Construction of an inter-organ signalling network model for understanding type 2 diabetes

A THESIS

SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS OF THE DEGREE OF

DOCTOR OF PHILOSOPHY IN BIOLOGY

BY

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INDIAN INSTITUTE OF SCIENCE EDUCATION AND RESEARCH, PUNE

2018

Certificate

Certified that the work incorporated in the thesis entitled "**Construction of an inter-organ signalling network model for understanding type 2 diabetes**" submitted by **Shubhankar Atish Kulkarni** 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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Declaration

I declare that this written submission represents my ideas in my own words and where others' ideas have been included; I have adequately cited and referenced the original sources. I also declare that I have adhered to all principles of academic honesty and integrity and have not misrepresented or fabricated or falsified any idea/data/fact/source in my submission. I understand that violation of the above will be cause for disciplinary action by the Institute and can also evoke penal action from the sources which have thus not been properly cited or from whom proper permission has not been taken when needed.

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Acknowledgements

It is difficult to list the people I met and their support I got over half a decade in just a few lines. Writing acknowledgements, hence, was one of the toughest tasks to do.

Firstly, I would like to express my sincere gratitude to my advisor Prof. Milind Watve and his scientific superpower of 'connecting the dots'. I am grateful to him for not only helping me with my work, but also inculcating in me, the right scientific attitude.

I am thankful to my research advisory committee: Dr. Sutirth Dey (IISER, Pune), Dr. Pranay Goel (IISER, Pune) and Dr. Anu Raghunathan (NCL, Pune); for their encouragement and criticism alike, throughout my Ph.D. I am also grateful to the library officials Ms. Tanuja Sapre and Ms. Namrata Shinde for their prompt help in accessing journal papers and books.

Next, I extend my gratitude to the current and former lab-members of MGW lab for making work more fun-filled. I thank the diabetes sub-group in the lab – (soon to be Dr.) Manawa Diwekar-Joshi, Dr. Pramod Patil, Dr. Harshada Vidwans and Akanksha Ojha; with whom I shared BILD-related work and the famous Saturday Breakfast. I thank my other lab members Dr. Ulfat Baig, Dr. Uttara Lele, Dr. Abhijeet Bayani, Neha Shintre, Chinmay Kulkarni, Poorva Joshi, Vibishan B., Dr. Ruby Singh, Tejal Gujrathi, Ketaki Holkar, Anagha Pund, Kajol Patel, Dr. Alok Bang, Trupti Bhingarkar, Ojas S.V., Ketaki Bhagwat, Poortata Lalwani and Nazneen Gheewalla for their support and help.

I thank my fellow guinea pigs – the first Integrated Ph.D. batch of the Biology department of IISER, Pune – Chaitanya Mungi, Ajay Labade, Ketakee Ghate, Sayali Choudhary and Roopali Pradhan for being there throughout. Other people whom I met here and made long-lasting friendship with are Dr. Manasi Khasnis (and family), Niraja Bapat, Vibha Singh, Harshini Tekur, Aditi Maduskar and Devika Bodas.

Finally, I would like to thank my family: my parents and my wife, for their patience and their unconditional support. I couldn't have managed to make it through without it.

Shubhankar

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List of Abbreviations

ACCORD	Action to Control Cardiovascular Risk in Diabetes Study Group
ADVANCE	Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation
α-MSH	α-Melanocyte Stimulating Hormone
baPWV	brachial-ankle Pulse Wave Velocity
BDNF	Brain Derived Neurotrophic Factor
BMI	Body Mass Index
CART	Cocaine and Amphetamine Regulated Transcript
CBF	Cerebral Blood Flow
CI	Confidence Intervals
CNS	Central Nervous System
CRH	Cortico-Releasing Hormone
DEN	Differential Expression Network
DEXA	Dual Energy X-ray Absorptiometry
df	Degrees of Freedom
dL	decilitres
DMN	Default Mode Network
DNA	Deoxy-ribonucleic Acid
DOHAD	Developmental Origin of Health And Disease
DPP-4	Dipeptidyl Peptidase – 4
EGF	Epidermal Growth Factor
FFA	Free Fatty Acids
FGF	Fibroblast Growth Factor
FIRKO	Fat specific Insulin Receptor Knock-Out
GABA	γ-Aminobutyric Acid
GLP-1	Glucagon-Like Peptide – 1
GLUT-4	Glucose transporter – 4
GnRH	Gonadotropin-Releasing Hormone
GWAS	Genome-Wide Association Studies
HbA_{1c}	Haemoglobin A_{1c}
HFD	High Fat Diet

HIV	Human Immunodeficiency Virus
HOMA	Homeostatic Model Assessment
ICMR	Indian Council of Medical Research
IGF-1	Insulin-like growth factor – 1
IL-6	Interleukin 6
IQ	Intelligence Quotient
KIR	Inward Rectifier Potassium channel
K+	Potassium ion
KEGG	Kyoto Encyclopaedia of Genes and Genomes
kg	kilograms
LIRKO	Liver specific Insulin Receptor Knock-Out
MeSH	Medical Subject Headings
mg	milligrams
MIRKO	Muscle specific Insulin Receptor Knock-Out
MODY	Maturity Onset Diabetes of the Young
MRI	Magnetic Resonance Imaging
NGF	Nerve Growth Factor
NICE-SUGAR	Normoglycemia in Intensive Care Evaluation–Survival Using Glucose Algorithm Regulation
NOS	Nitric Oxide Synthase
p	p value
PAT	Peripheral Arterial Tone
PS	Perturbed State
ROS	Reactive oxygen species
SFRP-5	Secreted Frizzled Related Protein 5
SGLT-2	Sodium Glucose Co-Transporter 2
siRNA	Small Interfering Ribonucleic Acid
SMC	Simple Matching Coefficient
SNP	Single Nucleotide polymorphisms
SS	Steady State
STITCH	Search Tool for Interactions of Chemicals
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SUR1	Sulphonylurea Receptor 1

T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TCI	Temperament Character Inventory
TNF-α	Tumour Necrosis Factor - α
UKPDS	United Kingdom Prospective Diabetes Study
UPGMA	Unweighted Pair Group Method with Arithmetic mean
USD	United States Dollar
VADT	Veterans Affairs Diabetes Trials
VO_2max	Maximum Volume of Oxygen
WHO	World Health Organisation
WHR	Waist to Hip Ratio

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Abstract

Type 2 diabetes mellitus (T2DM) is believed to be irreversible although no component of the pathophysiology is irreversible. We show here with a network model that the apparent irreversibility is contributed by the structure of the network of inter-organ signalling. A network model comprising all known inter-organ signals in T2DM showed bi-stability with one insulin sensitive and one insulin resistant attractor. The bi-stability was made robust by multiple positive feedback loops suggesting an evolved allostatic system rather than a homeostatic system. Certain evolutionary hypotheses do suggest existence of multiple stable states in a population which are adapted to different environmental conditions and social roles. Similarly, the bi-stability in this case and the preponderance of positive feedbacks in the network suggest co-existence of the diabetic state and the healthy state. The robustness was unlikely to have arisen due to one or a few nodes or links since deleting individual nodes and randomly adding links to the network did not disturb the bi-stability. Sensitivity analysis showed that this result wasn't due to chance alone or due to any of the assumptions or contradictions. In the absence of the complete network, impaired insulin signalling alone failed to give a stable insulin resistant or hyperglycaemic state. The model made a number of correlational predictions, many of which were validated by empirical data. The current treatment practice targeting obesity, insulin resistance, beta cell function and normalization of plasma glucose failed to reverse T2DM in the model. However certain behavioural and neuro-endocrine interventions like up-regulations of dopamine, ghrelin, oestrogen and osteocalcin ensured a reversal. These results suggest novel prevention and treatment approaches which need to be tested empirically. The model also shows a difference in steady-state and perturbed-state causality and suggests that making steady-state predictions from perturbed-state data might have led to a confused cause-effect relationship in the field. Finally, a design of a network-level clinical study has been suggested with the kind of analysis used to interpret such a dataset.

Chapter 1

Type 2 diabetes (T2DM) research: Has it hit a wall?

1.1 Demography

With over 415 million people affected, diabetes needs no formal introduction. This number is estimated to rise up to 642 million by 2040 (Mathers and Loncar, 2006). Out of the 415 million, 91% are affected by Type 2 Diabetes Mellitus. Another 318 million people are estimated to have impaired glucose tolerance which may lead to T2DM. The global expenditure on treating diabetes is more than 650 billion USD per year (majority of the countries spend between 5% and 20% of their health expenditure on treating diabetes). India ranks second with about 69.2 million adults affected with diabetes which indicates that every sixth diabetic is an Indian. But more importantly, she ranks first in the number of people having impaired glucose tolerance (36.5 million people) indicating the highest rate of potential increase in the number of diabetics (International Diabetes Federation, 2015).

The number of people affected with diabetes was about 151 million in the year 2000 with a prevalence of 4.6% (International Diabetes Federation, 2000). The emergency is evident with this increase in prevalence to up to 8.8% in the year 2015 (International Diabetes Federation, 2015). This apparent increase may be partly contributed by increase in the number of health check-ups, at least in the lower economy group. Nonetheless, a state of emergency persists and effective strategies to halt and more optimistically cure diabetes are warranted.

1.2 Pathophysiology of T2DM

Classification of diabetes was attempted way before the formal classification (since 1880) was accepted world-wide. A broad division of fat versus thin diabetics is found in ancient Indian literature (Sushruta Samhita) (Tattersall, 2010). Later in 1936, diabetes was classified as insulin sensitive and insulin insensitive (Himsworth and Lond, 1936). What became known as type 2 diabetes was often referred to as mild diabetes (Cook and Sepinwall, 1975). For a few decades between 1970s and 1990s diabetes was classified as insulin-dependent and non-insulin-dependent. Two major types of diabetes were globally recognized after the World Health Organisation (WHO) published its second report in 1981 (Bajaj et al., 1980); with type 1 being the former Insulin-Dependent Diabetes Mellitus and type 2 being Non-Insulin Dependent Diabetes Mellitus

characterized by central obesity and insulin resistance (Bajaj et al., 1980; Dobretsov et al., 2007). Today, we know that T1DM is characterized by an auto-immune reaction, wherein body's own defence mechanism destroys the pancreatic β-cells leading to deficit of the hormone insulin. Thereby, the body cannot produce the amount of insulin it needs. A daily supply of insulin is vital in this case. A third transient type of diabetes called the Gestational Diabetes was also recognized by the WHO in 1981 (Bajaj et al., 1980). It is diagnosed during pregnancy, usually after the 24th week and normally disappears after the birth of the child. Women with gestational diabetes have elevated blood glucose levels as compared to healthy pregnant women. Although the blood sugar returns to normal after pregnancy, women with gestational diabetes have a higher probability of developing T2DM later in life (Kim et al., 2002). The focus of the thesis is on T2DM, which is the most common type of diabetes observed world-wide. Pathophysiology of T2DM is believed to comprise 5 main steps:

a. Genetic, environmental and dietary factors lead to obesity
b. Obesity causes insulin resistance in the body
c. To overcome the insulin resistance, pancreatic β-cells produce more insulin
d. This chronic overproduction leads to failure of β-cells and thereby, insulin insufficiency
e. This leads to overt hyperglycaemia and chronic hyperglycaemia then leads to the complications of T2DM (Defronzo et al., 2015; Watve, 2013)

The pathology of T2DM includes microvascular (Retinopathy, Neuropathy and Nephropathy) and macrovascular (Atherosclerosis, Cardiovascular and Cerebrovascular) complications (Fowler, 2008). Retinopathy is observed in 34.6% of the diabetics. This includes patients with proliferative Diabetic Retinopathy, Diabetic Macular Oedema and Vision-threatening Diabetic Retinopathy (Yau et al., 2012). A study in India concluded that 19.1% of the diabetics show Diabetic Neuropathy. Diabetic autonomic neuropathy may lead to silent myocardial infarction resulting in death in 25% to 50% patients within 10 years of development of the disease (Bansal et al., 2006). Worldwide, 25% to 50% of the diabetics also develop Diabetic nephropathy (Tang, 2010). In UKPDS (UK Prospective Diabetes Study, 1998), 2% patients showed

microalbuminurea at diagnosis which elevated to 25% at the end of 10 year follow-up (Adler et al., 2003).

The main mechanism in case of the macrovascular complications is atherosclerosis; it leads to either cardiovascular diseases or cerebrovascular complications (Fowler, 2008). The risk of Coronary Heart Disease is increased 2 to 4 fold if the individual has T2DM. In a 7-year long population-level study, the per cent incidence of Myocardial Infarction with T2DM was 20% as compared to 3.5% for non-diabetics. Similarly, the frequency of a diabetic having a stroke is 3 times higher than a non-diabetic. This was even observed in the Multiple Risk Factor Intervention Trial of 347978 men (Beckman et al., 2002).

1.3 Treatment options

The diagnosis and treatment of diabetes dates back to 600 B.C. when Sushruta, an Indian physician recognized this disease and suggested a treatment regime based on diet changes and exercise (Tipton, 2008). The next major breakthrough for diabetes treatment came in 1921, after the discovery of insulin by Frederick Banting, John Macleod and their team (Karamitsos, 2011). The current treatment options for T2DM are based on the above noted pathophysiology (Table 1.1). Usually, the treatment includes one or more of these drugs in combination depending upon the requirement of the patient, his/her risk factors and the proven contra-indications for the drugs.

Table 1.1: Lines of pharmacological treatment for T2DM

Line of Treatment	**Drug classes used**
Suppression of liver gluconeogenesis	Biguanides (Cheng and Fantus, 2005)
Increasing insulin sensitivity	Biguanides (Cheng and Fantus, 2005); Thiazolidinediones (Cheng and Fantus, 2005)
Enhancement of insulin production	Sulphonylureas (Cheng and Fantus, 2005); Glucagon Like Peptide-1 (GLP-1) analogues (Cernea and Raz, 2011); Dipeptidyl Peptidase – 4

	(DPP-4) inhibitors (Cernea and Raz, 2011)
Insulin supplementation	Insulin
Reduction in obesity	Intestinal lipase inhibitors (Cheng and Fantus, 2005); α-glucosidase inhibitors (Cheng and Fantus, 2005)
Reduction in free fatty acids	Thiazolidinediones (Cheng and Fantus, 2005); Intestinal lipase inhibitors (Cheng and Fantus, 2005);
Other means of normalizing blood glucose	Alpha glucosidase inhibitors (Cheng and Fantus, 2005); Sodium Glucose Co-Transporter – 2 (SGLT-2) Inhibitors (Inzucchi et al., 2015)

1.3.1 Non-pharmacological treatment options that are usually prescribed alongside drugs:

a. Diet: Suggestive guidelines have been published by World Health Organization and other international organizations for diabetics. Differences in the guidelines arise based on a person's race, geographic location, etc. Indian Council of Medical Research (ICMR) also has a set of dietary guidelines for the management of T2DM (Indian Council of Medical Research, 2005).

b. Exercise: The ICMR and WHO guidelines suggest physical activities like yoga, brisk walking or any other equivalent forms of exercise (Bajaj et al., 1980; Indian Council of Medical Research, 2005).

c. Bariatric surgery: Bariatric surgery has shown promising results in decreasing body weight as well as increasing insulin sensitivity and thereby, glycaemic control (Madsbad et al., 2014). But it is not advised to non-obese patients due to surgical risks.

d. Stress management: Psychological intervention to develop a positive attitude and lead a healthy life is also part of the treatment in some cases. This also includes development of family support and creation of a healthy environment for the patient (Indian Council of Medical Research, 2005).

1.3.2 Prospective treatment options:

a. β-cell regeneration and replacement: β-cell loss is observed in T1DM as well as T2DM patients. β-cell regeneration has shown great promise in T1DM patients and the same lines of treatment can be applied to T2DM patients with severe β-cell loss. Serpin B1 has recently shown β-cell proliferation in a mouse model of insulin resistance (Ouaamari et al., 2016). On the other hand, replacement strategies that exploit reprogramming of cells to induce pluripotency (for T2DM patients) are gaining impetus (Ohmine et al., 2012).

b. Other investigational drug therapies: Other upcoming therapies that target a range of parameters associated with metabolic syndrome have shown some promise in reducing the Haemoglobin A_{1c} (HbA_{1c}) levels in patients during preliminary clinical trials. Some mentionable ones include Colesevelam, a bile sequesterant which was used to treat hyperlipidemia, led to a reduction in HbA_{1c} levels by 1% over 12 weeks (Zieve et al., 2007); Ranolazine, a sodium potassium channel inhibitor, which was used in the treatment of angina has shown to reduce the HbA_{1c} levels by 0.6% over 4 months (Morrow et al., 2009); Salsalate, an anti-inflammatory agent has shown to reduce the fasting glucose by 13% as compared to placebo over a month of treatment (Fleischman et al., 2008); and bromocriptine, a dopamine agonist has been shown to reduce the HbA_{1c} levels by 0.6% over an year and free fatty acids and plasma triglycerides levels by 30% (Scranton et al., 2007).

c. Behavioural intervention: A new regime of specific behaviours to combat T2DM is recently gaining impetus. It is based on the behavioural pattern adapted by our ancestral hunter-gatherer society. Lack of such behaviours that were a part of the ancestral society may have a negative effect on the physiology. A series of games and aggressive exercises developed to mimic these behaviours enhance not only muscle mass and bone strength, but a plethora of neuro-endocrinal parameters that have a cumulative effect on the correlates of T2DM (Watve, 2013). This work is also backed by a pilot volunteer trial which shows improvement in insulin sensitivity in participants following this behavioural therapy (Belsare et al., 2010). Boxing is considered one such aggressive exercise and the effect of that on blood pressure parameters was shown in a trial in

Sydney (Cheema et al., 2015). Another trial conducted in the University of Glasgow has shown similar beneficial effects of aggressive exercises on insulin sensitivity indices over non-aggressive ones (Rashid, 2010).

1.4 Challenges to the classical pathophysiology of T2DM

1.4.1 Gaps, flaws and paradoxes in the classical theory: A number of recent studies have exposed many gaps, flaws and paradoxes in the classical thinking of the pathophysiology of T2DM. Some examples of the experiments that cast serious doubt on the mainstream theory are:

a. The muscle tissue is responsible for majority of insulin dependent glucose uptake in the body. If insulin receptors in the muscle are specifically knocked out in mice (MIRKO mice) making the muscle tissue maximally insulin resistant, insulin levels are expected to increase to compensate the insulin resistance as explained by the mainstream theory. But in MIRKO mice insulin levels remain surprisingly normal (Kim et al., 2000). If the main insulin dependent tissue, i.e. muscle, is insulin resistant and there is no compensatory insulin rise, the plasma sugar level should go up according to the mainstream theory. However, fasting plasma glucose remains unaltered in MIRKO mice. Similarly, fat-cell specific insulin receptor knockout (FIRKO) mice are lean, non-diabetic and have a longer lifespan. So muscle and fat cell insulin resistance in experimental animals and lack of compensatory insulin response are not sufficient to cause diabetic hyperglycaemia (Blüher et al., 2002, 2003; Kim et al., 2000).

b. In the liver-specific insulin receptor knockout (LIRKO) mice, both insulin and glucose levels are increased early in life but after a few weeks, the fasting sugar levels return to normal although liver insulin resistance remains high and there is no further rise in insulin levels (Johnson et al., 1972; Michael et al., 2000). Therefore, extreme liver insulin resistance does not seem to sustain a long-term rise in plasma glucose.

c. If reduced glucose uptake is responsible for increased plasma glucose, hyperglycaemia should be accompanied by subnormal intracellular glucose concentrations in insulin-dependent tissues. However, in diabetes,

hyperglycaemia has been shown to be associated with increased total glucose transport and raised levels of glucose in muscle cells (Farrace and Rossetti, 1992; Nolte et al., 1995).

d. In the mainstream thinking, it is noted that compensatory hyperinsulinemia exerts extra insulin production 'load' on β-cells, which is believed to lead to β-cell dysfunction. However, the β-cell number is known to increase associating with insulin resistance (Bernal-mizrachi et al., 2001; Brüning et al., 1997; Devedjian et al., 2000; Hardikar et al., 2015). Until it is demonstrated that the fold increase in insulin levels is substantially greater than the fold increase in β-cell number, it cannot be assumed that an average β-cell has an extra load. In some of the rat models, the fold increase in β-cell number is greater than the fold increase in fasting insulin (Bernal-mizrachi et al., 2001; Devedjian et al., 2000). In some other models, fold increase in fasting insulin is not greater than 20 % of the fold increase in β-cell mass (Brüning et al., 1997). Insulin transcription in β-cells is actually reduced rather than increased in the hyperinsulinemic state (Hardikar et al., 2015). In humans, although data on β-cell number in insulin resistance state are scanty, the picture is similar (Van Assche et al., 1978; Butler et al., 2010). Greater rise in insulin production as compared to increase in β-cell number has never been clearly demonstrated in humans. Therefore, there is no evidence that β-cell dysfunction is induced by compensatory insulin response.

e. Although evidence suggests that Glucose transporter – 4 (GLUT-4) is the major insulin-dependent glucose transporter in muscle (Abel et al., 2001; Stenbit et al., 1997), mice deficient in GLUT-4, have normal blood glucose level demonstrating that if insulin-dependent glucose uptake is impaired, alternative pathways compensate for the loss so that the total glucose uptake by muscle is hardly affected (Fam et al., 2012; Katz et al., 1995; Ryder et al., 1999).

f. There is increasing evidence, from human as well as animal models of early life insulin resistance, that rise in insulin levels precede insulin resistance (Chakravarthy et al., 2008). A number of mechanisms exist by which a rise in insulin secretion can decrease insulin sensitivity but no mechanism is known by which insulin resistance can give rise to increased insulin response in a normoglycaemic state. A number of researchers have shown with a variety of

evidence that hyperinsulinemia is primary, and insulin resistance appears to compensate for hyperinsulinemia, contrary to the mainstream thinking (Corkey, 2012; Dubuc, 1976; Garvey et al., 1986; Nankervis et al., 1985; Pories and Dohm, 2012; Shanik et al., 2008; Watve, 2013; Weyer et al., 2000). If hyperinsulinemia is not a compensatory response to insulin resistance, the hypothesis of inadequate insulin compensation leading to hyperglycaemia also gets undermined.

g. If insulin secretion is experimentally suppressed in an insulin-resistant state, it should lead to increased plasma glucose according to the mainstream thinking. A number of independent experiments in rodents and humans using different means such as diazoxide (Alemzadeh et al., 1993, 1996, 2002, 2004, 2008; Schreuder et al., 2005), octreotide (Velasquez-Mieyer et al., 2003), a SUR1/Kir 6.2 K+ adenosine triphosphate channel opener (Alemzadeh et al., 2004), a combination of insulin-siRNA and human insulin degrading enzyme (Hwang et al., 2007) and dietary means (protein deficiency)(Schteingart et al., 1979) have shown that whenever insulin production is suppressed, insulin sensitivity increases and blood sugar remains normal. This demonstrates that insulin resistance and inadequate compensation are unlikely to be necessary and sufficient to cause hyperglycaemia in T2DM.

h. Hyperglycaemia is believed to be the cause of diabetic complications according to the mainstream thinking. However, apart from correlations, there is no other evidence for the causal role of glucose in the pathogenesis of complications. Early signs of vascular endothelial dysfunction (Hadi and Suwaidi, 2007), autonomic neuropathy (Dobretsov et al., 2007) and retinopathy (Nguyen et al., 2007) are now shown to often precede hyperglycaemia and hence, the cause-effect relationship appears to be confused in the mainstream thinking. Also, aggressive normalization of glucose did not reduce the risk of macrovascular complications in many large scale clinical trials (Max Miller et al., 1976; Stratton et al., 2000; Turner et al., 1998) and marginal reductions in the risk of complications with treatment were independent of the glucose levels (Holman et al., 2008).

The central question raised by the collection of experimental results outlined above is whether the classical theory of T2DM stands falsified. Some have clearly claimed falsification (Pories and Dohm, 2012; Watve, 2013) and wondered why the treatment is

still based on a theory which is clearly falsified (Watve, 2017). Among the community of clinical diabetologists there is a slow response to falsifying evidence, but certain changes have started happening. For example, the American Diabetes Association relaxed the HbA_{1C} targets for T2DM treatment and advocated not to aim for tight glycaemic control in elder patients and people prone to hypoglycaemic episodes (American Diabetes Association, 2018). However, any major qualitative change in clinical thinking in response to the experimental falsification is conspicuously absent.

1.4.2 The success and failure of treatment: Since the mainstream thinking forms the basis of the treatment for T2DM, it is no surprise why the latter has yielded only modest results. The mainstream thinking mainly revolves around fat tissue, muscle, pancreas and liver, and ignores almost every other tissue and organ of the body including the brain. This gluco-insulinocentric thinking may have been gained the central importance due to the burden of history. The dramatic discovery of insulin and early success in saving lives of T1DM patients portrayed insulin as the only relevant factor in glucose regulation and all other factors were ignored in spite of experimental demonstrations of their importance. The same treatment when applied to patients with T2DM has not shown similar dramatic results. On the contrary, some of them have aggravated the disease parameters in the participating patients (see details below).

The other possible reason of failure is too much emphasis on obesity as the causal factor. Although obesity and insulin resistance are consistently correlated across studies, the correlations are weak and the modal variance explained is less than 10% (Vidwans and Watve, 2017). Trials apparently “successful” in remission of T2DM (Lean et al., 2018) start with a set of patients with high Body Mass Index (BMI) only. The success claimed in this trial may not be applicable for the large number of normal weight and thin type 2 diabetics. The large number of factors other than fat that contribute to insulin resistance are largely ignored by the mainstream clinical thinking.

Hypoglycaemia is a major cause of clinical trial dropouts and even mortality in some cases. Hypoglycaemia was seen to increase in the ACCORD (The Action to Control Cardiovascular Risk in Diabetes Study Group), the ADVANCE (Action in Diabetes and Vascular Disease: Preterax and Diamicron Modified Release Controlled Evaluation) as well as the VADT (Veterans Affairs Diabetes Trials) studies after insulin therapy (Duckworth et al., 2009; Skyler et al., 2009; The Action to Control Cardiovascular Risk in

Diabetes Study, 2008). The hazards ratios for severe hypoglycaemia were 3.00, 1.86 and 3.52 in ACCORD, ADVANCE and VADT, respectively (Boussageon et al., 2017).

Insulin plays two different types of roles in the body – metabolic and mitogenic. In T2DM, the metabolic function seems to be affected while mitogenic functions appear to remain normal. Hence, exogenously injected insulin may pose greater problems with respect to the mitogenic activity of insulin (Lebovitz, 2011). Implications of this can be seen in a retrospective analysis study which found an odds ratio of 2.99 (CI 1.34-6.65, P = 0.007) for hepatocellular carcinoma in patients treated with sulphonylureas (insulin secretagogues) as against an odds ratio of 0.33 (CI 0.1 – 0.7, P = 0.006) for patients treated with metformin (Donadon et al., 2009). A retrospective case controlled study associating insulin therapy with risk of pancreatic cancer shows that after adjusting for age, sex, BMI, race, alcohol consumption, smoking, duration of diabetes and family history of cancer, the odds ratio of developing pancreatic cancer was 4.99 (P < 0.001) in patients having insulin therapy against those without insulin therapy (Li et al., 2009). Another analysis showed that the odds ratio of developing pancreatic cancer associated with sulphonylurea use was 1.3 (95% CI 1.1 - 1.6, P = 0.012) and 1.9 (95% CI 1.5 – 2.4. P < 0.0001) associated with insulin use (Bowker et al., 2006). The hazards ratio of tobacco smoking lung cancer was observed to be 4.9 (Osaki et al., 2007) and that of daily alcohol consumption and liver cancer was 1.52 (Schwartz et al., 2013). Compared to these, odds ratios observed for insulin treatment and cancer seem grave.

Studies analysing the effectiveness of insulin therapy have also identified that intensive insulin therapy designed to attain normal glucose levels led to a 90 – day mortality that was increased to 14% compared to individuals with moderate insulin therapy (The NICE-SUGAR Study, 2009). Another study showed similar results with increase in mortality as HbA_{1C} decreased from 7.5% to 6.4% (Currie et al., 2010). In the ACCORD study, the hazards ratios for all-cause mortality and cardiovascular mortality were 1.14 and 1.35, respectively per decrease in HbA_{1C} of only 1.1% (Boussageon et al., 2017). Weight gain is another problem associated with insulin therapy with the ACCORD study reporting more than 10 kg weight gain in 28% of the intensively treated patients and 14% in the moderately treated patients during the mean treatment period of about 3.5 years (The Action to Control Cardiovascular Risk in Diabetes Study, 2008).

Also, no long term randomized controlled trials indicate improved outcomes in insulin-treated T2DM patients in comparison with other treatments (Lebovitz, 2011). In the UKPDS 10 year follow-up study, it was observed that HbA_{1C} progressively rose from ~6.3% to 8.0% (Turner et al., 1998). The percentage of patients maintaining HbA_{1C} < 7% after 3 years was 47%, after 6 years was 37%, after 9 years was 28% and did not differ from the individuals treated with sulphonylureas at the end of the study (Turner et al., 1999).

1.4.3 Why T2DM is irreversible? Currently, T2DM is believed to be incurable. There can be three possible reasons for irreversibility of a condition.

1. There is something in the pathophysiology that is irreversible by itself: Except for advanced stages of complications, nothing in the baseline pathophysiology of T2DM is irreversible. Earlier β-cell loss was believed to be irreversible, but later studies showed good regeneration capacity *in vitro* as well as *in vivo* (Ouaamari et al., 2016). A concept that emerged to explain the failure of glycaemic control to reduce diabetic complications is 'hyperglycaemic memory' (Lee et al., 2016). Past hyperglycaemia somehow keeps a 'memory' which is sufficient to maintain the processes leading to complications even after glucose target has been met. This is used to explain why large scale clinical trials have failed to reduce the complications. However, none of the components of downstream pathways of hyperglycaemia are shown to be irreversible. Evidence to show that complications arise even before hyperglycaemia sets in exists (Dobretsov et al., 2007; Hadi and Suwaidi, 2007; Nguyen et al., 2007) suggesting other possible explanations for the apparent irreversibility.
2. The condition is reversible but we don't have the technology to reverse it: If insulin resistance and inadequate insulin production were the causes, we have technologies for both. There are insulin sensitizing drugs, there are insulin secretagogues and there is insulin supplementation. There also exists extreme sophistication in programmable insulin pumps and β-cell transplants. But nothing works in the long run, although they show beneficial short term effects. So this reason for irreversibility doesn't appear to be satisfactory.

3. Our understanding of the pathophysiology is either incomplete or utterly wrong, so that the current lines of treatment are equivalent to 'barking up the wrong tree'. Since the first two are weak, this possibility needs to be explored more seriously.

1.5 Relationship between T2DM and lesser known bodily parameters

As mentioned earlier, there are other players in the body which have a substantial role in the functioning of insulin and plasma glucose. Though their interactions with insulin and their possible role in T2DM and also its treatment have been worked upon in parallel, it has not been included in the mainstream hypothesis. Some of them have been noted here.

a. Brain: Role of brain in the regulation of glucose homeostasis was recognized since 1854 when a French physiologist, Claude Bernard, showed increased blood sugar levels after puncturing the floor of the fourth ventricle in the rabbit brain (Tups et al., 2017). The importance of brain got side-lined after the discovery of insulin in 1921. Other experiments that highlight the central role on glucose homeostasis are the ones showing that vagotomy (removing the vagus nerve) leads to increased endogenous glucose production by the liver (Matsuhisa et al., 2000) and the autonomic control of the pancreas where sympathetic stimulation decreases and parasympathetic stimulation increases the secretion of insulin (Ahrén, 2000). The cross talk between the sympathetic and the parasympathetic systems has been well documented (Campfield and Smith, 1983). Role of the brain is dependent on sensing of glucose levels in the surrounding fluid; and modulating food intake and glucose production based on that (Tups et al., 2017).

b. Adiponectin: Adiponectin is a hormone secreted by the adipose tissue. It has been shown to carry out many protective functions against T2DM including its direct role in maintaining insulin sensitivity (Kubota et al., 2002). It promotes β-cell survival in the pancreas and decreases glucose output and lipogenesis in the liver. In the adipose tissue, it enhances the adipocyte number, activates the lipid metabolism genes and shows anti-inflammatory effects (Turer and Scherer, 2012). It is associated with a reduced risk of myocardial infarction in men

(Pischon et al., 2004). In mouse models of renal dysfunction (which is usually associated with overt T2DM), treatment with adiponectin has shown to improve it by correcting the albuminurea and the podocyte foot process effacement (Ohashi et al., 2007; Sharma et al., 2008). When infused centrally for a long-term, adiponectin improved peripheral insulin sensitivity, β-cell mass, lipid metabolism; increased energy expenditure and decreased visceral fat in 90% pancreatectomised rats (Park et al., 2011).

c. Glucagon: Glucagon, a hormone produced by the pancreas, functions majorly to increase liver glucose production to maintain the plasma glucose content. It was shown that although there was β-cell destruction, glucagon receptor knockout mice did not become diabetic; whereas hyperglycaemia was observed in the wild type mice with equivalent β-cell destruction (Lee et al., 2011). In diabetic patients, glucagon suppression led to correction of diabetic symptoms like ketoacidosis, even after insulin treatment was stopped (Gerich et al., 1975; Raskin and Unger, 1978). The glucagon suppression has gained importance to the point that Roger Unger et al (Unger and Cherrington, 2012), in a review, urged this technique to be transformed in to a therapy and also suggested a glucagonocentric makeover to the pathophysiology of diabetes. On the contrary, glucagon is also suggested as a treatment option accompanying the other anti-hyperglycaemic agents due to its function in reducing hypoglycaemic episodes, which are common in patients on anti-hyperglycaemic therapy (Kedia, 2011).

d. Leptin: Similar to adiponectin, leptin is a hormone secreted by the adipose tissue. It is known to act centrally and reduce food intake (Schulz et al., 2012). It also decreases the amount of adipose tissue and loss of leptin action leads to diet-induced obesity. Centrally injected leptin decreases glucose-stimulated insulin secretion and this decrease is dose dependent. It also induces uptake of glucose by muscles and heart (Morton and Meek, 2012). Even subcutaneous injection of leptin normalizes the fasting glucose levels in T2DM rats (Cummings et al., 2011). Taken together, leptin treatment ameliorates the symptoms of T2DM in many animal models (Kalra, 2012).

e. Testosterone: Testosterone is intricately involved with the diabetic parameters, which is evident, since hypogonadal men show symptoms of T2DM. Testosterone

therapy in hypogonadal men led to reduction in HbA_{1c} from 8.08% to 6.14% and also reduced the fasting glucose levels from 128 mg/dl to 101 mg/dl (Haider et al., 2014).

f. Dopamine: Dopamine has receptors on the pancreatic β-cells which when activated leads to inhibition of insulin release (Rubí et al., 2005). Dopamine injections also lead to reduction in food intake and fasting glucose levels in rats with diet-induced obesity (de Leeuw van Weenen et al., 2011).

g. Osteocalcin: Osteocalcin increases insulin-dependent glucose uptake in wild type mice and long term osteocalcin treatment significantly improved body mass and glucose homeostasis (Ferron et al., 2008; Rached et al., 2010). It maintains β-cell proliferation and insulin sensitivity too (Lee et al., 2007).

h. Physical fitness: The cardiorespiratory fitness and several other measures of physical fitness are good predictors of T2DM, independent of obesity (Patil et al, manuscript under preparation). However, this is not integrated in mainstream clinical thinking.

i. Behaviour-metabolism links: There are over 70 neuroendocrine, metabolic and other mechanisms that link behaviour with the pathophysiology of T2DM (Watve, 2013). However, behaviour is not a part of mainstream clinical thinking.

This is certainly an incomplete list. A large number of genetic, epigenetic, neuronal, behavioural, hormonal and metabolic factors are associated with T2DM and their interrelationships and causal roles are grossly underexplored. It is possible that studying the inter-relationship between the large number of inter-related factors might be the key towards a new understanding of T2DM.

