starvation proteins are broken down much before depletion of fat stores in humans, unlike migratory birds (Pasquet *et al.*, 1992; Pond, 1998). Thus, humans do not seem to be adapted to efficient storage and reutilization of fats. The difference between the physiology of obese versus lean individuals appears to be in the inability to utilize stored fat during starvation rather than more efficient storage of fat (Pasquet *et al.*, 1992; Pond, 1998). Interestingly this tendency has been called ‘spend thrift’(Reinhardt *et al.*, 2015). in contrast with classical ‘thrift’. It is hard to perceive how the inability to utilize stored fat would be selected for by feast and famine.

5. Conditions for evolution of lifetime programming

The thrifty phenotype or foetal-programming hypothesis suggests that there is lifetime programming based on the intrauterine conditions. There are two possible advantages of thrifty programming for an individual under developmental constraints (i) short term survival advantage in the foetal and early infant stages and (ii) a lifetime predictive, adaptive advantage. Lifelong rigid programming for a short-term advantage is difficult to explain since the body shows substantial age-related plasticity in different endocrine, metabolic and genetic pathways. The conditions under which lifetime programming can evolve have been examined recently (Baig *et al.*, 2011; Nettle, Frankenhuis and Rickard, 2013; Bateson, Gluckman and Hanson, 2014; Nettle and Bateson, 2015). A threshold predictive correlation between birth time and lifetime conditions is required for such a programming mechanism to evolve. Such predictive correlation is certainly not seen in climatic causes of variability in food availability (Baig *et al.*, 2011). If there are other causes of correlation they have not been explicitly stated and examined. Even a short-term advantage, if any, has also not been conclusively demonstrated so far. Hence, predictive adaptive programming is currently little more than speculation.

6. Multiple organ involvement not coherently explained

Insulin resistant and/or obese individuals show a variety of system level changes which have effects on diverse bodily functions including wound healing, ovulation, spermatogenesis, sexual behaviour, angiogenesis, innate immunity, tissue architecture, iron metabolism, memory and other cognitive brain functions, anxiety, aggression and related behaviours (Watve, 2013). The classical thrifty hypotheses focus only on energy homeostasis and offer no explanations for the changes that are not directly relevant to it. Changes in immunity are explained only weakly by stating that infectious diseases could accompany famines and hence, along with thrift, inflammation could have been facilitated (Fernández-Real and Ricart, 1999; Fernández-Real and Pickup, 2008). However, there is no evidence that the inflammatory tendency associated with insulin resistance offers any advantage in fighting infections. There are very little attempts to offer ultimate level explanations for the other associated systemic alterations.

7. What is the mechanism of thrift?

Neel clearly stated that high insulin production was the mechanism of thrift. After the picture was turned upside down with insulin resistance on the central stage, this mechanism did not seem logical. However, no alternative mechanism was proposed with sufficient clarity. Even today any clear physiological mechanism of achieving thrift is not on the horizon despite many false alarms.

It is also necessary to be clear about the two or more functional components of thrift. It can refer to higher intake of food or reduced expenditure of energy or both. It also matters whether the reduced expenditure of energy is specific to feast conditions or continues during famine as well. There is little clarity about this, and these components are used interchangeably according to convenience of the argument.

In response to the realized inadequacy of the thrifty hypotheses there appear to be two lines of developments. One is to refine the thrift hypothesis or suggest new versions of the thrift hypothesis that seem to escape at least some of the inadequacies or flaws. The other is to suggest alternative hypotheses for the evolution of obesity and insulin resistance independent of thrift.

6.3.4 Refined versions of thrift

1. Thrift could have evolved as a response to subacute nutrient conditions and not for the extreme feast and famine conditions. At subacute stress, selection for survival may be weak, but the selection for fecundity might be at work (Prentice, Hennig and Fulford, 2008; Stipp, 2011). This assumption might actually bypass the criticism about the actual frequencies and intensities of famines faced.

2. Wells argued that foetal programming confers more advantage to the mother than the foetus (Wells, 2003; Wells, 2007). Optimization of the maternal inputs per foetus is the main advantage gained by the mother by limiting the nutrients. If the nutrition conditions are limiting, thrift might improve the survival chances of the subsequent foetus by increasing the provisions available with the mother herself. The offspring also adapt to this maternal strategy and thus thrift is beneficial to both. According to this model, there is no implicit requirement of

particular conditions during birth other than dependence of mother. It does not require a birth-time-life-time correlation. However, it still does not explain the need for lifetime programming. Also, this explanation assumes thrift to mean reduced energy requirement. If thrift means increased food intake, this hypothesis does not work. This exemplifies why we need better clarity about which form of thrift we are talking about (Wells, 2003; Wells, 2007).

3. Thrift may be manifested in several other forms affecting several other body processes apart from energy intake and expenditure. “There is more to fat than thrift and there is more to thrift than fat”- from Wells (Wells, 2012) sets the stage for other possible forms of thrift namely differential energy allocation, altered growth rate, altered reproductive strategies or immune function. This is an attempt to modify the concept of thrift to make it more accommodative. This can potentially explain the multi-system involvement in obesity and metabolic syndrome. However this direction is likely to make the original concept of thrift no more identifiable. It is likely to be a multi-dimensional adaptation to a set of environmental and social challenges apart from nutritional challenges alone. This set of adaptation is no more captured by the word ‘thrift’ alone. It may be an adaptive response by the mother not only to her nutritional status but to her social position, predatory pressures, parasite loads and other factors affecting health and reproductive strategies.

All these and more such refined hypotheses are intelligent attempts to deal with the inadequacies of the classical versions of thrift hypotheses. However, refinement can handle all the issues. There are however alternatives to thrift in litareture.

6.3.5 Alternatives to thrift

a. According to Speakman (Speakman, 2008) genetic drift rather than selection is responsible for spreading the obesity genes. Freedom from predation at some stage of human evolution released the selective pressure against obesity and hence the obesity genes could drift. It is a matter of chance that some of them became common in the population.

b. Corbett et al postulated (Corbett, McMichael and Prentice, 2009) that the genotype leading to obese, diabetic, and PCOS-prone individuals today had the advantage of better fertility in famine conditions. The insulin-sensitive or "non-thrifty" genotype has better fertility under conditions of food abundance. As result with increasing food security in modern times the original insulin resistant genotype is being replaced by insulin sensitive one. What we see today is perhaps transient polymorphism (Corbett and Morin-Papunen, 2013).

c. Slow versus fast life histories: A "grow fast die young" type of life history (Stipp, 2011) is likely to be implicated by intrauterine conditions. After intrauterine growth retardation, if an individual undergoes rapid compensatory growth in early childhood, the mechanisms involved in facilitating early growth might become detrimental later. Insulin is a growth factor and high levels of insulin may be needed to facilitate early growth. Many of the pathways and molecules implicated in early growth including mTOR and AMPK have a role in the pathogenesis of T2DM too (Stipp, 2011).

d. Behavioural switch hypothesis: There can be more than one alternative strategy to cope with an environmental or social challenge. For example, the response to a conspecific competitor can be either aggressive or befriending and accordingly the hormonal responses differ (Rosati and Hare, 2012, 2013) The behavioural switch hypothesis depends upon two interconnected behavioural strategies namely hawk versus dove (aggressive versus socially manipulative) in social competition and r versus K (large number of offspring with little investment in each versus fewer ones with greater investment in each) in reproduction. Aggression anticipates injuries and extrinsic death. Hence, aggressive individuals need to invest more in reproduction than in longevity. There is a trade-off between fecundity and lifespan too. Typically, aggressive individuals are more insulin sensitive and have lower levels of insulin, cortisol and cholesterol (Golomb, Stattin and Mednick, 2000; Hillbrand *et al.*, 2005; Zhang, 2005; Watve, 2013). All the key molecules involved in T2DM including insulin, leptin, cholesterol, cortisol have demonstrable cognitive and behavioural functions (Belsare *et al.*, 2010; Watve, 2013). Therefore, according to the hypothesis individuals with dove or diplomat tendencies and/or limited reproductive capacity/opportunities become insulin resistant and store more lipids to increase the lifespan. Over 70 molecular signals and pathways connect behaviour with metabolism and immunity

(Kulkarni, Sharda and Watve, 2017). A physically aggressive lifestyle is more injury prone and therefore the immune challenges of the body are different from those of an aggression avoider. Developmental constraints predispose the individual to certain behavioural and reproductive strategies and therefore the metabolism is programmed to suit the strategy adopted (Watve, 2013). The behavioural switch hypothesis further says that food intake regulation pathways have evolved for optimizing foraging strategies in presence of predator or other foraging risks. When feeding is detached from foraging and foraging is detached from risks, the regulation pathways fail to work setting the stage for susceptibility to obesogenic environment.

A further spinoff of the behavioural switch hypothesis is the behavioural deficiency or "vitaction" deficiency hypothesis. According to this hypothesis human physiology is fine-tuned to a set of behaviours typical of a hunter-gatherer lifestyle. Human behaviours are extensively linked to neuroendocrine pathways. The expression and levels of many growth factors, hormones, neuropeptides, and other signals is behaviour dependent. As a result, certain behaviours are essential for normal metabolism and physiology. These behaviours, comparable to essential amino acids or vitamins, are called "vitactions" (Watve et al unpublished).

A chronic deficiency of behaviours such as risk (related to foraging), physical aggression, adventure, rapid nerve-muscle coordination leads to the deficiency of certain neuronal pathways and expression of some signal molecules (Watve, 2013; Baig *et al.*, 2019). This deficiency reflects on to a network of metabolic changes (Kulkarni, Sharda and Watve, 2017). Of particular importance is the deficiency of growth factors and angiogenic factors that lead to impaired vascular endothelial function. Impaired vascular function is responsible for reduced circulation and nutrient supply to the brain. The brain in turn responds by compensatory increase in blood pressure, blood sugar etc.

We also have some non-adaptive interpretations of the intrauterine effects. The developmental constraints hypothesis is non-adaptive. In addition, there is a reverse causation interpretation of the association of birth weight and later life metabolic states. It proposes that having an insulin resistant genotype increases

the chances of surviving intrauterine undernourishment (Nair, Nair and Chacko, 2009). Therefore, low birth weight may not be causal to insulin resistance but will show a statistical association. This is non-programming interpretation of the association but depends on the assumption that insulin resistance helps in survival under nutrient limitations. It is necessary for evolutionary interpretations to compare their merits against non-adaptive or non-evolutionary interpretations as well.

6.4 Expectations from an evolutionary hypothesis

Now we have several alternative hypotheses, and the relative merits of these hypotheses can be evaluated. A logical way to evaluate the alternative hypotheses is to first delineate our expectations from an evolutionary hypothesis. Having done so we can examine which of the hypotheses fulfils most or all of the expectations listed below.

1. Explain polymorphism/ individual propensity: The hypothesis should be able to explain the population variability in propensity to obesity and/or T2DM. This needs to be done at two stages- a. the hypothesis should in principle allow polymorphism or phenotypic variability and b. it should quantitatively account for the polymorphisms observed in the population.
2. Explain intrauterine effects: Since the evidence for intrauterine and trans-generational effects is robust and consistent across studies, the hypothesis should adequately account for these effects.
3. Account for poor performance of GWAS: The hypothesis should explain why GWAS poorly explains obesity or T2DM. Currently the variance explained by all the known hits is tiny and more genomic data are unlikely to improve the picture substantially.
4. Account for non-monotonic relationships: The relationship of adiposity with insulin resistance is non-monotonic. Lipoatrophy is associated with high insulin resistance (Pardini *et al.*, 1998) and so is high adiposity. Similarly, fertility association with obesity is inverted U shaped, fertility going down at low (Frisch, 1987) as well as high adiposity (Nguyen *et al.*, 2007; Polotsky *et al.*, 2010). The evolutionary hypothesis should take into account this non-monotonicity. Most

hypotheses talk about only one arm of the U shape according to convenience of the argument.

5. Make testable predictions across species: Insulin resistance is not uniquely human. It is detected in some non-human primates and dolphins without any obesogenic intervention (Kaufman and Vermeulen, 2005; Lord, Bond and Thompson, 2009; Venn-Watson, 2014). Therefore, a sound evolutionary hypothesis could not be human specific. It has to account for observed patterns of naturally occurring variation in insulin resistance in other animal species too. Although we have limited access to ancestral human ecology, we have a wide variety of animal species adapted to a variety of nutritional and other ecological conditions. Therefore, it should be possible to test any hypothesis by cross species correlations. For example, the feast and famine hypotheses can be tested in species that hibernate or undertake long distance migrations in which they do not feed. Although such data are difficult to obtain and analyse, we can at least make differential testable predictions from all the extant hypotheses that can potentially be tested with non-human species. An exercise of making such predictions itself can bring better clarity in thinking. A good example of this is the association of insulin signalling with longevity. Across a wide variety of species impairment of insulin signalling increases lifespan (Kaletsky and Murphy, 2010). But in humans, insulin resistance is associated with a series of disorders. This apparent contradiction warrants caution if any hypothesis presumes insulin resistance as a mechanism of early aging.

6. Explain the positive association between obesity, and insulin resistance: Most hypotheses stop at explaining obesity and assume that the further chain of events is inevitable. Obesity is assumed to inevitably lead to insulin resistance and the cascade of changes that follow. However, for a good evolutionary hypothesis it is necessary to explain why obesity leads to insulin resistance, whether this association is true across species or is restricted to humans, rodents and a few other mammals. The inevitability of insulin resistance following obesity is debatable because the mechanism by which fat induces insulin resistance is not clear. A variety of hypotheses have been floated but each of them has either failed to gain experimental support or has faced one or more flaws or paradoxes (Watve, 2013) In different species of mammals, the association between diet obesity and insulin resistance has taken different shapes and different causal relations (Ojha

and Watve, 2018). Therefore, the classical assumption that obesity leads to insulin resistance stands on shaky grounds and needs serious rethinking on both proximate and ultimate levels.

7. Answer fundamental questions: It is well known that insulin plays an important role in nutrient uptake by certain tissues but not others. Asking a question "why" at this level is necessary for EM. Why do some cells need an external signal to pick up nutrients from the supply line while others don't? Why are there so many different glucose transporters in the body, some being insulin dependent and others independent? Unless an evolutionary hypothesis addresses such fundamental questions, t cannot be said to be sufficiently "evolutionary".

8. Proximate-ultimate complementarity: For a healthy biological theory one needs to have a logical and complete picture at the ultimate level and one at the proximate level which should complement each other. Most hypotheses appear to perform surreptitious skipping between proximate and ultimate levels. For example, a hypothesis may stop at explaining obesity at ultimate level of explanation and then say the rest follows because there are proximate mechanisms by which obesity induces insulin resistance, inflammation and so on. All the components of metabolic syndrome including fat accumulation, fat distribution, chronic systemic inflammation, insulin resistance, β-cell function, alterations in cognition and behaviour, fertility and reproduction related changes, cholesterol metabolism, atherosclerosis, altered bone remodelling, vascular functions and wound healing mechanisms need to be explained separately and coherently at the ultimate level, separately and empirically at the proximate level in such a way that the two levels complement each other. Most of the evolutionary hypotheses today stop applying ultimate reasoning at a convenient stage leaving the rest to proximate mechanisms. It is possible that there is some inevitability in proximate mechanisms. Ultimate explanations are not needed for inevitable processes, but in such cases the inevitability needs to be demonstrated at a fundamental level.

9. Suggest better clinical practices for prevention, control and reversal: If EM does not add useful insights into clinical practice it remains a luxurious intellectual exercise. A useful evolutionary hypothesis should be able to suggest improvements in the approaches to prevent, control or treat a disease. To call it a contribution of evolutionary hypothesis it is necessary that the suggestion is different from what pathophysiological theories would visualize in the absence of

evolutionary hypotheses. These suggestions need to be testable and should undergo randomized clinical trials. Only after going through the acid test of RCT an evolutionary hypothesis can be said to be complete and clinically useful.

10. Weigh the paradoxes, flaws and contradictory evidence about insulin resistance with logical coherence: We have seen in the earlier chapters that the classical picture of glucose regulation by insulin and the theory of insulin resistance faces several challenges. This means that the target of evolutionary explanation is itself changing. If what we want to explain undergoes a change then it is imperative that how we explain it would also change. Any evolutionary hypothesis needs to integrate all the new evidence and the changing picture and accommodate it in a coherent way.

6.5 Paradoxes in the pathophysiology of T2DM itself which need to be accommodated by an evolutionary hypothesis

The mainstream thinking that has dominated the field comprises four causal steps namely (i) obesity causes insulin resistance, (ii) β-cells compensate for insulin resistance by producing more insulin (iii) insufficient compensation by dysfunctional β-cells leads to hyperglycaemia and (iv) chronic hyperglycaemia leads to complications of diabetes. Although this is still the mainstream thinking, one also finds several discordant notes in literature in the form of paradoxes, inadequacies, and contradicting evidence. Some examples of the conflicting issues are as follows.

6.5.1 The causal role of obesity

Although fat is generally assumed to be causal to insulin resistance several findings have challenged this assumption. Classes of individuals that are obese but metabolically normal exist and their estimated prevalence among obese can be up to 51 % (Rezende *et al.,* 2014). Thin and insulin resistant individuals are also common (Teixeira *et al.*, 2015). Some animal models of obesity such as transgenic mice overexpressing PEPCK in adipose tissue accumulate large amounts of fat but do not become insulin resistant (Franckhauser *et al.,* 2002). Adenovirus induced obesity is paradoxically accompanied by increased insulin sensitivity (Pasarica *et al.,* 2006). Fat accumulation in MIRKO mice is clearly a consequence rather than cause of muscle insulin resistance (Kim *et al.,* 2000). All

these examples demonstrate that the association of adiposity with insulin resistance is not necessarily causal and inevitable. There must be specific reasons why obesity is associated with insulin resistance in humans. The reasons and the direction of causality need to be elucidated at both proximate and ultimate levels. Regarding the mechanism(s) by which adiposity induces insulin resistance there are a large number of contradictions and paradoxes (Watve, 2013). The classical Randle hypothesis was refuted by experiments (Bajaj *et al.*, 2007; Turner *et al.*, 2007). The adipokines hypothesis is flawed by the fact that adipose tissue produces both pro and anti-inflammatory adipokines which have both insulin sensitizing and insulin resistance action. The intra-muscular-triglyceride hypothesis is contradicted by the finding that athletes have high IMTGs but they are insulin sensitive (van Loon, 2004). The topmost paradox is that in the gold standard of measurement of insulin resistance, the hyperinsulinemic clamp technique, infusion of insulin rapidly depletes free fatty acids and the measurement of insulin resistance is done after FFA depletion. Therefore, this insulin resistance is unlikely to be caused by standing levels of FFAs (Watve, 2013). Thus, the central assumption of many evolutionary hypotheses that obesity is causal to insulin resistance stands on very slippery grounds. Currently it is neither substantiated nor refuted. Making such an assumption the basis of an evolutionary theory is like raising a building on a weak foundation.

6.5.2 Hyperinsulinemia first

A number of researchers have pointed out that hyperinsulinemia appears before insulin resistance and therefore it may not be a compensatory response to insulin resistance (Dubuc, 1976, 1981; Garvey, Olefsky and Marshall, 1986; Weyer *et al.*, 2000). We have seen in chapters 2 to 5 that the classical concepts of insulin resistance and the relationship between fasting glucose and fasting insulin stand seriously challenged. This challenge undermines the foundation of HOMA-IR as a measure of insulin resistance and all arguments based on this assumption. Currently there is no answer to this question how a normoglycemic hyperinsulinemic state is achieved. A possible and evidence-based solution to the paradox is that hyperinsulinemia is primary and leads to compensatory insulin resistance (Ratzmann, Ruhnke and Kohnert, 1983; Teuscher *et al.*, 1987; Lustig *et al.*, 2003, 2004; Lustig, 2006; Shanik *et al.*, 2008). The suggestion and the

evidence that hyperinsulinemia precedes insulin resistance is concordant with Neel's thrift but conflicts with modern thrift. If hyperinsulinemia is not a compensatory response to insulin resistance, it is illogical to assume that inadequate compensation leads to hyperglycaemia.

6.5.3 Compensatory hyperinsulinemia

It is classically believed that β-cells need to secrete excess insulin during the compensatory response leading to exhaustion or stress which leads to β-cell dysfunction. However, whether individual ß-cells produce excess insulin has rarely been tested. In the hyperinsulinemic state there is substantial rise in the β-cell population and some recent experiments show that insulin gene transcription levels in individual β-cells in the hyperinsulinemic state is lower than that in the healthy state (Hardikar *et al.*, 2015. Therefore, the classical belief of β-cell exhaustion or stress of overwork is not supported by evidence. This is compatible with Neel's rejection of the β-cell exhaustion idea. But in that case alternative mechanisms for β-cell dysfunction need to be sought.

6.5.4 Complications of diabetes

Doubts are raised on the classical faith that chronically increased levels of glucose lead to the complications of diabetes. Many studies have shown that early signs of complications arise prior to hyperglycaemia (Miller *et al.*, 1999; Leeson *et al.*, 2001; Carnethon *et al.*, 2003; Meigs, 2004; Ribeiro *et al.*, 2008). On the other hand, normalizing glucose did not reduce the rate of complications or mortality in many large-scale clinical trials (Shaughnessy, 2003; The NICE-SUGAR Study Investigators, 2009; Bonds *et al.*, 2010). In fact, mortality rates increased on normalizing glucose in some studies (The NICE-SUGAR Study Investigators, 2009; Bonds *et al.*, 2010). This casts serious doubts on whether hyperglycaemia is central to the pathophysiology of T2DM or is just one of the outcomes of a complex process.

6.6 Conclusion: Need of a new evolutionary hypothesis

These are a few of the existing debatable issues about the current perspective about the pathophysiology of T2DM. An evolutionary hypothesis cannot decline the responsibility of resolving these apparent paradoxes. At the minimum it is at

least necessary to be aware of these paradoxes and be open for the emergence of a different picture.

We know today that not only the levels of glucose and insulin are altered in T2DM but the levels of several dozen other molecules are altered (Watve, 2013; Kulkarni, Sharda and Watve, 2017). Apart from the burden of history, there are no other grounds to assume that glucose and insulin are central to all the observed changes. It is likely that glucose and insulin are only two of the players in the complex network of changes. Causal pathways in the complex correlational network are yet to be established clearly. We also know that a number of signals affect liver glucose production independent of insulin. Right from the days of Claude Bernard, the brain is known to play an important role in glucose homeostasis and the role of nervous system in T2DM is highlighted by many studies from time to time (Bernard *et al.*, 1998; Obici *et al.*, 2002; Pickup and Williams, 2002; Schwartz, 2005; Carey, Kehlenbrink and Hawkins, 2013).