1.6 Research approaches for T2DM

Most biology before the turn of 19th century was observational. A strong foundation of experimental biology was laid by the turn of the century. The second half of the 20th century added a number of novel tools. Today, a given question in biomedicine can be addressed with multiple tools that complement each other.

a. Morphology and anatomy: Earlier research mostly comprised of morphological and anatomical studies in animals or in human cadavers. Even today, post-mortem analysis of T2DM patients is quite common (Clark et al., 1988). The fact that β-cell population was never completely destroyed in type 2 diabetes was revealed by post mortem histology of the pancreas. Newer micro-imaging techniques have led to identification of intricate differences between healthy and diseased cells (Costes et al., 2011).

b. Cell and molecular biology: Elucidation of specific pathways, signalling and determining functional roles of genes is achieved through cell and molecular biology. Different gene manipulation techniques have enabled the loss of function and gain of function mutations which can be used to determine the exact functions / effects of that particular gene.

c. *In vivo* animal experiments: *In vivo* experiments were also common earlier. This trend continues even today. *In vivo* animal experiments are considered next to actual human trials.

d. Clinical trials: Clinical trials are an essential part of any new treatment option. They also feedback research to improve the treatment strategy.

e. Theoretical work: Hypothesis building is a prelude to all experiments. Theoretical work also helps inter-disciplinary research where scientists from one field can apply their experience to problems in other fields. Theories make a logically coherent picture from experimental and observational facts using joining-the-dots approach. Theories also help designing experimental work further. It also comes to the rescue when actual experimental work is not possible in that particular setting.

f. Statistical tools: Use of statistical tools is not limited to calculating t-test and p values, but to develop new tools that can answer questions in biology where experimental data are limited.

g. Mathematical modelling: Mathematical models can give a predictive vision in biology. At times models have predicted phenomena or principles ahead of experiments. Models are often more important in falsifying hypotheses than supporting them. This is because a process that is mathematically possible need

not be true in real life but something that is mathematically impossible cannot exist in real life. Biological experiments are usually backed by a model which demonstrates working of the experimental phenomenon or its role in a bigger system.

h. Omics: More recent omics tools including genomics, transcriptomics, proteomics and metabolomics give extensive data. These high-throughput analysis techniques are cost and time effective. Obesity and T2DM were believed to have a strong genetic component earlier. Genomic studies have now revealed the limited role of genetics in both (Boehnke et al., 2010; Morris et al., 2012).

Network models: In the field of diabetes research, these tools have been used extensively. The inquiry started with the view that defect in a single organ, gene, molecule or pathway is responsible for a disorder. Glucose was the first molecule to be associated with diabetes. Insulin was the second. Over decades, a realization that it is a multi-organ multi-system phenomenon became stronger. Still glucose and insulin were believed to be the central molecules and others the consequences of their dys-regulation. However, the demonstration that a large number of molecules, cells and signals are altered in T2DM and some of the changes precede glucose dys-regulation, have raised more possibilities. With the increasing number of systems and signals involved, the classical simpler hypotheses-driven approaches are proving inadequate. Network modelling is a relatively recent promising tool that can integrate a large number of players interacting with each other. Therefore, it is likely to be a tool to get new insights in to type 2 diabetes. With the increase in computational power, handling larger datasets has become easier. Network models provide a bird's eye view of the underlying problem. Inferences made from such models can be experimentally tested.

Our approach in this thesis belongs to the network modelling category. Since much of the thinking regarding T2DM has revolved around insulin and its action, there is extensive work on the intracellular insulin signalling pathways (Defronzo, 2004). A sound understanding of the orchestration of organs is required to understand the disease. We need to be open to the possibility that insulin and glucose are not central players but only two of the links in a complex network of signals. In order to get a good understanding of T2DM, we need to consider all demonstrated interactions between

molecules and other signals involved in T2DM without any prejudice and construct a comprehensive model.

In this thesis, we constructed a multi-organ multi-signal interactive network model to study its behaviour. We focus on the relatively neglected network of inter-organ signalling in an attempt to throw light on how the organ cross-talk shapes the pathophysiology of T2DM. The intended outcome is to come up with alternative possibilities for the treatment approach that can suggest new lines of work for experimental and translational research.

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Chapter 2

Review of type 2 diabetes (T2DM) network models

This chapter summarizes the network studies from the literature, laying out a foundation for the work in the thesis. As mentioned in the previous chapter, network modelling is extensively used by researchers to get an idea how a complex system might work. The results of the model may suggest novel experimental approaches or help interpret the results. Classically, experimental designs address specific systems or pathways. For extrapolating the effects of an intervention to other systems or the organism as a whole, network model is a useful tool. This review discusses the network approaches used in this field so far and identifies gaps and unaddressed questions where greater inputs of efforts are needed.

2.1 Literature search

I systematically searched the Pubmed database using the search term – *'type 2 diabetes AND network [title/abstract]'*. I selected research papers that were available online as full articles or at least the abstracts on or before 30th June, 2017. The details of the search are presented in Table 2.1.

Table 2.1: Search and selection details of 'type 2 diabetes network models' search

Search details	*("diabetes mellitus, type 2"[MeSH Terms] OR "type 2 diabetes mellitus"[title/abstract] OR "type 2 diabetes"[title/abstract]) AND network[title/abstract]*
Hit obtained after first round	1200
Second round selection details	Deleted papers – - Had words like 'capillary network', '….affected by a network of transcription factors', social network', 'actin network', 'microtubule network', 'fibrin network' actual cell connectivity network - Were reviews, book chapters, commentaries or poster presentations - 'Network' was in the name of a cohort group or trial or a database

	- Had used artificial neural networks as a method for analysis
shortlisted after second round	298

2.2 Classification of networks

The concept of networks and their applications was pioneered in 1969 (Bachman, 1969), but its use in the field of diabetes appears to begin by 1996. My search ended by end of June, 2017. I then classified these publications based on 3 different criteria.

2.2.1 Causal or correlational networks (see Table 2.2): Most of the curated networks were correlational. Only 9% of the networks were causal (had causal interactions between its nodes). Out of the causal networks, about 74% were constructed based on predicted causality. Here, causal relations are inferred using tools like Ingenuity Pathway Analysis (Padilla et al., 2014) or Causal Reasoning (Enayetallah et al., 2011), but are not empirically demonstrated. Demonstrated causal networks were only a few and will be discussed later in this chapter.

2.2.2 Qualitative or quantitative networks (see Table 2.2): Again, majority of the networks were qualitative (97.4 %). The small number of quantitative networks were built of DNA microarray data (Nogiec et al., 2015) or were simulations assuming a range of parameter values (Banerjee and Bose, 2008).

2.2.3 Classification based on data source (see Table 2.2): These classification types and the proportion of publications per type are presented in Figure 2.1. Maximum hits were papers involving gene- gene interactions followed by drug meta-analyses. Some notable experiments are discussed in brief later in this chapter.

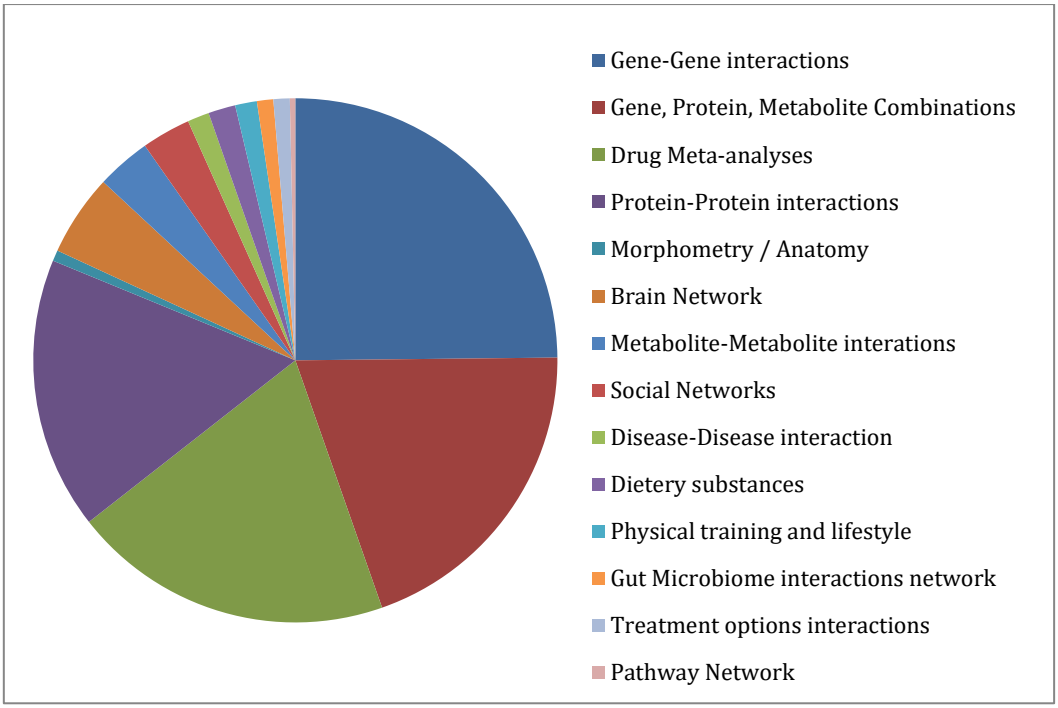

Figure 2.1: Types of T2DM network models. Types of T2DM network models and their proportions amongst selected publications based on the source of data used for the study.

Table 2.2: Networks observed in the literature classified according to their causality and their quantitative or qualitative nature

Classification according to the data source	Classification according to the data type		If causal		Qualitative or Quantitative	
	Causal	Correlational	Predicted Causal	Demonstrated Causal	Boolean or Qualitative	Quantitative
Gene-Gene interactions	1	73	1	0	73	1
Gene, Protein, Metabolite Combinations	12	47	12	0	59	0

Drug Meta-analyses	0	59	0	0	59	0
Protein-Protein interactions	6	44	6	0	49	1
Morphometry or Anatomy	1	1	1	0	1	1
Brain Network	0	15	0	0	15	0
Metabolite-Metabolite interations	1	9	1	0	9	1
Social Networks	2	7	2	0	9	0
Disease-Disease interaction	0	3	0	0	4	0
Dietery substances	0	5	0	0	5	0
Physical training and lifestyle	1	2	1	0	3	1
Gut Microbiome interactions network	0	3	0	0	3	0
Treatment options interactions	3	0	0	3	0	3

Pathway Network	0	1	0	0	1	0

2.2.4 Classification based on purpose: The use of the network model was considered to classify the publications. These 7 types of purposes and respective examples of network models are explained here in brief.

1. Identifying novel disease markers:

   Most of the shortlisted network models were based on gene-gene interactions and most of these gene-gene interaction network models were developed in order to make GWAS studies more useful in identifying important disease markers (Hale et al., 2012) and drug targets (Vaquero et al., 2012) for T2DM.

   On similar lines, several protein-protein interaction studies were devoted to identify proteins whose expression is altered in T2DM and related diseases (Padilla et al., 2014) or potential targets for treatment (Wang et al., 2015b). The gene-gene interaction networks focussed on identifying genes that are linked to more than one diseases (Dong et al., 2014). Even protein-protein interactions were used to identify genes common to two different diseases with T2DM being one of them (Lepedda et al., 2016).

2. Prioritizing Disease genes:

   Gene identification was started with the hope of finding a handful of genes that individually or in combination regulate the salient features of T2DM. This turned out to be another problem in itself with more and more GWASs identifying a growing number of genes. This propelled studies that built methods to prioritize these genes based on their importance in being associated with T2DM. Along with gene-gene interaction and protein-protein interaction data, gene-protein-metabolite composite networks were also used in prioritizing candidate target genes (Chen et al., 2013).

   An attempt at prioritizing genes was done using Differential Expression Networks (DENs). Instead of the traditional use of differentially expressed genes, Sun and colleagues (Sun et al., 2013) used differentially expressed interactions

(or edges in a network). Their network was composed of the interactions between the differentially expressed genes as well as the corresponding genes. The traditional method picks out differential genes individually and hence ignores their interactions and dependencies. The new method does not have that shortcoming. The authors compared DENs across three different tissues – adipose, muscle and liver and found that metabolic dysfunction is maximally observed in liver. Furthermore, they compared the DENs in the three tissues across 4, 8, 12, 16, 20 week time points in rats. They compared the expression patterns of genes involved in the insulin resistance pathway and found that insulin hyper-secretion precedes insulin resistance. The question whether hyperinsulinemia is a cause or a consequence is debated for long in T2DM research (Corkey, 2012) and recent experiments hint towards hyperinsulinemia being the cause rather than the consequence (Shanik et al., 2008) thus fortifying the results by Sun and colleagues.

Using the STRING repository, Vitali and colleagues (Vitali et al., 2013) constructed a T2DM protein-protein network and designed a method to rank the potential drug targets. The ranking is based on the reachability of the node, its global effect (i.e. the effect that node has on the other potential drug targets in the network) and its synergistic effect (i.e. the effect of that target on the network in synergy with 2 other drug targets). Using this ranking method, they identified potential drug targets from the network. Insulin-like growth factor 1 receptor ranked first and the other targets' scores were not even close.

A more sophisticated model to enrich or prioritize disease genes was developed by Wang and colleagues (Wang et al., 2015a). The model used the KEGG database to construct directed regulatory biological networks. The network contains all the genes that were previously known to be associated with the disease of interest (T2DM is one of their case-studies). The authors defined control paths, which are chains of interactions of nodes that can lead the network system to a diseased state in a time dependent manner. They calculated the perturbation influence of the disease genes which depends on the number of downstream nodes affected by perturbing the gene of interest. This index was then used to enrich the genes associated with the disease phenotype.

3. Understanding interactions:

In one of the initial attempts to study gene-gene interactions, Banerjee and Bose (Banerjee and Bose, 2008) proposed a minimalistic two-gene network model where the two genes activate each other's synthesis processes and one of them also has an auto-repression loop. Using kinetic equations, the authors ran simulations with these two genes which form the core beta-cell transcriptional network. They also mimicked situations where one of these genes has a mutation and explain the development of Maturity Onset Diabetes of the Young (MODY). The model is useful in isolated systems and forms the basis for modelling more complex cases. With only two genes involved, this model does not consider the interactions of these two genes with the others, which might play an influential role in determining the outcome of the two-gene interaction.

A pathway network includes relationships between different pathways. It gives a broader image of the dialogue within a tissue or multiple tissues. With this in view, Gao and colleagues (Gao et al., 2012) combined multiple gene expression and quantitative trait measurements to determine the state of a pathway in each tissue. After doing this for all the pathways considered in the study, they constructed a pathway co-expression network. After doing topological analysis, they identified gluconeogenesis / glycolysis in the liver and insulin signalling pathway in the muscles to be the most important pathways since they showed the highest degrees and the betweenness centralities. Two more variables, age and strain (obese or lean), were added to the study (gene expression data were taken at 4 weeks and 10 weeks of age from both the strains). They found that along with pathways involved in metabolism, oxidative stress interacts with many other pathways in all the different groups. In case of 10 week diabetic mice (mice that became diabetic at or before 10 weeks), inflammation pathways were relatively more interactive. This study highlighted the role inflammation and oxidative stress pathways in T2DM.

Do and colleagues (Do et al., 2015) tried to identify the trans-boundary relationships between the metabolites present in saliva, blood and urine. They listed out metabolites of interest and calculated the partial correlations between them. Then they overlaid the 3 networks (the 3 biofluids) of partial correlations

and observed the similarities. They also calculated the partial correlations between the same metabolite across the 3 fluids. They found higher overlap of within-fluid correlations between plasma and urine than plasma and saliva and saliva and urine. Also, more plasma-urine correlations were observed than the other two combinations.

Considering that only metabolite related information of a patient is insufficient, Qiu and colleagues (Qiu et al., 2016) constructed an ancestry-specific (from information on ancestry) human phenotypic network which was centred on T2DM. They believed that analysis on the all-inclusive human ancestral information can be misleading. What they finally showed is that different populations have different phenotypes associated with T2DM and thereby the pathways involved in the pathogenesis of this disease are different for these different populations.

4. Identifying components of medicines and their effects:

   Of particular interest is the study where Luo and colleagues (Luo et al., 2013) constructed a directed (causal) T2DM network with proteins, metabolites and their associated drugs using the STITCH database. The algorithm they used links proteins to each other based on the directed edges. The edges are of two types – positive and negative and hence the downstream node is either up-regulated or down-regulated. They have T2DM as a node in the network. Based on the up- or down-regulation of that node, they pick out targets that lead to T2DM and highlight the drugs that affect these targets. The signal transduction process in this network is not time dependent – all the nodes are linked at the same time and hence, there is no transduction of signals, *per se*. Another drawback of the method is that they have not considered feedbacks in the network. Feedbacks are extremely important components of physiology, which if ignored may give highly misleading picture. If a node has a feedback loop, the algorithm ignores it leading to loss of perturbation information. Using this method, the authors identify important components of a traditional Chinese medicine and their underlying mechanisms. Later in 2016, another similar study was conducted using pure protein-protein interactions (Gong et al., 2016).

Using the STRING (protein-protein interaction) database, Perez-Lopez and colleagues (Perez-lopez et al., 2015) constructed a human interactome for T2DM. They measured the propagation of perturbations in this interactome and also listed the side-effects of the various drugs used to treat T2DM. Their results show that drug targets were better propagators of perturbations than other non-target proteins. Moreover, drugs that had side-effects were better propagators of perturbations than those having no side-effects. This also reflected in the centrality of nodes. The nodes in T2DM network had lesser centrality when compared to protein-protein interactions for other diseases. This study does not pin-point any specific targets for treatment of T2DM but gives features of the networks that have higher chances of identifying such targets within them.

Further studies included use of gene-gene interactions to observe a network-wide effect of certain dietary substances (Wang et al., 2015c) and traditional medicines (Yang et al., 2015).

5. Association of T2DM with non-bodily chemicals:

Studies based on diets and their meta-analyses were included in this class. Some of these papers said that the results are inconclusive with the current data available and hint at the requirement of more studies (Carter et al., 2013) and some others were just study protocols without actual data (Schwingshackl et al., 2016). Recently, gene-protein-metabolite interactions were used in identifying converging mechanisms that link some common organic pollutants to T2DM (Ruiz et al., 2016).

A different approach using non-bodily chemicals was used by Taboureau and colleagues (Taboureau and Audouze, 2017) to identify associated diseases. They listed chemical contaminants and their associated diseases from The Distributed Database. They constructed a network out of those associations. Based on the contaminants shared by two different diseases, they assigned a score for the disease pair. A minimal network was then constructed consisting of only the significant disease-disease associations. They claimed that such a network can highlight uncharacterized associations between different diseases.

6. Diagnosis of T2DM:

Li and colleagues (Li et al., 2016) tried to further subtype T2DM patients based on medical records and genotypic similarities. They constructed a network with associations between different genetic variants and different diabetes-related disease phenotypes. They could identify 3 different subtypes – 1) characterized by diabetic retinopathy and neuropathy; 2) cardiovascular diseases and cancer malignancy and 3) neurological diseases, cardiovascular diseases, HIV infections and allergies. They also identified SNP markers for these 3 subtypes.

As gut microbiome studies started showing differences in diabetics and non-diabetics, they were incorporated in diagnostic assays too. In a classical study by Mahana and colleagues (Mahana et al., 2016), gut bacterial association networks were used to identify differences between control and mice fed with antibiotics. They showed a difference in network topology between the two groups. The control mice gut microbiome network was more robust in the sense that the clusters in the network resembled the naturally occurring microbial phyla. The antibiotic treatment disrupted these clusters and new ones were formed. They identified specific developments like predominance of a certain taxon after antibiotic treatment. Another variable was introducing high fat diet (HFD) to both these groups. Even HFD led to disruption of the clusters observed in the control group but the level of disruption was not as great as that of the antibiotic group. The authors then mapped these clusters to different metabolic diseases (insulin resistance and non-alcoholic fatty liver disease) the host could develop and tried to determine if the network structures could help predict the development of those diseases before-hand.

Apart from using metabolite-metabolite networks for metabolite and ionic profiling of T2DM subjects (Sun et al., 2012), they have been used to make better and earlier diagnosis of T2DM (Carter et al., 2016).

Brain network does not necessarily imply neuronal connections but it is more of an association network of different regions in the brain. The association is based on the regions of the brain that are active simultaneously in a given brain state. The default state of the brain is called the wakeful resting state. The associations

between the different regions of the brain in this default state together forms the Default Mode Network (DMN). Most of the studies here compared the DMNs of healthy and diabetic individuals and they found topological differences between the two (Chen et al., 2016; Yang et al., 2016a). Some studies showed differences in networks other than DMN like left frontal parietal network, sensorimotor network (Chen et al., 2015) and cortical white matter network (Zhang et al., 2016) between healthy and diabetic individuals. It is also noted that there is loss of small-world architecture of the brain network in diabetic patients (Zeng et al., 2015). Moreover, diabetics with poor glycaemic control had lower network efficiency and longer path lengths in the brain network (Kim et al., 2016) and the loss of functional connectivity in several DMN regions was associated with insulin resistance too (Xia et al., 2015a). Impaired connectivity is associated with duration of T2DM too (Yang et al., 2016b).

Although most of these studies propose that disruptions in the brain network can be the reason behind cognitive impairment in T2DM, another recent study has shown altered connectivity in the brain network of diabetics even without cognitive impairment (Yang et al., 2016b). Cross –sectional data alone cannot explain what leads to what. Is T2DM causing the disruptions in the brain network or is it the other way around. Bussel and colleagues (van Bussel et al., 2016) studied brain functional networks of diabetics as well as pre-diabetics and had an interesting result. The brain functional network is mildly disrupted in pre-diabetics and to a relatively greater extent in diabetics. But the clustering coefficient and the local efficiency were seen to be increased in the both diabetics and pre-diabetics. They hypothesize that this particular re-organization of the network is a compensatory mechanism for the brain to make up for the cognitive losses. Another group that looked at brain connectivity differences in obese (glucose tolerant and non-glucose tolerant alike) and lean subjects during fasting and fed states found that the fasting brain hypothalamus connectivity with the pre-frontal cortex was stronger in obese versus lean individuals. Feeding dampened the connectivity in the lean subjects while there was no effect in the obese ones. Moreover, there wasn't any difference in the connectivity between glucose tolerant and non-tolerant obese subjects (Lips et al., 2014).

Cerebral blood flow (CBF) is associated with cognitive scores. A decrease in CBF was observed in T2DM patients and more so in patients with poorly controlled blood pressure in the regions of DMN (Xia et al., 2015b). The authors also mention that hypertension is associated with decreased CBF and low blood pressure with increased CBF. One can hypothesize that increase in blood pressure is a mechanism of the brain to increase the CBF to make up for the cognitive impairment. Longitudinal and brain region-specific studies need to be conducted to convert the associations into causal relationships.

7. Management of T2DM:

   Management of T2DM works at different levels like prevention, treatment through drugs, sharing information and experiences and studying demographics.

   The basic therapy for both prevention and treatment is physical training. This includes meta-analyses trying to determine the relative importance of certain physical training regimes and behaviours. Pillay and colleagues (Pillay et al., 2015) looked at about 161 different behavioural programme studies for T2DM and concluded that different self-management education programmes were ineffective in lowering the HbA_{1C} levels (≥ 0.4%) on their own, but could do that in combination with support programmes (clinical, behavioural, psychosocial or educational programmes).

   A meta-analysis gives the association between different parameters under study. A network is constructed with these parameters as nodes and their correlations with each other as links. Such a network meta-analysis (of 14 trials including 915 participants) focusing on physical training regimes (resistance training and aerobic exercises) concluded that a combination of both gave the best results with respect to reduction in HbA_{1C} levels (Schwingshackl et al., 2014).

   In case of treatment of T2DM, Goede and colleagues (Goede et al., 2016) helped determine exact dosage of insulin for a patient based on the patient's diet, blood glucose, etc. The authors argued that the plasma glucose measurement time intervals of 15 to 60 min do not provide an adequate resolution to visualize the detailed dynamic events. They built an electric circuit model which mimics the physiological process of glucose homeostasis starting from meal ingestion,

absorption to the cross-talk between the liver, pancreatic alpha and beta cells. 5 min interval glucose measurements were used as input data for the model. The model could accurately determine the insulin dosage needed for the particular patient. Another effort to construct a similar model to determine insulin dosage was developed using a causal probabilistic network (Tudor et al., 1998).

Although some studies concluded that there was no significant difference between the efficacies of various anti-hyperglycaemic classes (Gross et al., 2011), most of the others hint at combination therapy being better off than single drug treatment (Man et al., 2016; Orme et al., 2014) or in combination with diet and exercise (Stevens et al., 2015). This might also be due to the failure of one drug after a certain period of time (DeFronzo et al., 2013). Some papers studied the effect of certain specific drugs (Fahrbach et al., 2016) and others compared the adverse effects of drugs and their efficacy (Sun et al., 2015) and also their cost-effectiveness (Schubert et al., 2017). Lastly, a few papers are actually protocols suggested to conduct such network meta-analyses (Schubert et al., 2017).

Publications with just the term 'social network' were deleted in the second round of selection but the ones which involved social network models were considered and are discussed here in detail. Ninomiya and colleagues (Ninomiya et al., 2017) demonstrated for the first time an association between psychosocial factors and diabetic nephropathy. They measured psychosocial condition of diabetic patients using different indicators and concluded that high level of social support was associated with lower risk of diabetic nephropathy. Using a network approach, they found out that out of the different indicators they measured, two associations were the strongest – happiness score and social support; and happiness score and optimism score. In general, social support is crucial in patient health care.

Other studies involved a novel data mining approach, where Akay and colleagues (Akay et al., 2015) collected information, from a public forum of diabetic patients, of the use and experience of the drug sitagliptin. The forum had users sharing their experiences (both positive and negative) about the drug and authors constructed a network of the flow of information among the different users. They identified influential users and proposed that such studies can help

gather better healthcare information. Another study looked at socio-spatial knowledge networks in a multi-ethnic rural society. The authors collected geographical information regarding a patient's clinic, healthcare providers, social gatherings, etc. and where healthcare knowledge can be improved for different target ethnic communities (Cravey et al., 2001).

2.3 Limitations of network models so far

1. Causality: The network models so far have used data based on correlations to extract the links for the network. If causal links are used, they are mostly predicted causal. Very few studies have used demonstrated causal links (Goede et al., 2016; Tudor et al., 1998).
2. Data restriction: Different levels that show a cross-talk amongst them are genes, transcription factors, proteins, lipids, hormones, enzymes, tissues, organs, neurons, behaviours and mental states. Interactions among these different levels might be important in the pathophysiology of a disease. But network models commonly use a single level. Malpique and colleagues (Malpique et al., 2014) used three levels namely genes, proteins and metabolites which is the upper limit so far. In the case of complex diseases, where a single gene, protein or organ cannot be said to be the unique cause of the disease, a multilevel approach might give greater insights.
3. Although some of the network models attempt to evaluate targets or treatments, they stop at glucose control as the only end point. It has been already demonstrated that controlling glucose levels does not reduce the complications of T2DM (Max Miller et al., 1976; Stratton et al., 2000; Turner et al., 1998). Therefore, this marker is inadequate and better system-level indicators of reversal are required.
4. Do not explain the flaws and paradoxes of T2DM: The studies are focussed on finding novel targets for treatment, but do not address the primary concern, i. e. the flaws and paradoxes in the classical theory of T2DM. Every new approach should be able to address these flaws and adequately explain them. Understanding the underlying mechanism of T2DM itself could be the best

objective of a model. Once the disease process is better understood, potential treatment options will surface soon.

2.4 Objectives of this thesis

1. Explain the irreversibility: Even though none of the individual processes in the pathophysiology of T2DM are irreversible, T2DM is considered to be irreversible (Watve, 2013). A good model of T2DM should provide adequate explanation for the irreversibility.
2. Explain flaws and paradoxes: The flaws and paradoxes are mentioned in Chapter 1: Section 1.4.1. A new approach should be able to explain these flaws and come up with an alternative theory.
3. Examine whether reversal is possible: After understanding why T2DM seems irreversible, we need to find if it can be permanently or durably reversed by using an effective approach.
4. Identify novel promising treatment options: If reversal is possible, novel targets need to be identified that can do so. New approaches to prevent, control or potentially 'cure' T2DM is a desirable outcome of a comprehensive model. These targets have a better chance of proving useful since they have been extracted using a new approach that satisfactorily escapes the prevalent flaws and paradoxes.

Since T2DM involves multiple organs of the body, it is necessary to include them in the model. They are classically considered effects, but may actually have a causal role in the process.

As we saw in this chapter, networks have been used in various aspects associated with T2DM starting from the basic understanding of the disease initiation and progression to its efficient diagnosis, treatment and management. In each of these aspects described above, there is consistent growth in the gain of knowledge and thereby, the understanding of T2DM. Networks, with their capacity to integrate a huge number of components and their interactions have now become a crucial part of analysis of any study. The literature studied here has only a few demonstrated causal models while a

large percentage is the predictive causal models. Such models do carry an advantage when the kind of input data you require is not present in the literature. But in case of T2DM, after decades of research, a large number of interactions are already studied and these demonstrated links can give better results than the ones based purely on predictive causality.

We know that neurotransmitters (Thoa et al., 1972) and hormones (Calcagnoli et al., 2013) affect behaviours on one hand and also interact with enzymes (Rubí et al., 2005) and metabolites (Cincotta et al., 1997) in the body. The interactions known today are beyond the conventional gene-gene and protein-protein interactions and many other types of parameters affect and are affected by them. In this regard, networks comprising behavioural, endocrine, neuronal and metabolite factors together hold a better promise in understanding the underlying problem. In light of serious challenges posed against the classical theory, such broad-level networks may give rise to interpretations which are at a substantial deviation from the classical theory.

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Chapter 3

Materials and methods: The network model

This chapter gives a detailed rational and methodology for the construction of the T2DM network and an algorithm which helped make perturbations to the constructed network. This chapter also deals with the sensitivity analysis done to figure out under what conditions the major result of the model, i.e. the network bi-stability, is robust and thereby appreciate the limitations of the model too. The possible applications of the network model and their results are explained in Chapter 4.

3.1 The need of a holistic network

The network literature study in Chapter 2 showed that there are very few demonstrated causal networks. Most of the networks use bioinformatics tools or databases which either give associations between the components or a predicted causality between them. Moreover, these networks usually consider only a single level, for example, a gene-gene interaction. Data acquisition is comparatively easier in this case given the vast range of bioinformatics databases and efficient tools for data extraction. We know today that multiple levels come into play to establish a well-orchestrated regulation of any system in the body. Even in case of a disease, this co-ordination is disrupted, but nonetheless, it still exists. Trying to understand a disease with all these levels together gives better promise of predicting its cause and finding ways to treat it. What has not been shown earlier is a network of demonstrated causality between the nodes across multiple such levels such as hormones, enzymes, neurotransmitters, tissues, behaviours, cytokines, etc. Data acquisition in such a case is not easy since no single database exists where all these nodes and their demonstrated interactions have been listed. Data needs to be gathered using generalized search tools and evaluated and compiled manually.

The understanding of T2DM appears to have hit a wall. T2DM as a condition is classically considered irreversible although all of its individual component processes are reversible. Many new genes and alleles associated with T2DM are discovered everyday adding intricate details to the already vast and complex picture but a comprehensive understanding of the pathophysiology of T2DM still remains remote (Watve, 2013).

I have tried to approach this situation with a network based on demonstrated causality and consisting nodes from multiple levels including hormones, enzymes, neurotransmitters, tissues, behaviours, cytokines, etc. The main aim of this thesis is to address the anomalies and missing links in the field. Identification of novel targets and approaches to prevent, control and reverse T2DM is a secondary aim. Networks can be more appreciable if we can find these answers here.

3.2 Identifying nodes and links of the network

3.2.1 Identification of nodes: I started with the classical theory of T2DM involving the 3 main variables classically believed to be central to T2DM namely plasma insulin levels, insulin resistance and plasma glucose levels. I then searched literature for signals that affected or were directly affected by one or more of the three (direct effectors) and further for signals that affected or were directly affected by the direct effector signals (indirect effectors). Since specific behaviours are also known to trigger certain hormones and growth factors among the direct effectors, behaviours were also included in the list of signals. Thus, my definition of signals includes nutrients, metabolites, hormones, growth factors, cell populations, behaviours and neuronal signals (Figure 3.1).

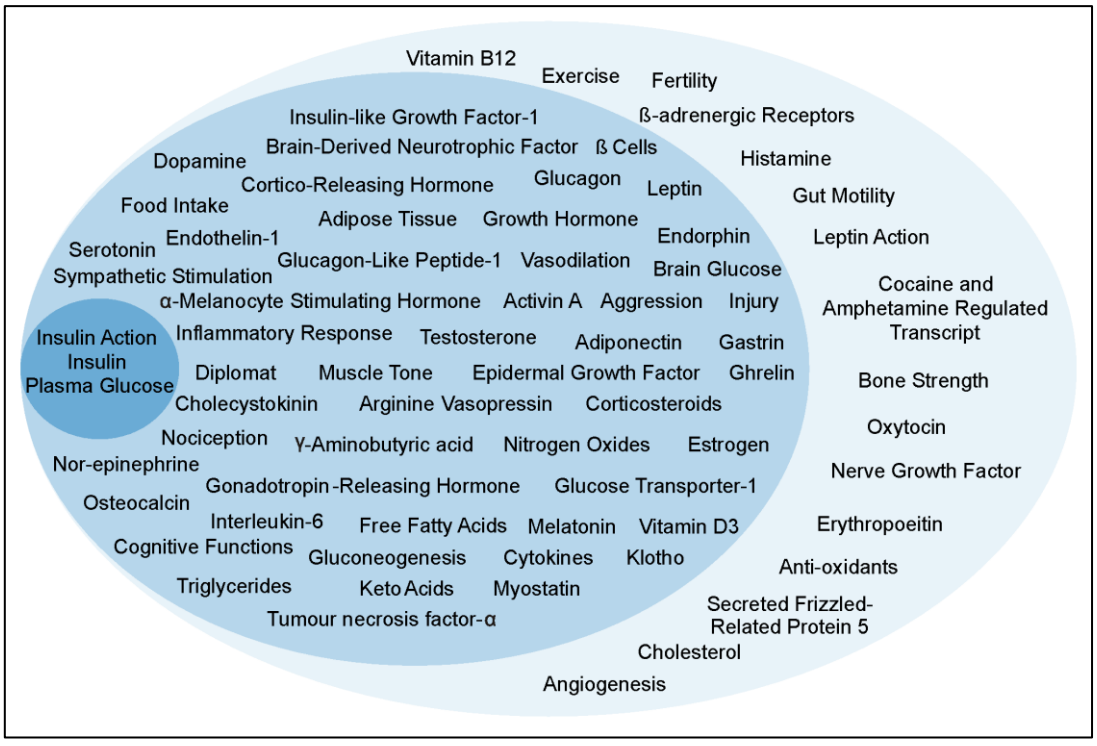

Figure 3.1: Signals in their respective tiers. First tier (innermost circle) includes players classically believed to be central to T2DM. Second tier (intermediate whorl) includes the players that directly affected or were directly affected by the players in the first tier. The third tier (outermost whorl) included players that affected those in the second tier or were affected by them. Reproduced from (Kulkarni et al., 2017).

3.2.2 Inclusion and Exclusion criteria: After listing a large number of possible interactions, I applied the following inclusion and exclusion criteria and redundancy filters.

1. Since the focus was on signalling between cell types and organs, I excluded strictly intracellular pathways. I treated the cells and organs as black boxes but accounted for all signals going in and out. Complex signals and pathways do exist within a cell but their net outcome is reflected in the cell's interaction with rest of the system. It is this part of the system that the model addresses.
2. If two or more signals shared the same upstream signal/s and the downstream effect/s, they were merged into one. From a known linear signalling pathway, only one molecule was listed. Components of inflammation like macrophage accumulation and reactive oxygen species were merged into one due to similar effects they show on the rest of the molecules in the network. Gonadotropin-releasing hormone is responsible for the release of Leutinizing hormone and Follicle stimulating hormone and hence they were grouped under the former. Growth factors were grouped together with the exceptions of Nerve growth factor (NGF), Insulin-like growth factor 1(IGF-1) and Epidermal growth factor (EGF) who deemed a different node. All these exceptions have a set of specific interactions, not displayed by other growth factors in the network. NGF up-regulates Brain Derived Neurotrophic Factor (BDNF) (Tirassa et al., 2003) and nociception (Gearing et al., 2013). IGF-1, along with the other classical growth factor-related interactions, has a positive effect on muscle mass (Barton-Davis et al., 1998), bone strength (Yakar et al., 2002) and a negative effect on growth hormone (Yamashita and Melmed, 1987). EGF has a positive effect on BDNF (Tirassa et al., 2003) and fertility (Tsutsumi et al., 1993a) and a negative effect on nociception (Andres et al., 2010). Myokines were grouped under cytokines except for BDNF, Secreted Frizzled Related Protein 5 (SFRP-5) and Interleukin 6

(IL-6). BDNF is involved in improving cognitive functions (Gray et al., 2006). SFRP5, unlike the other cytokines, decreases inflammation (Ouchi et al., 2010). IL6 plays an additional role in up-regulating glucagon-like peptide 1 (Kahles et al., 2014) and reducing aggression (Alleva et al., 1998). However, if there was a branching point in a pathway, it was listed as a signal. BDNF, SFRP5 and IL6 are branching points since they show some effects not shown by the other cytokines.

3. Only the signals having both upstream and downstream effects from other nodes of the network were included with the exception of a few important ones. Signals β-adrenergeic receptors, leptin action, melatonin, vitamin D3, exercise and vitamin B12 have downstream effects in the network but do not have an upstream link reaching them from the network. These nodes were deemed important either because of their involvement in the classical theory as in the case of β-adrenergeic receptors and exercise, or their significant effects on insulin sensitivity as in the case of vitamin D3 (Borissova et al., 2003) and melatonin (de Oliveira et al., 2012). Leptin resistance is argued by a few to be an important player in obesity and metabolic syndrome and hence, it was included in the model (Zhou and Rui, 2013).
4. The receptors of all the hormones included in the network were grouped along with their respective hormones expect for insulin and leptin. Insulin resistance and leptin resistance are observed in T2DM and they are phenomena that happen at the receptor level and not at the hormone production level. Hence, insulin action and leptin action are separate nodes and require a discrete place in the network.
5. If a given signal had different actions in different organs they were considered different nodes. For example, glucose in blood and that in the brain were treated as separate nodes. GABA in pancreas and in CNS has different effects on the other nodes in the network. Hence, they were considered are two separate nodes.

3.2.3 Identification of links: All the signals have a functional meaning. So, a down-regulation means loss or decrease in the signal. Whether it is because of structural change or any other change is considered irrelevant. The source data to extract possible

interactions amongst the listed signals were publications reporting interventional studies giving causal evidence for a positive effect (up-regulation) or a negative effect (down-regulation) of a given signal on another signal of interest. All searches were made in 'Google Scholar' and 'BioMedNet' using the name(s) of the target nodes and "regulation of", "expression of" and "affected by" as key words. Correlations and associations were not considered as evidence for an interaction. All published interactions were treated with equal weighting. No weighting of interactions was done by number of studies / publications, validation, reliability, impact factor or level of current acceptance. Since most of the interventional data comes from non-human species, I included all experiments with humans, rodents or other mammalian hosts (see Appendix I for model organisms used in the reference for each link).

Finally, 330 interactions among 72 signals were identified from 493 publications and incorporated in the model. A network was constructed using these signals and interactions (Figure 3.2). All signals were treated as organ-specific nodes and the interactions formed the directional links (in the network) between these nodes.

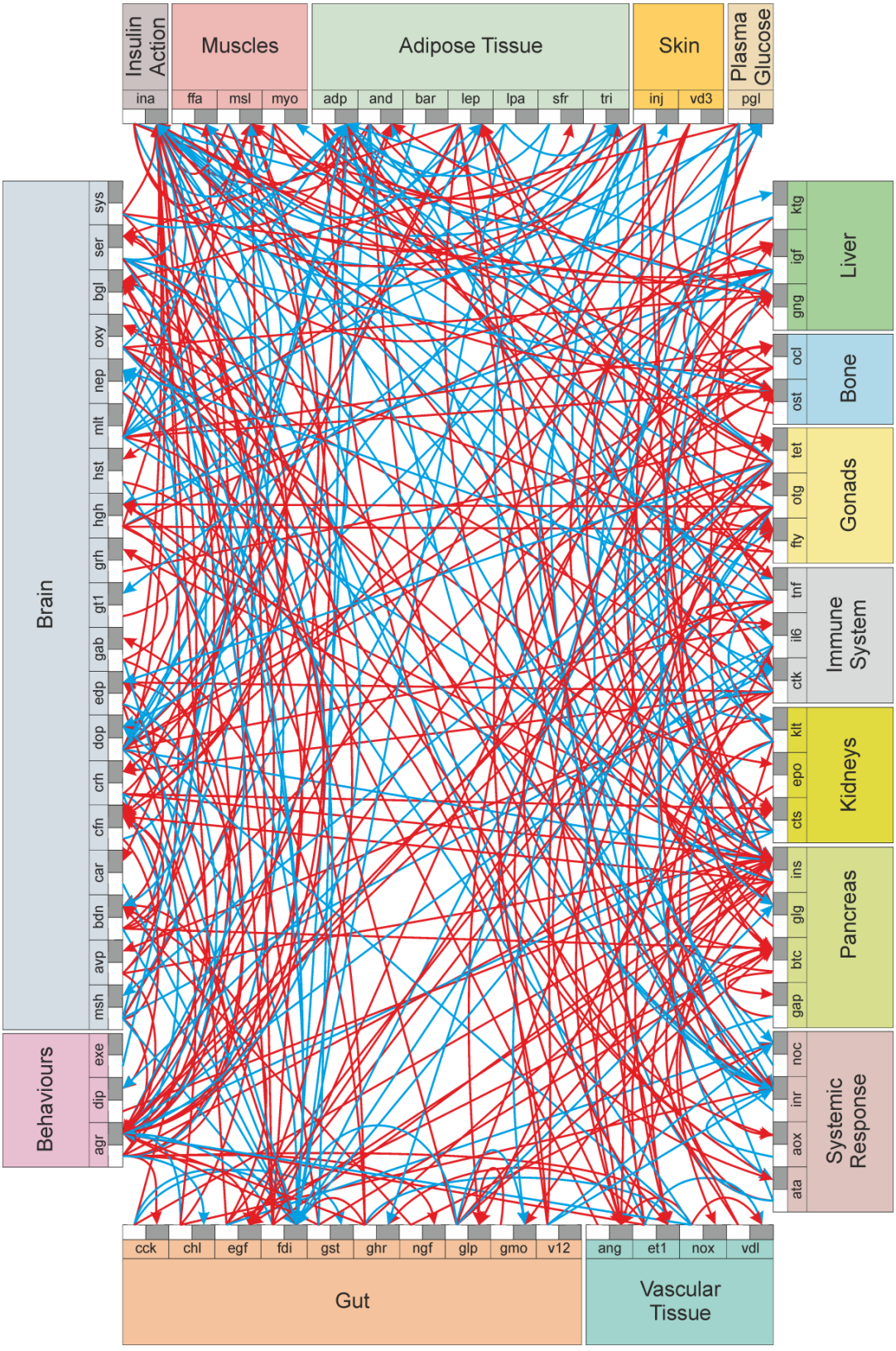

Figure 3.2: The inter-organ signalling network involved in the pathogenesis of T2DM. Each organ (coloured rectangles) displays the signals it produces. The outbound (white rectangle) and inbound (black rectangle) portals for each signal are shown. Red

arrows indicate up-regulation interactions and cyan, down-regulation interactions. Three letter codes for the node as in Table 3.2. Reproduced from (Kulkarni et al., 2017).

3.2.4 Inclusion of behaviour in the network: As stated in chapter 2, most networks include genes or molecules as nodes. During data generation as described above, it was realized that there are certain links to behaviour. For example, insulin decreases risk taking or increases memory and cognitive function (Benedict et al., 2007; Kern et al., 2011). Although it may be uncommon to include behavioural characteristics as nodes in a physiological network, it is not unexpected for T2DM, since there are hypotheses relating behavioural and reproductive strategies to insulin resistance (Corbett et al., 2009; Watve, 2013; Watve and Yajnik, 2007). The following nodes related to reproductive and behavioural syndromes were incorporated in the model.

1. Physical exercise versus physical aggression: I differentiated the two nodes exercise and physical aggression for the following reasons. The node exercise, here, refers to calorie consumption aspect of exercise. This is the classical concept of exercise which is generally prescribed to counter T2DM. However, exercise also has a number of direct neuroendocrine effects which are less appreciated. Most active sports include components mimicking hunter-fighter behaviour including hitting, kicking, chasing, attacking, defending, etc. Apart from calorie burning, such exercises have many behavioural components that trigger specific neuro-endocrine signals. For example, running and chasing may be energetically the same but behaviourally and endocrinologically different. A number of studies show that insulin sensitivity can increase substantially by exercise even without loss of weight or total fat which demonstrates that exercise has direct effects on insulin sensitivity independent of weight loss (Belsare et al., 2010; Duncan et al., 2003; Heijden et al., 2010; Ross, 2003). Since the question whether the beneficial effects of exercise are through the energy pathway or through the behavioural pathway is important, I segregated the energy component and behavioural components of exercise as two different nodes namely ‘exercise’ and ‘aggression’. These two nodes were treated as separate and independent nodes with exercise having links to energy pathway and aggression having links to behavioural pathways.

2. Diplomat behaviour: Diplomat behaviour refers to social manipulation and the ability to do so. Physical aggression and diplomat behaviour play diametrically opposite roles in the pathophysiology of Type 2 Diabetes and related disorders (Watve, 2013; Watve and Yajnik, 2007). Insulin is shown to affect social behaviour in humans independent of glucose (Kern et al., 2001).

3. Fertility: Both male and female sex hormones and reproductive processes have links with insulin sensitivity. Since both oogenesis and spermatogenesis have similar links to insulin resistance syndrome, I included a single node fertility to make the model gender neutral. The node fertility refers to differences during gestation and otherwise (Barkley et al., 1979; Kurachi and Oka, 1985; Tsutsumi et al., 1993a)/ differences during lactation and otherwise (Neumann et al., 1993)/ proliferation of granulosa cells (Maillard et al., 2010)/ number of healthy embryos (Čikoš et al., 2010)/ oocyte quality (Kuro-o et al., 1997; Richards et al., 2012)/ embryonic development (Richards et al., 2012)/ litter size (Gill-Sharma et al., 1993)/ rate of abortion (Tsutsumi et al., 1993a)/ number of implantation sites (Gill-Sharma et al., 1993; Tsutsumi et al., 1993b, 1993a)/ uterine weight (Tsutsumi et al., 1993b, 1993a)/ ovarian atrophy (Kuro-o et al., 1997; Ohnishi and Razzaque, 2010)/ mammary gland size (Okamoto and Oka, 1984) or amount of milk produced (Okamoto and Oka, 1984) in females; and genital atrophy (Kuro-o et al., 1997; Ohnishi and Razzaque, 2010)/ testis size (Gill-Sharma et al., 1993)/ histology of testes (Gill-Sharma et al., 1993; Kuro-o et al., 1997)/ sperm count (Noguchi et al., 1990) or sperm motility (Breier et al., 1996) in males. Infertility is significantly associated with T2DM and a number of components of fertility are affected by insulin resistance and vice versa (Bener et al., 2009; Conn et al., 2000).

4. Insulin action and leptin action are two nodes in the network which represent the action of insulin and leptin at the receptor level. When the state value of these nodes is +1, they represent the insulin sensitive or the leptin sensitive state and when their state value is -1, they represent the insulin resistant or the leptin resistant states, respectively. Since there are different signals affecting the levels of these hormones and the resistant or sensitive state, both the hormone level and their action are considered different nodes. Both insulin and leptin

resistance are known phenomena in T2DM. Thus, the action node represents the resistance-sensitivity axis. This was not considered necessary for all other hormones.

5. Logical interactions – Some interactions were added which did not have a specific reference, but were obvious, logical or evident. For example, food intake increases plasma glucose, plasma free fatty acids, muscle energy, or fat storage are such obvious links. It is also logical that increased capillary density would increase the transport surface and thereby increase glucose transport. There were nine such logical interactions in the model for which references are not cited.

3.3 Topological properties of the network

Basic terminologies used to describe a network (Barabási and Pósfai, 2016) –

- Directed network: A network in which the links have a direction associated with them. The T2DM network is a directed one.
- Undirected network: A network in which the links have no direction, that is, they just show an association between the two corresponding nodes.
- Degree: The number of links connected to a node.
- Indegree: The number of links directed towards a node.
- Outdegree: The number of links originating from an individual node.
- Density: Density of a network is the ratio of the number of links to the number of possible links in the network (Wasserman and Faust, 1994).
- Path length: The number of links on a path between two nodes.
- Shortest path length: The path between two nodes with the fewest number of links.
- Diameter: The longest shortest path length in the network is called the diameter.
- Clustering coefficient: Clustering coefficient is defined as the ratio of the number of links between the node of interest and its neighbours and the total number of possible links (Aparicio et al., 2015).

- Closeness centrality: the mean geodesic (i.e., shortest path) distance between the node and all the other nodes reachable from it (Newman, 2005).
- Betweenness centrality: Betweenness centrality of a node is the sum of the fraction of all-pairs shortest paths that pass through it (Brandes, 2001).

Cytoscape (version 3.5.1) was used to calculate the network characteristics (Table 3.2). Cytoscape is widely used to construct and analyse biological networks (Su et al., 2014). Before the analysis of the network, one needs to clearly indicate whether the analysis should be done considering the network as a directed one or an undirected one. The kind of analysis used differs in both these settings. For the T2DM network, I used the settings used to analyse a directed network. Individual node characteristics and the inferences thereby are discussed in detail in Chapter 4: Section 4.3.1. This network is a single component which means that all the nodes are connected to each other, directly or indirectly. The average path length gives the speed of information travel in the network. More than 90% of the nodes are connected to each other directly, which means that the information travels pretty fast (within 10^1 time steps). A typical node in the network is connected to approximately 8 other nodes (irrespective of the direction of the link). There also exist multi-edge node pairs in the network which form a direct feedback loop.

The topological properties do not take into consideration the directionality of the network and hence are a snapshot of the network architecture. I focus more on the translational outcomes of the network which I get by making perturbations to this directional network. The perturbations will help me get a timely series of the changes in the network. This will be more useful in understanding the pathophysiology of the disease and identifying novel target nodes. I have used these topological properties to find out if any of these explain the results from perturbation simulations or at least correlate with them. This issue is discussed in Chapter 4 Section 4.3.1. Also, these properties give a sense of the complexity of the model to the reader.

Table 3.1 Topological properties of the network.

Characteristic	Value
Clustering coefficient	0.162

Network diameter	7
Shortest path length	1
Shortest paths	4615 (90.27%)
Average path length	2.989
Average degree	8.5
Network density	0.0645
Multi-edge node pairs	24

3.4 Perturbation simulations

I developed a perturbation algorithm for the network. The algorithm enabled me to select a specific node from the network and perturb it, i.e. change its levels in the network. This perturbation mimics the change in the concentration or mass or any other measure of the node in an organism. Once the levels of the selected node are changed, the effects of this perturbation are passed on to the downstream nodes. Their levels change in turn and the cycle continues. This algorithm was developed to study the possible causes of T2DM and also possible effective lines of treatment.

A combination of Microsoft Excel 2007 for data input (addition of links to the network) and output (network perturbation results) and Visual Basic Application for executing the links was used to construct a network perturbation model (See Appendix II for code). The signals were treated as nodes that can have one of three states namely 0 or baseline, +1 or up-regulated and -1 or down-regulated. Also, the directional links were of three different kinds namely up-regulatory or positive (which increased the state of the downstream node by 1), down-regulatory or negative (which decreased the state of the downstream node by 1) and basal level (which did not change the state of the downstream node). A zero signal here does not mean that there is no signal; it rather denotes that there is basal level signalling going on between the two nodes. Although the model considers only discrete states, it does not indicate extreme states. For example, -1 state of β-cell mass does not mean complete destruction of β-cells. In T2DM,

a substantial proportion of β-cells survives lifelong (Maclean and Ogilvie, 1955). Therefore, even in the -1 state of β-cells, insulin-producing capacity is not assumed to be completely lost; instead, it is subnormal.

After constructing the network, I studied the effects of different kinds of perturbations in the network. At the beginning, all nodes were at a default state of zero. Whenever a node was manually up or down-regulated, the state of that node changed to +1 or -1, respectively. All the directional links starting from that node were activated to change the states of the recipient nodes (first generation nodes). Subsequently, directional links from these first generation nodes were activated to change the states of nodes further downstream (second generation nodes). The event of activation of one generation of nodes was termed as a 'cycle'. Whenever a node received activated signals from more than one other node, the signals were added arithmetically to give a net signal strength. Based on the net positive or negative value of the signal strength, the state of the node was changed by +1 or -1, respectively; but without exceeding the state limits of -1 to +1. If the net signal strength was zero or normal in a given cycle, then the node returned to its normal default state. Thus, at any given time, the direction of change in the state of a node was solely determined by the net input signal. However, the step length for any change was restricted to unity, i.e. the state -1 could not become +1 in a single step.

Mathematically, the function of each node in every cycle can be explained as follows.

If $Si \neq 0$, then the downstream nodes of 'i' get activated.

$si = \sum eji \times Sj$; where 'S' is the state of the node 'i', 's' is the cumulative signal it received and 'eji' is the link pointing from node j to i.

Depending upon the cumulative signal, the node is assigned a state.

If $si > 0, Si(t) = Si(t-1) + 1$

If $si = 0, Si(t) = 0$

If $si < 0, Si(t) = Si(t-1) - 1$; where 't' is the cycle number

The state is then bound to limits -1 to +1

If $Si(t) \leq -1, Si(t) = -1$

If $Si(t) \geq 1, Si(t) = 1$

For example, to simulate the effects of primary hyperinsulinemia, the state of insulin in the starting cycle was made 1 where all the other nodes had a state of zero to mimic the basal level signalling across the remaining network. In the first cycle, the direct effects of insulin were executed. Hence, only those nodes that were immediate downstream of insulin altered their state to +1 or -1 depending upon whether they received up-regulation or down-regulation link, respectively, from the insulin node. In the current example, β-cells, leptin, klotho, EGF, cognitive functions, endothelin-1, gonadotropin -releasing hormone, nitric oxides and gut motility were up-regulated (state changed to +1); and keto acids and adiponectin were down-regulated (state changed to -1). In the second cycle, the immediate effects of these first generation nodes were executed. Thus in every cycle, the effects radiated, and because all the nodes lay in a network, in a few cycles, every node was affected in some way or the other. The recorded output was the state of each node after each cycle.

A stable state of a node was described as a consistent resultant state of the node which remained so throughout further cycles. Also, any perturbation from the state should lead back to the same stable state. If a node changed its state with a repeated cyclic pattern of a fixed periodicity throughout the cycles, it was termed as a node in stable oscillation. If a node changed its states with unpredictably altering periodicity, it was termed as a node in a chaotic state. The stable state of the system was defined as a state in which every node was in a stable state or in short term deterministic oscillations. Further, for the definition of a stable state, it was necessary that if the system was point perturbed starting with that particular state, it returned to the same state. If an apparently stable state obtained after one perturbation did not return to it after any other point perturbation, it was called pseudo-stable state. A chaotic state of the system was defined by one or more nodes being in a chaotic state. Whenever there were stable oscillations or chaos, the average of the last hundred cycles was taken as the 'mean final state' for a node.

3.4.1 Kinds of Perturbations: The time duration (in cycles) of a perturbation was set to unity, also called as a point perturbation. Here, I modified the perturbation algorithm to accommodate another perturbation time variant, a sustained perturbation. I used these two types of perturbations separately or in combination.

a. Point perturbations: After the perturbation was made in the starting cycle, the perturbed node came back to basal state after the first cycle; and then its state was allowed to be decided by the links it received eventually from other nodes.

b. Sustained perturbations: The state of a starting perturbation node was changed and the changed state was maintained independent of any link it received subsequently.

3.4.2 Null model simulation: A null model was constructed as a control to check if the results I get are an artefact of the perturbation algorithm. The null model had the same algorithm as that of the perturbation simulations and same number of nodes and links in the original model but the links placement was completely randomized. Every node received a random number of outgoing links form the range zero to ten. The downstream node for every link was randomized and could be any of the 71 other nodes. The positive or negative effect on the downstream node was also randomized between zero (down-regulation) and one (up-regulation).

3.5 Perturbation simulation results

3.5.1 Bi-stability after point perturbations: For all point perturbations, after 20 – 25 cycles, the system invariably reached a stable state. The changes in states of all the nodes after a point perturbation (up-regulation in insulin node) are shown in Figure 3.3. Simulations were run for 5000 cycles but those only up to 30 cycles are shown here. A stable state was achieved in less than 20 cycles and none of the nodes changed their states after this point. Additionally, this can be appreciated in Figure 3.4, where, after cycle number 20, there is no change in the network. Furthermore, there were only two observed stable states that the system reached. Either chaos or a homeostatic return to the starting (all zero) state was never observed in the system. The two stable states did not drift further after any point perturbation and were thus true stable states by definition. If instead of zero, starting states of all nodes were randomly assigned, the same two stable system states were obtained.

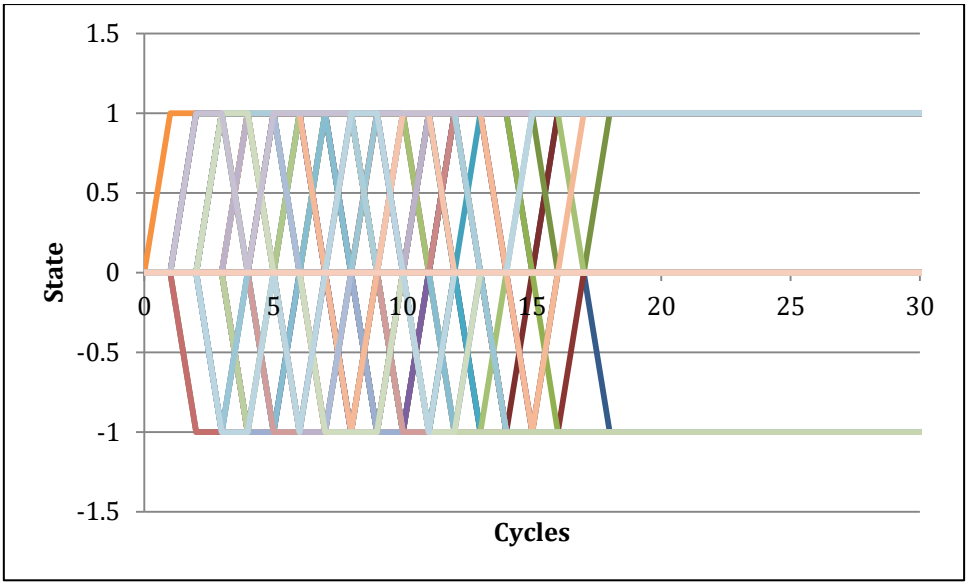

Figure 3.3: States of the nodes after a perturbation. The states of all the 72 nodes are plotted across time (in cycles).

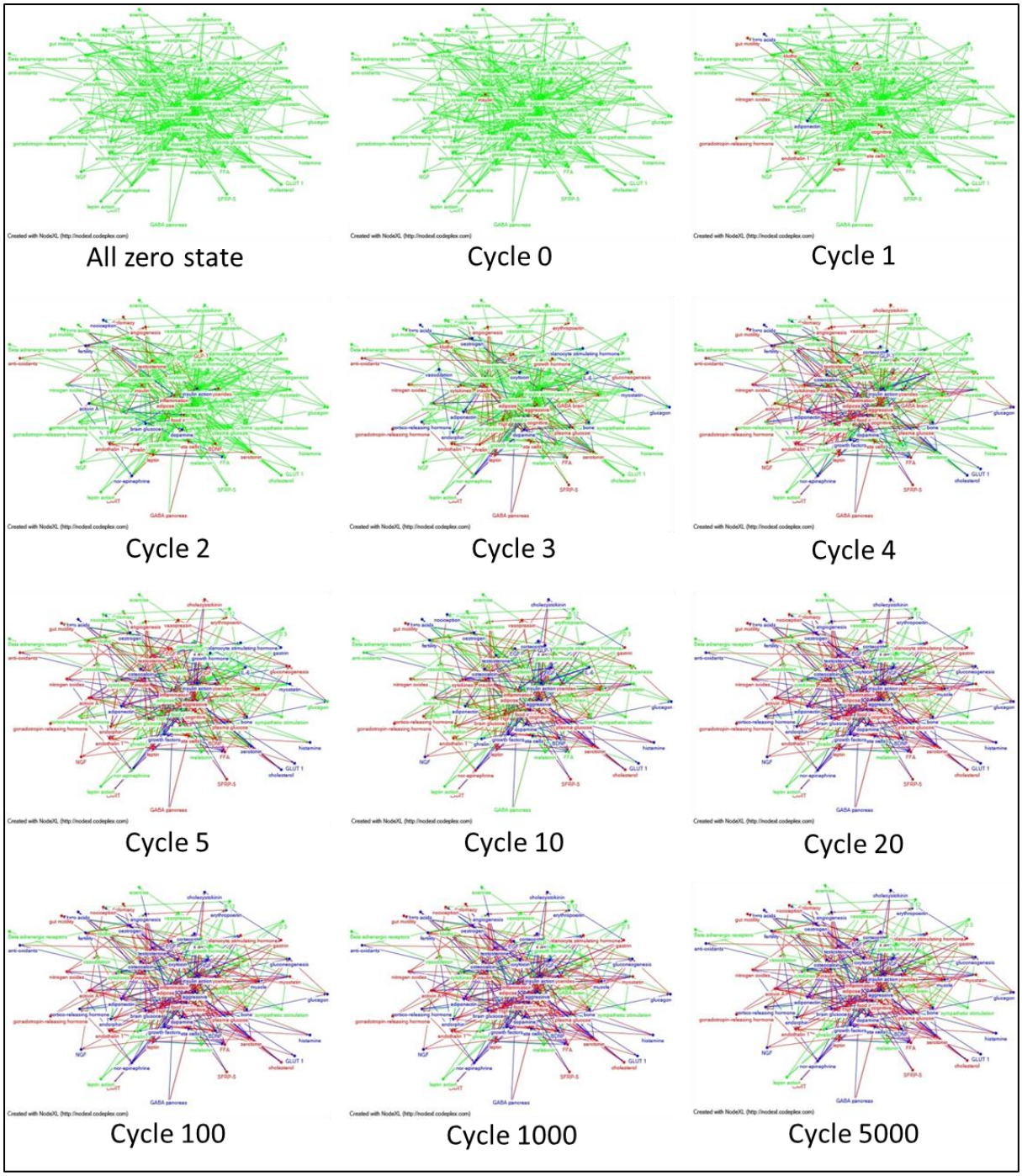

Figure 3.4: Perturbation of the network. Node insulin is up-regulated (manually perturbed) and its effect on the overall network is shown in terms of up-regulations and down-regulations of the different nodes and links. Every cycle shows the activated generation of nodes at that point in time. Perturbation is made in cycle zero, and the model was run up to 5000 cycles. Up-regulated nodes and links are shown in red, down-regulated in blue and basal level in green. NodeXL Basic (version 1.0.1.380) was used to construct these network diagrams.

In the two alternative stable system states, the states of all nodes including insulin action were stable, consistent and exactly opposite (in terms of +1 or up-regulated and -1 or down-regulated) to each other. For example, the state of any node A was +1 (positive) in one of the stable states and -1 (negative) in the other. Some of the nodes had a state of zero in both the stable states. This can be because those nodes received exactly equal number of positive and negative links in every cycle or because, as mentioned earlier, they did not have an upstream link from the network. Even then, these nodes which do not have an upstream link reaching them from the network, their contribution to the results is important since their own perturbation leads to either of the two stable states through their downstream nodes. Since insulin resistance is conventionally believed to be central to T2DM, I called the two stable states as insulin sensitive and insulin resistant attractors. The former was characterized by low adiposity, cholesterol, glucose levels and inflammatory markers; and high adiponectin. The latter had a diametrically opposite picture (Table 1). The nodes which, when up-regulated, led to the insulin sensitive attractor were collectively called the insulin sensitive basin of attraction and those which led to the insulin resistant attractor, when up-regulated, were collectively called the insulin resistant basin of attraction.

Table 3.2: Attractors for the point perturbations.

Serial Number	**Signals/ Nodes**	**Three Letter Code**	**State in the insulin resistant attractor**	**State in the insulin sensitive attractor**
1.	Activin A	*ata*	1	-1
2.	Adiponectin	*and*	-1	1
3.	Adipose Tissue	*adp*	1	-1
4.	Aggression	*agr*	-1	1
5.	α-Melanocyte Stimulating Hormone (α-MSH)	*msh*	1	-1
6.	Angiogenesis	*ang*	-1	1

7.	Anti-oxidants	*aox*	-1	1
8.	Arginine Vasopressin	*avp*	0	0
9.	β-Adrenergic Receptors	*bar*	0	0
10.	β Cells	*btc*	-1	1
11.	Bone Strength/ Bone Mass	*ost*	-1	1
12.	Brain-Derived Neurotrophic Factor (BDNF)	*bdn*	-1	1
13.	Brain Glucose	*bgl*	-1	1
14.	Cholecystokinin	*cck*	-1	1
15.	Cholesterol	*chl*	1	-1
16.	Cocaine and Amphetamine Regulated Transcript (CART)	*car*	1	-1
17.	Cognitive Functions	*cfn*	1	-1
18.	Cortico-Releasing Hormone (CRH)	*crh*	-1	1
19.	Corticosteroids	*cts*	-1	1
20.	Cytokines	*ctk*	0	0
21.	Diplomat Behaviour	*dip*	1	-1
22.	Dopamine	*dop*	-1	1
23.	Endorphins	*edp*	-1	1
24.	Endothelin-1	*et1*	1	-1
25.	Epidermal Growth Factor (EGF)	*egf*	-1	1
26.	Erythropoeitin	*epo*	-1	1
27.	Exercise	*exe*	0	0

28.	Fertility	*fty*	-1	1
29.	Food Intake	*fdi*	1	-1
30.	Free Fatty Acids	*ffa*	1	-1
31.	γ-Aminobutyric acid (GABA) pancreas	*gap*	-1	1
32.	γ-Aminobutyric acid (GABA) brain	*gab*	0	0
33.	Gastrin	*gst*	1	-1
34.	Ghrelin	*ghr*	0	0
35.	Glucagon	*glg*	-1	1
36.	Glucagon-Like Peptide-1 (GLP-1)	*glp*	0	0
37.	Gluconeogenesis	*gng*	-1	1
38.	Glucose Transporter-1 (GLUT-1)	*gt1*	-1	1
39.	Gonadotropin-Releasing Hormone (GnRH)	*grh*	1	-1
40.	Growth Hormone	*hgh*	0	0
41.	Gut Motility	*gmo*	1	-1
42.	Histamine	*hst*	-1	1
43.	Inflammatory Response	*inr*	1	1
44.	Injury (Growth Factors)	*inj*	-1	1
45.	Insulin	*ins*	1	-1
46.	Insulin Action	*ina*	-1	1
47.	Insulin-like Growth Factor (IGF-1)	*igf*	-1	1

48.	Interleukin-6	*il6*	0	0
49.	Keto Acids	*ktg*	-1	1
50.	Klotho	*klt*	0	0
51.	Leptin	*lep*	1	-1
52.	Leptin Action	*lpa*	0	0
53.	Melatonin	*mlt*	0	0
54.	Muscle Strength/ Muscle Mass	*msl*	-1	1
55.	Myostatin	*myo*	1	-1
56.	Nerve Growth Factor (NGF)	*ngf*	-1	1
57.	Nitric Oxide	*nox*	1	-1
58.	Nociception	*noc*	1	-1
59.	Nor-epinephrine	*nep*	-1	1
60.	Oestrogen	*otg*	-1	1
61.	Osteocalcin	*ocl*	-1	1
62.	Oxytocin	*oxy*	-1	1
63.	Plasma Glucose	*pgl*	1	-1
64.	Secreted Frizzled-Related Protein 5 (SFRP-5)	*sfr*	1	-1
65.	Serotonin	*ser*	1	-1
66.	Sympathetic Stimulation	*sys*	0	0
67.	Testosterone	*tet*	-1	1
68.	Triglycerides	*tri*	1	-1
69.	Tumour necrosis factor-α (TNF-α)	*tnf*	1	-1

70.	Vasodilation	*vdl*	0	0
71.	Vitamin B12	*v12*	0	0
72.	Vitamin D3	*vd3*	0	0

3.5.2 Null model simulation results: The bi-stability thus obtained is unlikely to be a statistical generality since a null model with the same number of nodes and links but with randomization of link placements rarely gave bi-stability. Out of 1000 null model simulations, 931 ended in a chaotic state. Stability was observed in 69 of them out of which, 12 showed a single stable state; 49 showed bi-stability, 4 showed tri-stability and the remaining 4 showed tetra-stability. The uncommon occurrence of bi-stability ($p < 0.05$) in the null model implies that the observed bi-stability in the network is unlikely to have arisen by chance alone.

3.5.3 Results for Sustained Perturbations: I perturbed each node singularly, in a sustained manner, and observed the downstream effects. Sustained perturbation of the nodes in the network did not affect bi-stability. A fraction of these perturbations led to stable short repetitive oscillations in the states of some nodes. Out of the 72 nodes, 49 sustained perturbations gave identical results as respective point perturbations. Remaining 23 sustained perturbations showed some changes in the attractor signatures as compared to their respective point perturbations. These changes were obvious since the system is not suspended or allowed to settle in case of sustained perturbations. The constant perturbation throughout the model run mimics a constant supply (in case of sustained up-regulation perturbation) or a constant deficit (in case of sustained down-regulation perturbation) of that particular sustainably perturbed node. This sustained force leads to deviations in the attractor signatures from the original result. Nevertheless, bi-stability was maintained in all sustained perturbations.

3.5.4 Combining Sustained and Point Perturbations: With each of the sustained perturbations in the background, every other node was point perturbed one at a time and simulations were run for a minimum of 300 cycles. Out of the 72 sustained perturbations, 61 led to bi-stability although the signatures of the attractors changed occasionally. These 61 included glucose, insulin and adiposity. This means that forcibly controlling any of these did not assure a healthy state. The state could drift depending

on other perturbations. This is compatible with the clinical results for treatments targeting weight loss, insulin signalling or plasma glucose reduction which show inconsistencies across studies and very limited evidence for effective reversal of the state (see chapter 1). In contrast, sustained up-regulations of ten other nodes gave rise to a single insulin sensitive attractor and these were aggression, adiponectin, dopamine, ghrelin, growth hormone, melatonin, muscle strength, oestrogen, osteocalcin, and testosterone. And sustained up-regulation of serotonin invariably led to the insulin resistant attractor. Sustained up-regulation of any of the 10 nodes or down-regulation of serotonin never allowed the system to become insulin resistant. Not only that, but aggression, dopamine, ghrelin, muscle strength, oestrogen and osteocalcin were able to completely reverse the states leading to the insulin sensitive attractor if the simulations began from the insulin resistant attractor as the starting condition. Hence, these nodes can be identified as potential therapeutic targets and are discussed in detail in Chapter 4 Section 4.4.

3.5.5 Clustering the nodes: Although with combinations of perturbations, the signatures of attractors could change, there were significant associations between the states of several nodes. I clustered the nodes based on Simple Matching Coefficient (SMC) between pairs of nodes defined as the number of times the states of the two nodes matched across all possible combinations of perturbations. This led to a SMC matrix of 72 × 71 nodes to which the basic set of 72 point perturbations and 72 singular sustained perturbations were added to make the total 5256. All the scores were normalized by this total number 5256. Hence, every possible pair of nodes had a score from zero to one. To view this scoring as a distance between the two nodes under consideration, I subtracted that number from one. Hence, the pairs of nodes having a score nearer to zero mean that the nodes in the pair are strongly correlated and hence closer to each other and the pairs having a score of one denotes the longest possible distance and thereby no correlation between the nodes in that pair. These scores were used to construct a frequency distribution. Since the histogram showed two distinct peaks, it indicated clear clustering (Figure 3.5). The two peaks in the frequency distribution of pair-wise distances correspond to the intra-group distance and the inter-group distance, respectively. I considered the first dip, i.e. 0.4 in the histogram as a threshold and listed all the pairs which had a distance less than that threshold. Clustering was

made by associations starting with the first pair till the list was exhausted. In this way, the following 3 different clusters were obtained.

1. *and*, *agr*, *ang*, *aox*, *bdn*, *btc*, *cck*, *cts*, *crh*, *dop*, *egf*, *edp*, *epo*, *fty*, *gap, glg*, *gng*, *gt1*, *hst*, *igf*, *inj*, *ina*, *ktg*, *msl*, *ngf*, *nep*, *otg*, *ost*, *ocl*, *oxy*, *bgl*, *tet*
2. *ata*, *adp*, *msh*, *car*, *chl*, *cfn*, *dip*, *et1*, *ffa*, *fdi*, *gst*, *grh*, *inr*, *ins*, *lep*, *myo*, *nox*, *pgl*, *sfr*, *ser*, *tnf*, *tri*, *gmo*, *noc*
3. *avp*, *bar*, *ctk*, *gab*, *ghr*, *hgh*, *il6*, *klt*, *lpa*, *mlt*, *sys*, *vdl*, *vd3*, *exe*, *glp*, *v12*

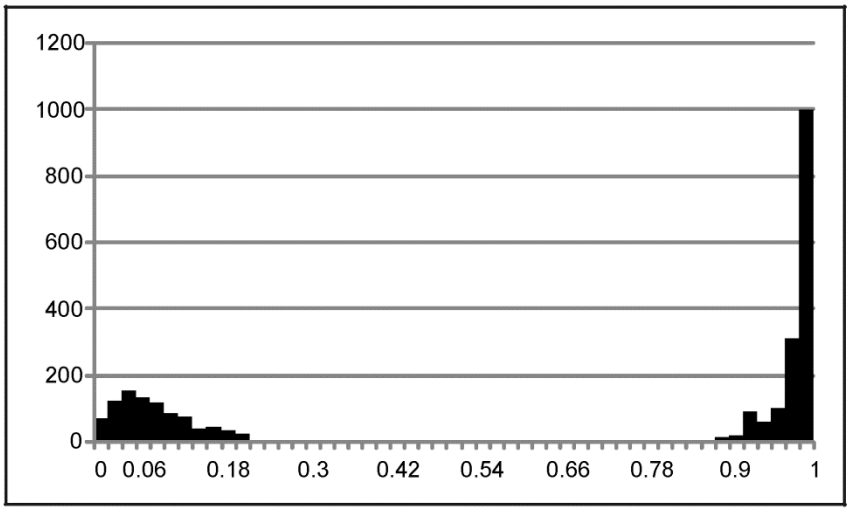

Figure 3.5: Frequency distribution of distances of pairs of nodes. I tried to validate this clustering result by comparing it to another readily used clustering technique - DendroUPGMA (http://genomes.urv.cat/UPGMA/), an open source online software to cluster the nodes in the network and plot a dendrogram (Figure 3.6). The software uses UPGMA (Unweighted Pair Group Method with Arithmetic mean) for clustering. I used the input data type as similarity matrix and fed in the 72 × 72 matrix with the original scores out of 5184 for each pair of nodes. Clusters identified by both the clustering protocols were identical. Reproduced from (Kulkarni et al., 2017).