On this background it is necessary that an evolutionary hypothesis explains why brain and so many other signals are involved in regulating glucose. Signals coming from a variety of tissues and organs appear to form a complex network. Why didn't the different homeostatic systems evolve to be modular and independent of each other? Why did a complex network evolve? Why did a cross talk between reproduction, muscle power, cognitive functions and glucose homeostasis evolve? Why a molecule like insulin plays so many apparently unrelated roles in the body including cognition, behaviour, ovulation, protein synthesis, cell growth factor etc.? A good evolutionary hypothesis needs to take into account the bigger and more complex picture of T2DM. So far most evolutionary hypotheses of T2DM are too naïve to do so mainly because they have set the classical naïve picture of T2DM as their target of explanation. After having set the goals for an evolutionary hypothesis of T2DM, we can discuss with examples how they can be applied to evaluate different hypotheses. Currently it may not be possible to test every hypothesis on every issue since most of the hypotheses are not explored enough to see whether they fulfil every expectation. But the discussion below can direct thinking in the right direction so

that the proponents as well as followers of every hypothesis attempt to develop the respective hypothesis towards meeting the expectations.

6.6.1 Population variability

We have discussed above the limitations of the thrift family of hypotheses in explaining population variability. Stable polymorphism is possible under certain conditions including heterozygote advantage, co-evolutionary arms race, negative frequency dependent selection or rock-paper-scissor like dynamics. Thrifty gene does not fit into any of the conditions needed for stable polymorphism. The drifty gene hypothesis and fertility selection hypothesis also fails to explain stable polymorphism. A transient polymorphism may be possible in principle but these hypotheses do not make any quantitative predictions about polymorphism that can be quantitatively tested with empirical data.

The thrifty phenotype and behavioural switch hypotheses do not explain the population variance based on genetic polymorphism. Thrifty phenotype assumes the developmental history to shape individual propensity. The behavioural switch is compatible with genetic polymorphism, developmental history, trans-generational effects, social ranking as well as individual choice as a source of population variance. Negative frequency dependence of the hawk and dove game is central to the hypothesis but it may act through genetic or phenotypic mechanisms. We have seen that genome studies explain only a tiny part of population variability which exposes the inadequacy of all genetic hypotheses. This may give an indirect advantage to the phenotypic hypotheses. But none of the phenotypic hypotheses have so far attempted to make quantitative predictions about population variance.

6.6.2 Intrauterine effects

None of the genetic hypotheses account for the intrauterine and trans-generational effects, whereas the phenotypic hypotheses do. The thrifty phenotype hypothesis actually originates from explaining intrauterine effects. However, it suffers from conceptual ambiguity. There are two possible interpretations of developmental effects namely developmental constraints and developmental programming. Developmental constraints assumes that undernourishment during intrauterine life

permanently affects tissues such as pancreatic islets which makes the individual more susceptible to develop β cell dysfunction in later life (Hales and Barker, 2001). The evidence for developmental constraints on ß-cells is contradictory since many studies have shown that individual born after intra uterine growth retardation have a larger β cell number at birth (Chakravarthy *et al.*, 2008). The developmental programming hypothesis assumes that there is predictive adaptive programming (PAP) of metabolism. PAP is under criticism on several grounds (Bogin, Silva and Rios, 2007; Wells, 2007; Rickard and Lummaa, 2007; Baig *et al.*, 2011) the main problem is in examining conditions that can lead to evolution of PAP and the demonstration that these conditions were faced by human ancestors to evolve predictive adaptive thrift. Evidence in baboons as well as humans contradicts PAP in favour of developmental constraints (Hayward, Rickard and Lummaa, 2013; Lea *et al.*, 2015). Currently we are unable to resolve between the two alternative views. The behavioural switch hypothesis relies on developmental programming, but the programming is not for thrift. The programming is for anticipated social position and optimum behavioural strategy. Since a positive correlation between mother's and offspring's social position is demonstrated (Dewsbury, 1990; Holekamp and Smale, 1991; East *et al.*, 2009). a PAP can evolve in principle as a social adaptation.

6.6.3 Insulin-glucose relationship:

While the prevalent belief is that insulin action affects fasting glucose and insulin resistance alters this relationship, we have seen that this assumption stands seriously challenged. It has been facing challenges in various forms over the past few years (Corkey, 2012; Pories and Dohm, 2012). For example, there is increasing evidence for the 'hyperinsulinemia first' viewpoint (Ratzmann, Ruhnke and Kohnert, 1983; Teuscher *et al.*, 1987; Lustig *et al.*, 2003, 2004; Lustig, 2006; Shanik *et al.*, 2008). Although there is more evidence in favour of this view, it is not yet the mainstream view. Neel's thrift, fast life history (Stipp, 2011). and behavioural switch hypotheses (Watve, 2013) subscribe to hyperinsulinemia first hypothesis whereas others depend upon insulin resistance first. Now since we have shown with rigor that the concepts of compensatory hyperinsulinemia and insulin regulated fasting glucose are not supported, evolutionary hypotheses critically dependent on it can be eliminated.

Of particular interest is the behavioural switch hypothesis since it depends upon the cognitive and behavioural role of insulin. This hypothesis proposes that when the brain needs more insulin, it upregulates β cell-population and thereby plasma insulin through parasympathetic signals. Parallel to this brain also directly regulates fasting glucose by autonomic inputs to the liver. This is compatible with the model 1c (figure 1, Chapter 5) in which there is a shared input to FG as well as FI but the two do not affect each other directly in the fasting steady state.

6.6.4 Obesity-IR association:

A related ambiguity is whether obesity induces IR or the other way round or both are correlated due to a common causal pathway. Neel argued that the 'diabetic tendency' (that can be possibly interpreted as hyperinsulinemia) was responsible for obesity. After turning the concept upside down, the later versions of thrift believe that obesity induces insulin resistance. In either case the association is not strong and in meta-analysis obesity parameters explain only about 15% of insulin resistance, if we accept HOMA-IR as the measure (Vidwans and Watve, 2017). The behavioural switch hypothesis suggests that both might originate independently, triggered by the behavioural strategies adopted by an individual (Watve, 2013; Baig *et al.*, 2019). which may explain why the correlation is consistent but weak in terms of variance explained.

6.6.5 Causes of β-cell dysfunction:

Since there is no evidence for β-cell overwork or exhaustion, one needs to look for alternative causes of β-cell dysfunction. Oxidative stress is a candidate, but there are inconsistencies with the timeline. Increased glucose is blamed for increased oxidative stress, but glucose level is unlikely to increase without β-cell dysfunction. The behavioural switch hypothesis suggests an alternative, in which sympathetic suppression of release of insulin vesicles from β-cells leads to increased retention time of insulin accompanied by amylin. The increased retention time of amylin increases the probability of amyloid formation resulting in increased β-cell damage (Watve, Bodas and Diwekar, 2014). These two alternative hypotheses increased oxidative stress and increased retention time induced amyloid damage make diametrically opposite testable predictions about β-cell population dynamics. The oxidative damage mechanism gives rise to a

positive feedback vicious cycle. If hyperglycaemia induced oxidative stress leads to β-cell dysfunction, insulin secretion will be further inadequate leading to further dysregulation of glucose increasing oxidative stress further. When such a cycle begins, it will end in complete destruction of β-cells. On the other hand, the sympathetic suppression hypothesis leads to a negative feedback cycle leading to limited destruction of β-cells. The hypothesized mechanism is that inadequate glucose supply to brain activates the sympathetic nerves to suppress insulin vesicle release increasing the retention time of insulin-amylin and thereby inducing amyloidogenesis. Amyloidogenesis kills some of the β-cells resulting into reduced insulin and increased glucose, the increased plasma glucose supplies more glucose to brain which normalizes sympathetic response and arrests the β-cell damage process. By this hypothesis β-cell damage will never be complete in T2DM. Post-mortem examinations of long-standing type 2 diabetic patients have shown that a substantial β-cell mass is conserved even after decades of diabetes (Clark *et al.*, 1990; Porte and Kahn, 2001; Butler *et al.*, 2003). This favours the behavioural switch hypothesis but an in-depth probe on β-cell dynamics is needed to resolve between the two opposite predictions of different hypotheses.

6.6.6 System level effects

Most evolutionary hypotheses do not attempt to explain the complex cross talk amongst different systems of the body and the brain involved in T2DM. Most treat the involvement of other organs as an inevitable side effect of altered glucose homeostasis. However, apart from the burden of history there is no other evidence to ensure that their role in T2DM is only consequential and not causal. The only possible exception is the behavioural switch hypothesis which expects alterations in multiple systems independent of glucose homeostasis.

6.6.7 Compatible with complex T2DM picture

Many evolutionary hypotheses including later versions of thrifty gene, drifty gene, thrifty phenotype, assume a central role of obesity. Exceptions are Neel's thrift, behavioural switch, and fertility selection hypotheses. As the awareness about the multi-organ involvement and cross talk in T2DM is increasing, there are more attempts to explain the complexity. However, most evolutionary hypotheses are yet far away from reaching this goal.

6.6.8 Suggests change in clinical practice and randomized clinical trials

"The epistemic yardstick of clinical importance is the possibility of causal intervention." (Cournoyea, 2010). Possible clinical or epidemiological useful suggestions are made by the thrifty phenotype and behavioural switch hypotheses. Thrifty phenotype hypothesis suggests that improvement of maternal nutrition would be necessary and sufficient to reduce prevalence of T2DM across a generation. This prediction is certainly testable but would take a long time to see results. The behavioural switch hypothesis states that specifically designed behavioural interventions should be able to prevent and perhaps reverse T2DM. This prediction is certainly testable using a carefully designed clinical trial but has not been tested yet.

It is not exceedingly difficult to realize that almost all the hypotheses are quite primitive and have not made sufficient attempts to work towards these goals. Table 1 summarizes the status of the different hypotheses as perceived today. It is possible that some of the hypotheses have the potential to explain more than what they did when they were proposed. It is necessary for the proponents of the hypotheses as well as others interested in evolutionary insights that we attempt to progress in this direction. While doing so we are likely to reject certain hypotheses based on poor performance scores on the multiple expectation scale. But this process itself is likely to lead us to resolve between hypotheses and narrow down on the most appropriate one(s). The process should culminate in finding a theory that highlights the root cause of the disorder and accordingly suggest a line of treatment that targets the root causes rather than the symptoms.