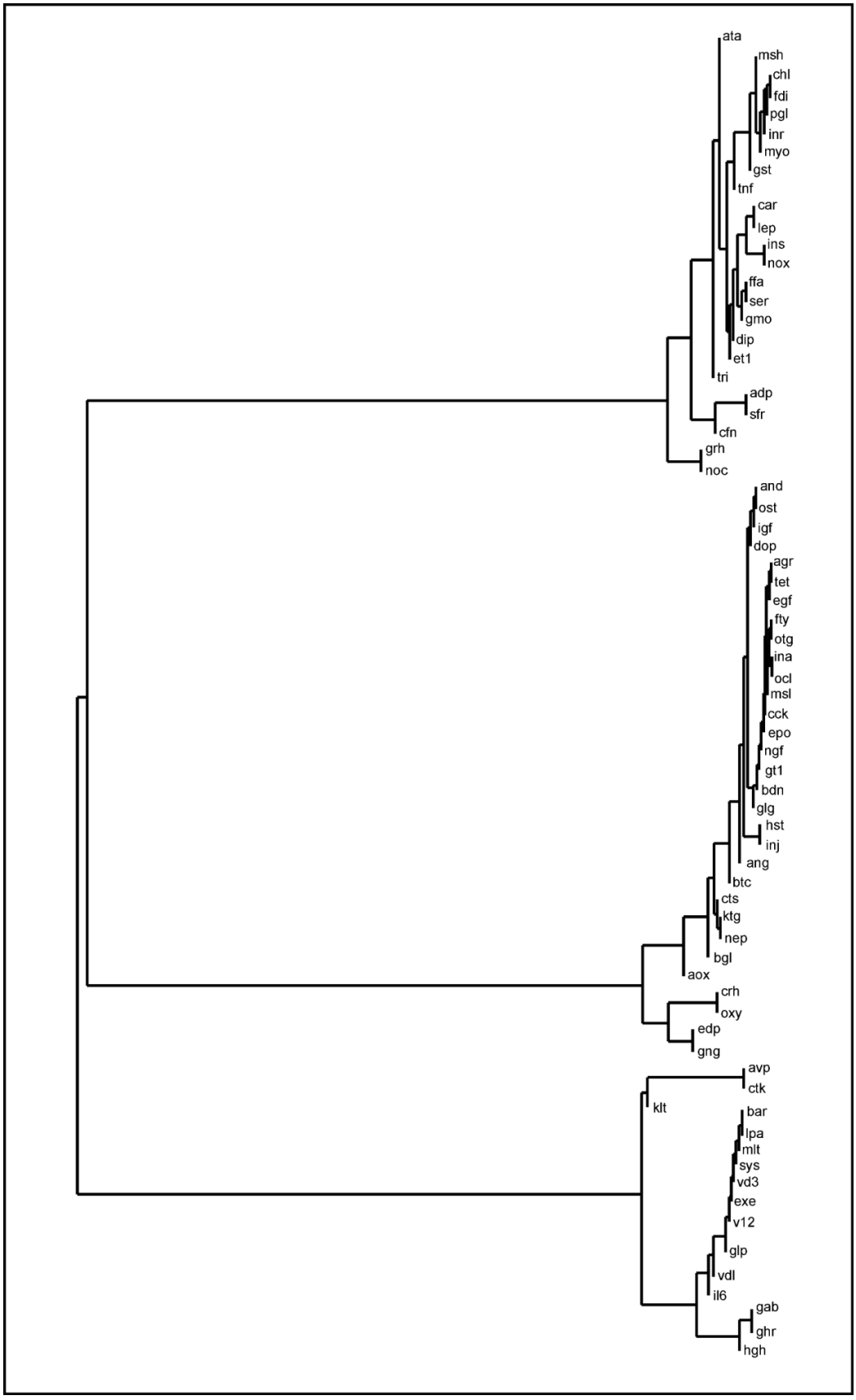

Figure 3.6: Dendrogram generated by DendroUPGMA. Reproduced from (Kulkarni et al., 2017).

3.5.6 Validation of the Network Model: These clusters suggested a way of validating the model. The nodes in the first cluster were correlated to insulin sensitivity in the model. The nodes in the second cluster were related to insulin resistance and those in the third cluster had a basal (zero) state and were correlates to neither insulin sensitivity nor insulin resistance in the model. I checked if these one to one correlations of all these individual nodes with either insulin sensitivity or insulin resistance are also observed in real life data. I found that about 80.35% of these correlations do exist in the literature, which in a way validates the model.

I expected all the other nodes in a cluster too, to be positively correlated to each other in real life data. Currently, there are no studies that provide quantitative data on all the nodes together. However, different studies have looked at different correlations. Of particular value are correlations between nodes that do not have a direct link between them but they lie in the same cluster in the above classification. Demonstrated correlations compatible with this expectation include myostatin to leptin (Lin et al., 2002), TNF-α to triglycerides, plasma glucose to cholesterol (Hotamisligil et al., 1995), vitamin D3 to vasodilation (Ertek et al., 2012) and growth hormone to klotho (Schmid et al., 2013).

I tested the robustness of the bi-stability of the model by random addition of a link between two randomly chosen nodes also. In 1,000 such random addition trials, bi-stability was not altered except for 8 specific link additions. In 6 out of the 8, there were 3 stable states instead of 2 and in only 2 cases there were multiple stable states. None of the additions resulted in chaos or homeostatic return to the starting state. Out of these 8 links, 2 links were against what is known in the literature. These are cholesterol down-regulating vitamin D3 and aggression up-regulating vasopressin. It is known that cholesterol is a precursor in the vitamin D3 pathway (Bikle, 2014) and vasopressin up-regulates aggression (Ferris and Delville, 1994). About the other 6 links, which were fertility down-regulating ghrelin, nitric oxides up-regulating ghrelin, ghrelin up-regulating serotonin, anti-oxides up-regulating vitamin D3, TNF-α up-regulating vasopressin and triglycerides up-regulating ghrelin; there was no information available. It was observed that a link with ghrelin was observed in 4 out of the 8 times. Taken together, it demonstrates further that the bi-stability is robust and unlikely to be because of some critical missing link.

3.6 Sensitivity analysis of the network model

3.6.1 Assumptions of the Network Model: To test the sensitivity of bi-stability to the underlying assumptions of the model, I relaxed the assumptions one by one and in combinations to see whether bi-stability was an artefact caused by some of them.

When I changed the mode of signal additions from simple arithmetic addition to qualitative addition, i.e. when a given node received both non-zero up-regulation and non-zero down-regulation links, the net signal strength was treated as zero. When a node received only positive signals, the node was up-regulated and when it received only negative signals, it was down-regulated. This invariably resulted in to chaos with every node and no long term tendency towards being up-regulated or down-regulated. A null model with qualitative additions invariably gave chaos. Therefore, this result appears to be more of a statistical generality of this mode of addition than any specific character of this network. The qualitative addition never allowed a sustained departure from the zero state. In the context of T2DM, this would mean that a stable insulin resistant or diabetic state may never be obtained. In reality, long term stability of insulin resistant or diabetic state is common and reversal is difficult. The qualitative addition mode did not appear to represent a realistic picture.

In the original model, the step length (amount of state change a node can undergo) was always unity as mentioned above. As it changed from -1 to 0 in one cycle, the signals changed according to the new states and the next step was decided by the new set of signals. I relaxed this step length assumption and two other variants were considered.

1. Direct step length: This variation allowed a direct leap from -1 to +1 if the net signal was greater than zero or vice versa. This variation of the model did not consider change in signals during state transition.

2. Short step length: In this variation, the states as well as steps were fine grained with a resolution of 0.1 so that twenty different states for each node were possible between -1 and +1. Each link when activated led to a change of 0.1 in the downstream effector node. If multiple signals reached a node in a cycle, their cumulative signal strength was calculated by adding them arithmetically. If it was lesser than the previous state, 0.1 was deducted from

it and if it was greater than the previous state, 0.1 was added to it. I examined whether the results were sensitive to the step length.

For the unit and direct leap step lengths, bi-stability was observed and the composition of the two attractors remained identical. There were subtle changes in the basins of attraction though. When the steps were fine grained for the short step length, bi-stability was maintained, but the stable states of the nodes took intermediate stable values instead of +1 and -1. Between unit step and fine grained step, the basins of attraction were over 80% similar. When direct leap was allowed, bimodality and composition of attractors remained the same and the basins of attraction were similar to unit step model by over 80%. Since bi-stability and attractor composition were not sensitive to the step length, for further analysis I used the unit step model alone which was faster as well as accommodated changes in signals during transition.

Thus, relaxing some of the assumptions did not affect bi-stability and relaxing certain others gave rise to unrealistic chaotic results. None of the assumptions gave rise to good homeostatic control where the system returned to its ground state on its own. This demonstrated the robustness of bi-stability and the soundness of the set of assumptions used in the model.

3.6.2 Contradictions of the Network Model: A surprising finding of the search for links was that some of the classical beliefs were not supported by interventional evidence. For example, there is no evidence for compensatory hyperinsulinemia (Watve, 2013). I have discussed each one of them in detail here. I made point perturbations to the network model independently with either of these contradicting links from each contradicting pair. I also encountered twelve other contradicting reports, where some studies had reported up-regulation while others observed down-regulation effect between the same directional node pair. I treated the contradictory links similar to the insulin resistance - hyperinsulinemia link i.e., the model was run separately assuming positive link or assuming negative link between the node pair.

1. Compensatory hyperinsulinemia: I found no interventional evidence that muscle insulin resistance was compensated by hyperinsulinemia. Lack of evidence for this widely held assumption is acknowledged (Corkey, 2012; Shanik et al., 2008; Watve, 2013) but the assumption continues to be a part of mainstream thinking.

Strictly going by the inclusion criteria of the model, I should not have included this link in the model. However, since compensatory hyperinsulinemia is a widely held belief, I decided to run (make point perturbations to the network model and observe any changes in the results) the model independently with and without this link. The difference in the outcomes of the two models could potentially give us the importance of this link. The interesting and surprising finding was that having or not having the compensatory hyperinsulinemia link did not affect the bi-stability of the network or the signatures of the two attractors. Since some researchers have argued for compensatory insulin resistance in response to primary hyperinsulinemia (Shanik et al., 2008), I reversed the causal arrow between insulin resistance and insulin levels which again did not affect bi-stability.

2. Link between obesity and insulin resistance: The link between obesity and insulin resistance is also laden with contradictory evidence but the mainstream thinking is that obesity increases insulin resistance. Similar to the insulin resistance – hyperinsulinemia link, reversing between the assumptions that obesity causes insulin resistance or insulin resistance causes obesity, or deleting the obesity-insulin resistance link altogether, did not affect bi-stability or the attractor signatures except for the state of obesity (i.e. the node 'adipose tissue') itself.

3. Irreversibility of β-cell damage: The apparent irreversibility of β-cell damage is also debated. Although classically β-cells were believed not to regenerate once lost, experiments over the last two decades have shown that β-cells have good regeneration capacity *in vitro* and *in vivo* including *de novo* regeneration from ductal ascinar cells (Yamaoka, 2002). I operated the model independently assuming β-cell -1 state to be reversible as well as irreversible. When I operated the model assuming β-cell dysfunction to be reversible, in the insulin resistant attractor, the state -1 remained stable and up-regulating the state of β-cells, transiently (point perturbation) or sustainably, did not bring the system back to the insulin sensitive state. This suggests a possible solution to the β-cell paradox, that is, why β-cell dysfunction appears to be irreversible in T2DM when the cells have good regeneration capacity. In the model, other signals coming from the

network kept β-cell function down-regulated. Alternatively, I assumed β-cell dysfunction to be irreversible, that is, when β-cells achieved a state of -1, it was retained -1 through all further cycles. Even under this assumption, bi-stability was attained and the composition of the attractors was substantially the same.

4. Necessity of insulin action node: When insulin and insulin action together down-regulated plasma glucose, bi-stability was unaltered but when insulin alone down-regulated glucose independent of insulin action, the system oscillated with large periodicity (up to 32 cycles) and there were multiple resultant states. Therefore, inclusion of the insulin sensitivity-resistance axis was one of the critical conditions for the bi-stability of the system.

5. Up- versus down-regulation contradictions: I also encountered twelve other contradicting reports, where some studies had reported up-regulation while others observed down-regulation effect between the same node pair. These contradiction pairs are shown in Table 3.3. I treated the contradictory links similar to the insulin resistance - hyperinsulinemia link i.e., the model was run separately assuming positive link or assuming negative link between the nodal pair.

Table 3.3: Up- versus down-regulation contradiction pairs.

Sr. No.	Contradiction pair		Default link considered between the two nodes
	Link originating at	Link ending at	
1.	Endorphin	Food intake	end → fdi
2.	GABA brain	Aggression	gab → agr
3.	GABA brain	Food intake	gab ⊣ fdi
4.	Oestrogen	Insulin action	otg ⊣ ina
5.	Oestrogen	Inflammatory response	otg → inr
6.	Oxytocin	Corticosteroids	oxy → cts
7.	SFRP-5	Adipose tissue	sfr ⊣ adp

8.	Testosterone	Insulin action	tet → ina
9.	Leptin	Serotonin	lep → ser
10.	Oxytocin	Aggression	oxy → agr
11.	Nitrogen oxide	Aggression	nox ⊣ agr
12.	Plasma glucose	β-cells	pgl ⊣ btc

For 11 out of the 12 up versus down-regulation contradictions examined, the system still retained bi-stability with the up-regulation or down-regulation arrows. Eight out of the 11 contradicting interactions that retained bi-stability showed no effects on the attractor signatures although the basins of attractions altered marginally (< 15%) in some of them. One of the 11 interactions (plasma glucose – β-cells pair) showed marginal effects in both the attractor signatures (<5%) and the basins of attraction (<10%) but retained the bi-stability. Two of the 10 interactions (leptin-serotonin pair and oxytocin-aggression pair) brought about marginal changes in the attractor signatures. The only up versus down-regulation contradiction that affected bi-stability was when endothelial nitric oxide synthase (e-NOS) and neuronal nitric oxide synthase (n-NOS) action were considered a single node. Different studies have found either up-regulating (Demas et al., 1999; Gammie and Nelson, 1999) or down-regulating (Chiavegatto et al., 2001; Demas et al., 1997; Kriegsfeld et al., 1997; Nelson et al., 1995) action of NOS on aggression. Bi-stability was retained for the down-regulation link but not for the up-regulation link. After segregating the actions of e-NOS and n-NOS, bi-stability was retained. Since different studies report up or down-regulating action of n-NOS on aggression, the model was run with either of the links at a time. With both types of links, bi-stability was maintained but the inclusion of n-NOS in the basin of attraction was affected.

6. Reactive oxygen species (ROS): ROS is considered an important player in the pathophysiology of T2DM. During redundancy filtering, ROS was filtered out since it was tightly linked to inflammation and both shared identical incoming and outgoing links. But since ROS is believed to be an important player, I

simulated keeping ROS as a separate node. This change again did not affect bi-stability and up-regulation of ROS led to the insulin resistant state.

7. Glucagon – insulin link: Glucagon has a direct up-regulation effect on insulin secretion (Ohneda et al., 1975), but through the agency of kisspeptin, it has a down-regulation effect (Song et al., 2014), making the net effect zero. The signal between glucagon and insulin was therefore filtered out. However, since insulin and glucagon are believed to be central molecules to T2DM, I operated the model with and without these links singly and in combination. The bi-stability remained robust to the inclusion or exclusion of these links.

3.7 A comparison with the classical theory

The classical theory of insulin resistance states that obesity leads to insulin resistance, insulin resistance tends to increase plasma glucose which stimulates increased insulin secretion. This increased insulin secretion brings glucose back to normal leading to an insulin resistant-hyperinsulinemic-normoglycaemic stable state. Failure of compensatory hyperinsulinemia owing to β-cell exhaustion or dysfunction results in to hyperglycaemia. I included only adipose tissue, insulin, insulin action, β-cell mass and plasma glucose (Figure 3.7) as nodes in the model and included all known and classically believed links.

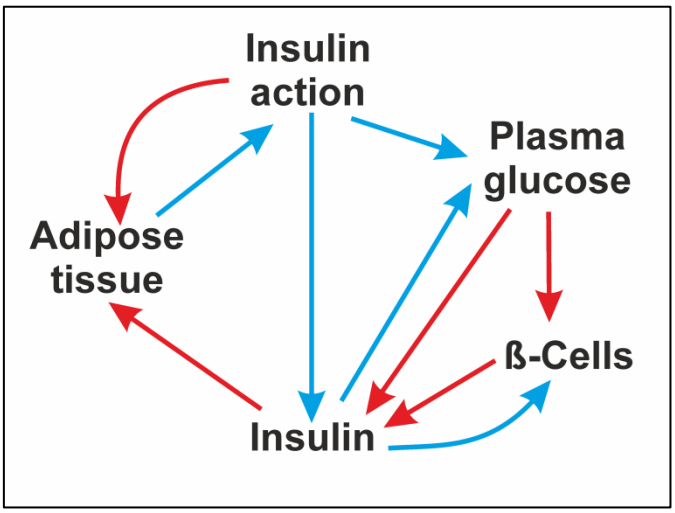

Figure 3.7: Classical model. Interactions among adipose tissue, insulin action, plasma glucose, plasma insulin and β-cell mass according to the classical theory are shown with

red arrows indicating up-regulation links and cyan, down-regulation links. Reproduced from (Kulkarni et al., 2017).

In this classical model, I failed to see bi-stability under any condition. After any point perturbation in any of the five nodes, the system returned to the initial basal state in not more than 4-5 cycles or showed stable oscillations around the initial basal state. This is a typical behaviour of a homeostatic system. No point perturbation could change the basal state and lead to a stable insulin resistant state (Figure 3.8). Being a smaller and simpler system, it is easier to visualize the reasons. For example, when I up-regulated adipose tissue mass, insulin resistance and subsequently, plasma glucose increased. This increased insulin levels and subsequently glucose returned to normal. As glucose returned to normal, insulin could not remain elevated. Thus a normoglycaemic-hyperinsulinemic state was not stable. The states of the same nodes as observed in the T2DM network are showed in figure 3.9. Even when I applied the slow step algorithm to the classical network, an insulin resistant stable state was not attained. When insulin was up-regulated in the slow step length classical model, it led to a positive change in adipose tissue and negative changes in plasma glucose and beta cells; and thus, it eventually decreased, ultimately oscillating around zero with the other nodes (Figure 3.10).

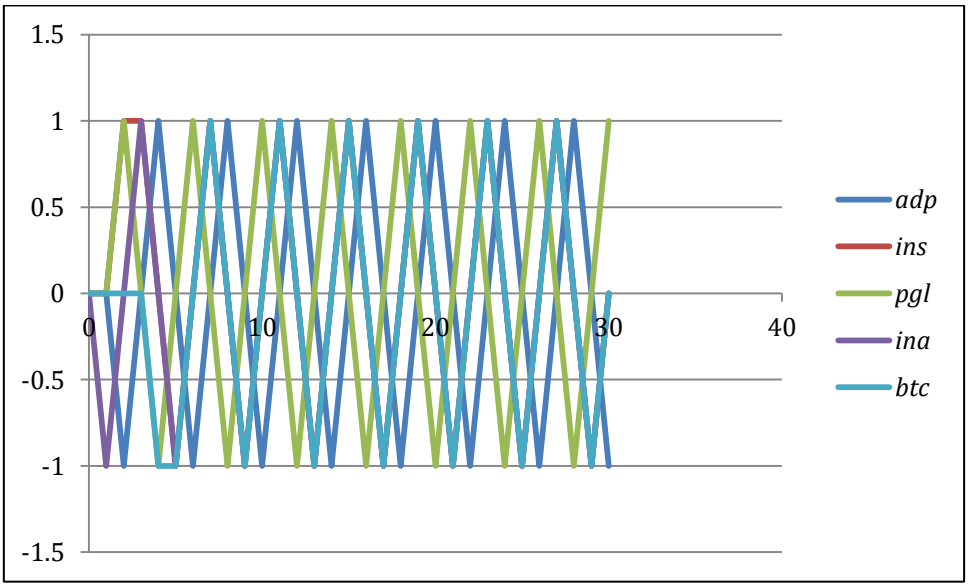

Figure 3.8: Changes of states of nodes in the classical model.

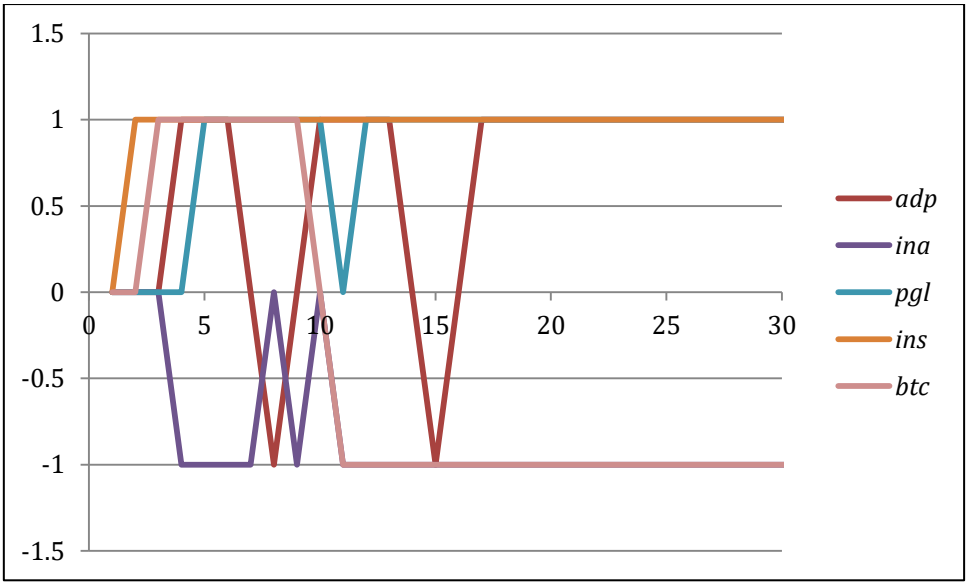

Figure 3.9: Changes of states of nodes in the T2DM network model.

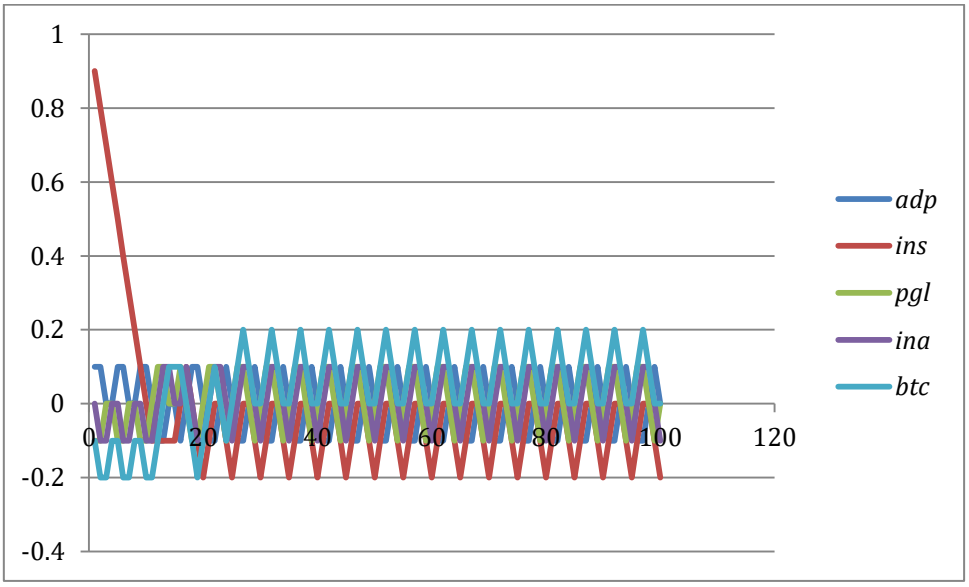

Figure 3.10: Changes of states of nodes in the classical model with slow step length.

Further in a state of high insulin resistance, the lipogenic action of insulin was suppressed and therefore, adipose tissue was reduced. Reduction in adipose tissue normalized insulin resistance and thus the system was back to its starting state. Even if I assumed that chronic overproduction of insulin affected β-cell function, β-cell mass remained in a homeostatic state since glucose is known to stimulate β-cell proliferation. Further, owing to the other homeostatic loops, both glucose and insulin returned to

normal thereby removing β-cell stress. Inclusion of glucotoxicity, that is, considering *pgl* to *btc* a negative regulator, did not drift the system away from homeostasis. Assuming β-cell loss as irreversible, that is, fixing *btc* state to -1 resulted into oscillation of insulin between zero and -1 states but glucose remained normal because of feedback loops operating through *adp* and *ina*. All the links in this small network made effective negative feedback loops and therefore, the system failed to give a persistent insulin resistant state under any condition.

This suggests that the stability of the insulin resistant state observed in T2DM may not be contributed by any defect in the restricted network involving insulin, glucose, obesity and β-cell. It appears to be contributed by the structure of the extended network.

3.8 Discussion

The model essentially demonstrates that the pathophysiology of type 2 diabetes is orders of magnitude more complex than the classical picture of insulin resistance and relative insulin deficiency causing hyperglycaemia. Insulin and glucose have been the two molecules central to classical thinking but apart from the burden of history, there are no other grounds to treat insulin and glucose to be more important in T2DM than any other nodes of the network. The behaviour of the system is decided more by the network structure than by one or a few key molecules. In a network structure, it is possible to reach all nodes by starting from any random node. Therefore, although I started assembling the network from insulin and glucose, it does not mean the network is gluco-insulino-centric.

The model also accounts for foetal programming. If we consider the all zero baseline state of the system as a foetal condition and the point and sustained perturbations as stimuli faced in embryonic or early life, these stimuli can drive the system to one of the two states which are difficult to reverse. Unlike the classical belief that obesity is cause of T2DM, we observe that there are many different ways in which the process can begin. The set of such perturbations that can lead to the diabetic state is the insulin resistant basin of attraction. This may account for Developmental Origin of Health And Disease (DOHAD) in adulthood (Barker, 1998) or predictive adaptive response (Gluckman et al., 2005). Since the model is based entirely on experimental data and it appropriately

accounts for many realistic phenomena, the unexpected outcomes of the model need to be considered seriously as new possibilities. Empirical work in this direction is needed to test whether they work in reality.

Limitations of the model mainly come from four of its attributes. Firstly, there might be some important links that are yet to be discovered and are therefore missing in the network. I have been updating the model intermittently through my work and adding newly identified links to it. Secondly, the experiments from which data are taken are carried out on different model systems. All the identified links did not come solely from human studies since such data are not available. Hence, studies from other mammalian systems were also included to identify links from. Hence, the model represents a mammalian system. The use of the other model systems to extract the links is justified since there were no qualitative differences between the two. This means that for a particular link, the rate of the up-regulation of the downstream mode may differ in different model systems but the fact that the former node affects the latter in a positive manner stands true. Since my model considers only the qualitative nature of the link, these intra-organismic differences are insignificant in my case.

Thirdly, the model is qualitative and discrete. A node can attain only three distinct states namely +1, 0 and -1. The reason behind generation of a qualitative model was again lack of data. Not all links from the network come from a single model system and as mentioned above, the links might differ quantitatively across the different systems. If such data becomes available, a similar model which can use it can be developed. This is explained in some detail in Chapter 5 Section 5.4. Lastly, since the model is qualitative, time-related predictions cannot be made. Some links in the model might be faster-acting than the others, but this distinction has not been made in the model for the same reason that link reaction rates for all the links are not available in the same organism. Nevertheless, the model made correct correlational predictions between pairs of variables that did not have a direct causal connection suggesting thereby that the network model works reasonably well despite the limitations. This suggests that the novel and unexpected predictions of the model need to be tested empirically.

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222.

Chapter 4

Implications and applications of the network model

This chapter deals with the applications of the model starting with finding nodes that might be important for the bi-stable nature of the network. Then the reasons for the robustness of the bi-stability are discussed. Lastly, the novel therapeutic ways are mentioned that, at least in this model, lead to the healthy state.

4.1 What makes the bi-stability robust?

Since there were only two resultant attractors in the baseline model, the nodes could be classified as the ones whose up-regulation led to the insulin sensitive attractor and the other whose up-regulation led to insulin resistant attractor. Notably, point up-regulation of 40 of the 72 nodes, led to a stable state in which they remained up-regulated. This is a positive feedback effect. Sixteen of the nodes resumed the zero state although they drove the system to one of the two stable states. The remaining 16 showed an overcompensation-like response, i.e. point up-regulation of these 16 nodes led to a state in which they were down-regulated. Overall, the network had a preponderance of positive feedback circuits which explains the robust bi-stable behaviour of the system.

If the network is redrawn segregating the two groups of nodes, one consisting of nodes in the insulin sensitive basin of attraction and the other consisting of those in the insulin resistant basin of attraction (Figure 4.1, see also Table 4.1), it can be appreciated that there are significantly more positive links within group as compared to between groups and there are significantly more negative links between groups as compared to within groups (chi square =37.33619, df= 3, $p< 0.0001$). This makes the bi-stability and the dichotomous grouping of the nodes very robust. Within group positive and between groups negative links will stabilize and reinforce the attractors; whereas within group negative and between groups positive links will tend to destabilize the attractors. Since there were 216 stabilizing and 114 destabilizing links, there is no wonder that the two attractors were highly stable and not sensitive to changing a few nodes or links (Figure 4.2).

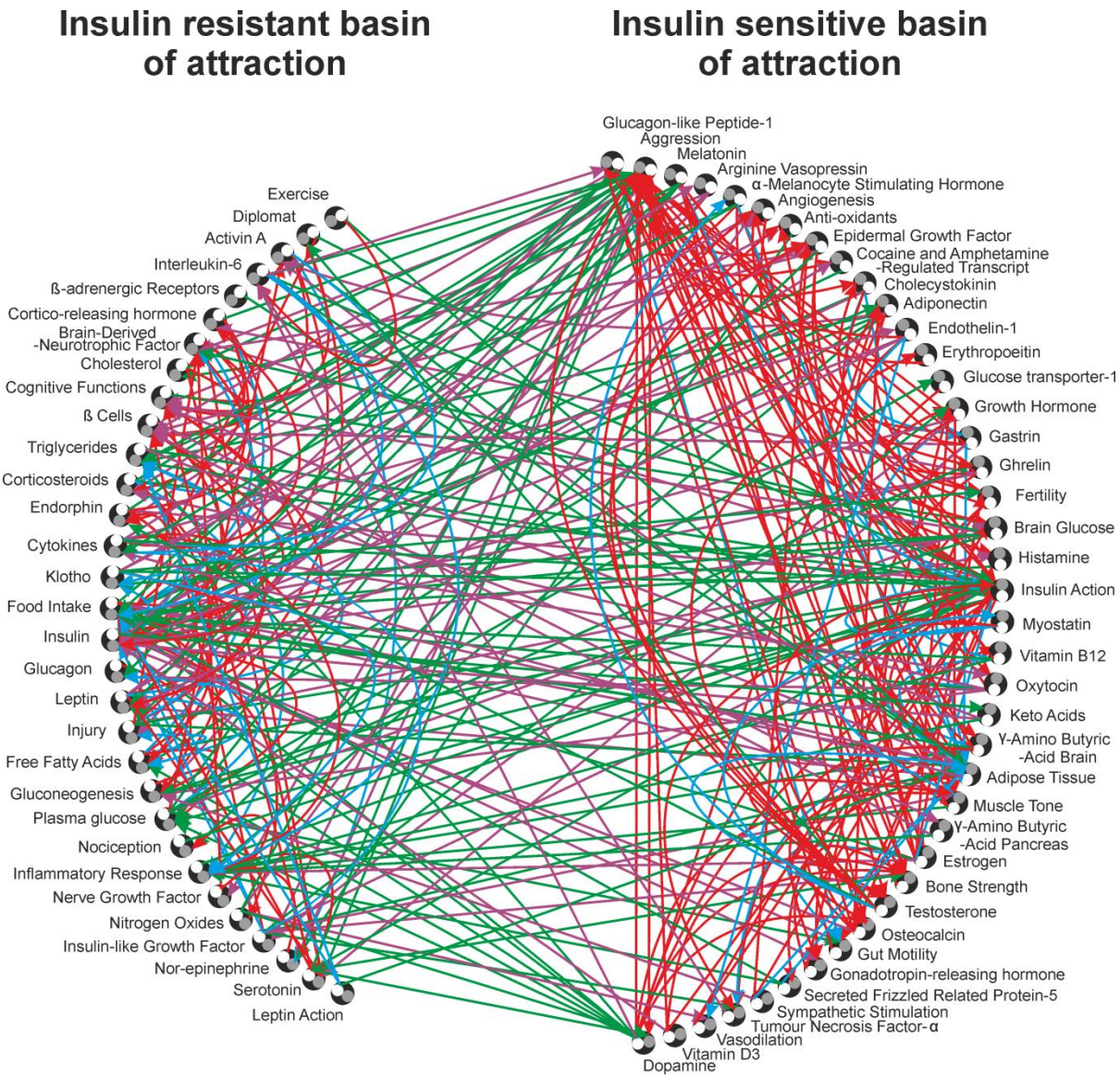

Figure 4.1: Basins of attraction. T2DM signalling network segregated according to the point perturbations leading to the two attractors. The outbound (white circle) and inbound (grey circle) portals are shown for each node. Red arrows indicate intra-group up-regulation links; cyan, intra-group down-regulation links; purple, inter-groups up-regulation links; green, inter-groups down-regulation links. Reproduced from (Kulkarni et al., 2017).

Table 4.1: Placement of nodes in different organs and the two basins of attraction.

Organs	**Insulin Sensitive Basin of Attraction**	**Insulin Resistant Basin of Attraction**
Adipose Tissue	adp, and, sfr	bar, lep, lpa, tri
Behaviour	agr	dip, exe
Bone	ost, ocl	
Brain	msh, avp, car, dop, gab, gt1, grh, hgh, hst, mlt, oxy, bgl, sys	bdn, cfn, edp, crh, nep, ser
Gonads	fty, otg, tet	
Gut	cck, egf, gst, ghr, glp, gmo, v12	chl, fdi, ngf
Immune System	tnf	ctk, il6
Insulin Action	ina	
Kidneys	epo	cts, klt
Liver	ktg	gng, igf
Muscles	msl, myo	ffa
Pancreas	gap	btc, glg, ins
Plasma Glucose		pgl
Skin	vd3	inj
Systemic Response	aox	ata, inr, noc
Vascular Tissue	ang, et1, vdl	nox

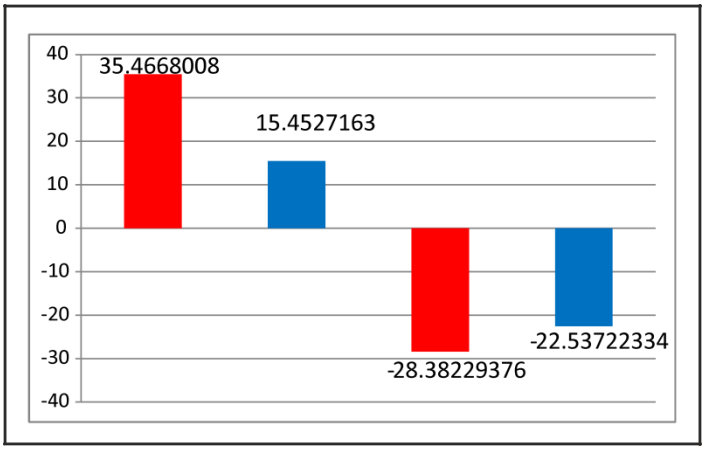

Figure 4.2: Link Statistics. The bars represent the deviation from the expected number of links per cluster, the expected being calculated assuming independence. First two columns show the stabilizing links and the next two columns show the destabilizing links for the two clusters. The red and blue bars represent the insulin sensitive and the insulin resistant basins of attraction, respectively. Reproduced from (Kulkarni et al., 2017).

4.1.1 Cyclic loop analysis. Another addition to the network model was the algorithm to extract cyclic loops from the network. A loop is a chain of links starting and ending in the same node. This gave us a clear idea of the fraction of loops that led to a positive feedback (activating the starting node through the loops) and the fraction of loops that led to a compensation-like response (inhibiting the starting node) for each node. The total number of loops goes on increasing from 46 one-membered loops (only one intermediate node the loops passes through) to 24,26,640 nine-membered loops. The rise in the number of loops with increase in the number of members is exponential (Figure 4.3). There was an obvious correlation between the number of links a node makes with the other nodes and the number of loops it has (Figure 4.4). It was also observed that as the number of members in the loops goes on increasing, the ratio of positive feedbacks to the negative ones goes on decreasing from over 1.4 to unity. I compared this with a null model with same number of nodes and links, but random placement of links across the nodes. It was observed in case of the null model, that there was no bias towards more positive feedbacks (Figure 4.5).

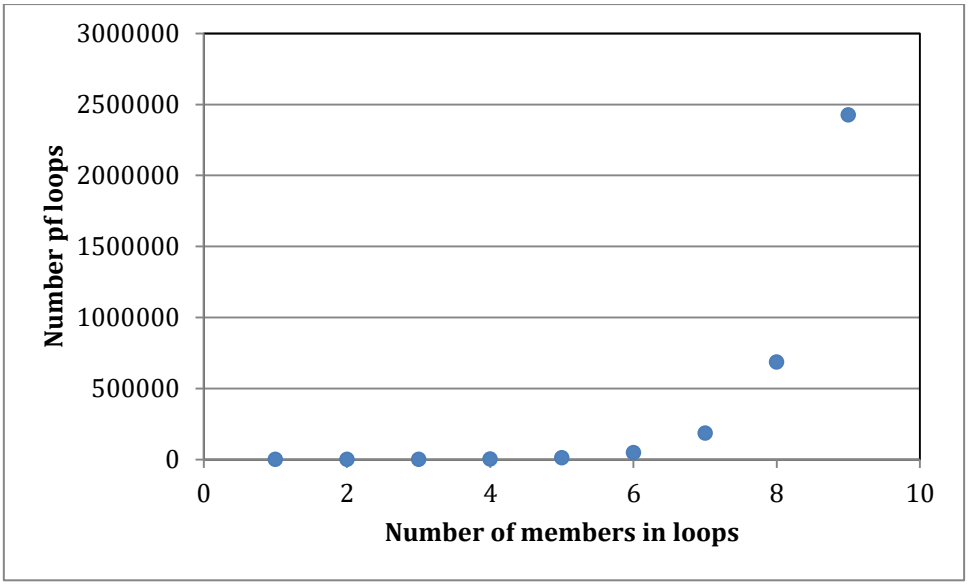

Figure 4.3: Total links vs number of members per loop

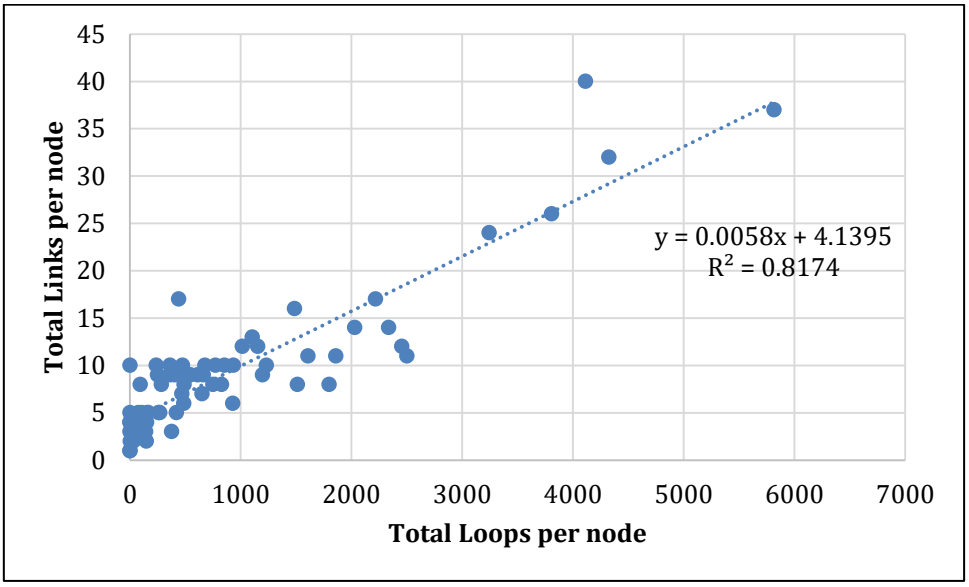

Figure 4.4: Total links vs total loops per node

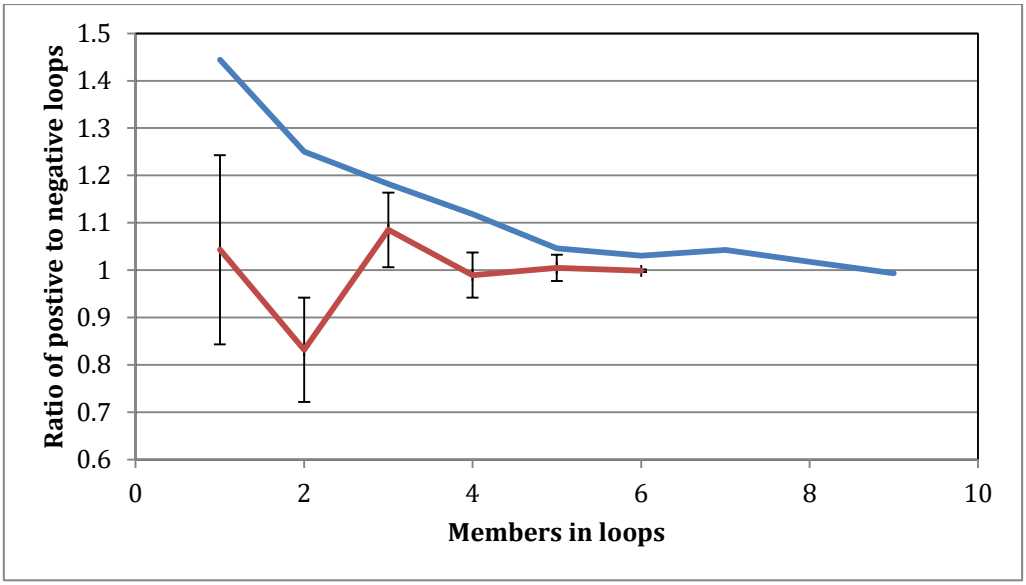

Figure 4.5: Positive/ negative loops vs number of members in the loop. Cyan line, the T2DM network model; red, across 3 different null models.

In the T2DM network, the smaller loops are more positive and thereby responsible for the reinforcement of the two attractors. As the members go on increasing, the loops go on becoming less biased. Thus, the preponderance of small positive feedback loops appears to be responsible for the stability of the two attractors.

4.2 Why bi-stability may have evolved

The classical clinical thinking is that the body has a homeostatic system operative under healthy conditions and disease is a departure from this state. The structure of the network on the other hand suggests something else. Here, both the states are stable and reinforced by multiple stabilizing mechanisms. This suggests that there needs to be some strong selective force giving the network the bi-stable behaviour. Multiple stabilizing loops are unlikely to have arisen by chance. The two states might be adaptive under two different contexts and the body has evolved sufficient plasticity to enter one or the other state as guided by the environment. This view is qualitatively different from the concept of a single homeostatic state and a pathological departure from it.

Evolutionary medicine is a relatively recent school of thought in medicine that studies the evolutionary origins of health and disease. A number of evolutionary hypotheses for obesity and T2DM have been proposed (Baig et al., 2011; Corbett et al., 2009; Mankar et

al., 2008; Neel, 1962; Watve and Yajnik, 2007). A common concept underlying most evolutionary hypotheses of T2DM is that insulin resistance is an adaptive state. The hypotheses differ in proposing what is adaptive and under which set of conditions. For example, the thrift family of hypotheses presumes obesity as an adaptation to fluctuating food availability and insulin resistance an inevitable outcome of obesity (Neel, 1962), the behavioural switch hypothesis proposes that the two states are fine tuned to two different behavioural strategies namely hawk and dove. The hawk resembles a 'stronger' phenotype accompanied by insulin sensitivity, higher muscle mass and 'r' type of reproduction strategy while the dove resembles a 'smarter' phenotype accompanied by disinvestment in the muscle and progeny with the help of insulin resistance. This hypothesis also proposes that the transition between the two states is possible and beneficial when the environment changes (Watve and Yajnik, 2007). The fertility selection hypothesis puts fertility in the forefront and proposes that in case of food shortage or abundance, body physiology adapts in order to maximise fertility (Corbett et al., 2009).

The different hypotheses are not completely mutually exclusive although they make certain differential predictions (Watve and Diwekar-Joshi, 2016) and can be tested differentially. I do not intend to test them here, but would rather highlight their common grounds that insulin resistance is an adaptive strategy. Once a strategy is adapted, the body physiology, as a whole, needs to fine tune itself, which involves a number of organs and systems. Therefore, a network needs to evolve that can bring about the finer level adjustments in a coordinated manner. Perhaps this logic underlies the evolution of cross talks between organs and evolution of networks. If the homeostasis view is correct, we would expect modular homeostatic mechanisms for every controlled variable with little, if any, cross talk between systems. Since one change needs to be accompanied by several other changes in the physiology, a network with two or more stable states is expected to evolve.

If bi-stability is evolved as alternative adaptive states, why one of them leads to pathological outcomes is an important question. The possible answers to this are also discussed in literature on evolutionary medicine. One possible reason is a supernormal response shown by the alternative steady state. The response of the body's physiology is adapted to a range of specific environmental conditions. In the example below (Figure

4.6), the optimum response to the environmental feature is sigmoid but within the range that the species faced during evolutionary time, the relationship is almost linear. It is likely therefore, that the evolved physiological response is linear. Now, if one faces an environment beyond the range for which it evolved, there would be an exaggerated response which is far from the optimum. Since the body has never come across such an environment before, the logical response it gives is the one shown with the red dotted line. This is called a supernormal response. A number of features of the modern life style are substantially different from the environment in which the human body evolved. Therefore, physiological responses that were once adaptive might turn out to be pathological (Watve, 2013).

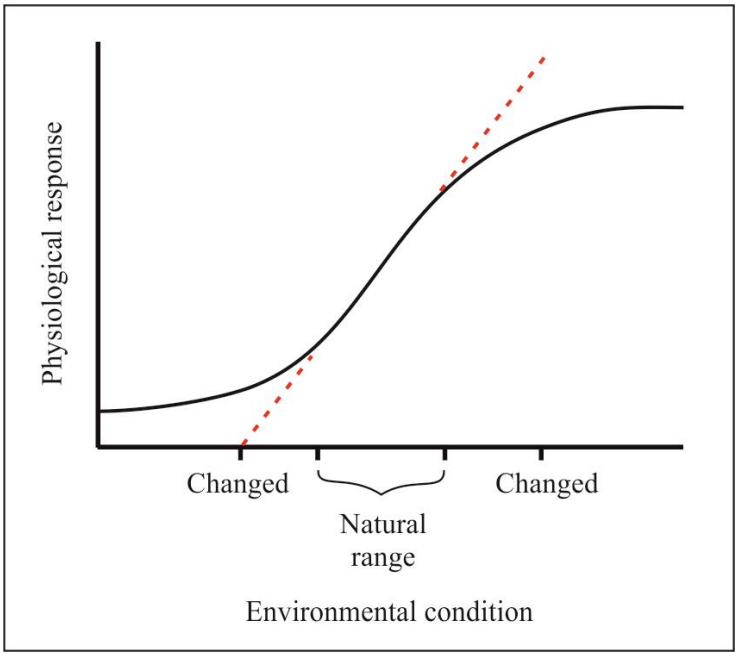

Figure 4.6: Supernormal response. Black line, natural physiological response; red dotted, supernormal response.

The other possibility is unusually long term sustenance of the alternative state. The complications of T2DM arise only after a long term existence of the state. It is possible that this rarely happened in the evolutionary history of the species and as a result there was little selective pressure to evolve mechanisms to arrest them. Today, the increased lifespan on the one hand and early onset of the condition on the other allow sufficiently long time for the complications to become serious.

4.3 Identifying applicable features of the network model

Having established a model whose results can be interpreted in basic biological terms, we move on to further understand whether it makes any useful translational predictions. The most evident way to do this is to try and find a single node or a cluster that is crucial in deciding the behaviour of the system. Such a key node can be potentially a good treatment target. If a target assures an insulin sensitive state in spite of other perturbations, it would make a good potential target.

4.3.1 Is There Any Key Node? To check the sensitivity of the model to the nodes involved in the network and also to highlight the important nodes which when removed lead to the collapse of bi-stability, I deleted each node one at a time and observed the effect of perturbing every other node. A node under focus was frozen to the zero state all the time. This turned all the incoming as well as outgoing links from the node ineffective and thereby the node was cut-off from the rest of the network. This analysis also suggested whether tight homeostatic control over any node is sufficient for homeostasis of the entire system. I found that in 71 of the 72 deletions, there was no deviation from bi-stability. The system showed a deviation from bi-stability only when the node fertility (*fty*) was deleted. Deletion of *fty* led to multiple stable states; some being insulin sensitive and others being insulin resistant. Most of the correlates of insulin resistance remained similar except that high cholesterol was now associated with insulin sensitivity. To check whether any particular outgoing link of *fty* was responsible for this effect, I deleted each of them individually. None of the links made by *fty* when individually deleted affected the bi-stability. It seems to be a compound effect of the 4 upstream links to *fty* namely up-regulation by adiponectin (Čikoš et al., 2010), EGF (Tsutsumi et al., 1993), oestrogen (Gill-Sharma et al., 1993) and growth hormone (Breier et al., 1996) and down-regulation by klotho (Ohnishi and Razzaque, 2010); and 3 links downstream to *fty* namely up-regulation of EGF (Kurachi and Oka, 1985), oestrogen (Barkley et al., 1979) and oxytocin (Neumann et al., 1993). It is interesting to note that freezing glucose to the normal state did not ensure homeostasis of the entire network suggesting that glucose homeostasis is not central and critical to the behaviour of the network.

I tried to find out the reason behind why fertility came out to be the only node which when deleted led to the collapse of bi-stability. The nodal parameters such as closeness

centrality, betweenness centrality, clustering coefficient (Figure 4.7), indegree and outdegree (Figure 4.8) were calculated (Cytoscape 3.5.1) to see if they could explain this behaviour shown by *fty*. None of these parameters could help explain that at least individually. I also calculated the edge betweenness centrality (the number of shortest paths that go through an edge); but none of the edges that *fty* makes were highlighted in the analysis (Figure 4.8). I therefore conclude that the answer lies in the network structure as a whole rather than with any single parameter.

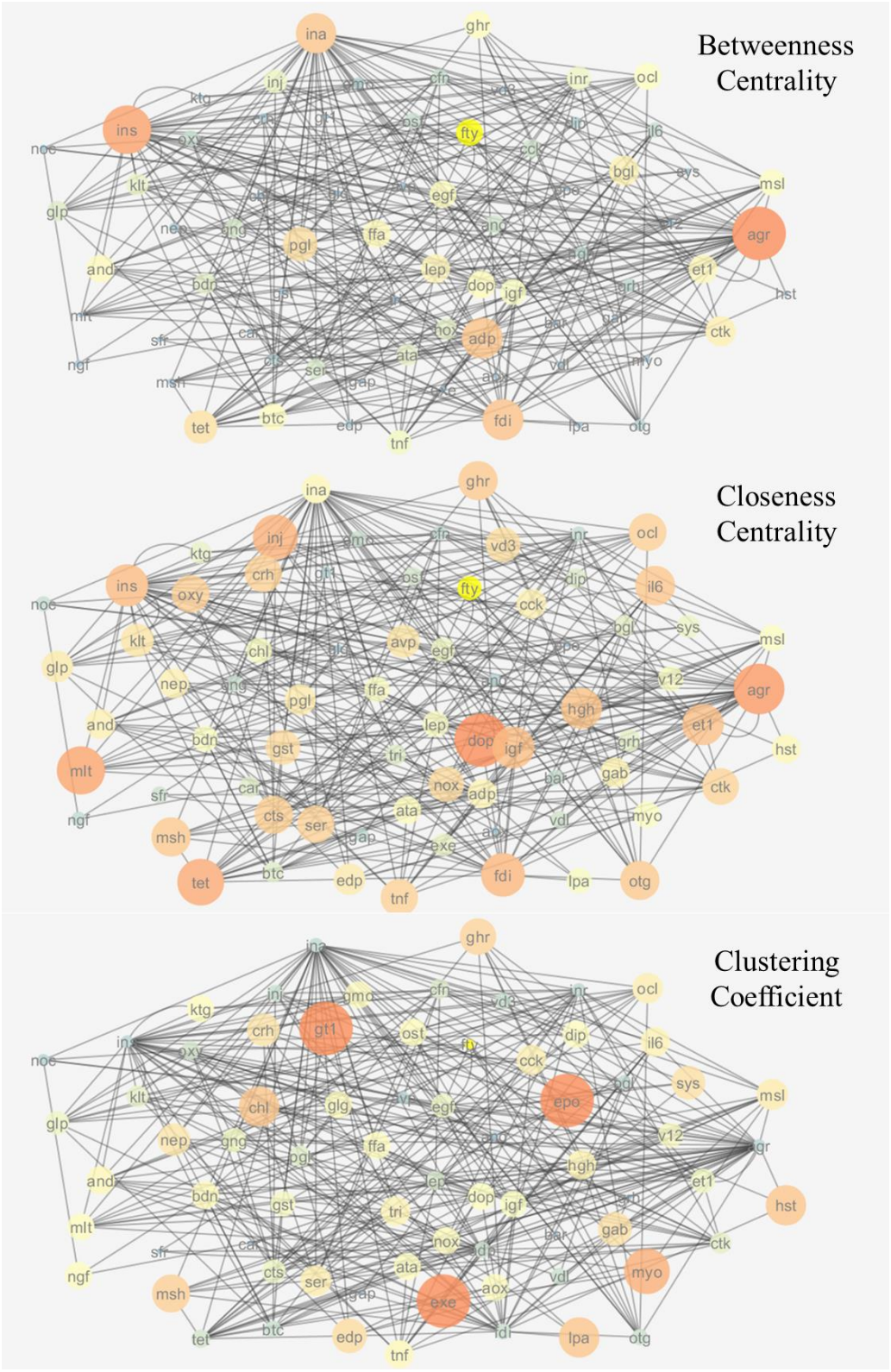

Figure 4.7: Nodal properties of closeness centrality, betweenness centrality and clustering coefficient. Node size corresponds to value. Also, the colour from blue to red represents the values from low to high. Node fertility is highlighted in yellow.

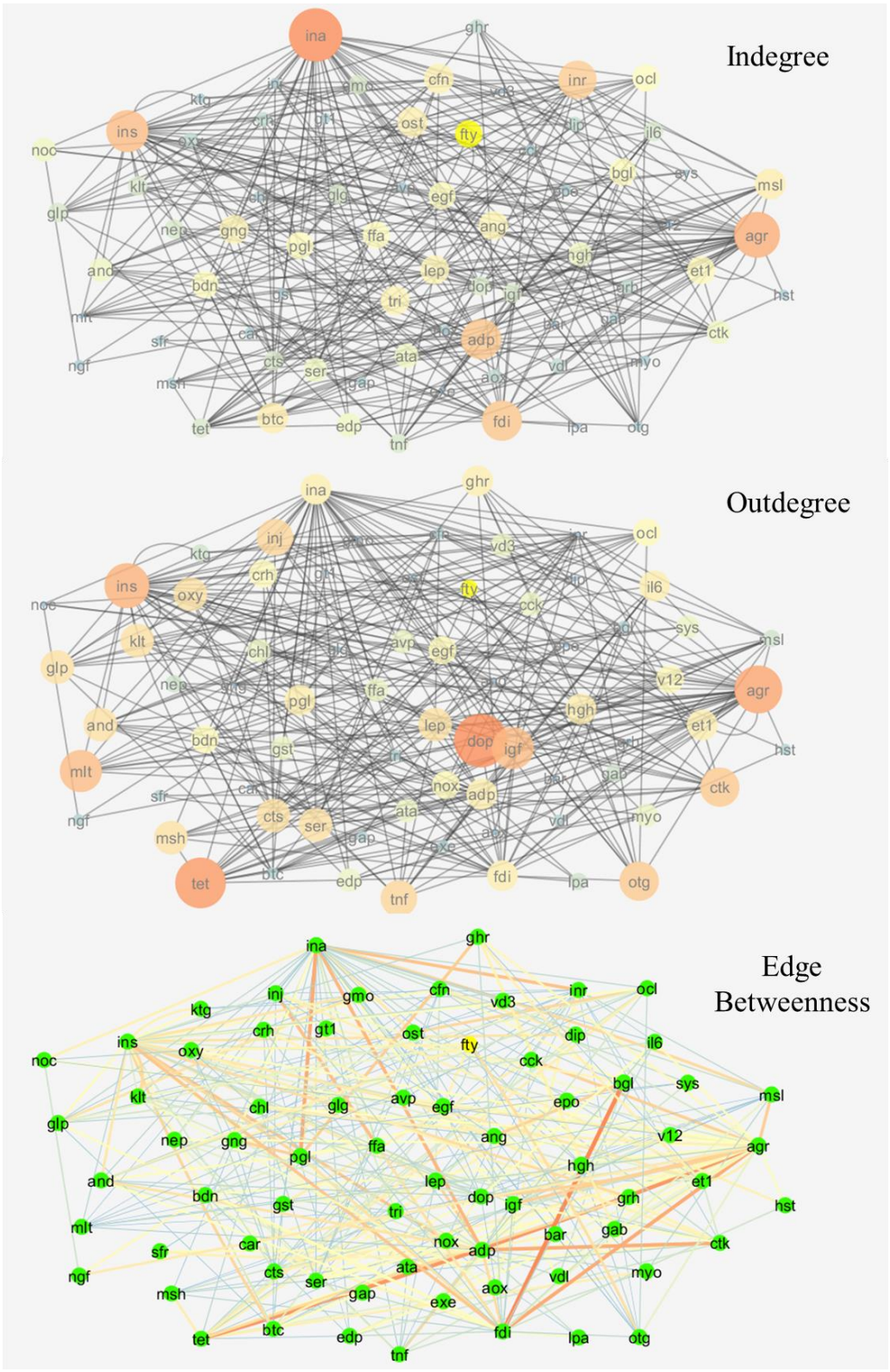

Figure 4.8: Nodal properties of indegree, outdegree and edge betweenness. Edge width corresponds to value. Also, the colour from blue to red represents the values from low to high. Node fertility is highlighted in yellow.

Fertility being the critical node might be surprising for the classical theory of type 2 diabetes which holds obesity to be the driver of the series of changes. It is not a surprise for some of the evolutionary hypotheses which involve selection for certain reproductive strategies to be a driving principle behind the evolution of insulin resistance (Corbett et al., 2009; Watve and Yajnik, 2007). These hypotheses are discussed later in this chapter.

4.3.2 Is there a key node combination? In addition to single node deletion, I deleted combinations of nodes by randomly freezing to zero 10% of the nodes at a time. Out of 1000 such simulations, bi-stability was conserved 81% of the times. Among the remaining 19%, there was complete loss of stability 1.1% of the times. Among the deleted combinations that led to loss of stability the nodes aggression, dopamine and fertility were overrepresented. Among the other non-bi-stability outcomes, 2.2% was contributed by uni-stability where the states of the nodes were at and around the basal zero state indicating that the network was in a robust homeostatic state. Among the combinations of deletions that gave robust homeostasis, fertility and leptin were overrepresented suggesting that these nodes in combination are critical for the bi-stable behaviour of the system. It is interesting to note that glucose did not appear in this list indicating that ensuring glucose homeostasis along with a few other key nodes does not assure homeostasis of the entire system. See Table 4.2 for the list of combinations of deletions that led to homeostatic uni-stability and complete loss of stability. In the remaining 15.7% cases tri, tetra or penta-stability was obtained in which some states were insulin sensitive and others resistant.

Table 4.2: List of deletions of the 10% of the nodes that led to uni-stability and complete loss of stability.

Simulation No.	**Uni-stability**						
1	ktg	fty	and	gmo	bdn	glp	adp
2	klt	noc	fty	sys	lep	pgl	hst
3	bgl	lep	gst	ctk	ghr	fty	gmo
4	myo	ins	avp	agr	and	epo	lpa

5	et1	mlt	igf	chl	inj	ost	tri
6	dop	gap	msh	lep	adp	bgl	agr
7	btc	fty	ctk	grh	lep	inj	otg
8	avp	btc	myo	oxy	dop	lpa	hst
9	cts	hst	ost	lep	fty	msl	exe
10	exe	hgh	msl	hst	and	oxy	chl
11	noc	avp	vd3	fdi	msh	oxy	fty
12	chl	egf	il6	fty	ngf	glp	cfn
13	oxy	sys	dip	btc	gst	fty	and
14	ang	fty	oxy	msl	gt1	chl	hst
15	ser	ktg	mlt	exe	gt1	otg	hst
16	otg	epo	lpa	car	chl	hst	ocl
17	agr	adp	ins	tnf	ost	nox	epo
18	aox	fdi	lep	glp	fty	and	ina
19	myo	ina	bgl	dop	nep	et1	epo
20	fdi	klt	ctk	avp	inr	ina	gmo
21	igf	gap	ina	ffa	bgl	edp	myo
22	mlt	dip	ocl	dop	sfr	gst	ina
Simulation No.	**Complete loss of stability**						
1	otg	gng	chl	tet	sys	pgl	gab
2	fty	et1	dip	ser	agr	exe	igf
3	agr	bar	fdi	vd3	gap	oxy	msl
4	fdi	ocl	crh	and	ina	msl	ang

5	dop	v12	ghr	noc	ngf	ins	fty
6	tet	inj	aox	gap	vdl	dop	otg
7	vdl	dop	ocl	glp	agr	ser	gap
8	ata	chl	fty	inj	pgl	ina	bar
9	ina	mlt	oxy	bar	ocl	ata	dop
10	vd3	chl	ang	hst	fty	cck	inr
11	mlt	agr	nep	et1	aox	gmo	myo

4.4 Towards robust targets for treatment of T2DM

The combined perturbation simulation results give us possible new insights into long term effectiveness of a treatment. The critical question here is if a treatment target is sustainably locked into a desired state, how the network behaves in presence and absence of other perturbations. An ideal treatment target could be one which when locked should keep the system in an insulin sensitive state irrespective of any other perturbations. The different approaches currently targeted for treatment are suppression of liver gluconeogenesis, restoration of β-cell mass, incretin action, enhancement of insulin production, insulin supplementation, reduction in obesity, reduction in plasma free fatty acid levels, normalizing plasma glucose, reducing oxidative stress and exercise. None of these treatments was able to ensure an insulin sensitive state by sustained perturbation. The states were rather decided by the accompanying point perturbations. Thus, none of these treatments were able to reverse the diabetic state in the long run although transient suppression of plasma glucose could be obtained with many of them. One major line of attempted treatment is to improve the β-cell function or introduce a new population of healthy β-cells. The critical underlying questions are whether β-cell regeneration in T2DM is reversible and whether improving β-cell function can reverse T2DM. In the model, sustained up-regulation of β-cells did not ensure a stable insulin sensitive state. Therefore, the effectiveness of this approach in the treatment of T2DM is questionable.

In contrast, there were 10 nodes namely aggression (*agr*), testosterone (*tet*), dopamine (*dop*), oestrogen (*otg*), osteocalcin (*ocl*), melatonin (*mlt*), ghrelin (*ghr*), muscle strength (*msl*), adiponectin (*and*) and growth hormone (*hgh*) which when sustainably up-regulated, ensured insulin sensitivity. These nodes, when sustainably up-regulated from the all zero basal state, led to the insulin-sensitive state. All these nodes connect to insulin sensitivity by multiple pathways with positive regulatory pathways far outnumbering negative regulatory pathways (Table 4.3). For example, aggression links directly and indirectly to the first tier players (from Figure 3.1) which are insulin, glucose and insulin action through EGF (Brand et al., 2002; Hakonen et al., 2011; Sánchez et al., 2007), IGF-1 (O'Connell and Clemmons, 2002; Sapolsky and Spencer, 1997), dopamine (Erp and Miczek, 2000; de Leeuw van Weenen et al., 2011), muscle mass (Schwarz and Peever, 2011), bone strength (Bliziotes et al., 2000), adiponectin (Borcherding et al., 2011; Kubota et al., 2002), testosterone (Albert et al., 1991; Sattler et al., 2014) and other intermediates. Similar role is shown to be played by oestrogen in females (Albert et al., 1991, 1992). Osteocalcin, a marker of bone formation (Falahati-nini et al., 2000), also increases insulin sensitivity in humans (Lee et al., 2007). Melatonin is also known to enhance insulin sensitivity (Sartori et al., 2009), and also aggression (Jasnow et al., 2002). Thus, most of the above mentioned nodes that could ensure insulin sensitive state were closely related to aggression and aggression may hold the key to an insulin sensitive state as suggested by Belsare et al.(Belsare et al., 2010), Watve (Watve, 2013) and Watve and Yajnik (Watve and Yajnik, 2007).

Table 4.3: Number of pathways from the novel target to insulin action

Novel target	Total pathways	Positive / negative ratio
and	49	3.090909091
agr	140	1.955555556
dop	167	2.1
ghr	154	1.375
hgh	107	2.225806452
mlt	138	1.976744186

msl	41	3
otg	110	2.678571429
ocl	68	1.56
tet	135	1.62
ser	99	0.446153846

Table 4.3 Footnotes: All pathways that link the 11 promising nodes to insulin sensitivity were mapped and listed. The 10 nodes whose up-regulation increases insulin sensitivity, have a greater proportion of positive regulatory pathways. Serotonin, whose down-regulation increases insulin sensitivity, had a greater proportion of negative regulatory pathways.

I further examined how much time did each of the potential candidate nodes took for a reversal from insulin resistant to sensitive state. As mentioned earlier, an ideal treatment target could be one which when locked should either keep the system in an insulin sensitive state or lead the system to it irrespective of any other perturbations. In this race, oestrogen was the fastest actor which made the transition in 3 cycles followed by ghrelin (4 cycles), aggression (5 cycles), dopamine (7 cycles), muscle strength (24 cycles) and osteocalcin (59 cycles). If serotonin was down-regulated for at least 10 cycles, it also pushed the system from insulin resistant to insulin sensitive state. The system stays in the insulin sensitive state, even when the sustained perturbation is lifted, after the specified number of cycles. Applying a combination of interventions could reduce the number of cycles required for transition from insulin resistant to sensitive state. A minimum of 3 nodes were required to be simultaneously up-regulated for bringing up the transition in one or two cycles. These combinations are listed in Table 4.4. Once the system attained the insulin sensitive state by any of these above combinations of interventions, it could sustain itself against any point perturbations even when the interventions were withdrawn.

Table 4.4: Combinations of three nodes that led to insulin sensitive state when up-regulated simultaneously for a single cycle

Combination	Node 1	Node 2	Node 3
1	aggression	dopamine	testosterone
2	aggression	dopamine	serotonin*
3	aggression	dopamine	ghrelin
4	aggression	dopamine	muscle strength
5	aggression	dopamine	melatonin
6	aggression	dopamine	growth hormone
7	aggression	dopamine	oestrogen
8	aggression	testosterone	serotonin*
9	aggression	testosterone	ghrelin
10	aggression	testosterone	muscle strength
11	aggression	testosterone	melatonin
12	aggression	testosterone	growth hormone
13	aggression	testosterone	oestrogen
14	aggression	ghrelin	serotonin*
15	dopamine	testosterone	oestrogen
16	dopamine	oestrogen	growth hormone

Table 4.4 footnotes: Serotonin needs to be down-regulated to lead to an insulin-sensitive state.

When these interventions were applied assuming β-cell degeneration to be irreversible, individual up-regulation of *agr*, *dop*, *otg*, *ocl* and *ina*; and down-regulation of *ser* could still lead to the insulin sensitive state. When these interventions were applied when both β-cell and insulin levels were kept fixed at -1, the results were identical. Thus, the question whether β-cell degeneration is reversible or irreversible did not seem to be central to the reversal of an insulin resistant state to a sensitive one.

From the above analysis where the three-membered combinations were identified which led to an insulin sensitive state, it is evident that aggression (*agr*) is the strongest node. *agr* appeared in more than 80% of these three-membered combinations followed by dopamine (*dop*) which appeared in 61% of the combinations. Hence, I selected these two candidates to understand what determines the transition from the insulin resistant to the sensitive state. When *agr* was sustainably up-regulated and the model was run for an up-regulation of all the other factors, only a single attractor was obtained. Moreover, it was an insulin sensitive attractor since the state of insulin action (*ina*) was 1. Sustained up-regulation of *dop* also gave the same result. When *agr* was sustainably down-regulated and *dop* was sustainably up-regulated, the same insulin sensitive attractor was obtained. But when *dop* was sustainably down-regulated and *agr* was sustainably up-regulated, only insulin resistant attractor was obtained. Since, both *agr* and *dop* are pro-insulin sensitivity nodes, up-regulating them lead to insulin sensitivity. But when they were forced in opposite directions, abnormal network behaviour was observed. State of insulin action always correlated with dop. Hence, the state of the stronger of the two prevails; which means that if *dop* is up-regulated and *agr* is down-regulated, the resultant attractor is insulin sensitive. From the above examples, we can infer that *dop* is the stronger node and dopamine dominates over aggression.

The reason for this behaviour lies in the network structure. The strength of a node can also be determined by looking at the closeness centrality of that node; i.e. the prompt influence of that node on the rest of the network. The closeness centrality (given by cytoscape 3.5.1) of *dop* is 0.48, which is greater than that of *agr* (0.46) and also the highest amongst all the nodes in the network. Also, the outdgree of *dop* is 14, which is greater than that of *agr* (12) and again, the highest in the network.

4.5 Discussion

Despite the limitations of the model owing to its qualitative nature, the results are realistic in multiple ways. Running the model under different sets of assumptions, accommodating contradictory empirical results and the sensitivity analysis demonstrates that the model is robust and the results are not the artefactual outcome of any particular assumption. The model was able to predict the clinically observed correlates of insulin resistance accurately. The classically perceived treatments

targeting liver glucose production, insulin sensitivity, insulin secretion including incretin action and β-cell function failed to bring about a transition in the steady state in the model although they could temporarily improve glucose control. This matches with the clinical observations that all these lines of treatments have largely failed to cure diabetes or even control hyperglycaemia in the long run (Defronzo, 2004). Many large scale clinical trials have revealed that normalizing blood glucose is not effective in avoiding diabetic complications (Max Miller et al., 1976; Stratton et al., 2000; Turner et al., 1998). This finding is compatible with the model. Further, the model demonstrates that it might be impossible, in principle, to prevent diabetic complications by a sole focus on normalizing glucose. The ineffectiveness of aggressive glucose normalization trials may not be because of failure to appropriately regulate glucose. Even if glucose is regulated without hypoglycaemic and other undesirable events, the complications may not be arrested since normalization of glucose alone does not reverse the network state.

The implications of these results to clinical diabetes are radical. The failure of the mainstream treatment to reverse T2DM is generally interpreted as inadequacy of the treatment. It is believed that the line of treatment is correct but the efficiency is inadequate. Often the blame for increased mortality on stringent sugar normalization is put on episodes of hypoglycaemia. The model, on the other hand, suggests that the failure of the treatment may not be inadequacy but an inappropriate direction of treatment. Therefore, rather than refining the technology of glucose regulation, we need to explore alternative treatment goals altogether.

Because of the anastomoses of the network, the function lost by deleting a link can be compensated by alternative paths. Since the number of links stabilizing the attractors far outnumber the ones destabilizing it, a few missing links are unlikely to alter the behaviour of the network. This may explain why knockouts such as MIRKO, or insulin suppressing agents failed to increase fasting glucose in experiments (Alemzadeh et al., 2004; Kim et al., 2000). It is possible in a network that one or a few nodes play a central role, but if this is true, it should have been detected by systematic deletion of nodes that I performed. The system was generally robust in this analysis and the only node whose deletion or freezing made any changes in the behaviour of the system was not related to energy homeostasis but to fertility and behaviour. This might be surprising for the classical theory of T2DM but is expected by some of the upcoming evolutionary

hypotheses for the origin of T2DM (Corbett et al., 2009; Watve and Yajnik, 2007). Unless a single node or single link makes a critical difference, a disorder is unlikely to originate in a single gene defect. Therefore, it is no wonder then that genome wide association studies are able to explain not more than 2% of obesity (Boehnke et al., 2010) and10% insulin resistance (Morris et al., 2012) at a population level.