Table 1: A format for comparative evaluation of different evolutionary hypotheses for type-2 diabetes: A complete evaluation may not be possible currently since a number of hypotheses have not been explored sufficiently to see whether they fulfil the expectations. The signs represent current perceptions about the hypotheses compared. '+' indicates that the hypothesis explains the point satisfactorily, '-' indicates that the hypothesis fails to explain the point and '?' indicates that the possibility is not yet explored or is debated (Watve and Diwekar-Joshi, 2016)

Evolutionary hypothesis → **Points explained by the evolutionary hypothesis ↓**	**Thrifty gene (Neel's)**	**Thrifty gene later**	**Thrifty phenotype**	**Refined thrift**	**Drifty gene**	**Fertility selection**	**Behavioural switch**
Polymorphism/variability	-	-	+	+	-+	+	+
Intra-uterine effects	-	-	+	+	-	+	+
Independence of FG-FI	-	-	-	-	?	+	+
Hyperinsulinemia first	+	-	-	-	?	-	+
GWAS limited success	-	-	+	+	-	-	+
Ultimate cause for Obesity-IR association	-	-	-	-	-	-	+
Non-monotonic associations of obesity	?	?	?	?	?	?	?
Differential tissue insulin dependence	?	?	?	?	?	?	+
β-cell amyloid and persistence	+-	+-	+-	+-	+-	+-	+
System level effects	?	?	?	?	?	?	+
Proximate ultimate complementarity	?	?	?	?	?	?	+
Resolve paradoxes in T2DM pathophysiology	+-	-	-	-	-	-	+
Suggests change in clinical practices	-	-	+	?	-	?	+
Randomised Clinical Trial	-	-	Possible	-	-	-	Possible

6.7 Inter-compatibility and combination of hypotheses

No single hypothesis explains the evolution of propensity to obesity and T2DM. More than one hypothesis must be considered simultaneously to account for the underlying pathophysiology. To do so, we need to analyse the inter-compatibility of the different hypotheses. For example, developmental programming and behavioural strategies have some compatibility with each other in the context of intra uterine or trans-generational effects. Neel's thrift and later versions of thrift are not compatible in the context of mechanism and time course of development of the insulin resistant state. Only compatible hypotheses can be combined to give logically coherent explanations. Therefore, a detailed context specific compatibility analysis needs to be an important intrinsic part of the development of evolutionary theory of T2DM. But this step is possible only after the primary evaluation step described above. After eliminating the ones that get rejected by the analysis, the remaining ones can be tested for inter-compatibility and it can be asked whether a combination of them fulfils the expectations better than one.

Collectively, the obesity cantered hypotheses for the origin of T2DM perform poorly on this matrix. Hypotheses that presume reproductive and behavioural origins of the condition certainly look more promising. But we need to wait until all the alternative hypotheses are explored in sufficient depth to see whether they fulfil the predictions which are unexplored or untested at present.

6.8 Importance of evolutionary biology in medicine

Through this piece of work, we have shown the gaps and inadequacies in the evolutionary hypotheses of T2DM. There are, however, strong reasons to believe that evolutionary logic, if appropriately used, will give important insights into medicine in general and T2DM and other life-style related disorders. Evolutionary biologists have the right capabilities needed to overcome these problems. They are trained rigorously in quantitative thinking and have theoretical and mathematical knowledge and ability to join dots and make synthesis from disjointed findings. Importantly, they can differentially handle proximate and ultimate causation. Unfortunately, so far these strengths have not been adequately used in EM, particularly in the context of T2DM. If the theoretical rigor seen in many other

fields of evolutionary biology is brought into evolutionary medicine that is likely to bring a fundamental and useful revolution in medicine.

6.9 References

Baig, U. *et al.* (2011) 'Can Thrifty Gene(s) or Predictive Fetal Programming for Thriftiness Lead to Obesity?', *Journal of Obesity*, 2011, pp. 1–11. doi: 10.1155/2011/861049.

Baig, U. *et al.* (2019) 'Foraging theory and the propensity to be obese: an alternative to thrift', *HOMO*, 70(3), pp. 193–216. doi: 10.1127/homo/2019/1078.

Bajaj, M. *et al.* (2007) 'Effects of peroxisome proliferator-activated receptor (PPAR)-α and PPAR-γ agonists on glucose and lipid metabolism in patients with type 2 diabetes mellitus', *Diabetologia*, 50(8), pp. 1723–1731. doi: 10.1007/s00125-007-0698-9.

Barker, D. (1998) 'In utero programming of chronic disease', *Clinical Science*, 95(2), pp. 115–128. doi: 10.1042/cs0950115.

Bateson, P., Gluckman, P. and Hanson, M. (2014) 'The biology of developmental plasticity and the Predictive Adaptive Response hypothesis', *The Journal of Physiology*, 592(11), pp. 2357–2368. doi: 10.1113/jphysiol.2014.271460.

Belsare, P. V. *et al.* (2010) 'Metabolic syndrome: Aggression control mechanisms gone out of control', *Medical Hypotheses*, 74(3), pp. 578–589. doi: 10.1016/j.mehy.2009.09.014.

Bernard, C. *et al.* (1998) 'Pancreatic beta-cell regeneration after 48-h glucose infusion in mildly diabetic rats is not correlated with functional improvement', *Diabetes*, 47(7), pp. 1058–1065. doi: 10.2337/diabetes.47.7.1058.

Bogin, B., Silva, M. I. V. and Rios, L. (2007) 'Life history trade-offs in human growth: Adaptation or pathology?', *American Journal of Human Biology*, 19(5), pp. 631–642. doi: 10.1002/ajhb.20666.

Bonds, D. E. *et al.* (2010) 'The association between symptomatic, severe hypoglycaemia and mortality in type 2 diabetes: retrospective epidemiological analysis of the ACCORD study', *BMJ*, 340(jan08 1), pp. b4909–b4909. doi: 10.1136/bmj.b4909.

Boyko, E. J. (2000) 'Proportion of type 2 diabetes cases resulting from impaired fetal growth', *Diabetes Care*, 23(9), pp. 1260–1264. doi: 10.2337/diacare.23.9.1260.

Butler, A. E. *et al.* (2003) 'beta-Cell Deficit and Increased beta-Cell Apoptosis in Humans With Type 2 Diabetes', *Diabetes*, 52(1), pp. 102–110. doi: 10.2337/diabetes.52.1.102.

Cannon, W. (1934) 'The Wisdom of the Body', *Nature*, 133(3351), pp. 82–82. doi: 10.1038/133082a0.

Carey, M., Kehlenbrink, S. and Hawkins, M. (2013) 'Evidence for Central Regulation of Glucose Metabolism', *Journal of Biological Chemistry*, 288(49), pp. 34981–34988. doi: 10.1074/jbc.R113.506782.

Carnethon, M. R. *et al.* (2003) 'Influence of Autonomic Nervous System Dysfunction on the Development of Type 2 Diabetes: The CARDIA study', *Diabetes Care*, 26(11), pp. 3035–3041. doi: 10.2337/diacare.26.11.3035.

Chakravarthy, M. V. *et al.* (2008) 'Decreased Fetal Size Is Associated With -Cell Hyperfunction in Early Life and Failure With Age', *Diabetes*, 57(10), pp. 2698–2707. doi: 10.2337/db08-0404.

Clark, A. *et al.* (1990) 'Islet amyloid polypeptide in diabetic and non-diabetic Pima Indians', *Diabetologia*, 33(5), pp. 285–289. doi: 10.1007/BF00403322.

Corbett, S. J., McMichael, A. J. and Prentice, A. M. (2009) 'Type 2 diabetes, cardiovascular disease, and the evolutionary paradox of the polycystic ovary syndrome: A fertility first hypothesis', *American Journal of Human Biology*, 21(5), pp. 587–598. doi: 10.1002/ajhb.20937.

Corbett, S. and Morin-Papunen, L. (2013) 'The Polycystic Ovary Syndrome and recent human evolution', *Molecular and Cellular Endocrinology*, 373(1–2), pp. 39–50. doi: 10.1016/j.mce.2013.01.001.

Corkey, B. E. (2012) 'Diabetes: Have We Got It All Wrong?: Insulin hypersecretion and food additives: cause of obesity and diabetes?', *Diabetes Care*, 35(12), pp. 2432–2437. doi: 10.2337/dc12-0825.

Cournoyea, M. (2010) An epistemic and ethical critique of evolutionary medicine. Munk School Briefings, University of Toronto, ISSN 1715-3484.

Dewsbury, D. A. (1990) 'Fathers and sons: genetic factors and social dominance in deer mice, Peromyscus maniculatus', *Animal Behaviour*, 39(2), pp. 284–289. doi: 10.1016/S0003-3472(05)80872-3.

Drake, A. and Walker, B. (2004) 'The intergenerational effects of fetal programming: non-genomic mechanisms for the inheritance of low birth weight and cardiovascular risk', *Journal of Endocrinology*, 180(1), pp. 1–16. doi: 10.1677/joe.0.1800001.

Dubuc, P. U. (1976) 'The development of obesity, hyperinsulinemia, and hyperglycemia in ob/ob mice', *Metabolism*, 25(12), pp. 1567–1574. doi: 10.1016/0026-0495(76)90109-8.

Dubuc, P. U. (1981) 'Non-Essential Role of Dietary Factors in the Development of Diabetes in ob/ob Mice', *The Journal of Nutrition*, 111(10), pp. 1742–1748. doi: 10.1093/jn/111.10.1742.

East, M. L. *et al.* (2009) 'Maternal effects on offspring social status in spotted hyenas', *Behavioral Ecology*, 20(3), pp. 478–483. doi: 10.1093/beheco/arp020.

Fernández-Real, J.-M. and Ricart, W. (1999) 'Insulin resistance and inflammation in an evolutionary perspective: the contribution of cytokine genotype/phenotype to thriftiness', *Diabetologia*, 42(11), pp. 1367–1374. doi: 10.1007/s001250051451.

Fernández-Real, J. M. and Pickup, J. C. (2008) 'Innate immunity, insulin resistance and type 2 diabetes', *Trends in Endocrinology & Metabolism*, 19(1), pp. 10–16. doi: 10.1016/j.tem.2007.10.004.

Franckhauser, S. *et al.* (2002) 'Increased Fatty Acid Re-esterification by PEPCK Overexpression in Adipose Tissue Leads to Obesity Without Insulin Resistance', *Diabetes*, 51(3), pp. 624–630. doi: 10.2337/diabetes.51.3.624.

Frisch, R. E. (1987) 'Body fat, menarche, fitness and fertility', *Human Reproduction*, 2(6), pp. 521–533. doi: 10.1093/oxfordjournals.humrep.a136582.

Garvey, W. T., Olefsky, J. M. and Marshall, S. (1986) 'Insulin Induces Progressive Insulin Resistance in Cultured Rat Adipocytes: Sequential Effects at Receptor and Multiple Postreceptor Sites', *Diabetes*, 35(3), pp. 258–267. doi: 10.2337/diab.35.3.258.

Gesink Law, D. C., Maclehose, R. F. and Longnecker, M. P. (2006) 'Obesity and time to pregnancy', *Human Reproduction*, 22(2), pp. 414–420. doi: 10.1093/humrep/del400.

Gluckman, P. D., Hanson, M. A. and Spencer, H. G. (2005) 'Predictive adaptive responses and human evolution', *Trends in Ecology & Evolution*, 20(10), pp. 527–533. doi: 10.1016/j.tree.2005.08.001.

Golomb, B. A., Stattin, H. and Mednick, S. (2000) 'Low cholesterol and violent crime', *Journal of Psychiatric Research*, 34(4–5), pp. 301–309. doi: 10.1016/S0022-3956(00)00024-8.

Guglielmo, C. G. (2010) 'Move That Fatty Acid: Fuel Selection and Transport in Migratory Birds and Bats', *Integrative and Comparative Biology*, 50(3), pp. 336–345. doi: 10.1093/icb/icq097.

Hales, C. N. *et al.* (1991) 'Fetal and infant growth and impaired glucose tolerance at age 64.', *BMJ*, 303(6809), pp. 1019–1022. doi: 10.1136/bmj.303.6809.1019.

Hales, C. N. and Barker, D. J. P. (1992) 'Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis', *Diabetologia*, 35(7), pp. 595–601. doi: 10.1007/BF00400248.

Hales, C. N. and Barker, D. J. P. (2001) 'The thrifty phenotype hypothesis', *British Medical Bulletin*, 60(1), pp. 5–20. doi: 10.1093/bmb/60.1.5.