Reproductive capacity is measured differently in males and females. Since I wanted to make the model gender independent, I added the node fertility to it which comprised all the parameters related to the reproductive capacity pertaining to both males and females. The inclusion of fertility in males and that in females in a single node was justified since the interactions between fertility and insulin action are parallel in males and females. Fertility showed no correlation with the topological properties of the network. We argue that fertility is specifically evolved under certain pressures. In the model, fertility stands at the interface of insulin sensitivity and resistance. Removing fertility out of the equation results in a homogenous cluster of nodes and no two insulin sensitive and resistant groups are observed. This brings us to the notion that these two groups exist because of fertility. In case of an insulin sensitive individual, fertility is more and hence the investment per offspring is less. In case of an insulin resistant individual, fertility is less which increases the investment in the offspring (Watve, 2013). Fertility determines the fate of the progeny and it is intricately associated with insulin action. Change in either has an effect on the other.

Clinically, the first important realization of the study is that a large number of signals can potentially influence insulin sensitivity and the current emphasis on obesity alone is perhaps overplayed and unwarranted. The means of transiting from the insulin resistant attractor to the insulin sensitive one revealed by the model are substantially different from the traditional line of thinking in clinical practice or in drug discovery. The model shows that none of the current lines of treatment are able to make this transit. Instead, the model suggests some non-conventional lines of treatment. Of particular interest is the role of exercise. Sustained physical activity alone did not have effects comparable to aggression in the model. Physical activity has been classically considered to affect energy balance and reduce adiposity. Physical aggression, on the other hand, has many other direct endocrine effects (Belsare et al., 2010) and this effectively assured insulin sensitivity in the model. This raises the possibility that

exercises work more effectively through the behavioural neuro-endocrine pathways rather than through calorie consumption. In reality, many types of exercises have some or the other behavioural components and thereby stimulate the neuro-endocrine pathways (Chae and Kim, 2009; Farrell et al., 1987; Nexø et al., 1988; Stranahan et al., 2009; Volek et al., 1997) in addition to burning calories. A testable prediction of the model is that different exercises can be expected to have different endobolic effects even if the caloric requirement is matched (Belsare et al., 2010; Whyte et al., 2010).

The insights provided and the suggestion of new promising treatment targets is a result of our model not shared by earlier network models of T2DM. This difference is likely to be the result of inclusion of multi-organ and multi-level nodes and links. Inclusion of genetic, physiological, metabolic, endocrine, immunological and neurobehavioural mechanisms in the model elucidates elements that were not visualized at a single or a few levels at a time. Inclusion of neurobehavioural elements is particularly remarkable feature of our model which the classical thinking had not seriously incorporated in spite of some evidence pointing in that direction. Some evolutionary medicine hypotheses (Baig et al., 2011; Corbett et al., 2009; Mankar et al., 2008; Neel, 1962; Watve and Yajnik, 2007) are centred around behavioural and reproductive strategies and they are particularly relevant here. Looking at a broader picture has not only made more data available but made new dimensions visible.

We can no more view complex disorders by piecemeal and expect to treat the disorder effectively. The behaviour of a network can be substantially different from the behaviour of smaller pieces of the network. The model suggests molecular targets such as adiponectin, growth hormone, melatonin and testosterone for prevention of T2DM; and dopamine, ghrelin, oestrogen and osteocalcin for prevention as well as treatment of T2DM. There is some attention given to these targets as potential treatment options (Achari and Jain, 2017; Oh et al., 2016; Zanatta et al., 2014) and some of them are at an early clinical trial stage (Cincotta et al., 1999; Jones et al., 2011). However, if the end point is still visualized as glucose normalization, the trials may not go on the right direction. A radical conceptual change is needed to explore the new lines of treatment.

A quick glance at the new set of targets reveals that all of them are related to behaviour. Adiponectin affects food intake (Tambascia et al., 2008), aggression affects dopamine (Ferrari et al., 2003), serotonin (Erp and Miczek, 2000) and testosterone (Elias, 1981).

Dopamine affects aggression (Ossowska et al., 1996) and food intake (Davis et al., 2009). Ghrelin affects aggression (Chen et al., 2015) and food intake (Abizaid et al., 2006). Growth hormone affects aggression (Matte, 1981) and so do melatonin (Jasnow et al., 2002) and oestrogen (Ogawa et al., 2000). Oestrogen also affects food intake (Musatov et al., 2007). Serotonin affects aggression (Cleare and Bond, 1997) and food intake (Hrboticky et al., 1985). Testosterone affects diplomat behaviour (Eisenegger et al., 2010) and aggression (Kriegsfeld et al., 1997). Therefore, it is likely that behavioural intervention may have a better promise. The concept of behavioural intervention is already there on the horizon (Carter et al., 2014; Schwingshackl et al., 2016), but currently it talks mainly about eating behaviours alone. Behaviours related to aggression or adventure are linked to elements of metabolic syndrome by a large number of pathways compiled in the appendix I of Watve (Watve, 2013) which suggests that behavioural interventions in this direction could affect physiology of many systems of the body. It is quite likely that a paradigm shift is awaiting round the corner in the field and we need to be open to this possibility.

4.6 References

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Chapter 5

Steady-state and perturbed state causality and the design of a network clinical study

The T2DM network model constructed and used here is based on causal links that are demonstrated by interventional experiments. However, biological causality is more complex and what an experiment demonstrates may have limited implications in the context of the system. As shown below, causality in a perturbed system can be substantially different than causality in a steady state. This chapter explores the difference between steady state (SS) versus perturbed state (PS) causality in homeostatic systems. This concept is then applied to the T2DM network. On the one hand, it explains how a network may shape the difference between steady state and perturbed state causality. On the other, it explores theoretical and empirical ways of inferring steady state causality in a multivariate system and then how a clinical study on T2DM needs to be designed to get the necessary data for this approach.

Determining the relationship between SS and PS causality as well as inferring causality from multivariate steady state data are aspects of on-going team work in our lab. I have contributed to the teamwork as well as benefited from it. In this chapter, I will describe the relevant outcomes from the teamwork and then apply it to my model.

5.1 Introduction to steady state and perturbed state causality

Researchers in our lab realized that the nature of causality in a PS can be qualitatively different from that in a SS and the inability to distinguish between the two appears to have misguided research in biomedicine and even treatment in practicing medicine (Diwekar-joshi *et al*, manuscript under preparation).

A well worked out theoretical model that can be used to distinguish clearly between perturbed and steady state causation is the logistic population growth curve. The population trajectory is decided by two parameters of the model, the intrinsic growth rate of the population r and the carrying capacity K of the environment for this species. The carrying capacity determines the size of the population at equilibrium. The intrinsic growth rate of the population does not determine the equilibrium population but it determines the time taken to reach the equilibrium or steady state (see Figure 5.1). Non-zero positive r is essential for attaining the equilibrium or returning to it if

perturbed. Thus, *r* has a causal role in attaining the equilibrium if disturbed but has no causal role in deciding the position of the equilibrium. Thus, in a perturbed state, *r* decided the fate of a population but once a steady state is reached, *r* loses its causal relevance.

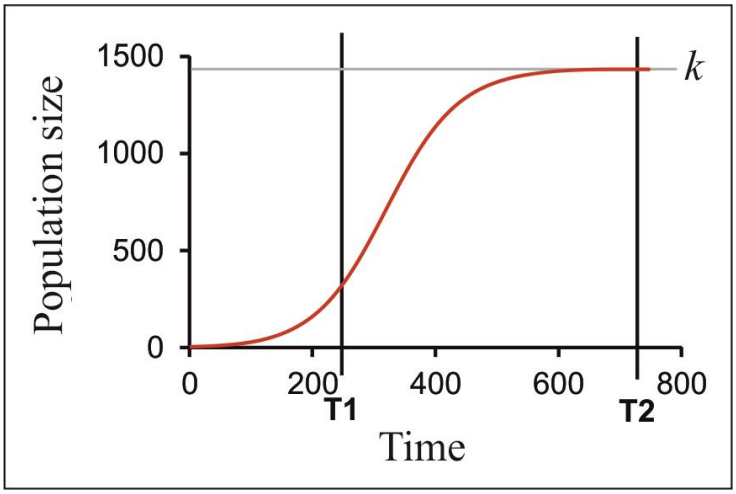

Figure 5.1: Simulated population dynamics of a species. It can be seen that at time T1, the population is in proportion to its growth rate but at T2 approaching equilibrium, growth rate become increasingly irrelevant in determining population size. Thus, growth rate is an important determinant of population size in a perturbed state but not at a steady state.

This has relevance to experimental biology. The distinction between SS and PS causality is important in building the philosophy of experimental physiology and medicine. Most biological experiments are perturbation experiments and by using them, we try to infer about the biological system which is often in a homeostatic steady state. Thus, we use a method of perturbation and try to infer causality in a steady state. This is a major philosophical problem in experimental biology. A knock-out experiment that disables homeostatic control implies that the gene function may be necessary for achieving homeostatic control but does not necessarily imply that the gene determines the steady state levels of the controlled variable.

A largely overlapping concept is that of driver versus navigator causality. In order to reach a destination both types of causalities are required. A driver is necessary to reach the destination but the driver does not decide the destination. In the examples

discussed above, the *r* of logistic equation shows driver causality whereas *K* is the navigator. In a knock-out experiment, knocking out either the driver or the navigator disables the act of reaching the destination. Therefore, a knock-out experiment is insufficient to infer whether the gene has a driver function or a navigator function or both.

5.1.1 Demonstration of a causal mechanism does not imply SS causality: In a healthy warm blooded animal, the body temperature is maintained constant by homeostatic mechanisms. If homeostasis is efficient, one may not find any correlation between environmental temperature and body temperature. In this sense, the environmental temperature does not determine body temperature. Nevertheless, heat transfer between the body and the environment exists and can be demonstrated. This example shows that demonstration of a causal mechanism is not sufficient to establish a causal role in a steady state.

5.1.2 Need for distinguishing between SS and PS causality in T2DM: Insulin was discovered in the context of type 1 diabetes (T1DM) which was the commoner form of diabetes that time. In T1DM, insulin producing β-cells are destroyed by an autoimmune mechanism and therefore, insulin production is almost absent. Similarly, complete pancreatectomy or complete destruction of β-cells by high doses of streptozotocin lead to loss of glucose control. But this does not tell us whether insulin is a driver or navigator or both. Without any clarity on this distinction, it was assumed that insulin action decided the steady state glucose levels and a change in this steady state fasting glucose is a result of a change in insulin signalling.

A number of experiments showed later that either suppressed or raised insulin levels do not affect fasting glucose levels. Muscle or fat cell specific insulin receptor knockouts also do not show raised levels of fasting glucose. Systematic review and meta-analysis of such experiments in rodents as well as humans and a set of primary experiments from our lab (Diwekar-Joshi *et al*, manuscript under preparation; Diwekar-Joshi, thesis under preparation) have now demonstrated that insulin acts like *r* of the logistic equation. It is necessary to reach a steady state of fasting glucose; it decided the rate at which the steady state is achieved; but it does not decide the steady state glucose level. Thus, one major assumption in the theory as well as treatment strategy of T2DM was wrong. For

decades together, diabetes research was misled owing to a philosophical failure to distinguish between the SS and PS causality. A number of concepts such as the homeostatic model assessment (HOMA) indices for measuring insulin resistance and β-cell responsiveness are based on the assumption that insulin regulates the fasting glucose levels. All experiments have only shown that insulin signalling is necessary to achieve glucose steady state (driver cause). No experiment conclusively demonstrates that insulin signalling decides the steady state glucose levels (navigator cause). The latter is only an assumption which is challenged by a series of experiments; but the field is confused about the interpretations of these experiments because of the inability to distinguish between the two types of causes.

But intensive glucose control studies have shown us that insulin cannot reduce steady state glucose levels (Max Miller et al., 1976; Stratton et al., 2000; Turner et al., 1998). Perturbation experiments are usually conducted in translational research to identify novel targets. We need to be careful in assuming a similar causal relationship between the variables under study at the steady state and using these therapeutic targets. In case of T2DM, targets that can reduce the blood glucose levels at the steady state need to be identified.

5.2 SS PS in the network model

Having made clear the distinction between SS and PS causality, we will return to the network model. To build the network model, I have used a set of experiments which demonstrate a causal link but the experimenters have not distinguished between SS and PS causality in the experiments. So the input information is incomplete in a way. However, the network itself is likely to give us some insights into why some demonstrated PS causal relationships may fail to remain causal in a steady state.

In a network setting, two variables may be connected to each other by multiple pathways. It is possible that there is a direct up-regulation link but the net effect through the network is nil or even down-regulation (Figure 5.2). Therefore, having a direct up-regulation causal link does not ensure that the relationship between the two

will be in the same direction. Further, when the network reaches a steady state, the two may not have a positive correlation with each other. We know that correlation does not ensure causation, but absence of correlation certainly means absence of effective causation. In the absence of steady state correlation, the causal link cannot be demonstrated in a steady state. This is likely to be at least one of the reasons why a PS causal link may disappear in a steady state.

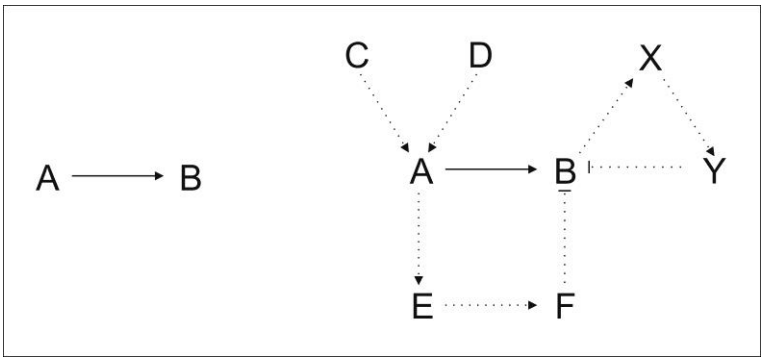

Figure 5.2: Direct and indirect causal links in a network system.

5.2.1 Identifying the vanishing SS causal relations in the model: Going by this approach, I identified nodal pairs who have a causal link at the perturbed state and are correlated at the steady state, those who have a causal link at the perturbed state but are not correlated at the steady state and those who do not have a causal link at the perturbed state but are correlated at the steady state.

The steady state relationships were identified in Chapter 3 Section 3.3.5. The results in the clustering methods employed are displayed below.

1. *and*, *agr*, *ang*, *aox*, *bdn*, *btc*, *cck*, *cts*, *crh*, *dop*, *egf*, *edp*, *epo*, *fty*, *gap*, *glg*, *gng*, *gt1*, *hst*, *igf*, *inj*, *ina*, *ktg*, *msl*, *ngf*, *nep*, *otg*, *ost*, *ocl*, *oxy*, *bgl*, *tet*

2. *ata*, *adp*, *msh*, *car*, *chl*, *cfn*, *dip*, *et1*, *ffa*, *fdi*, *gst*, *grh*, *inr*, *ins*, *lep*, *myo*, *nox*, *pgl*, *sfr*, *ser*, *tnf*, *tri*, *gmo*, *noc*

3. *avp*, *bar*, *ctk*, *gab*, *ghr*, *hgh*, *il6*, *klt*, *lpa*, *mlt*, *sys*, *vdl*, *vd3*, *exe*, *glp*, *v12*

The nodes in each cluster are positively correlated to each other. The nodes in clusters one and two are negatively correlated to each other and not correlated to the nodes in

cluster three. The perturbed state causal relations are tabulated in Appendix I. I compared these perturbed state causal relations with their respective steady state correlations and listed the nodal pairs in three categories.

1. The nodal pairs that have a PS causal link and correlate in the SS:

Table 5.1: Nodal pairs that have a PS causal link and correlate in the SS.

Node 1	**Node 2**
ata	*ins*
adp	*ata*
adp	*lep*
adp	*sfr*
adp	*tnf*
and	*fty*
and	*ina*
agr	*bdn*
agr	*cck*
agr	*cts*
agr	*dop*
agr	*egf*
agr	*edp*

Node 1	**Node 2**
egf	*ina*
edp	*bdn*
edp	*ina*
et1	*ins*
et1	*lep*
epo	*ang*
fty	*egf*
fty	*otg*
fty	*oxy*
fdi	*adp*
fdi	*ffa*
fdi	*ins*
fdi	*pgl*

Node 1	**Node 2**
lep	*inr*
lep	*ser*
msl	*agr*
msl	*ina*
myo	*adp*
myo	*tnf*
ngf	*bdn*
nep	*crh*
otg	*agr*
otg	*ang*
otg	*fty*
otg	*ocl*
ost	*ocl*

agr	*igf*
agr	*ngf*
agr	*tet*
ang	*bgl*
bdn	*btc*
bdn	*ina*
btc	*gap*
cck	*ina*
chl	*cfn*
chl	*inr*
chl	*ser*
cfn	*dip*
cts	*gng*
crh	*cts*
crh	*edp*
ctk	*avp*
dip	*ins*
dop	*and*
dop	*agr*

gap	*btc*
ghr	*gab*
ghr	*hgh*
glg	*gng*
gt1	*bgl*
hst	*agr*
igf	*btc*
igf	*ina*
igf	*msl*
igf	*ost*
il6	*glp*
inj	*ang*
inj	*btc*
inj	*hst*
inj	*ina*
ins	*cfn*
ins	*et1*
ins	*grh*
ins	*lep*

ocl	*and*
ocl	*ina*
ocl	*tet*
oxy	*agr*
oxy	*cts*
oxy	*glg*
oxy	*gng*
pgl	*ata*
pgl	*ins*
ser	*cfn*
ser	*inr*
tet	*agr*
tet	*ang*
tet	*aox*
tet	*egf*
tet	*epo*
tet	*ina*
tet	*msl*
tet	*ocl*

dop	*ina*
dop	*msl*
dop	*ost*
dop	*ocl*
egf	*bdn*
egf	*btc*
egf	*fty*

ins	*nox*
ins	*gmo*
ina	*msl*
ina	*ost*
lep	*car*
lep	*cfn*
lep	*et1*

tnf	*ata*
tnf	*et1*
tnf	*inr*
tnf	*lep*
tri	*adp*
exe	*il6*
v12	*hgh*

Since correlation does not always ensure causality, I can only say that there are 117 pairs that might have the same causal relationship in the SS and PS.

2. The nodal pairs that do not have a causal link demonstrated in the PS but correlate in the SS:

Table 5.2: Nodal pairs that do not have a causal link demonstrated in the PS but correlate in the SS.

Node 1	**Node 2**
adp	*and*
adp	*ctk*
agr	*chl*
agr	*et1*

Node 1	**Node 2**
gab	*agr*
gap	*inr*
ghr	*et1*
ghr	*fdi*

Node 1	**Node 2**
mlt	*ins*
mlt	*lep*
mlt	*pgl*
mlt	*tri*

agr	*ser*
and	*ata*
and	*ffa*
and	*fdi*
and	*inr*
and	*tri*
aox	*inr*
ata	*btc*
ata	*ctk*
avp	*cfn*
avp	*ins*
avp	*agr*
avp	*gng*
bar	*adp*
bdn	*cfn*
bdn	*fdi*
bdn	*ser*
bgl	*cfn*
bgl	*fdi*

ghr	*ins*
ghr	*agr*
glp	*fdi*
glp	*ins*
glp	*gmo*
glp	*btc*
glp	*glg*
glp	*ina*
glp	*ost*
gmo	*glp*
gng	*pgl*
grh	*tet*
gst	*agr*
gst	*btc*
gst	*ina*
hgh	*ins*
hgh	*agr*
hgh	*fty*
hgh	*igf*

mlt	*agr*
mlt	*ina*
mlt	*msl*
mlt	*ost*
mlt	*ocl*
msh	*agr*
msh	*ina*
msh	*msl*
msh	*ctk*
msl	*inr*
myo	*ina*
myo	*msl*
ngf	*noc*
noc	*ina*
nox	*agr*
nox	*ang*
nox	*dop*
nox	*nep*
nox	*vdl*

btc	*ins*
cck	*fdi*
cck	*gst*
cck	*ins*
cfn	*bgl*
chl	*agr*
crh	*fdi*
crh	*ins*
ctk	*et1*
ctk	*inr*
ctk	*lep*
ctk	*crh*
ctk	*edp*
ctk	*ina*
ctk	*oxy*
cts	*ffa*
cts	*inr*
cts	*ins*
cts	*tri*

hgh	*ina*
hgh	*msl*
hst	*fdi*
igf	*adp*
igf	*fdi*
igf	*ins*
igf	*tnf*
igf	*ctk*
igf	*hgh*
il6	*adp*
il6	*fdi*
il6	*inr*
il6	*agr*
il6	*ina*
ina	*adp*
ina	*pgl*
ina	*tri*
inj	*adp*
inj	*inr*

ocl	*ins*
ocl	*glp*
otg	*fdi*
otg	*inr*
otg	*gmo*
otg	*hgh*
oxy	*adp*
oxy	*fdi*
oxy	*noc*
pgl	*glg*
pgl	*gt1*
pgl	*bgl*
pgl	*ghr*
ser	*agr*
ser	*dop*
ser	*ina*
ser	*ost*
sys	*adp*
sys	*egf*

dop	*ffa*
dop	*fdi*
dop	*ins*
dop	*lep*
dop	*pgl*
dop	*tri*
dop	*il6*
edp	*fdi*
egf	*noc*
et1	*agr*
et1	*ina*
et1	*ghr*
et1	*vdl*
exe	*adp*
fdi	*agr*
fdi	*msl*
ffa	*bdn*
ffa	*gng*
ffa	*inj*

inj	*ins*
inr	*ina*
ins	*and*
ins	*btc*
ins	*egf*
ins	*ktg*
ins	*klt*
klt	*adp*
klt	*inr*
klt	*ins*
klt	*ang*
klt	*aox*
klt	*fty*
klt	*ina*
ktg	*cfn*
ktg	*inr*
ktg	*ins*
lep	*ang*
lpa	*adp*

sys	*gng*
sys	*ina*
tet	*adp*
tet	*msh*
tet	*dip*
tet	*myo*
tnf	*ina*
tnf	*nep*
tnf	*il6*
tnf	*klt*
tri	*ina*
v12	*inr*
v12	*noc*
v12	*igf*
v12	*ost*
vd3	*ins*
vd3	*agr*
vd3	*ina*
vd3	*ost*

ffa	*ina*
gab	*fdi*
gab	*grh*

lpa	*fdi*
lpa	*tri*
mlt	*adp*

vdl	*ina*
vdl	*bgl*

There are 191 such pairs that do not have a direct PS causal link but are correlated in SS. It is important to note this because false causal relationships may be inferred because of these correlations.

3. The nodal pairs that have a PS causal link but do not correlate in the SS:

Table 5.3: Nodal pairs that have a PS causal link but do not correlate in the SS.

Node 1	**Node 2**
ata	*btc*
adp	*and*
adp	*ctk*
and	*ata*
agr	*et1*
msh	*agr*
msh	*ina*
msh	*msl*

Node 1	**Node 2**
gab	*agr*
gab	*grh*
gst	*agr*
gst	*btc*
gst	*ina*
ghr	*agr*
ghr	*fdi*
ghr	*ins*

Node 1	**Node 2**
mlt	*ost*
mlt	*ocl*
ngf	*noc*
nox	*ang*
nox	*vdl*
otg	*hgh*
otg	*inr*
ocl	*ins*

avp	*agr*
avp	*cfn*
avp	*gng*
avp	*ins*
bdn	*cfn*
bdn	*ser*
btc	*ins*
cck	*ins*
cts	*ffa*
crh	*ins*
ctk	*crh*
ctk	*edp*
ctk	*et1*
ctk	*inr*
ctk	*lep*
ctk	*oxy*
dop	*il6*
edp	*fdi*
et1	*agr*

gng	*pgl*
grh	*tet*
hgh	*agr*
hgh	*fty*
hgh	*igf*
hgh	*ins*
hgh	*msl*
ins	*btc*
ins	*egf*
ins	*klt*
ina	*adp*
ina	*tri*
ktg	*cfn*
ktg	*ins*
klt	*adp*
klt	*ang*
klt	*aox*
klt	*ins*
lep	*ang*

ocl	*glp*
pgl	*bgl*
bgl	*cfn*
sys	*egf*
sys	*gng*
sys	*ina*
tnf	*il6*
vdl	*ina*
vdl	*bgl*
vd3	*agr*
vd3	*ins*
vd3	*ina*
vd3	*ost*
glp	*btc*
glp	*ins*
glp	*ina*
glp	*ost*
gmo	*glp*
v12	*igf*

et1	*ghr*
ffa	*gng*
fdi	*msl*

mlt	*agr*
mlt	*ina*
mlt	*msl*

v12	*ost*

There are 88 such pairs that do not have the same causal relationship in SS and PS. The total number of possible nodal pairs in the 72-node network is 5112. Out of these, only 117 may have the same causal relationship in the steady and the perturbed states (Figure 5.3).

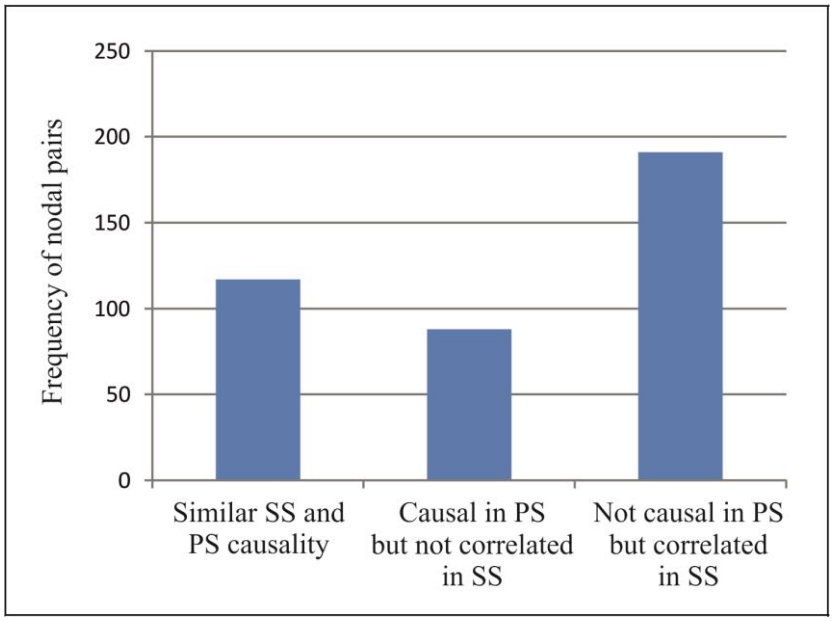

Figure 5.3: Nodal pairs that have similar and dissimilar causality in SS and PS.

5.2.2 Inferring SS-PS from sustained perturbations: Another approach to study SS causality experimentally is to hold a perturbation constant for a long time sufficient to reach a steady state. On introducing the perturbation if the target variable changes, it is a demonstration of PS causality. On sustaining a constant perturbation, if the system reaches a steady state again with the same level of the target variable, there is no SS causality although there is PS causality. On the other hand, if the target variable reaches an altered level in the new stable state as a result of the sustained perturbation, there is both PS and SS causation. Such experiments with insulin and glucose indicate that there

is a negative feedback PS causality between the two but no evidence for SS causality (Diwekar-Joshi *et al*, manuscript under preparation; Diwekar-Joshi, thesis under preparation). It is difficult to find such experiments among other pairs of variables. Nevertheless, we can test what happens on sustained perturbations in the network model. We have seen the results of sustained up-regulation of insulin in the model which does not alter bi-stability. This means that the steady state level of glucose is decided by factors other than insulin. This result is compatible with other work in our lab which shows that glucose and insulin have only a PS causal relationship and not SS. This is an important realization and several lines of evidence converge on the conclusion that insulin and glucose do not have a steady state relationship with each other although they influence each other in a perturbed state.

5.3 Predicting a SS causal network from empirical data: Is it possible?

Since in a steady state, the variables remain at a constant level over time, longitudinal studies are of no use in inferring causality. Some of the methods of causal analysis such as Granger causality (Granger, 1969) necessarily depend upon longitudinal data. In a steady state, only cross-sectional data can be obtained. In such data, we may find a number of inter-correlated variables but correlations give little information about causation directly.

A team in our lab has worked out a solution to this problem over the past few years which I was a small contributor to (Chawala *et al*, manuscript in revision). The novel philosophy, concept and method of inferring causation are described in a summary form in the next section. Later, I will also explore the application of these methods to the T2DM network in section 5.4.

5.3.1 Correlation to causation between 3 variables: In a cross-sectional dataset, determining causality is difficult. We (Chawala *et al*, manuscript in revision) have developed a set of rules that use regression parameters from a cross-sectional dataset and help deduce causality between them. Cross-sectional correlations between two variables are insufficient to determine causality. But in a homeostatic system with three

or more inter-correlated variables, it is possible to make causal inferences from steady state data. We initially listed all possible pathways between the three variables (Figure 5.4).

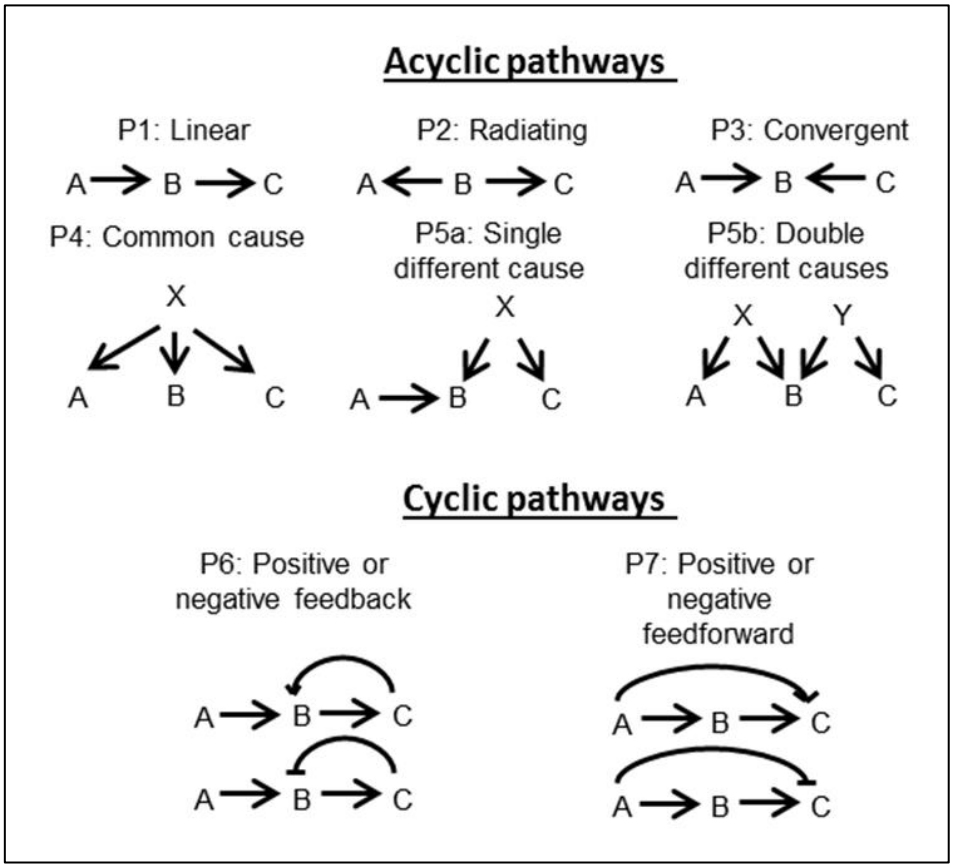

Figure 5.4: Possible pathways between three variables (Reproduced from Chawala *et al*, manuscript in revision)

Based on hypothesized pathways, we can write specific causal equations for each. The causal equations are derived from the hypothesized pathway, while the regression equations can be obtained from the given cross-sectional data using regression and correlation analysis. Causal equations are similar to structural equations. However, they differ in their interpretation and treatment. In structural equations, the left hand terms are effects and right hand terms are causes, and the two cannot be algebraically transferred without changing causal interpretations. In our approach, after finding equilibrium solutions, we can carry out algebraic operations freely in order to obtain testable predictions. The parameters of the regression equation are not necessarily identical to those of the causal equations.

For example, for a hypothesized pathway Y = mX + C, m is the causal slope, while the regression slope would be underestimated if there is post-effect variability in X, and such a bias in the slope is important in making and testing predictions. Similarly, we show that the parameters of causal equations hold pathway-specific relationships with the parameters of regression equations based on which, pathway-specific predictions about the regression correlation parameters can be made. We describe four general predictions across all pathways and formulate a null hypothesis for each. In addition, there are certain pathway specific predictions too, which are not discussed here. The four general predictions are:

1. Whether the coefficient of determination r^2_{AC} can be estimated from the product of r^2_{AB} and r^2_{BC}.

2. Whether slope M_{ca}can be estimated from the product of the slopes M_{ba} and M_{cb}.

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. 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}.

Let us consider the linear pathways as an example here. The causal equations for a linear pathway are:

$A = input = \acute{A} + e_a$

$B = m_1 A + e_1 + k_1$

$C = m_2B + e_2 + k_2$

Where e_a, e_1, e_2 are errors, not correlated to each other.

Regression parameters can be derived from the causal equations as follows. Since in regression of *B* on *A*, the slope = *cov (A, B)/var A*,

$$M_{ba} = \frac{\sum e_a e_b}{\sum e_a^2} = \frac{\sum e_a(m_1 e_a + e_1)}{\sum e_a^2} = \frac{m_1 \sum e_a^2}{\sum e_a^2} = m_1$$

$$M_{cb} = \frac{\sum e_c e_b}{\sum e_b^2} = \frac{\sum (m_2 e_b + e_2) e_b}{\sum e_b^2} = \frac{m_2 \sum e_b^2}{\sum e_b^2} = m_2$$

$$M_{ca} = \frac{\sum e_c e_a}{\sum e_a^2} = \frac{\sum (m_2 m_1 e_a + m_2 e_1 + e_2) e_a}{\sum e_a^2} = \frac{m_2 m_1 \sum e_a^2}{\sum e_a^2} = m_2 m_1$$

$E_{ba} = e_b - M_{ba} e_a = m_1 e_a + e_1 - m_1 e_a = e_1$

$E_{cb} = e_c - M_{cb} e_b = m_2 e_b + e_2 - m_2 e_b = e_2$

$E_{ca} = e_c - M_{ca} e_a = m_2 e_b + e_2 - m_2 m_1 e_a = m_2 e_1 + e_2$

For linear equations, there is little difference between the causal equations and regression equations (Table 5.4). The regression equations therefore become

$B = M_{ba}A + E_{ba} + K_{ba} = m_1 A + e_1 + k_1$

$C = M_{ca}A + E_{ca} + K_{ca} = m_2 B + e_2 + k_2$

$C = M_{ca}A + E_{ca} + K_{ca} = m_1 m_2 A + (m_2 e_1 + e_2) + (m_2 k_1 + k_2)$

Table 5.4. Relationship between the causal and regression equations for linear pathway.

Slopes	**rors**
$M_{ba} = m_1$	$E_{ba} = e_1$

$M_{cb} = m_2$	$E_{cb} = e_2$
$M_{ca} = m_1 m_2$	$E_{ca} = m_2 e_1 + e_2$

Prediction R1: Based on the equations above and Table 5.4, it can be shown that

$r_{AC} - r_{AB} r_{BC} = 0.$

Prediction R2: From Table 5.4, it is obvious that the slope M_{ca} can be predicted from the product $M_{cb} M_{ba}$; $M_{ca} - M_{cb} M_{ba} = m_1 m_2 - m_2 m_1 = 0.$

Prediction R3: From Table 5.4, as there is no covariance between e_1 and e_2,

$r^2_{E_{ba} E_{cb}} = r^2_{e_1 e_2} = 0$

Prediction R4: For a linear pathway, it can be shown that

(a) $r_{BC} > r_{E_{ba} C}$ and further,

(b) (b) $\frac{r^2_{BC} - r^2_{E_{ba} C}}{r^2_{BC}} = r^2_{AB}$

Likewise, predictions were drawn for every pathway in Figure 5.3 (Table 5.5). We then test these predictions on a steady state cross sectional dataset and derive the plausible causal pathway between the 3 variables.

Table 5.5: Summary of predictions of all pathways considered (Reproduced from Chawala *et al*, manuscript in revision).