Hardikar, A. A. *et al.* (2015) 'Multigenerational Undernutrition Increases Susceptibility to Obesity and Diabetes that Is Not Reversed after Dietary Recuperation', *Cell Metabolism*, 22(2), pp. 312–319. doi: 10.1016/j.cmet.2015.06.008.

Hayward, A. D., Rickard, I. J. and Lummaa, V. (2013) 'Influence of early-life nutrition on mortality and reproductive success during a subsequent famine in a preindustrial population', *Proceedings of the National Academy of Sciences*, 110(34), pp. 13886–13891. doi: 10.1073/pnas.1301817110.

Hillbrand, M. *et al.* (2005) 'Serum Cholesterol Concentrations and Non-Physical Aggression in Healthy Adults', *Journal of Behavioral Medicine*, 28(3), pp. 295–299. doi: 10.1007/s10865-005-4665-y.

Holekamp, K. E. and Smale, L. (1991) 'Dominance Acquisition During Mammalian Social Development: The "Inheritance" of Maternal Rank', *American Zoologist*, 31(2), pp. 306–317. doi: 10.1093/icb/31.2.306.

Johnson, J. A. (1987) 'Dominance rank in juvenile olive baboons, Papio anubis: the influence of gender, size, maternal rank and orphaning', *Animal Behaviour*, 35(6), pp. 1694–1708. doi: 10.1016/S0003-3472(87)80062-3.

Kaletsky, R. and Murphy, C. T. (2010) 'The role of insulin/IGF-like signaling in C. elegans longevity and aging', *Disease Models & Mechanisms*, 3(7–8), pp. 415–419. doi: 10.1242/dmm.001040.

Kaufman, J. M. and Vermeulen, A. (2005) 'The Decline of Androgen Levels in Elderly Men and Its Clinical and Therapeutic Implications', *Endocrine Reviews*, 26(6), pp. 833–876. doi: 10.1210/er.2004-0013.

Kilpeläinen, T. O. *et al.* (2011) 'Genetic variation near IRS1 associates with reduced adiposity and an impaired metabolic profile', *Nature Genetics*, 43(8), pp. 753–760. doi: 10.1038/ng.866.

Kim, J. K. *et al.* (2000) 'Redistribution of substrates to adipose tissue promotes obesity in mice with selective insulin resistance in muscle', *Journal of Clinical Investigation*, 105(12), pp. 1791–1797. doi: 10.1172/JCI8305.

Kulkarni, S., Sharda, S. and Watve, M. (2017) 'Bi-stability in type 2 diabetes mellitus multi-organ signalling network', *PLOS ONE*. Edited by C. Cras-Méneur, 12(8), p. e0181536. doi: 10.1371/journal.pone.0181536.

Lea, A. J. *et al.* (2015) 'Developmental Constraints in a Wild Primate', *The American Naturalist*, 185(6), pp. 809–821. doi: 10.1086/681016.
Leeson, C. P. M. *et al.* (2001) 'Impact of Low Birth Weight and Cardiovascular Risk Factors on Endothelial Function in Early Adult Life', *Circulation*, 103(9), pp. 1264–1268. doi: 10.1161/01.CIR.103.9.1264.

Li, S. *et al.* (2010) 'Cumulative effects and predictive value of common obesity-susceptibility variants identified by genome-wide association studies', *The American Journal of Clinical Nutrition*, 91(1), pp. 184–190. doi: 10.3945/ajcn.2009.28403.

van Loon, L. J. C. (2004) 'Use of intramuscular triacylglycerol as a substrate source during

exercise in humans', *Journal of Applied Physiology*, 97(4), pp. 1170–1187. doi: 10.1152/japplphysiol.00368.2004.

Lord, L.-D., Bond, J. and Thompson, R. R. (2009) 'Rapid steroid influences on visually guided sexual behavior in male goldfish', *Hormones and Behavior*, 56(5), pp. 519–526. doi: 10.1016/j.yhbeh.2009.09.002.

Lustig, R. H. *et al.* (2003) 'Octreotide Therapy of Pediatric Hypothalamic Obesity: A Double-Blind, Placebo-Controlled Trial', *The Journal of Clinical Endocrinology & Metabolism*, 88(6), pp. 2586–2592. doi: 10.1210/jc.2002-030003.

Lustig, R. H. *et al.* (2004) 'Obesity, leptin resistance, and the effects of insulin reduction', *International Journal of Obesity*, 28(10), pp. 1344–1348. doi: 10.1038/sj.ijo.0802753.

Lustig, R. H. (2006) 'Childhood obesity: behavioral aberration or biochemical drive? Reinterpreting the First Law of Thermodynamics', *Nature Clinical Practice Endocrinology & Metabolism*, 2(8), pp. 447–458. doi: 10.1038/ncpendmet0220.

Matthews, D. R. *et al.* (1985) 'Homeostasis model assessment: insulin resistance and ?-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, 28(7), pp. 412–419. doi: 10.1007/BF00280883.

McCance, D. R. *et al.* (1994) 'Birth weight and non-insulin dependent diabetes: thrifty genotype, thrifty phenotype, or surviving small baby genotype?', *BMJ*, 308(6934), pp. 942–945. doi: 10.1136/bmj.308.6934.942.

McCarthy, M. I. and Zeggini, E. (2009) 'Genome-wide association studies in type 2 diabetes', *Current Diabetes Reports*, 9(2), pp. 164–171. doi: 10.1007/s11892-009-0027-4.

Meigs, J. B. (2004) 'Biomarkers of Endothelial Dysfunction and Risk of Type 2 Diabetes Mellitus', *JAMA*, 291(16), p. 1978. doi: 10.1001/jama.291.16.1978.

Miller, A. W. *et al.* (1999) 'Impaired Vagal Reflex Activity in Insulin-Resistant Rats', *Journal of Cardiovascular Pharmacology*, 33(5), pp. 698–702. doi: 10.1097/00005344-199905000-00004.

Moustaïd, N., Jones, B. H. and Taylor, J. W. (1996) 'Insulin Increases Lipogenic Enzyme Activity in Human Adipocytes in Primary Culture', *The Journal of Nutrition*, 126(4), pp. 865–870. doi: 10.1093/jn/126.4.865.

Mutch, D. M. and Clément, K. (2006) 'Unraveling the Genetics of Human Obesity', *PLoS Genetics*, 2(12), p. e188. doi: 10.1371/journal.pgen.0020188.

Nair, L., Nair, M. and Chacko, D. (2009) 'Markers of fetal onset adult diseases', *Indian Pediatrics*, 46(Suppl), pp. 48–54.

Neel, J. V (1962) 'Diabetes mellitus: a "thrifty" genotype rendered detrimental by "progress"?', *American journal of human genetics*, 14, pp. 353–62. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13937884.

Nesse, R. M. (2001) 'How is Darwinian medicine useful?', *Western Journal of Medicine*, 174(5), pp. 358–360. doi: 10.1136/ewjm.174.5.358.

Nettle, D. and Bateson, M. (2015) 'Adaptive developmental plasticity: what is it, how can we recognize it and when can it evolve?', *Proceedings of the Royal Society B: Biological Sciences*, 282(1812), p. 20151005. doi: 10.1098/rspb.2015.1005.

Nettle, D., Frankenhuis, W. E. and Rickard, I. J. (2013) 'The evolution of predictive adaptive responses in human life history', *Proceedings of the Royal Society B: Biological Sciences*, 280(1766), p. 20131343. doi: 10.1098/rspb.2013.1343.

Nguyen, R. H. N. *et al.* (2007) 'Men's body mass index and infertility', *Human Reproduction*, 22(9), pp. 2488–2493. doi: 10.1093/humrep/dem139.

O'Dea, K. (1991) 'Westernisation, insulin resistance and diabetes in Australian Aborigines', *Medical Journal of Australia*, 155(4), pp. 258–264. doi: 10.5694/j.1326-5377.1991.tb142236.x.

Obici, S. *et al.* (2002) 'Hypothalamic insulin signaling is required for inhibition of glucose production', *Nature Medicine*, 8(12), pp. 1376–1382. doi: 10.1038/nm1202-798.

Ojha, A. and Watve, M. (2018) 'Blind fish', *Evolution, Medicine, and Public Health*, 2018(1), pp. 186–189. doi: 10.1093/emph/eoy020.

Pardini, V. C. *et al.* (1998) 'Leptin Levels, β-Cell Function, and Insulin Sensitivity in Families with Congenital and Acquired Generalized Lipoatropic Diabetes1', *The Journal of Clinical Endocrinology & Metabolism*, 83(2), pp. 503–508. doi: 10.1210/jcem.83.2.4567.

Pasarica, M. *et al.* (2006) 'Human Adenovirus 36 Induces Adiposity, Increases Insulin Sensitivity, and Alters Hypothalamic Monoamines in Rats*', *Obesity*, 14(11), pp. 1905–1913. doi: 10.1038/oby.2006.222.

Pasquet, P. *et al.* (1992) 'Massive overfeeding and energy balance in men: the Guru Walla model', *The American Journal of Clinical Nutrition*, 56(3), pp. 483–490. doi: 10.1093/ajcn/56.3.483.

Pickup, J. C. and Williams, G. (2002) *Textbook of Diabetes*. 3rd edn. Wiley-Blackwell.

Polotsky, A. J. *et al.* (2010) 'Association of adolescent obesity and lifetime nulliparity—The Study of Women's Health Across the Nation (SWAN)', *Fertility and Sterility*, 93(6), pp. 2004–2011. doi: 10.1016/j.fertnstert.2008.12.059.

Pond, C. M. (1998) *The Fats of Life*. Cambridge University Press. doi: 10.1017/CBO9780511584633.

Pories, W. J. and Dohm, G. L. (2012) 'Diabetes: Have We Got It All Wrong?: Hyperinsulinism as the culprit: surgery provides the evidence', *Diabetes Care*, 35(12), pp. 2438–2442. doi: 10.2337/dc12-0684.

Porte, D. and Kahn, S. E. (2001) 'Beta-cell dysfunction and failure in type 2 diabetes: potential mechanisms', *Diabetes*, 50(Supplement 1), pp. S160–S163. doi: 10.2337/diabetes.50.2007.S160.

Prentice, A. M., Hennig, B. J. and Fulford, A. J. (2008) 'Evolutionary origins of the obesity epidemic: natural selection of thrifty genes or genetic drift following predation release?', *International Journal of Obesity*, 32(11), pp. 1607–1610. doi: 10.1038/ijo.2008.147.

Ramlau-Hansen, C. H. *et al.* (2007) 'Subfecundity in overweight and obese couples', *Human Reproduction*, 22(6), pp. 1634–1637. doi: 10.1093/humrep/dem035.

Rankinen, T. *et al.* (2006) 'The Human Obesity Gene Map: The 2005 Update', *Obesity*, 14(4), pp. 529–644. doi: 10.1038/oby.2006.71.

Ratzmann, K., Ruhnke, R. and Kohnert, K. (1983) 'Effect of pharmacological suppression of insulin secretion on tissue sensitivity to insulin in subjects with moderate obesity', *International Journal of Obesity*, 7, pp. 453–458.

Read, A. F. (1994) 'The evolution of virulence', *Trends in Microbiology*, 2(3), pp. 73–76. doi: 10.1016/0966-842X(94)90537-1.

Read, A. F. and Huijben, S. (2009) 'PERSPECTIVE: Evolutionary biology and the avoidance of antimicrobial resistance', *Evolutionary Applications*, 2(1), pp. 40–51. doi: 10.1111/j.1752-4571.2008.00066.x.