Prediction/ Rule → **Pathway ↓**	**R1**	**R2**	**R3**	**R4 a**	**R4 b**	**Pathway specific prediction**

P1 linear	$r^2_{AC} - r^2_{AB}.r^2_{BC} = 0$	$\lvert M_{ca}\rvert = \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} = 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} = r^2_{AB}$	
P2 radiating	$r^2_{AC} - r^2_{AB}.r^2_{BC} = 0$	$\lvert M_{ca}\rvert = \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} = 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} = r^2_{AB}$	
P3 convergent	$r^2_{AC} - r^2_{AB}.r^2_{BC} < 0$	$\lvert M_{ca}\rvert < \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} > 0$	$r_{Ebc,C}/r_{BC} > 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} = r^2_{AB}$	$r_{AC}=0$, $r^2_{AB}+r^2_{BC}<1$
P4 common cause	$r^2_{AC} - r^2_{AB}.r^2_{BC} > 0$	$\lvert M_{ca}\rvert > \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} < 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} >= r^2_{AB}$	Symmetry around A,B,C
P5 different cause	$r^2_{AC} - r^2_{AB}.r^2_{BC} < 0$	$\lvert M_{ca}\rvert < \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} > 0$	$r_{Ebc,C}/r_{BC} > 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} = r^2_{AB}$	$r_{AC}=0$ $r^2_{AB}+r^2_{BC}<1$
P6 feedback Negative	$r^2_{AC} - r^2_{AB}.r^2_{BC} > 0$	$\lvert M_{ca}\rvert > \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} < 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} >= r^2_{AB}$	
P6 feedback positive	$r^2_{AC} - r^2_{AB}.r^2_{BC} < 0$	$\lvert M_{ca}\rvert < \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} > 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} >=< r^2_{AB}$	
P7 feed-forward negative	$r^2_{AC} - r^2_{AB}.r^2_{BC} < 0$	$\lvert M_{ca}\rvert < \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} > 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} <= r^2_{AB}$	

P7 feed-forward positive	$r^2_{AC} - r^2_{AB}.r^2_{BC} > 0$	$\lvert M_{ca}\rvert > \lvert M_{ba}.M_{cb}\rvert$	$r_{Eba,Ecb} < 0$	$r_{Ebc,C}/r_{BC} < 1$	$\frac{\lvert r^2_{E_{ba}C} - r^2_{BC}\rvert}{\max(r^2_{E_{ba}C}, r^2_{BC})} >= r^2_{AB}$	

5.3.2 Correlation to causation in a network: The three-variable method described above can be used in a network setting. The nodes in the network can be divided into triplets of inter-correlated variables and each of these triplets can be considered as a three variable motif for the correlation to causation analysis. Bringing in a fourth one can provide additional insights which can be used for cross-checking or validating our first set of inferences. In complex causal networks, there can be many such cross-check and validation possibilities. For large networks, algorithms requiring massive computational power may be needed that may pin down one or a few network structures from the large number of possible ones using combinations of three-member motifs and cross validation facility among the motifs. Developing such algorithms is another area where massive team efforts are needed. But the principles on which it can be built are now in hand (Chawala *et al*, manuscript in revision).

Using this analysis, unlikely pathways can be rejected and the results from each triplet can be put together to construct a steady state causal network. This network can then be compared to the T2DM perturbed state network so the SS and PS state of every causal relation can be elucidated. Moreover, the steady state causal network constructed after applying the correlation to causation tool may give us a much better understanding of the pathophysiology of T2DM. The analytical methods used in the PS causality T2DM network model can then be applied in the SS causality T2DM network model to extract novel targets that can ensure reversal of T2DM.

5.4 Proposing a network clinical study

Unlike the classical belief, the network model suggests that changes in insulin and glucose may not be central to T2DM but may only be the presenting symptoms arising from a complex network of events preceding it. The cause-effect relationships and inter-connectedness of the multitude of changes is not incorporated in the classical theory. The network model is based on isolated experiments by a 'joining the dots' approach. In order to develop an insight into the causal relationships among the complex network, it is necessary to generate data on the different nodes of the network on the same set of subjects. Such data are unavailable currently in either experimental or epidemiological setting. All studies look at a small number of parameters at a time. So empirical data that could support, validate or make use of the model do not exist at present. Since the model results are radically different than the classical view and at the same time promising a new line of treatment for the reversal of T2DM, it is necessary to undertake an epidemiological study in the form of a 'network clinical study'. So far, network models have been only theoretical and have little direct association with empirical clinical work. A design and proposal for a clinical study that could directly connect itself to a network model and has a potential to be revolutionary in the field of T2DM is described below.

The key problem being investigated is the inference of causal relationships between the different variables in the pathophysiology of T2DM. This analysis will be able to identify reliable biomarkers for the diagnosis as well as prognosis of the disease as well as design either a universal or personalized line of treatment aimed towards complete reversal of T2DM.

The chain of events leading to T2DM may begin to appear early in life, although it might take a long time to manifest into symptoms. In order to capture different stages of the progression, the sample should contain individuals of various age classes and with different levels of risk and actual disease. The study also needs to capture the effects of diet, behaviour and other lifestyle related factors. Periodic blood samples obtained from the study group will be analysed for a large number of parameters including metabolomic, endocrine, cytokines/chemokines, growth factors, tissues and other molecular signals present in the T2DM network. Not all nodes in the network model are

amenable to analysis owing to the limitations of sampling. For example, the nodes in brain cannot be sampled. Nevertheless, all the nodes that are reflected in plasma levels, morphometry, clinical examination and behavioural assessments can be quantified. Therefore, although there will be a difference between the number of nodes in our network model and the number of variables that can be gathered in an empirical study, there will still be sufficient multivariate data to apply to a network model. A cross-sectional steady state dataset on these parameters can then be used to infer a causal network by the analytical methods that our lab has developed and are being refined further.

5.4.1 Recruitment of participants: The proposed study requires a large cross sectional sample of the population covering a wide range of endo-bolic conditions. The cross-sectional study will mainly generate the multivariate data needed by the network model and perform causal analysis. Cross sectional sampling: A targeted 1000 subjects spanning widely from sportsmen/ wrestlers/ boxers to completely sedentary non-exercising and non-aggressive adults; forest dwelling tribals to high density urban residents; lean to obese and highly insulin sensitive to long standing T2DM with complications will be involved in one-time sampling in which a multi-dimensional assessment of health status will be done (details of which follow).

5.4.2 Assessment Details: Assessment will be done along 4 different lines of enquiry - clinical, behavioural, fitness and physiological (metabolic, endocrine and immunological).

1. Clinical assessment: Clinical assessment will include detailed history taking, clinical (general and systemic) examination. Assessment of diabetic complications including retinal, sensory nervous and renal will be done. Assessment of autonomic function tests will be done with the help of established standard autonomic function tests including heart rate variability, valsalva ratio, slow deep breathing, heart rate response to standing/ tilt, blood pressure response to sustained hand grip, cold pressure test, mental arithmetic stress, etc. Vascular function assessment will be done by ankle-brachial Index, toe-Brachial Index, brachial-ankle pulse wave velocity (baPWV), vascular age & R-R variability. Endothelial function will be assessed using Endo-PAT. A full body fat assessment, body composition, lean tissue

mass, fat tissue mass and fractional contribution of fat measurements, will be done by Dual Energy X-ray Absorptiometry (DEXA). Trunk-to-leg fat ratio, total-to-trunk fat ratio, bone mineral density & bone mineral content will also be calculated. Cardiac function will be assessed by electrocardiography, 4-D echocardiography and cardiac stress test. MRI (structural & functional) will be used to assess cortical thickness, occurrence cortical atrophy and functional connectivity.

2. Behavioural assessment: The Watve and Yajnik hypothesis (Watve and Yajnik, 2007) of neurobehavioral origins depicts a soldier-diplomat dimension of personality or behavioural syndrome. A soldier personality is characterized by physical strength and aggression, adventurous behaviour and tolerance to physical pain and discomfort. A diplomat personality is physically weak, risk and aggression avoider, physical harm and pain avoider and having higher degree of social manipulation skills. In order to assess the difference in behaviour on various dimensions and its role in altering various biochemical parameters, assessment will be done using following methods - questionnaires, interviews, games and cognitive testing. Several standardized questionnaires assessing various parameters like quality of sleep (Pittsburgh sleep quality index), aggression (Buss Perry aggression questionnaire), novelty seeking (Temperament Character Inventory - TCI), sensation seeking (Arnett inventory), fear of pain, lifestyle questionnaire, risk taking, personality, social intelligence, co-operative-competition levels (competitive strategy scale), harm avoidance (TCI), thinking (rational/ experiential inventory) etc. will be used.

   There is evidence from previous studies highlighting differences in behaviour using various games in metabolic disorders like T2DM (Joshi et al., 2010). Behavioural games such as ultimatum game will also be used. Assessment of cognition-related parameters such as IQ, memory, executive function, attention, visuo-spatial abilities, fluency, information processing, decision making, musicality, empathy quotient, emotion recognition etc. will be assessed using validated Cognitive Tests Battery.

3. Physical fitness assessment: A complete physical assessment will be done using Global Physical Activity Questionnaire, Actigraph activity monitors, diet and calorie

assessment and a fitness test. The fitness test is a battery of tests designed to assess fitness along multiple dimensions - strength, flexibility, cardio-vascular capacity, balance and endurance. This battery is composed of various standardized tests like VO_2 max, grip strength using dynamometer, vertical jump test, sit and rise test and stork pose test to name a few. A total fitness score along with a resolution on above mentioned axis will be obtained. Posture will also be assessed while walking, standing and sitting with help of video recordings.

Along with that, anthropometric assessment will also be done. Following parameters will be obtained using World Health Organization (WHO) guidelines: height, weight, waist circumference, hip circumference, chest circumference, arm and leg length, bicipital and tricipital skin folds, Waist to Hip Ratio (WHR) and Body Mass Index (BMI).

4. Physiological parameters: These will be at the core of the study and main contributors to the network model. They would include assays for a variety of metabolic, hormonal and immunological markers including growth factors (EGF, NGF, FGF, BDNF, IGF, etc.), hormones (Insulin, glucagon, male and female sex hormones, cholecystokinin, cortisol, myostatin, adiponectin, erythropoietin, endothelin-1, vasopressin, cortico-releasing hormone, gastrin, ghrelin, growth hormone, kotho, leptin, melatonin, osteocalcin, oxytocin, vitamin D3, vitamin B12, glucagon-like peptide 1, etc.), cytokines (activin A, IL6, SFRP-5, TNF-α, etc.), metabolites (glucose, FFA, triglycerides, cholesterol, ROS, anti-oxidants, keto acids, etc.), autonomic and other neuronal function markers (α-MSH, dopamine, endorphin, CART, serotonin, GABA, histamine, nor-epinephrine, etc.).

   Although the limitation of sampling is that we can collect only blood, several organ-level signalling pathways have some reflection in blood. As a result, all nodes in the multi-organ model will not be represented in total, but some indirect information about them will be reflected. The resultant network will therefore be somewhat different than our current network model but could be analysed in a similar way and may have similar implications.

5.5 Discussion

Such study has not been done so far in spite of the huge amount of money spent on diabetes research all over the world. The reason for this is unlikely to be the availability of funds and appropriate tools. It is more likely to be lack of vision. Theoretical and empirical studies are inter-dependent. Development of theory is often limited by availability of data and design of new experimental and epidemiological studies is limited by the vision developed by theory. Since T2DM has been visualized as a fragmentary picture by all researchers, empirical studies giving a wide array of variables are conspicuously absent. A multi-level multi-organ network model has helped us giving a broader vision and raising the possibility that a broader vision can suggest novel potential breakthroughs as simple emergent properties of a complex model. Therefore, novel designs of empirical work need to be undertaken with a prospect of major conceptual breakthroughs.

5.6 Summary

Though the work in this thesis is focussed on construction and interpretation of a tool – 'a network model' to better understand T2DM, the thesis skims through the entire pipeline starting from defining a diseased state, developing a robust tool to understand the disease, making testable predictions out of it to finally setting up an empirical study that should accompany a network model. Translational output of any research is as important as the research itself; which is why I have put effort in establishing the entire pipeline.

I start with explaining what does a diabetic state in the network model mean. The insulin resistant attractor resembles a metabolic personality of a diabetic individual. This personality is characterized by the correlates of insulin resistance and the entire state represents the disease, not insulin resistance or glucose levels alone. The predicted correlates match well with clinical data on individuals with T2DM.

The answer to the apparent irreversibility of this diabetic state is two-fold. Firstly, diabetic state is re-enforced by multiple positive feedbacks. Presence of multiple

mechanisms to stabilize the insulin resistant state suggests that it is not just a pathological departure from the healthy state but an independent, adaptive and evolved state; which is difficult to come out of, but not entirely impossible. Secondly, the current treatment seems to target the visible consequences of the disease rather than the apparently invisible causal factors. The model reveals that glucose and insulin are not central or key nodes of the network. A change in glucose and insulin levels may be mere manifestations of T2DM and there appear to be multiple other regulatory mechanisms that actually 'lead' to the diabetic state. I have listed this set of parameters and they fall under the insulin resistant basin of attraction.

I then move on to explain the inadequacy and flaws in the current classical thinking. As I mentioned earlier in the thesis, there is growing evidence against different parts of the classical theory; but even in the light of these flaws, the treatment options given to patients haven't changed. They are still based on the classical theory. The flaws arise, in part, due to confused cause-effect relationships between the correlates of the disease. My network model takes into consideration only experimentally demonstrated links between the nodes and is thereby clear in the direction of causality. If the classical theory revolving around insulin levels, insulin resistance, glucose levels, β-cells and obesity is treated by the same approach, it cannot explain the existence of a stable insulin resistant state. This is because the loops form an efficient homeostatic system and it always oscillates stably around the normal.

Moving to the most important part of the thesis, I list the ways in which this diabetic state can be escaped or reversed and how an insulin sensitive state can be achieved. There are specific behaviourally regulated nodes in the network which, when up-regulated for sufficient duration, can prevent and in some cases reverse the diabetic state. This reversal is complete transformation of the state and not glucose normalization alone. The novel targets essentially reverse diabetes in all these different diabetic microphenotypes in the model and this is what we might expect to see as a reversal target in real life too. Since all the novel targets are behaviourally regulated, alteration of the behaviour is potentially a logical and healthier (without chemical intervention) option. So, reversal of diabetic state is possible at least in the model which raises hopes for a complete reversal in real life.

Testing these predictions empirically is beyond the scope of a single thesis and is adequate amount of work (both in magnitude and multitude) for a team comprising 'omics'-related researchers, physiologists, epidemiologists, medical practitioners and theoretical biologists. Our lab and other collaborators have come up with a design of a clinical study that can test the predictions from the model and provide a way to tackle such complex diseases. A network model is a powerful tool to handle the large number of interactions involved in the disease process. However, it still has its limitation of being qualitative. More quantitative data on these relationships and more powerful computational tools will enable handling a quantitative model. Further, a distinction between SS and PS causality can make a more refined executable model. My network model is a small step in this field of executable biology (Fisher and Piterman, 2010) and we hope to fine tune it to make it as realistic as possible. But without waiting for perfection in the model, it would be advisable to test empirically the novel suggestions for prevention, control and reversal that the model has made.

5.7 References:

Manuscripts not published yet:

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Appendix I

References for the links in the network with the respective model organisms used

Factor	Abbreviation	Effect		Model organism
Activin A	ata	btc1		Sprague-Dawley rat [1]
Activin A	ata	ctk0		HUVECs [2]
Activin A	ata	ins1		Cultured human pancreatic islets [3]
Activin A	ata	adp0		Human adipose progenitors [4]
adipose tissue	adp	ata1		Human adipose progenitors [4]
adipose tissue	adp	tnf1		Human adipose tissue [5]
adipose tissue	adp	ctk1		Human adipose tissue [6]
adipose tissue	adp	and1		Human adipose tissue [7]
adipose tissue	adp	lep1		Human adipose tissue [8]; Rat epididymal fat pad [9]; Human adipose tissue [10]; C57BL/6J mice [11]
adipose tissue	adp	sfr1		C57BL/6 mice [12]; Mice [13]
adiponectin	and	ina1		Mice [14]; Mice [15]
adiponectin	and	ata1		Primary human monocytes [16]
adiponectin	and	ffa0		C57BL/6J mice [17]
adiponectin	and	fdi0		Wistar rats [18]
adiponectin	and	inr0		Human aortic endothelial cells [19]
adiponectin	and	fty1		Bovine ovarian cells and embryo [20]; Mice embryos [21]; Human and C57BL/6J mice granulosa and cumulus cells [22]
adiponectin	and	tri0		C57BL/6J mice [17]
aggression	agr	chl0		Human subjects [23]
aggression	agr	egf1		Mice [24]; Osteoblast-like cell line [25]; Mice [26]
aggression	agr	ngf1		Mice [27]; Mice [28]; Mice [29]; Mice [30]; Mice [31]
aggression	agr	bdn1		Hamsters [32]
aggression	agr	dop1		Long-Evans rats [33]; Long-Evans rats [34]
aggression	agr	ser0		Long-Evans rats [33]; Long-Evans rats [34]
aggression	agr	edp1		Human subjects [35]; Human subjects [36]
aggression	agr	tet1		Human subjects [37]
aggression	agr	cts1		Osteoblast-like cell line [25]; Human subjects [37]
aggression	agr	cck1		Long-Evans rats [38]
aggression	agr	et11		Mice [39]
aggression	agr	igf1		Pudu deer [40]; Baboons [41]
alpha MSH	msh	tri0		OLETF rats [42]
alpha MSH	msh	ina1		OLETF rats [42]; Sprague-Dawley rats [43]
alpha MSH	msh	agr1		Mice [44]; Wistar rats [45]; Mice [46]
alpha MSH	msh	adp0		Sprague-Dawley rats [43]
alpha MSH	msh	msl1		Wistar rats [47]
alpha MSH	msh	ctk0		Blood samples [48]

alpha MSH	msh	fdi0		Long-Evans rats [49]; OLETF rats [42]; Sprague-Dawley rats [50]
angiogenesis	ang	bgl1		Logic
anti-oxidant	aox	inr0		Mice [51]
arginine vassopressin	avp	agr1		Hamsters [52]
arginine vassopressin	avp	cfn1		Human subject [53]; Human subjects [54]; Human subjects [55]
arginine vasopressin	avp	ins1		Human subjects [56]
arginine vassopressin	avp	gng1		Porton-Wistar rats [57]
BDNF	bdn	btc1		C57BL/KsJ-db/db mice [58]
BDNF	bdn	ina1		Mice [59]; Zucker fatty rats [60]; C57BL/KsJ-db/db mice [61]
BDNF	bdn	cfn1		Human subject [62]
BDNF	bdn	ser1		C57BL/6 mice [63]
BDNF	bdn	fdi0		Human subject [62]; C57BL/6 mice [64]; Mice [59]
beta adrenergic receptors	bar	adp0		Human adipocytes [65]; Beagle dogs [66]
beta cells	btc	ins1		Human and rat pancreatic islets [67]
beta cells	btc	gap1		Rats and Human insulinoma[68]
CART	car	fdi0		Mice [69]; Wistar rats [70]
cholecystokinin	cck	fdi0		129/SvEv mice [71]; Human subjects [72]; OLETF rats [73]; Sprague-Dawley rats [74]; Rhesus monkey [75]; Rats [76]; Mice [77]
cholecystokinin	cck	ina1		Mice [78]
cholecystokinin	cck	gst0		Pigs [79]
cholecystokinin	cck	ins1		Human subjects [80]
cholesterol	chl	ser1		Macaques [81]
cholesterol	chl	inr1		Mice [82]
cholesterol	chl	agr0		Monkeys [83]; Macaques [81]
cholesterol	chl	cfn1		Human subjects [84]
cognitive function	cfn	bgl0		Logic
cognitive function	cfn	dip1		Logic
corticosteroids	cts	tri0		Sprague-Dawleys rats [85]; Human subjects [86]
corticosteroids	cts	ffa1		Sprague-Dawleys rats [85]; Human subjects [86]
corticosteroids	cts	gng1		Hepatoma cells [87]; H4IIE rat hepatoma cells [88]; H4IIE rat hepatoma cells [89]
corticosteroids	cts	ina0		Rats [90]; Zucker rat [91]; Rat muscle tissue [92]; Mice [93]; Human subjects [94]; Human subject [95]; Human subjects [96]; Human subjects [97]
corticosteroids	cts	agr0		Mice [98]
corticosteroids	cts	inr0		Human subjects [99]; Normal human epithelial cells [100];

				Human subjects [101]; Human subjects [102]
corticosteroids	cts	ins0		Hamster HIT-15 Beta cells [103]; Mice pancreas [104]
cortico releasing hormone	crh	fdi0		Mice [105]
cortico releasing hormone	crh	cts1		C57BL/6J mice [106]; Rats [107]; Human subjects [108]; normal human epidermal melanocytes [109]; Human fetal adrenal cells [110]; Mice [111]
cortico releasing hormone	crh	agr0		Harlan-Sprague-Dawley mice [112]; Rats [113]
cortico releasing hormone	crh	ins1		Mice pancreatic islets [56]
cortico releasing hormone	crh	edp1		Pituitary [114]; Mouse pituitary cell line (ATt-20) [115]
cytokines	ctk	edp1		Mouse pituitary cell line (ATt-20) [115]; Mouse pituitary cell line [116]
cytokines	ctk	crh1		Baboons [117]; Rats [118]; Rats [119]
cytokines	ctk	lep1		Hamsters [120]; C57BL/6 mice [121]
cytokines	ctk	inr1		Logic
cytokines	ctk	oxy1		Rats [122]
cytokines	ctk	avp1		Rats [122]
cytokines	ctk	ina0		Human subject [123]; Human megakaryotic cell line CHRF-288-11 [124]
cytokines	ctk	klt0		C57/BL6 rats [125]
cytokines	ctk	et11		Porcine endothelial cells [126]
diplomat	dip	ins1		Chimpanzees [127]
dopamine	dop	nep0		Sprague-Dawley rats [128]
dopamine	dop	agr1		Rats [129]; Sprague-Dawley rats [128]
dopamine	dop	ost1		Mice [130]
dopamine	dop	ocl1		Human subjects [131]
dopamine	dop	ina1		C57BL6 mice [132]
dopamine	dop	msl1		Sprague-Dawley rats [133]
dopamine	dop	ins0		INS-1E cells and pancreatic islets [134]; C57BL/5J mice [135]
dopamine	dop	lep0		Human adipocytes [136]
dopamine	dop	il61		Human adipocytes [136]
dopamine	dop	and1		Human adipocytes [136]
dopamine	dop	ffa0		C57BL/5J mice [135]
dopamine	dop	tri0		C57BL/5J mice [135]
dopamine	dop	pgl0		C57BL/5J mice [135]
dopamine	dop	fdi0		Zucker rats [137]; C57BL/5J mice [135]
egf	egf	noc0		Dorsal root ganglia neurons [138]
egf	egf	btc1		Canine islets and murine bet cells [139]; FVB E1-DN

				mice [140]
egf	egf	bdn1		CD-1 mice [141]
egf	egf	ina1		Human adipose tissue [142]
egf	egf	fty1		Mice [143]; Mice [144]; Mice [145]; C3H/HeN mice [146]
endorphin	edp	bdn1		Sprague-Dawley rats [147]
endorphin	edp	ina1		Rats [148]
endorphin	edp	agr0		Mice [149]
endorphin	edp	fdi1	?	Rats [50]; Rats [150]
endorphin	edp	fdi0	?	Zucker rats [151]
endothelin	et1	ghr1		Holstein Steers [152]; Holstein Steers [153]
endothelin	et1	ina0		Rat muscles [154]; Sprague-Dawley rats [155]; Human subjects [156]
endothelin	et1	agr1		Mice [39]
endothelin	et1	ins1		Mice islets of Langerhans [157]
endothelin	et1	lep1		Adipocyte cell lines [158]
endothelin	et1	vdl0		Pigs, Humans and Rats [159]; Human epicardial coronary arteries [160]; Porcine endothelial cells [161]
EPO	epo	ang1		Human glioma [162];Human mesenchymal stem cells [163]
fertility	fty	egf1		Mice [164]
fertility	fty	oxy1		Rats [165]
fertility	fty	otg1		Mice [166]
FFA	ffa	bdn0		Human subjects [167]
FFA	ffa	gng1		Human subjects [168]
FFA	ffa	inj0		Human subjects [169]
FFA	ffa	ina0		Human subjects [167]; Human subjects [170]; Human subjects [171]; Human subjects [169]
food intake	fdi	adp1		Sprague-Dawley rats [172]
food intake	fdi	agr0		Meerkats [173]
food intake	fdi	msl1		Logic
food intake	fdi	ffa1		Logic
food intake	fdi	ins1		Rats [174]
food intake	fdi	pgl1		Logic
GABA Brain	gab	agr1	?	Mice [175]
GABA Brain	gab	agr0	?	Human subjects [176]; Mice [177]
GABA Brain	gab	grh1		Mice [178]
GABA Brain	gab	fdi0	?	Rats [179]; Rats [180]
GABA Brain	gab	fdi1	?	Sprague-Dawley rats [181]; Rats [182]
GABA pancreas	gap	inr0		Mice[183]
GABA pancreas	gap	btc1		Human islets and Rats[184]
gastrin	gst	btc1		Rat pancreas [185]
gastrin	gst	agr1		Mice [186]
gastrin	gst	ina1		Mice [187]
gastrin	gst	adp0		Mice [187]
ghrelin	ghr	agr1		BALB/c mice [188]

ghrelin	ghr	fdi1		Mice [189]; Rats [190]; Human subjects [191]; Wistar rats [192]; Mice and Rats [193]; Sprague-Dawley rats [194]; Sprague-Dawly rats [195]
ghrelin	ghr	hgh1		Holstein Steers [153]; Rats [196]; Rats [197]; Wistar rats [192]; Human subjects [198]; Mouse hypothalamus [199]; Human subjects [200]; Human subjects [191]
ghrelin	ghr	ins1		Sprague-Dawley rats [201]
ghrelin	ghr	gab1		Sprague-Dawley rats [202]; Rats and mice [203]
ghrelin	ghr	et10		Sprague-Dawley rats [204]
glucagon	glg	gng1		Rats [205]; Rats [206]; Bovine [207]
gluconeogenesis	gng	pgl1		Rats [205]; Rats [206]
glut1	gt1	bgl1		Rats [208]
GnRH	grh	tet1		European ground squirrels [209]; Sprague-Dawley rats [210]
growth hormone	hgh	msl1		Human subjects [211]; Human subjects [212]; Human subjects [213]
growth hormone	hgh	agr1		Mice [214]
growth hormone	hgh	ins1		Human subjects [215]; Human subjects [216]
growth hormone	hgh	ina0		Human subjects [215]; Human subjects [216]; 3T3-L1 adipocytes [217]; Human subjects [218]; Human subjects [219]; balb/c mice [220]
growth hormone	hgh	igf1		Rats [221]; Human subjects [219]; Human subjects [215]; Human subjects [222]; Mice [223]
growth hormone	hgh	fty1		Rats [221]
histamine	hst	fdi0		H1KO mice [224]; Sprague-Dawley rats [225]; Wistar King A rats [226]; Mice [227]
histamine	hst	agr1		Mice [228]
IGF 1	igf	ina1		Human subjects [229]; Human subjects [230]; Human subjects [231]; Sprague-Dawley rats [232]; Human subjects [222]; Human subjects [233]; Mice [234]; Mice [235]; Wistar rats [236]
IGF 1	igf	btc1		Mice [237]
IGF 1	igf	fdi0		Wistar rats [238]; Wistar rats [236]
IGF 1	igf	adp0		Sprague-Dawley rats [232]; Wistar rats [236]
IGF1	igf	msl1		C57BL/6 mice [239]; Mice [240]; Mice [241]; Rabbits [242]
IGF1	igf	ins0		Rat pancreatic beta cells [243]; Mice [235]
IGF1	igf	hgh0		Primary rat pituitary cells [244]; Sheep [245]; Mice [234]
IGF1	igf	ost1		Mice [246]
IGF1	igf	ctk0		Sprague-Dawley rats [247]
IGF1	igf	tnf0		Sprague-Dawley rats [247]
Il-6	il6	agr0		Mice [248]
Il-6	il6	ina0		Human adipose tissue [249]; Mouse hepatocytes [250]
Il-6	il6	adp0		Mice [251]

Il-6	il6	inr0		Human subjects[252]
Il-6	il6	glp1		Mice[253]
Il-6	il6	fdi0		Mice[254]
inflammatory response	inr	ina0		Mice [255]
injury (growth factors)	inj	btc1		Human pancreatic islets [256]; Mice [257]
injury (growth factors)	inj	hst1		Dogs [258]
injury (growth factors)	inj	adp0		Mice [259]
injury (growth factors)	inj	ins0		Mice [259]
injury (growth factors)	inj	ina1		Mice [259]
injury (growth factors)	inj	agr0		Mice [260]
injury (growth factors)	inj	inr0		Human fibroblast cells [261]
injury (growth factors)	inj	ang1		Mice [262]
insulin	ins	cfn1		Human subjects [263]; Human subjects [264]; Human subjects [265]; Human subjects [266]; Mice [267]
insulin	ins	ktg0		Rat adipose tissue [268]
insulin	ins	btc1		Canine islets and murine beta cells [139]
insulin	ins	lep1		Rat white adipose tissue [9]; Human subjects [269]
insulin	ins	klt1		COS-7 cells [270]
insulin	ins	egf1		C57BL/KsJ mice [271]; Mice [145]
insulin	ins	et11		Human subjects [272]
insulin	ins	grh1		GnRH expressing cell line [273]
insulin	ins	and0		Bovine adipocytes [274]; Human subjects [275]
insulin	ins	nox1		Bovine endothelial cells [276]; Human subjects [277]
insulin	ins	gmo1		Rats [278]
insulin action	ina	pgl0		Rats [279]; Rats [238]
insulin action	ina	gng0		Bovine [207]; Mice [280]; Mice [281]
insulin action	ina	msl1		Human muscle tissue[282]
insulin action	ina	ost1		Mice [283]; Mice and cell lines [284]
insulin action	ina	adp1		Mouse embryonic fibroblasts [285]; Mice [286]
insulin action	ina	tri1		Rats [287]; Mice [288]
keto acids	ktg	cfn1		Rats [289]
keto acids	ktg	ins1		Pancreatic beta cells[290]; Rat pancreatic islets [291]
Keto acids	ktg	inr0		Mammalian cell culture [292]
klotho gene	klt	fty0		Mice [293]; Mice [294]
klotho gene	klt	ina0		Mice [295]; Mice [296]
klotho gene	klt	aox1		Mice [297]
klotho gene	klt	ang1		Mice [298]

klotho gene	klt	inr0		KM mice [299]; Mice [300]
klotho gene	klt	adp1		Mouse 3T3-L1 cells [301]; Mice [295]
klotho gene	klt	ins1		MIN6 beta cells [302]
leptin	lep	inr1		Human adipocytes [10]
leptin	lep	car1		Wistar rats [70]; Sprague-Dawley rats [303]; Rats [304]
leptin	lep	ffa0		Rats [305]
leptin	lep	ang1		HUVECs and PAECs [306]; Normal HUVECs and HCASMCs [307]; Rats and human endothelial cells [308]; Wistar rats [309]
leptin	lep	cfn1		Mice [310]
leptin	lep	ser1	?	Black Swiss mice [311]
leptin	lep	ser0	?	Mice [312]
leptin	lep	et11		Rat portal vein [313];HUVECs [314]
leptin action	lpa	fdi0		Rats [304]; Mice [315]; Rat pancreatic islets [316]; Wistar rats [317]; Rats [318]
leptin action	lpa	adp0		Mice [315]; Mice [319]; Wistar rats [317]
leptin action	lpa	tri0		Mice [315]; Rat pancreatic islets [316]
melatonin	mlt	agr1		Syrian hamsters [320]
melatonin	mlt	ost1		MC3T3 cells [321]; Osteoblast-like cell line [322]
melatonin	mlt	ocl1		MC3T3 cells [321]
melatonin	mlt	adp0		Rats [323]; Sprague-Dawley rats [324]; Sprague-Dawley rats [325]; osteoblast-like cell line[322]
melatonin	mlt	lep0		Rats [326]; Rats [327]
melatonin	mlt	ins0		Rats [323]; Rats [326]; Sprague-Dawley rats [324]; Rats [327]
melatonin	mlt	pgl0		Sprague-Dawley rats [325]
melatonin	mlt	tri0		Sprague-Dawley rats [325]; Rats [327]
melatonin	mlt	ina1		Rats [328]; SAMP8/SAMR1 mice [329]; Mice [330]; Mice [331]; Rats [332]
melatonin	mlt	msl1		Rats [333]; Rats [334]
muscle strength	msl	agr1		Human subjects [335]
muscle strength	msl	ina1		Human subjects [335]
muscle strength	msl	inr0		Human subjects [252]
myostatin	myo	msl0		Mice [336]; Mice [337]; Mice [338]; Mice [339]; Sprague-Dawley rats [340]; Mice [341]
myostatin	myo	ina0		C57BL/6 mice [342]; Human subjects [343]; Mice [339]; C57BL/6 (B6) mice [344]
myostatin	myo	tnf1		C57BL/6 (B6) mice [344]
myostatin	myo	adp1		Mice[339]; Mice [341]; C57BL/6 (B6) mice [344]; C57BL/6 mice [342]
NGF	ngf	bdn1		Human and rat pancreatic islets [345]; CD-1 mice [141]
NGF	ngf	noc1		Dogs [346]; Lewis rats [347]; Human subjects [348]
NO	nox	vdl1		Human subjects [349]; Human subjects [277]
NO	nox	ang1		Mice [350]; Mice [262]
NO	nox	nep0		Wistar rats [351]
NO	nox	dop0		Wistar rats [351]

NO	nox	agr1	?	Mice [352]; Mice [353]
NO	nox	agr0	?	Mice [354]; Mice [355]; Mice [356]; Mice [357]
norepinephrine	nep	agr0		Sprague-Dawley rats [128]
norepinephrine	nep	ina0		Hamsters [358]
norepinephrine	nep	crh1		Rats [359]
oestrogen	otg	hgh1		Human subjects [360]; Rat osteosarcoma cells (UMR 106.01) [361]; Human subjects [362]
oestrogen	otg	ina1	?	Human subjects [363]; Rats [364]
oestrogen	otg	ina0	?	Human subjects [365]
oestrogen	otg	fdi0		C57BL/6J and Swiss Webster mice [366]
oestrogen	otg	agr1		Rats [367]; Rats [368]; California mice [369]; CD-1 mice [370]; C57BL/6J mice [371]; C57BL/6J mice [372]; C57BL/6J mice [373]
oestrogen	otg	ang1		Human endometrial cells and HMMECs [374]; BALB/c mice [375]; Mouse mammary tumour explants [376]; HUVECs and murine model [377]
oestrogen	otg	inr0	?	In vitro [378]
oestrogen	otg	inr1	?	BALB/c mice [375]
oestrogen	otg	gmo0		Colon muscle cells [379]
oestrogen	otg	ocl1		Human subjects [380]
oestrogen	otg	fty1		Holtzman strain rats [381]
bone strength	ost	ocl1		Normal human bone cells [382]
osteocalcin	ocl	ina1		Mice [383]; C57BL/6J mice [384 385]
osteocalcin	ocl	ins1		Mice [383]; C57BL/6J mice [384]
osteocalcin	ocl	and1		Mice [383]; C57BL/6J mice [384]
osteocalcin	ocl	tet1		129-Sv mice [386]; Mice [387]
osteocalcin	ocl	glp1		STC-1 cells and C57BL/6J mice [388]
oxytocin	oxy	agr1	?	Wistar rats [389]; Mice [390] Prairie voles [391]
oxytocin	oxy	agr0	?	Wild type Groningen rats [392]
oxytocin	oxy	cts1	?	Wistar rats [393]
oxytocin	oxy	cts0	?	Sprague-Dawley rats [394]; Human subjects [395]
oxytocin	oxy	adp0		Mice [396]; Mice [397]
oxytocin	oxy	fdi0		C57BL6 mice [398]
oxytocin	oxy	gng1		Rat hepatocytes [57]
oxytocin	oxy	glg1		Dogs [399]
oxytocin	oxy	noc0		Sprague-Dawley rats [400]
plasma glucose	pgl	ins1		Human subjects [401]
plasma glucose	pgl	ata1		Human subjects and HUVECs [2]
plasma glucose	pgl	ghr0		Human subjects [402]
plasma glucose	pgl	bgl1		Human subjects [403]
plasma glucose	pgl	glg0		Mongrel dogs [404]
plasma glucose	pgl	gt10		Large White pigs [405]; Rats [208]
brain glucose	bgl	cfn1		Logic
brain glucose	bgl	fdi0		C57BL/6NHsd mice [406]; Rats [407]
sfrp5	sfr	adp0	?	Mice [408]

sfrp5	sfr	adp1	?	Mice [12]
sfrp5	sfr	inr0		Mice [408]
serotonin	ser	cfn1		Mice [409]; Human subjects [410]; Human subjects [411]; Rats [412]; ICR mice [413]; Marmoset monkeys [414]; Wistar rats [415]
serotonin	ser	ina0		Hamsters [358]; C57BL/6 mice [416]; Naïve rat hepatoma cells [417]; Pigs [418]
serotonin	ser	ost0		Mice [419]; Human subjects [420]; Human subjects [421]; Mice [422]
serotonin	ser	agr0		Human subjects [423]; Rats [424]; Mice [425]; C57BL/6J mice [354]; Human subjects [426]; Human subjects [427]; Dogs [428]; Vervet monkeys [429]; Human subjects [430]; Dogs [431]; Mice [432]
serotonin	ser	inr1		C57BL/6 mice [433]; Mice [434]; HT-29 colon epithelial cells [435]; Mice [436]; C57BL/6J mice [437]
serotonin	ser	fdi0		Wistar rats [438]; Mice [439]; Mice [440]; Mice [441]; Human subjects [442]
serotonin	ser	dop0		Rats [443]
symapathetic stimulation	sys	gng1		Rats [206]
symapathetic stimulation	sys	ina1		Human subjects [444]
symapathetic stimulation	sys	adp0		Sprague-Dawley rat [445]; Human subjects [446]
sympathetic stimulation	sys	egf1		Human subjects [447]
testosterone	tet	edp0		Rats [448]
testosterone	tet	msh0		Rats [448]
testosterone	tet	egf1		Mice [449]; Mice [145]
testosterone	tet	myo0		C57BL6J mice [450]
testosterone	tet	msl1		Human subjects [451]; Human subjects [452]
testosterone	tet	ocl1		Human subjects [380]
testosterone	tet	adp0		Human subjects [453]; Human subjects [454]; Rats [455]; Human subjects [456]; Human subjects [457]
testosterone	tet	dip0		Human subjects [458]
testosterone	tet	aox1		Cerebellar granule cells [459]
testosterone	tet	epo1		Human subjects [460]
testosterone	tet	agr1		Mice [355]; Rats [367]; Rats [368]; CD-1 mice [370]
testosterone	tet	ina1	?	Human subjects [457]; Human subjects [453]
testosterone	tet	ina0	?	Rat skeletal muscle culture [461]
testosterone	tet	ang1		Sprague-Dawley rats [462]
TNF alpha	tnf	inr1		Mice [463]
TNF alpha	tnf	ina0		Murine 3T3-L1 or 3T3-F442A cells [464]; Human adipose tissue [249]
TNF alpha	tnf	ata1		Human leucocytes [465]; C57BL6/J mice [466]
TNF alpha	tnf	il61		SCID-HuRAg mice [467]; LS14 cell culture [468]

TNF alpha	tnf	lep1		C57BL/6J mice [469]; Human subjects [470]; C3H/HeOuJ mice [471]; Syrian hamsters [120]; C57BL/6 mice [121]
TNF alpha	tnf	klt0		C57/BL6 mice [125]; Mice and mouse embryonic adipocytes [472]
TNF alpha	tnf	et11		Bovine aortic endothelial cells [473]
TNF alpha	tnf	nep0		Rats [474]
triglycerides	tri	adp1		C57BL6 mice[475]
triglycerides	tri	ina0		C57BL6 mice[475]
vasodilation	vdl	bgl1		Logic
vasodilation	vdl	ina1		Sprague-Dawley rats [476]
vitamin D3	vd3	ina1		Human subjects [477]; Rats [478]
vitamin D3	vd3	ost1		Human subjects [479]
vitamin D3	vd3	agr1		Mice [480]
vitamin D3	vd3	ins1		Rat pancreas [481]; Rats [478]
Vitamin B12	v12	igf1		Mice [223]
Vitamin B12	v12	inr0		Mice [482]
Vitamin B12	v12	hgh1		Mice [223]
Vitamin B12	v12	ost1		Mice [223]
Vitamin B12	v12	noc0		Mice [482]
Exercise	exe	adp0		OM and S5B/P1 rats [483]
Exercise	exe	il61		Humans[484]
GLP-1	glp	ins1		Rat pancreatic ductal cells [485]; Human subjects [486]
GLP-1	glp	btc1		Human islets [487]; Rat pancreatic ductal cells [485]
GLP-1	glp	glg0		Wistar rats [488]
GLP-1	glp	gmo0		Human subjects [489]
GLP-1	glp	fdi0		Human subjects [489]
GLP-1	glp	ina1		Human subjects [489]
GLP-1	glp	ost1		Human subjects [490]; Sprague-Dawley rats [491]
Gut Motility	gmo	glp1		Human subjects [492]
Nociception	noc	ina0		Human subjects [493]

Table footnotes: A question mark in the fourth column indicates a contradicting interaction. Nine interactions which did not have a specific reference, but were obvious, logical or evident are labelled as 'Logic'. These interactions are explained in the thesis Chapter 3 section 3.2.4.