Reinhardt, M. *et al.* (2015) 'A Human Thrifty Phenotype Associated With Less Weight Loss During Caloric Restriction', *Diabetes*, 64(8), pp. 2859–2867. doi: 10.2337/db14-1881.

Rezende, L. F. M. de *et al.* (2014) 'Sedentary Behavior and Health Outcomes: An Overview of Systematic Reviews', *PLoS ONE*. Edited by A. Lucia, 9(8), p. e105620. doi: 10.1371/journal.pone.0105620.

Ribeiro, R. T. *et al.* (2008) 'Loss of Postprandial Insulin Sensitization During Aging', *The Journals of Gerontology Series A: Biological Sciences and Medical Sciences*, 63(6), pp. 560–565. doi: 10.1093/gerona/63.6.560.

Rickard, I. J. and Lummaa, V. (2007) 'The predictive adaptive response and metabolic syndrome: challenges for the hypothesis', *Trends in Endocrinology & Metabolism*, 18(3), pp. 94–99. doi: 10.1016/j.tem.2007.02.004.

Rosati, A. G. and Hare, B. (2012) 'Decision making across social contexts: competition increases preferences for risk in chimpanzees and bonobos', *Animal Behaviour*, 84(4), pp. 869–879. doi: 10.1016/j.anbehav.2012.07.010.

Rosati, A. G. and Hare, B. (2013) 'Chimpanzees and Bonobos Exhibit Emotional Responses to Decision Outcomes', *PLoS ONE*. Edited by G. di Pellegrino, 8(5), p. e63058. doi: 10.1371/journal.pone.0063058.

Sahlins, M. (1974) *Stone Age Economics*. Tavistock Publications.

Sallmen, M. *et al.* (2006) 'Reduced Fertility Among Overweight and Obese Men', *Epidemiology*, 17(5), pp. 520–523. doi: 10.1097/01.ede.0000229953.76862.e5.

Schwartz, M. W. (2005) 'Diabetes, Obesity, and the Brain', *Science*, 307(5708), pp. 375–379. doi: 10.1126/science.1104344.

Scott, L. J. *et al.* (2007) 'A Genome-Wide Association Study of Type 2 Diabetes in Finns Detects Multiple Susceptibility Variants', *Science*, 316(5829), pp. 1341–1345. doi: 10.1126/science.1142382.

Shanik, M. H. *et al.* (2008) 'Insulin Resistance and Hyperinsulinemia: Is hyperinsulinemia the cart or the horse?', *Diabetes Care*, 31(Supplement 2), pp. S262–S268. doi: 10.2337/dc08-s264.

Shaughnessy, A. F. (2003) 'What happened to the valid POEMs? A survey of review articles on the treatment of type 2 diabetes', *BMJ*, 327(7409), pp. 266–0. doi: 10.1136/bmj.327.7409.266.

Sladek, R. *et al.* (2007) 'A genome-wide association study identifies novel risk loci for type 2 diabetes', *Nature*, 445(7130), pp. 881–885. doi: 10.1038/nature05616.

Speakman, J. R. (2008) 'Thrifty genes for obesity, an attractive but flawed idea, and an alternative perspective: the "drifty gene" hypothesis', *International Journal of Obesity*, 32(11), pp. 1611–1617. doi: 10.1038/ijo.2008.161.

Stipp, D. (2011) 'Linking Nutrition, Maturation and Aging: From Thrifty Genes to the Spendthrift Phenotype', *Aging*, 3(2), pp. 85–93. doi: 10.18632/aging.100286.

Teixeira, P. J. *et al.* (2015) 'Successful behavior change in obesity interventions in adults: a systematic review of self-regulation mediators', *BMC Medicine*, 13(1), p. 84. doi: 10.1186/s12916-015-0323-6.

Teuscher, T. *et al.* (1987) 'Absence of diabetes in a rural west African population with a high carbohydrate/cassava diet', *The Lancet*, 329(8536), pp. 765–768. doi: 10.1016/S0140-6736(87)92797-8.

The NICE-SUGAR Study Investigators (2009) 'Intensive versus Conventional Glucose Control in

Critically Ill Patients', *New England Journal of Medicine*, 360(13), pp. 1283–1297. doi: 10.1056/NEJMoa0810625.

Thorleifsson, G. *et al.* (2009) 'Genome-wide association yields new sequence variants at seven loci that associate with measures of obesity', *Nature Genetics*, 41(1), pp. 18–24. doi: 10.1038/ng.274.

Turner, N. *et al.* (2007) 'Excess Lipid Availability Increases Mitochondrial Fatty Acid Oxidative Capacity in Muscle', *Diabetes*, 56(8), pp. 2085–2092. doi: 10.2337/db07-0093.

Venn-Watson, S. (2014) 'Dolphins and Diabetes: Applying One Health for Breakthrough Discoveries', *Frontiers in Endocrinology*, 5. doi: 10.3389/fendo.2014.00227.

Vidwans, H. B. and Watve, M. G. (2017) 'How much variance in insulin resistance is explained by obesity?', *Journal of Insulin Resistance*, 1(1). doi: 10.4102/jir.v2i1.22.

Virgin, C. E. and Sapolsky, R. M. (1997) 'Styles of male social behavior and their endocrine correlates among low-ranking baboons', *American Journal of Primatology*, 42(1), pp. 25–39. doi: 10.1002/(SICI)1098-2345(1997)42:1<25::AID-AJP2>3.0.CO;2-0.

Watve, M. (2013) *Doves, Diplomats, and Diabetes*. New York, NY: Springer New York. doi: 10.1007/978-1-4614-4409-1.

Watve, M., Bodas, A. and Diwekar, M. (2014) 'Altered autonomic inputs as a cause of pancreatic β-cell amyloid', *Medical Hypotheses*, 82(1), pp. 49–53. doi: 10.1016/j.mehy.2013.11.002.

Watve, M. and Diwekar-Joshi, M. (2016) 'What to expect from an evolutionary hypothesis for a human disease: The case of type 2 diabetes', *HOMO*, 67(5), pp. 349–368. doi: 10.1016/j.jchb.2016.07.001.

Weber, J.-M. (2009) 'The physiology of long-distance migration: extending the limits of endurance metabolism', *Journal of Experimental Biology*, 212(5), pp. 593–597. doi: 10.1242/jeb.015024.

Wells, J. C. (2012) 'A critical appraisal of the predictive adaptive response hypothesis', *International Journal of Epidemiology*, 41(1), pp. 229–235. doi: 10.1093/ije/dyr239.

Wells, J. C. (2003) 'The Thrifty Phenotype Hypothesis: Thrifty Offspring or Thrifty Mother?', *Journal of Theoretical Biology*, 221(1), pp. 143–161. doi: 10.1006/jtbi.2003.3183.

Wells, J.C. (2007) 'Flaws in the theory of predictive adaptive responses', *Trends in Endocrinology & Metabolism*, 18(9), pp. 331–337. doi: 10.1016/j.tem.2007.07.006.

Wells, J. C. (2007) 'The thrifty phenotype as an adaptive maternal effect', *Biological Reviews*, 82(1), pp. 143–172. doi: 10.1111/j.1469-185X.2006.00007.x.

Weyer, C. *et al.* (2000) 'A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia', *Diabetes*, 49(12), pp. 2094–2101. doi: 10.2337/diabetes.49.12.2094.

Williams, G. C. and Nesse, R. M. (1991) 'The Dawn of Darwinian Medicine', *The Quarterly Review of Biology*, 66(1), pp. 1–22. doi: 10.1086/417048.

Yilmaz, N. *et al.* (2009) 'The Relationship between Obesity and Fecundity', *Journal of Women's Health*, 18(5), pp. 633–636. doi: 10.1089/jwh.2008.1057.

Zhang, J. (2005) 'Association of Serum Cholesterol and History of School Suspension among School-age Children and Adolescents in the United States', *American Journal of Epidemiology*, 161(7), pp. 691–699. doi: 10.1093/aje/kwi074.

Chapter 7: Conclusions and Outlook

7.1 Conclusions

The main motivation of studying the insulin-glucose relationship was its dysregulation leading to T2DM which is a substantial burden on the health systems all over the world. There are several inconsistencies, flaws, and anomalies in the research about the pathophysiology and treatment of T2DM. A critical rethinking of fundamentals is necessary, and new studies related to this are coming up from several groups all over the world. With this in mind, we set out to critically re-examine the insulin-glucose relationship using multiple approaches which complement each other.

In the first approach, I looked at the literature for experiments in which insulin or insulin action has been increased or suppressed in a sustained manner. I conducted a systematic review of the literature in four different meta-analyses (Chapter 2). A meta-analysis of the literature in which the insulin degrading enzyme is inhibited, shows that the increase in insulin via this inhibition affects the post-meal glucose, but not the fasting glucose. Similarly, the IRKO, DZX and OCT meta-analyses look at the glucose levels after inhibition of insulin action or suppression of the insulin level. The results of these analyses also converged on the result that the alterations of insulin levels lead to a significant change in the GTT curve, but not the fasting glucose level. Thus, a change in insulin alters the perturbed state of glucose substantially more than it alters the steady state.

The next approach looked at the epidemiological data. If the parameters of glucose-insulin interaction are the same, the regression-correlation parameters between glucose and insulin should also remain the same in fasting versus post-meal conditions, although the range will be different. In epidemiological data, it was seen that the fasting correlation is substantially weaker than the post-meal correlation (Chapter 3). The regression slopes also differ indicating that the mechanisms that decide the fasting levels of glucose and insulin, and the ones that decide post-meal levels would be qualitatively and/or quantitatively different.

The STZ experiments (the third approach) agree with the epidemiological data in that the fasting and post-meal patterns are significantly different. Overall insulin explains glucose levels very poorly in fasting data, but much better in post-meal data (Chapter 4). The assumption that STZ acts specifically by β-cell destruction and has no other mechanism of action leaves many questions unanswered. Therefore, other possible mechanisms of action of STZ on glucose need to be explored.

In an attempt to infer causality from cross sectional data using the novel methods developed by Chawla et al (2018), the classical pathway for regulation of fasting glucose fails to get support. On the other hand, the null model that FG and FI do not affect each other but may have a common input such as autonomous nervous system is not rejected (Chapter 5).

Interpreting all experimental and epidemiological data together led to a convergent inference that, insulin has no role in determining steady state glucose level, but it can enhance the rate of return to a steady state after a perturbation. We make a fundamental distinction in causal relationships by showing that causation in a perturbed state can be distinct and different than causation is a steady state in homeostatic systems. On a more general scale, driver cause is one which takes a system to a state but does decide the attributes of that state. A navigator cause influences the attributes of the state, although it may not be sufficient to take the system to that state (figure 1). We outline the methodological norms for differentiating the two types of causalities (Chapter 5). By these norms, insulin is a driver for fasting glucose but not a navigator.

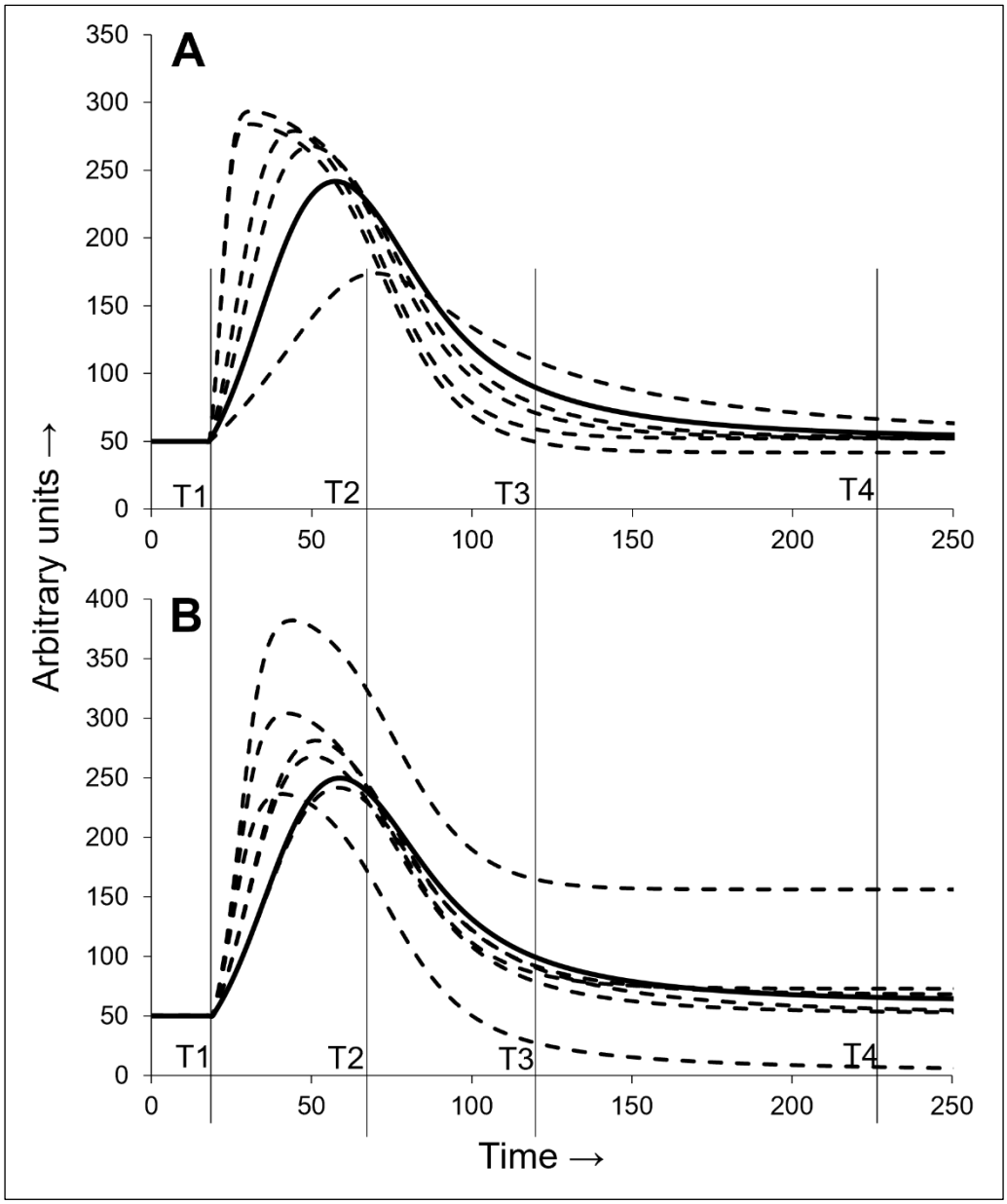


Figure 1: Distinct actions of a driver and a navigator on the steady and perturbed states of a homeostatic variable. In panels A and B, the solid line represents the normal levels of a homeostatic variable. The time points T1, T2 and T3 represent the perturbed state whereas the time point T4 represents the steady state of the variable. Panel A represents the action of a driver on the variable. The effect of the driver causes changes of the peak of the variable in the perturbed state, the steady state levels however remain the same, even though there is a delay in reaching the steady state levels. Panel B represent the action of a navigator on the variable. The effect of a navigator is that it changes the steady state level of the variable along with the perturbed state.

In this work, we have come up with a new understanding of insulin-glucose relationship. This novel view of the insulin-glucose relationship many implications to the hypotheses about evolutionary origins of type 2 diabetes. This new view substantially weakens the case for the obesity centred hypotheses. We have explored the criteria for the development of a sound theory for the evolutionary origins of a diseases with the specific example of type 2 diabetes.

This new relationship between insulin and glucose strengthens the behaviour and reproduction centred hypotheses to explain the evolutionary origin for type 2 diabetes.

7.2 Outlook

After revisiting the insulin-glucose relationship and highlighting the difference between this relationship at the steady state and perturbed state, the next steps would be to find the causal factors in the steady state. We highlight the role of insulin as a driver and not a navigator in the determination of steady state or fasting glucose. The next line of work world be to decipher the putative drivers which could determine the levels of fasting glucose.

The potential drivers and navigators which could regulate glucose apart from insulin have always been studied right from the time when Claude Bernard showed that damage to Medulla causes hyperglycaemia (Bernard, 1879). Like insulin, a variety of molecules and behaviours or actions have been associated with or implicated in glucose homeostasis over the years. The key is to try and identify if these factors act as a driver or navigator. To do this, effect of each factor on the steady state and perturbed state glucose must be known. Then and only then can we be sure if the factor is a driver or a navigator or even both. The best point to start to look for potential drivers and navigators would be to start with the molecules or behaviours identified to affect the insulin action in a steady state. Kulkarni et al 2017 have identified over 70 different molecules which affect insulin, insulin action and/or glucose levels in type 2 diabetes (Kulkarni, Sharda and Watve, 2017) using a network model. Moreover, they have also identified some key nodes in the network which take the system from an insulin resistant state to an insulin sensitive state. These key nodes could be checked for a driver/navigator function using a similar mode of study which has been used in the thesis. these are the molecules from the list of their key nodes that we could start with: testosterone, dopamine, oestrogen, osteocalcin, melatonin, ghrelin and adiponectin. (Kulkarni, Sharda and Watve, 2017). We could use a similar meta-analysis approach initially to assess if each of these factors act as a driver or navigator on glucose.

In this project, we have tried to delineate the causal relations between fasting glucose, fasting insulin and insulin resistance using the methods developed in our lab (Chawla et al, 2018). The same method could be employed to look at the relationship between fasting glucose and other putative drivers, provided steady state values are available for such variables. Kulkarni, Sharda and Watve 2017 have showed with an inter-organ signalling network model that several nodes which result in insulin sensitivity when perturbed sustainably. This list could be the first step/starting point for searching published literature for data sets with the steady state values for the variables identified as putative drivers. Thus, the final aim should be to find the putative drivers or combination of such drivers which are responsible for the fasting or steady state glucose levels. These could in turn suggest novel prevention and treatment approaches towards T2DM.

7.3 References

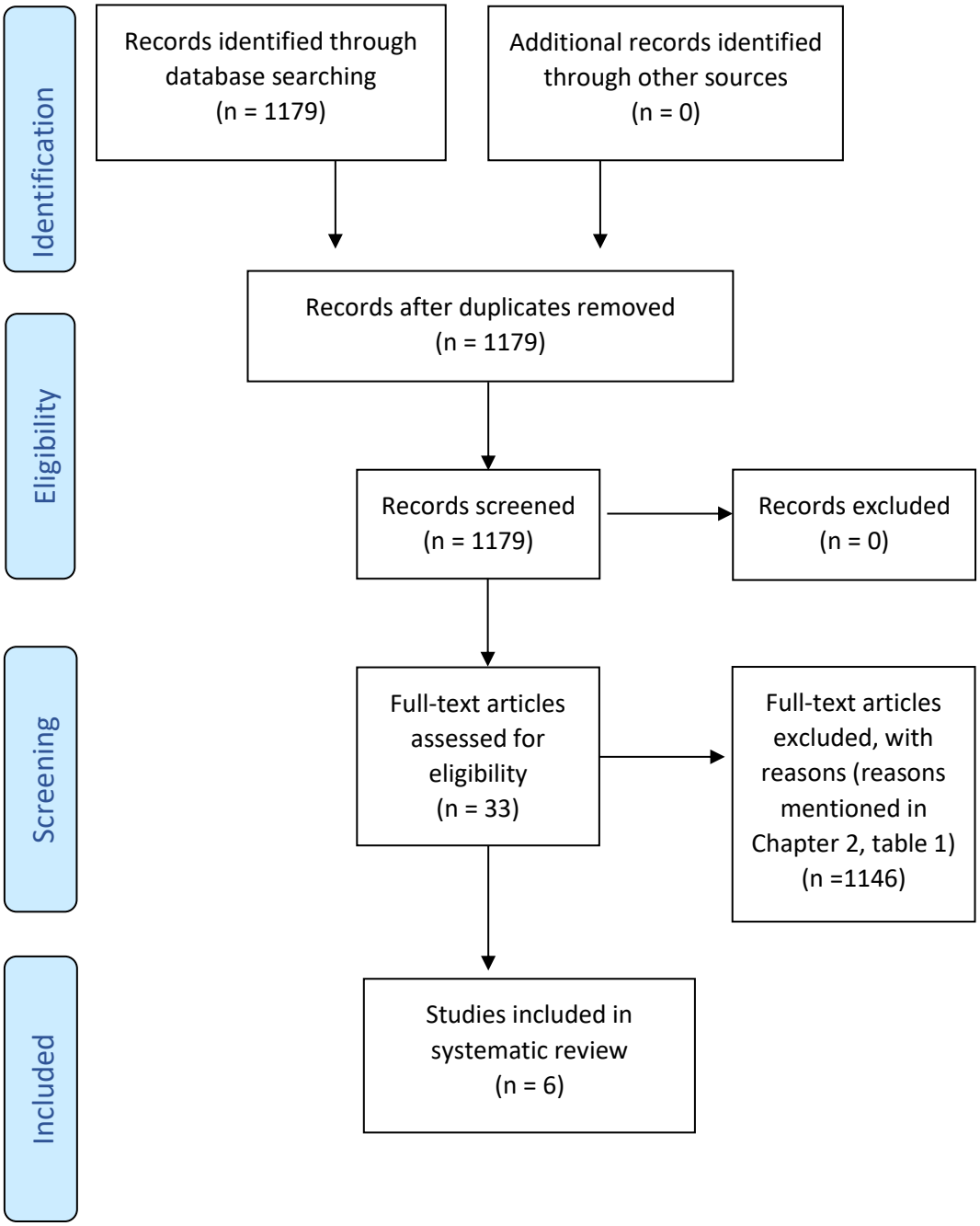
Bernard, C. (1879) ‘Leçons de physiologie opératoire’, *Librairie J.-B. Baillie et fils*, p. 650.

Chawla, S. *et al.* (2018) ‘Inferring causal pathways among three or more variables from steady state correlations in a homeostatic system’, *PLOS ONE*. Edited by M. Ruscica, 13(10), p. e0204755. doi: 10.1371/journal.pone.0204755.

Kulkarni, S., Sharda, S. and Watve, M. (2017) ‘Bi-stability in type 2 diabetes mellitus multi-organ signalling network’, *PLOS ONE*. Edited by C. Cras-Méneur, 12(8), p. e0181536. doi: 10.1371/journal.pone.0181536.

Appendix

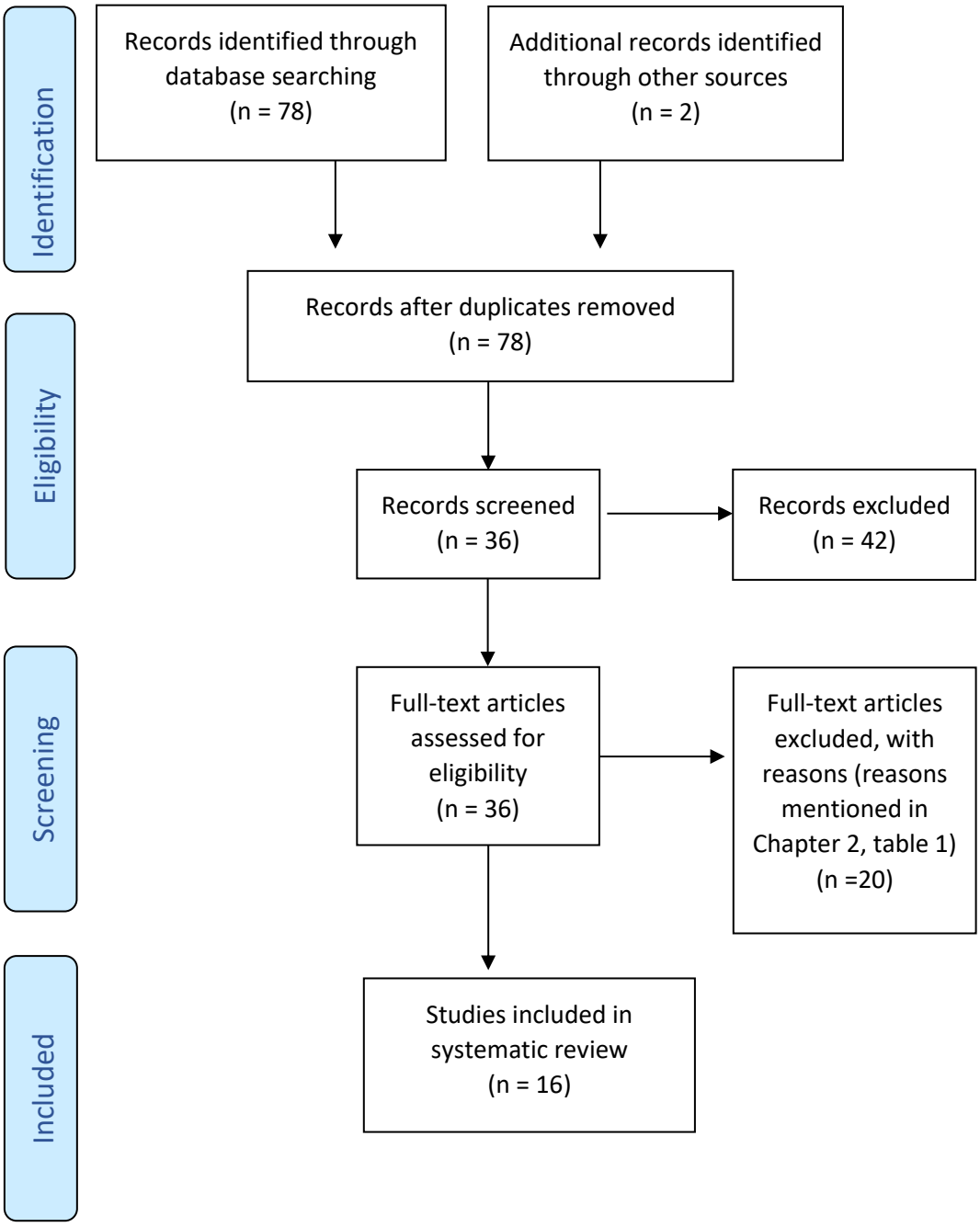
I.) PRISMA 2009 Flow Diagram: Insulin Degrading Enzyme (IDE) inhibition meta-analysis



Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

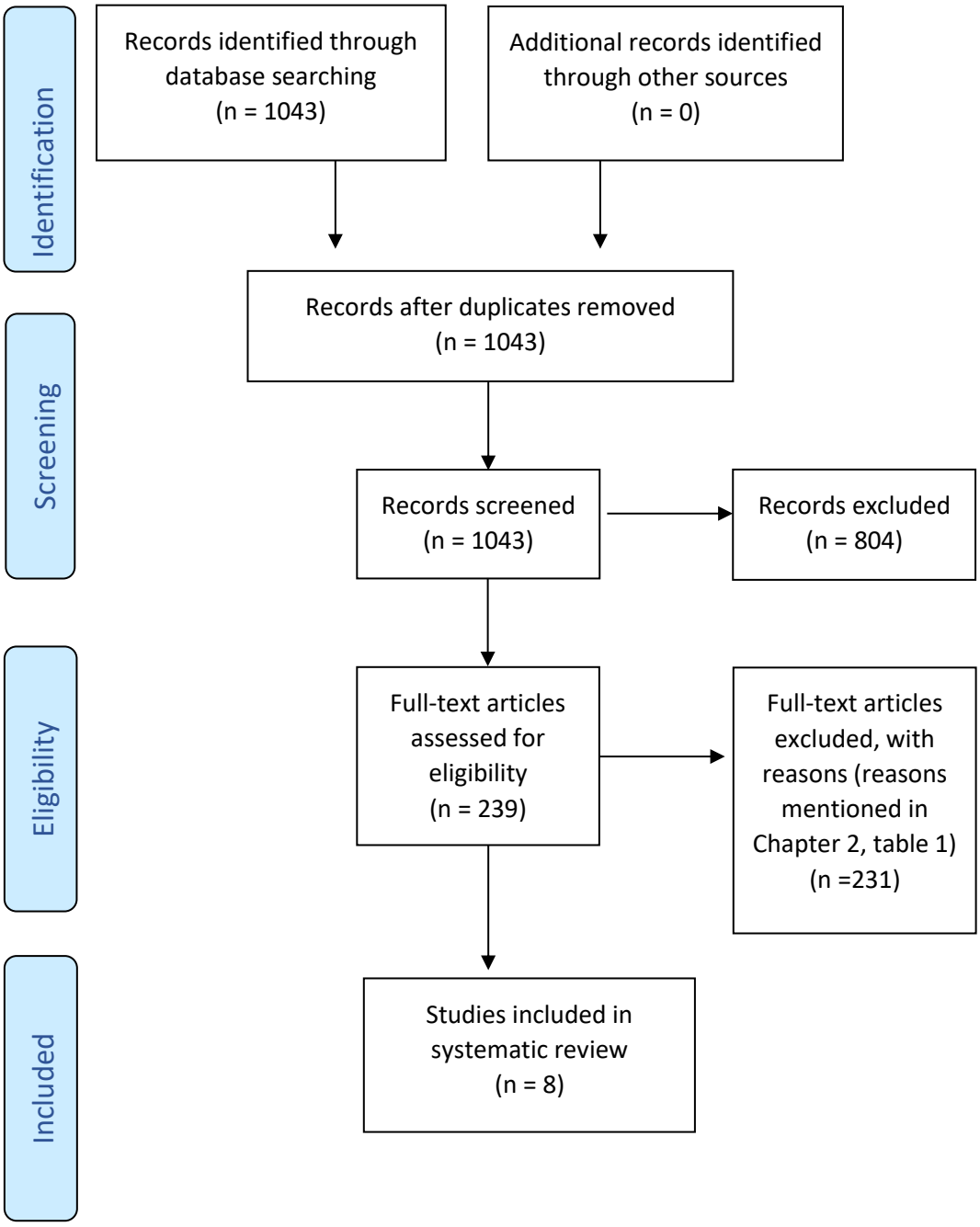
II.) PRISMA 2009 Flow Diagram: Insulin Receptor Knock-out (IRKO) meta-analysis



Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

III.) PRISMA 2009 Flow Diagram: Diazoxide (DZX) meta-analysis



Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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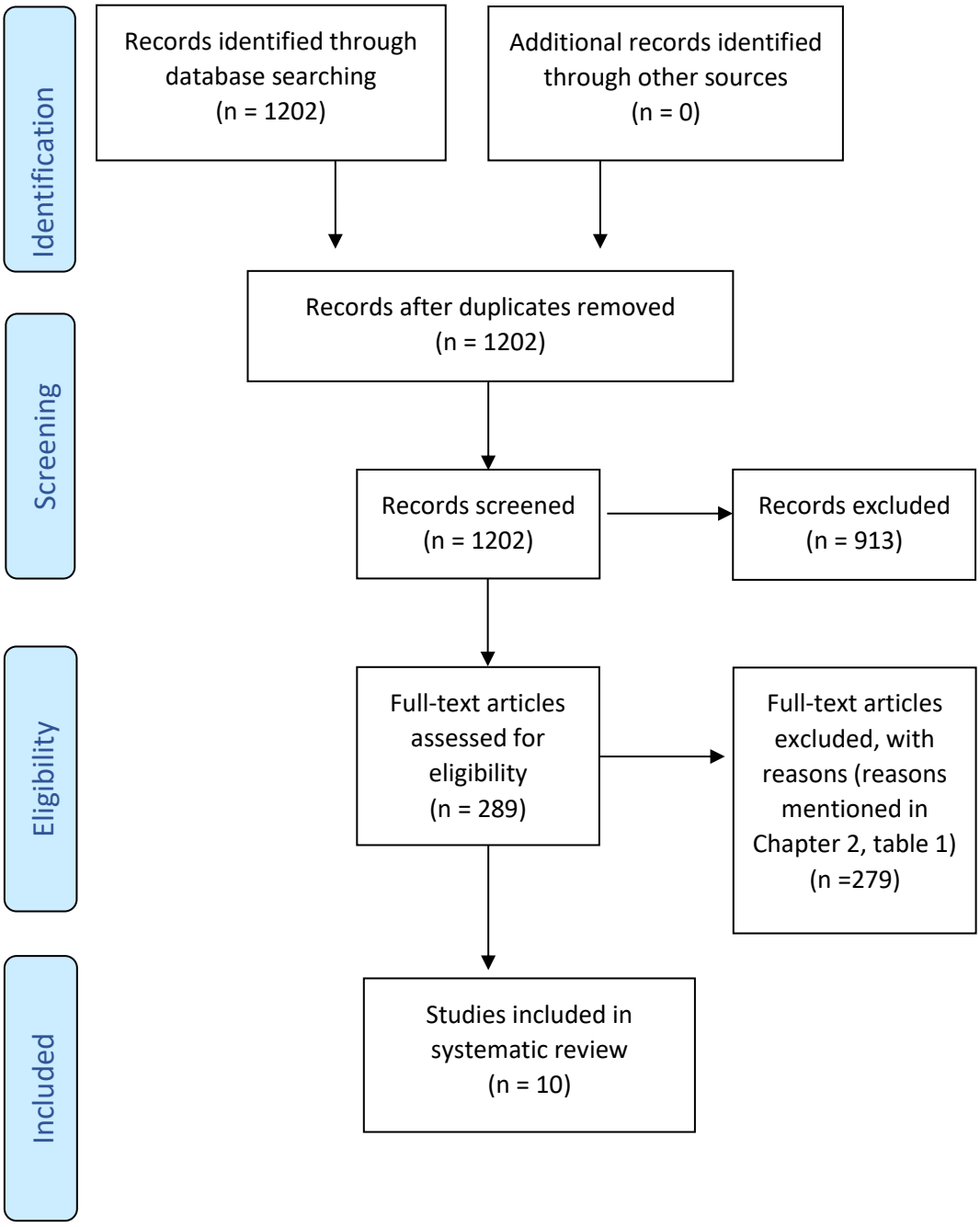
IV.) PRISMA 2009 Flow Diagram: Octreotide (OCT) meta-analysis



Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *P*referred *R*eporting *I*tems for *S*ystematic Reviews and *M*eta-*A*nalyses: The PRISMA Statement. PLoS Med 6(6): e1000097. doi:10.1371/journal.pmed1000097

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