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Appendix II

Interface of the network model and the code

As mentioned in Chapter 3, the model was constructed using Visual Basic Application (VBA), associated with Microsoft Excel 2010.

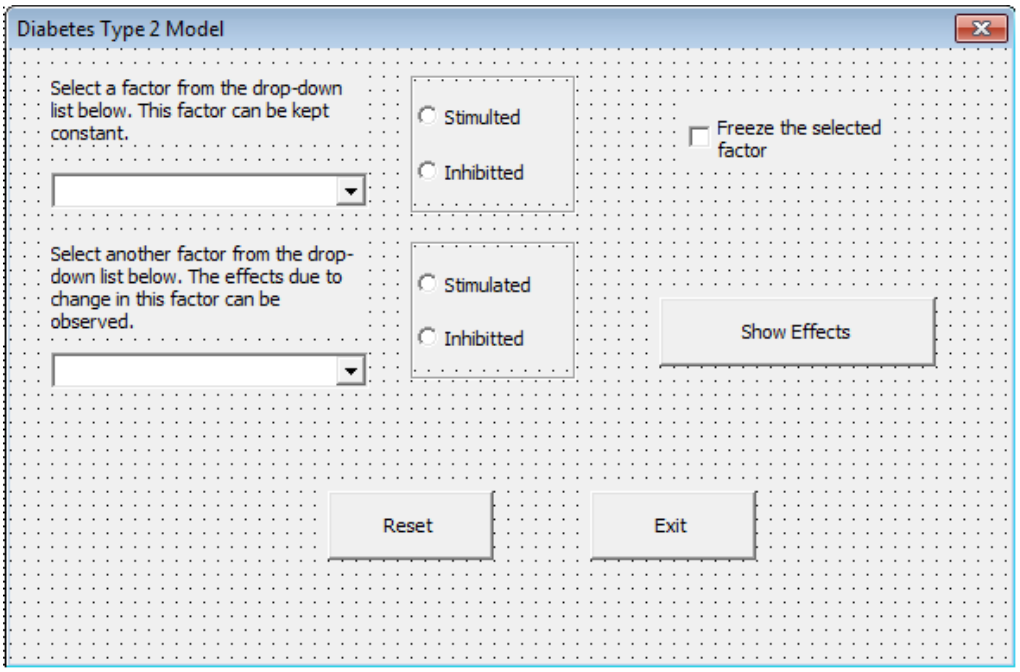

Figure AI.1: Network model user interface

Network model code

```
Private Sub cmdshoweffects_Click()

For everyfactor = 1 To 72

Call cyclethrough

Sheets("Sheet2").Cells(everyfactor, 1) = 1

For counter = 1 To 300

Call cycleperform(counter)

Next counter

For avg = 2 To 73

Sum = 0
```

```
For rangeit = 202 To 301
Sum = Sheets("Sheet3").Cells(rangeit, avg).Value + Sum
Next rangeit
Sheets("Sheet3").Cells(302, avg).Value = Sum
Sheets("Sheet3").Cells(303, avg).Value = Sheets("Sheet3").Cells(302, avg).Value / 100
Next avg
Sheets("Sheet3").Cells(302, 1) = "Sum (of last 100 cycles)"
Sheets("Sheet3").Cells(303, 1) = "Average (of last 100 cycles)"
For copydata = 2 To 73
Sheets("Sheet4").Cells((everyfactor + 1), copydata) = Sheets("Sheet3").Cells(303,
copydata)
Next copydata
Next everyfactor
End Sub


Sub cyclethrough()
Sheets("Sheet3").Cells(1, 1) = "Cycle"
For Row = 1 To 72
For col = 1 To 30
Sheets("Sheet2").Cells(Row, col) = Sheets("Sheet1").Cells(Row, col)
If Sheets("Sheet2").Cells(Row, 3).Value = comboboxtwo.Value Then
If optionbuttonfour.Value = True Then
Sheets("Sheet2").Cells(Row, 1) = -1
End If
If optionbuttonthree.Value = True Then
Sheets("Sheet2").Cells(Row, 1).Value = 1
 End If
```

```
End If
If chkboxone.Value = True Then
For freeze = 1 To 72
If Sheets("Sheet1").Cells(freeze, 3) = comboboxone.Value Then
Sheets("Sheet2").Cells(freeze, 1).Value = 0
If optionbuttonone.Value = True Then
Sheets("Sheet2").Cells(freeze, 1).Value = 1
End If
If optionbuttontwo.Value = True Then
Sheets("Sheet2").Cells(freeze, 1).Value = -1
End If
 End If
Next freeze
End If
'For sustained perturbation, add the perturbation here
'Sheets("Sheet2").Cells(1, 1).Value = 1
Next col
Sheets("Sheet3").Cells(1, (Row + 1)) = Sheets("Sheet1").Cells(Row, 4)
Sheets("Sheet4").Cells(1, (Row + 1)) = Sheets("Sheet1").Cells(Row, 4)
Next Row
End Sub


Sub cycleperform(counter)
For Row = 1 To 72
If Sheets("Sheet2").Cells(Row, 1) <> 0 Then
For col = 5 To 30
```

```
part1 = ""
part2 = ""
part1 = Left(Sheets("Sheet2").Cells(Row, col), 3)
If part1 = "" Then
Exit For
End If
part2 = Right(Sheets("Sheet2").Cells(Row, col), 1)
state = Sheets("Sheet2").Cells(Row, 1).Value
Call signalupdate(part1, part2, counter, state)
Next col
End If
Next Row
Call stateupdate(counter)
End Sub

Sub signalupdate(part1, part2, counter, state)
For Signalrow = 1 To 72
If Sheets("Sheet2").Cells(Signalrow, 4).Value = part1 Then
If state > 0 Then
If part2 = 0 Then
Sheets("Sheet2").Cells(Signalrow, 2).Value = Sheets("Sheet2").Cells(Signalrow, 2).Value
- 1
Else
Sheets("Sheet2").Cells(Signalrow, 2).Value = Sheets("Sheet2").Cells(Signalrow, 2).Value
+ 1
End If
End If
```

```
If state < 0 Then

If part2 = 0 Then

Sheets("Sheet2").Cells(Signalrow, 2).Value = Sheets("Sheet2").Cells(Signalrow, 2).Value
+ 1

Else

Sheets("Sheet2").Cells(Signalrow, 2).Value = Sheets("Sheet2").Cells(Signalrow, 2).Value
– 1

End If

End If

Exit For

End If

Next Signalrow

End Sub


Sub stateupdate(counter)

Sheets("Sheet3").Cells((counter + 1), 1).Value = counter

For staterow = 1 To 72

If Sheets("Sheet2").Cells(staterow, 2).Value > 0 Then

Sheets("Sheet2").Cells(staterow, 1).Value = Sheets("Sheet2").Cells(staterow, 1).Value +
1

End If

If Sheets("Sheet2").Cells(staterow, 2).Value < 0 Then

Sheets("Sheet2").Cells(staterow, 1).Value = Sheets("Sheet2").Cells(staterow, 1).Value - 1

End If

If Sheets("Sheet2").Cells(staterow, 2).Value = 0 Then

Sheets("Sheet2").Cells(staterow, 1).Value = 0

End If

If Sheets("Sheet2").Cells(staterow, 1).Value > 0 Then
```

```
Sheets("Sheet2").Cells(staterow, 1).Value = 1

End If

If Sheets("Sheet2").Cells(staterow, 1).Value < 0 Then

Sheets("Sheet2").Cells(staterow, 1).Value = -1

End If

Sheets("Sheet2").Cells(staterow, 2).Value = 0

If chkboxone.Value = True Then

For freeze = 1 To 72

If Sheets("Sheet1").Cells(freeze, 3) = comboboxone.Value Then

Sheets("Sheet2").Cells(freeze, 1).Value = 0

If optionbuttonone.Value = True Then

Sheets("Sheet2").Cells(freeze, 1).Value = 1

End If

If optionbuttontwo.Value = True Then

Sheets("Sheet2").Cells(freeze, 1).Value = -1

End If

End If

Next freeze

End If

'For sustained perturbation, add the perturbation here

'Sheets("Sheet2").Cells(1, 1).Value = 1

Sheets("Sheet3").Cells((counter + 1), (staterow + 1)).Value =
Sheets("Sheet2").Cells(staterow, 1).Value

Next staterow

End Sub
```

PLOS ONE

RESEARCH ARTICLE

Bi-stability in type 2 diabetes mellitus multi-organ signalling network

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Abstract

Type 2 diabetes mellitus (T2DM) is believed to be irreversible although no component of the pathophysiology is irreversible. We show here with a network model that the apparent irreversibility is contributed by the structure of the network of inter-organ signalling. A network model comprising all known inter-organ signals in T2DM showed bi-stability with one insulin sensitive and one insulin resistant attractor. The bi-stability was made robust by multiple positive feedback loops suggesting an evolved allostatic system rather than a homeostatic system. In the absence of the complete network, impaired insulin signalling alone failed to give a stable insulin resistant or hyperglycemic state. The model made a number of correlational predictions many of which were validated by empirical data. The current treatment practice targeting obesity, insulin resistance, beta cell function and normalization of plasma glucose failed to reverse T2DM in the model. However certain behavioural and neuro-endocrine interventions ensured a reversal. These results suggest novel prevention and treatment approaches which need to be tested empirically.

Citation: Kulkarni S, Sharda S, Watve M (2017) Bi-stability in type 2 diabetes mellitus multi-organ signalling network. PLoS ONE 12(8): e0181536. https://doi.org/10.1371/journal.pone.0181536

Editor: Corentin Cras-Méneur, University of Michigan, UNITED STATES

Received: December 7, 2016

Accepted: July 3, 2017

Published: August 2, 2017

Data Availability Statement: All relevant data are within the paper and its Supporting Information files.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Introduction

The classical thinking about the pathogenesis of Type 2 diabetes mellitus (T2DM) can be summarized in the form of five postulates: (i) Obesity results when net energy intake exceeds net energy expenditure. (ii) Obesity leads to insulin resistance. (iii) To compensate for the insulin resistance, more insulin is produced by the pancreatic β-cells. (iv) Chronically increased rate of insulin synthesis leads to 'exhaustion' or some form of dysfunction of β-cells which causes relative insulin insufficiency. This combination of insulin resistance and relative insulin insufficiency results in hyperglycaemia. (v) The pathophysiological complications of T2DM are a consequence of chronically elevated glucose levels in the blood [1,2].

A number of recent studies have exposed many gaps, flaws and paradoxes in this thinking [1–3]. The inability to cure diabetes can be attributed to these flaws and the clinical approach that uses this classical thinking in patient treatment. Since hyperglycemia was assumed to be the primary cause of the macrovascular and microvascular complications, treating hyperglycemia was the major course of treatment for T2DM patients. It was observed in many large scale

clinical trials that normalizing blood glucose is not sufficient to avoid diabetic complications [4].

One of the fundamental paradoxes of T2DM is that the diabetic state is known to be irreversible although no component of the pathophysiology is individually irreversible. Beta cell loss was considered irreversible for some time but they are shown to have good regeneration capacity[5–8]. Therefore, the reason why T2DM cannot be cured is not known. Experiments in rodents and humans using different means to suppress insulin production have shown that whenever insulin production was suppressed, insulin sensitivity increased and blood sugar remained normal [9–16]. Such experiments have raised doubts whether insulin resistance and inadequate insulin production is necessary and sufficient for hyperglycemia in T2DM.

Although T2DM is historically identified as a condition of increased plasma glucose levels owing to inadequate insulin action, we know today that not only insulin and glucose but a large number of metabolites, hormones, growth factors, neurotransmitters, neuropeptides, cytokines, behaviours and neuronal signals are up or down-regulated in this disorder. Whether alterations in these signals are causes or consequences of altered insulin signalling and hyperglycemia is not clearly known [2]. We need to be open to the possibility that insulin and glucose are not central players but only two of the links in a complex network of signals. In order to get a good understanding of the pathophysiology of T2DM we need to consider all demonstrated interactions between molecules and other signals involved in T2DM without any prejudice and construct a comprehensive model.

We constructed a multi-organ multi-signal interactive network model of pathophysiology of T2DM and studied its behaviour. We show here that a network explains the pathophysiology of T2DM better than a simplistic insulin and glucose centred model. The model was validated by testing many of its predictions and the results demonstrated that most of the characteristics of T2DM are contributed by the structure of the network rather than impairment of insulin signalling alone. Since the classical drug targets for the treatment of T2DM failed to ensure a complete cure [17], a systematic search for alternative markers and targets is needed and a network model is likely to give some directions for the search. In the model, interventions that could reverse the insulin resistant state were not related to obesity, beta cell functionality, insulin production or insulin action but to a set of behavioural and neuro-endocrine targets.

Materials and methods—The network model

Identifying nodes and links of the network

We started with the classical theory of T2DM involving the 3 main variables classically believed to be central to T2DM namely plasma insulin level, insulin resistance and plasma glucose level. We searched literature for signals that affected one or more of the three (direct effectors) and further for signals that affected the direct effector signals (indirect effectors). Since specific behaviours are also known to trigger certain hormones and growth factors among the direct effectors, behaviours were also included in the list of signals. Thus, our definition of signals includes nutrients, metabolites, hormones, growth factors, cell populations, behaviours and neuronal signals (Fig 1). All our signals have a functional meaning. So, a down-regulation means loss or decrease in the signal. Whether it is because of structural change or any other change, is considered irrelevant.

The source data to extract possible interactions amongst the listed signals were publications reporting interventional studies giving causal evidence for a positive effect (up-regulation) or a negative effect (down-regulation) of a given signal on another signal of interest. All searches were made in 'Google Scholar' and 'BioMedNet' using the name(s) of the target nodes and

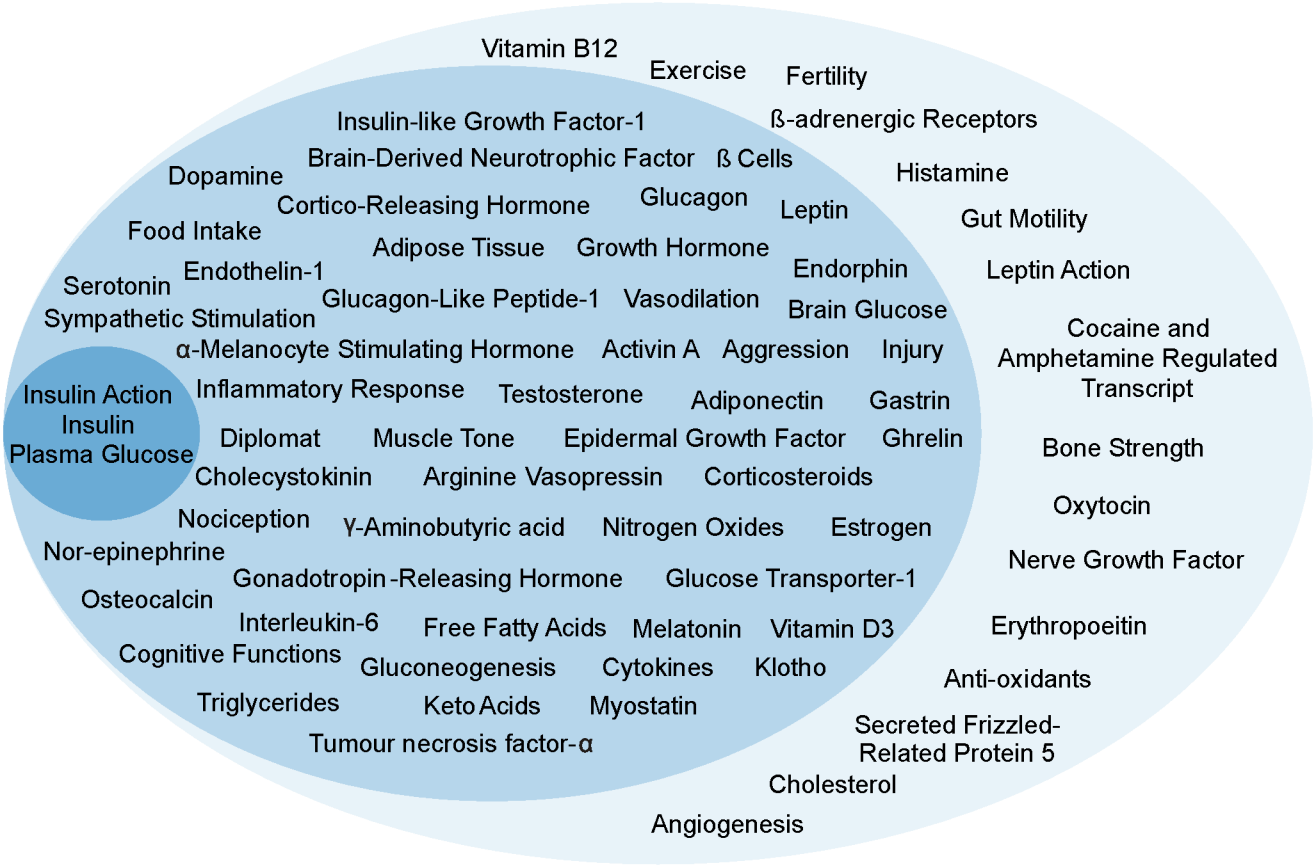

Fig 1. Signals in their respective tiers. First tier (innermost circle) includes players classically believed to be central to T2DM. Second tier (intermediate whorl) includes the players that directly affected or were directly affected by the players in the first tier. The third tier (outermost whorl) included players that affected those in the second tier or were affected by them.

https://doi.org/10.1371/journal.pone.0181536.g001

"regulation of", "expression of" and "affected by" as key words. Correlations and associations were not considered as evidence for an interaction. All published interactions were treated with equal weighting. No weighting of interactions was done by number of studies/ publications, validation, reliability, impact factor or level of current acceptance. Since, most of the interventional data comes from non-human species; we included all experiments with humans, rodents or other mammalian hosts (see S1 Table for model organisms used in the reference for each link).

After listing a large number of possible interactions, we applied the following inclusion and exclusion criteria and redundancy filters. Since our focus was on signalling between cell types and organs we excluded strictly intracellular pathways. If two or more signals shared the same upstream signal/s and the downstream effect/s, they were merged into one. From a known linear signalling pathway, only one molecule was listed. However, if there was a branching point in a pathway, it was listed as a signal. Only the signals having both upstream and downstream effects from other nodes of the network were included (see S1 Text for details).

Finally, 330 interactions among 72 signals were identified from 491 publications and incorporated in the model (see S2 Table for details of the nodes and links with references). A network was constructed using these signals and interactions (Fig 2). All signals were treated as organ specific nodes and the interactions formed the directional links (in the network) between these nodes. If a given signal had different actions in different organs they were

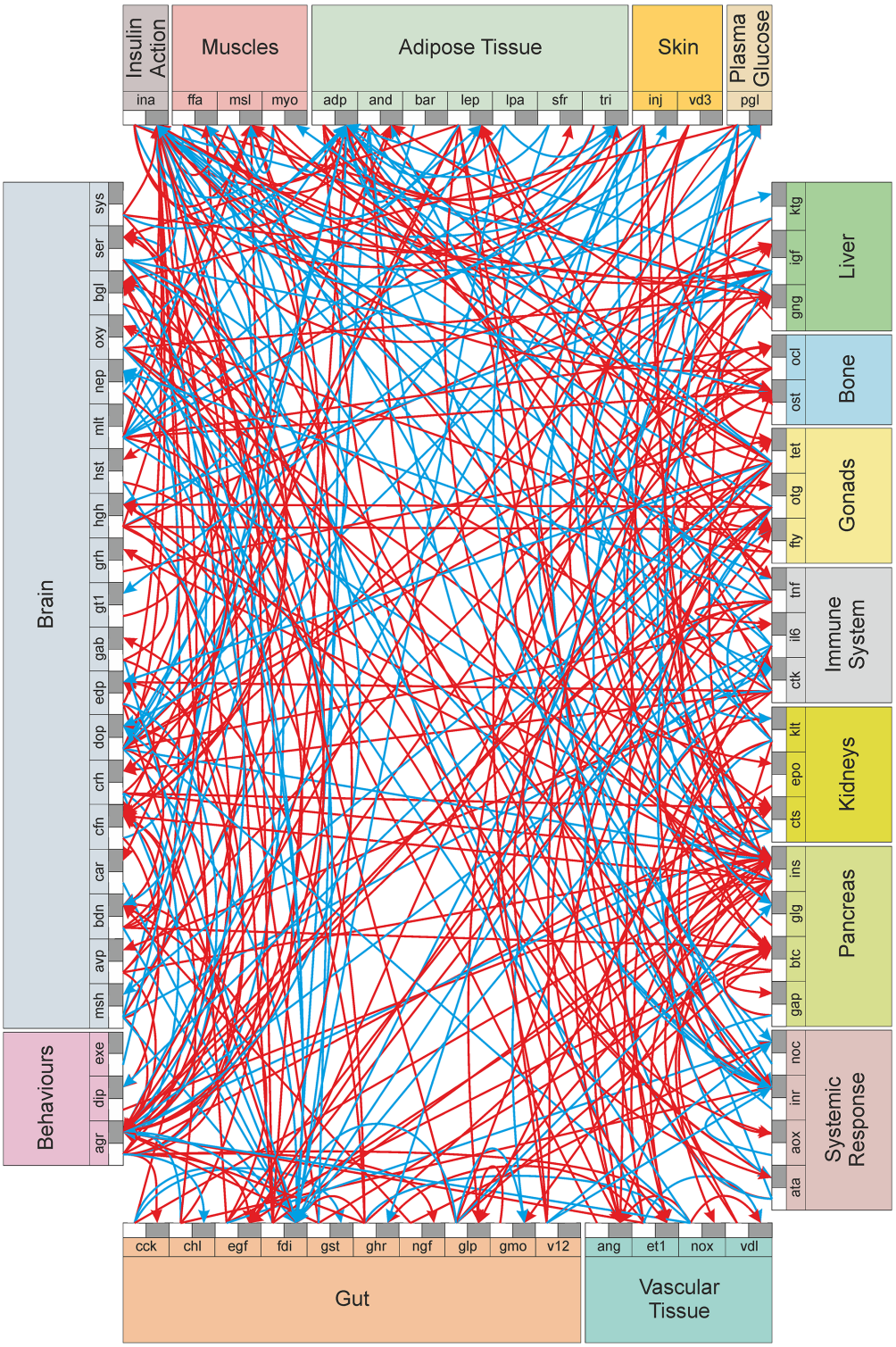

Fig 2. The inter-organ signalling network involved in the pathogenesis of T2DM. Each organ (coloured rectangles) displays the signals it produces. The outbound (white rectangle) and inbound (black rectangle) portals for each signal are shown. Red arrows indicate up-regulation interactions and cyan, down-regulation interactions. (See also S2 Table).

https://doi.org/10.1371/journal.pone.0181536.g002

considered different nodes. For example, glucose in blood and that in the brain were treated as separate nodes. A limitation of the study is that the network model may be currently incomplete due to lack of specific studies (studies yet to be pursued by the scientific community), publication bias or studies that we may have missed during literature survey. Currently, the model is only qualitative, in that it considers normal, up-regulated and down-regulated states as discrete states. Each of the links may have some quantitative dynamics which may be linear or non-linear which was not incorporated in the current model.

Perturbation simulations

A combination of Microsoft Excel 2007 for data input (addition of links to the network) and output (network perturbation results) and Visual Basic Application for executing the links was used to construct a network perturbation model. The signals were treated as nodes that can have one of three states namely 0 or baseline, +1 or up-regulated and -1 or down-regulated. Also, the directional links were of three different kinds namely up-regulatory or positive (which increased the state of the downstream node by 1), down-regulatory or negative (which decreased the state of the downstream node by 1) and basal level (which did not change the state of the downstream node). A zero signal here does not mean that there is no signal; it rather denotes that there is basal level signalling going on between the two nodes. Although the model considers only discrete states, it does not indicate extreme states. For example, -1 state of beta cell mass does not mean complete destruction of beta cells. In T2DM, a substantial proportion of beta cells survives lifelong [18]. Therefore, even in the -1 state of beta cells, insulin producing capacity is not assumed to be completely lost.

After constructing the network, we studied the effects of different kinds of perturbations in the network. At the beginning all nodes were at a default state of zero. Whenever a node was manually up or down-regulated, the state of that node changed to +1 or -1 respectively. All the directional links starting from that node were activated to change the states of the recipient nodes (first generation nodes). Subsequently directional links from these first generation nodes were activated to change the states of nodes further downstream (second generation nodes). The event of activation of one generation of nodes was termed as a 'cycle'. Whenever a node received activated signals from more than one other node, the signals were added arithmetically to give a net signal strength. Based on the net positive or negative value of the signal strength, the state of the node was changed by +1 or -1 respectively; but without exceeding the state limits of -1 to +1. If the net signal strength was zero or normal in a given cycle, then the node returned to its normal default state. Thus at any given time the direction of change in the state of a node was solely determined by the net input signal. However, the step length for any change was restricted to unity, i.e. the state -1 could not become +1 in a single step.

Mathematically, the function of each node in every cycle can be explained as follows.

If $Si \neq 0$, *then* $si = \Sigma eji$; where 'S' is the state of the node 'i', 's' is the cumulative signal it received and 'eji' is the link from node j to i.

Depending upon the cumulative signal, the node is assigned a state.

If $si > 0$, $Si(t) = Si(t-1) + 1$

If $si = 0$, $Si(t) = 0$

If $si < 0$, $Si(t) = Si(t-1) - 1$; where 't' is the cycle number

The state is then bound to limits -1 to +1

If $Si(t) \leq -1$,$Si(t) = -1$

If $Si(t) \geq 1$,$Si(t) = 1$

For example, to simulate the effects of primary hyperinsulinemia, the state of insulin in the starting cycle was made 1 where all the other nodes had a state of zero. In the first cycle, the

direct effects of insulin were executed. Hence, only those nodes that were immediate downstream of insulin altered their state to +1 or -1 depending upon whether they received up-regulation or down-regulation link respectively, from the insulin node. In the current example, β-cells, leptin, klotho, EGF, cognitive functions, endothelin-1, gonadotropin—releasing hormone, nitric oxides and gut motility were up-regulated (state changed to +1); and keto acids and adiponectin were down-regulated (state changed to -1). In the second cycle, the immediate effects of these first generation nodes were executed. Thus in every cycle, the effects radiated, and because all the nodes lay in a network, in a few cycles, every node was affected in some way or the other. The recorded output was the state of each node after each cycle.

In the model above, the step length was always unity. As it changed from -1 to 0 in one cycle, the signals changed according to the new states and the next step was decided by the new signals. The step length was altered in two other variations of the model. One allowed a direct leap from -1 to +1 if the net signal was > 0 or vice versa. This variation of the model did not consider change in signals during state transition. In another variation the states as well as steps were fine grained with a resolution of 0.1 so that twenty different states for each node were possible between -1 and +1. Each link when activated led to a change of 0.1 in the downstream effector node. Multiple signals led to a cumulative signal strength which changed the state of the node quantitatively between the limits of -1 and +1. We examined whether the results were sensitive to the step length.

We used two types of perturbations separately or in combinations. (i) Point perturbations, i.e., after the perturbation was made in the starting cycle, the perturbed node came back to basal state after the first cycle; and then its state was allowed to be decided by the links it received eventually from other nodes. (ii) Sustained perturbations, i.e., the state of a starting perturbation node was changed and the changed state was maintained independent of any link it received subsequently.

A stable state of a node was described as a consistent resultant state of the node which remained so throughout further cycles. If a node changed its state with a repeated cyclic pattern of a fixed periodicity throughout the cycles, it was termed as a node in stable oscillation. If a node changed its states with unpredictably altering periodicity, it was termed as a node in a chaotic state. The stable state of the system was defined as a state in which every node was in a stable state or in short term deterministic oscillations. Further for the definition of a stable state it was necessary that if the system was point perturbed starting with that state it returned to the same state. If an apparently stable state obtained after one perturbation did not return to it after any other point perturbation it was called pseudo-stable state. A chaotic state of the system was defined by one or more nodes being in a chaotic state. Whenever there were stable oscillations or chaos the average of the last hundred cycles was taken as the 'mean final state' for a node.

Some debatable links

A surprising finding of the search for links was that some of the classical beliefs were not supported by interventional evidence. For example we found no interventional evidence that muscle insulin resistance was compensated by hyperinsulinemia. Lack of evidence for this widely held assumption is acknowledged [2,19,20] but the assumption continues to be a part of mainstream thinking. Strictly going by the inclusion criteria of the model, we should not have included this link in the model. However since compensatory hyperinsulinemia is a widely held belief, we decided to run (make point perturbations to the network model and observe any changes in the Results) the model independently with and without this link. The difference in the outcomes of the two models could potentially give us the importance of this link. The

link between obesity and insulin resistance is also laden with contradictory evidence but the mainstream thinking is that obesity increases insulin resistance. We run the model separately with no link and with to and fro links between the two nodes.

The apparent irreversibility of beta cell damage is debated. Although classically beta cells were believed not to regenerate once lost, experiments over the last two decades have shown that beta cells have good regeneration capacity in vitro and in vivo including de novo regeneration from ductal ascinar cells [21]. We operate the model independently assuming beta cell -1 state to be reversible as well as irreversible. We also encountered eleven other contradicting reports, where some studies had reported up-regulation while others observed down-regulation effect between the same node pair. We treated the contradictory links similar to the insulin resistance—hyperinsulinemia link i.e., the model was run separately assuming positive link or assuming negative link between the node pair.

Results

Point perturbations

For all point perturbations, after 20–25 cycles, the system invariably reached a stable state. Further, there were only two observed stable states that the system reached. Chaos or a homeostatic return to the starting state was never observed in the system. The two stable states did not drift further after any point perturbation and were thus true stable states by definition. If instead of zero, starting states of all nodes were randomly assigned, the same two stable system states were obtained. The bi-stability thus obtained is unlikely to be a statistical generality since a null model with the same number of nodes and links but with randomization of link placements rarely gave bi-stability. Out of 1000 null model simulations, 931 ended in a chaotic state. Stability was observed in 69 of them out of which, 12 showed a single stable state; 49 showed bi-stability, 4 showed tri-stability and the remaining 4 showed tetra-stability. The uncommon occurrence of bi-stability ($p < 0.05$) in the null model implies that the observed bi-stability in the network is unlikely to have arisen by chance alone.

In the two alternative stable system states, the states of all nodes including insulin action were stable, consistent and exactly opposite (in terms of +1 or up-regulated and -1 or down-regulated) to each other. Since insulin resistance is conventionally believed to be central to T2DM we called the two attractors as insulin sensitive and insulin resistant attractors. The former was characterized by low adiposity, cholesterol, glucose levels and inflammatory markers; and high adiponectin. The latter had a diametrically opposite picture (Table 1). The nodes which, when perturbed (up-regulated), led to the insulin sensitive attractor were collectively called the insulin sensitive basin of attraction and those which led to the insulin resistant attractor, when perturbed (up-regulated), were collectively called the insulin resistant basin of attraction.

The model used three different step lengths. For all the three step lengths, bi-stability was observed and the composition of the two attractors remained identical. There were subtle changes in the basins of attraction though. When the steps were fine grained, although the nodes attained transient fractional values in the initial cycles, they ultimately settled at +1 or -1 and the attractors remained identical. Between unit step and fine grained step the basins of attraction were over 90% similar. When direct leap was allowed bimodality and composition of attractors remained the same and the basins of attraction were similar to unit step model by over 80%. Since bi-stability and attractor composition were not sensitive to the step length, for further analysis we used the unit step model alone which was faster as well as accommodated changes in signals during transition.

Table 1. Attractors for the point perturbations.

Serial Number	Signals/ Nodes	Three Letter Code	State in the insulin resistant attractor	State in the insulin sensitive attractor
1.	Activin A	ata	1	-1
2.	Adiponectin	and	-1	1
3.	Adipose Tissue	adp	1	-1
4.	Aggression	agr	-1	1
5.	α-Melanocyte Stimulating Hormone (α-MSH)	msh	1	-1
6.	Angiogenesis	ang	-1	1
7.	Anti-oxidants	aox	-1	1
8.	Arginine Vasopressin	avp	0	0
9.	β-Adrenergic Receptors	bar	0	0
10.	β Cells	btc	-1	1
11.	Bone Strength/ Bone Mass	ost	-1	1
12.	Brain-Derived Neurotrophic Factor (BDNF)	bdn	-1	1
13.	Brain Glucose	bgl	-1	1
14.	Cholecystokinin	cck	-1	1
15.	Cholesterol	chl	1	-1
16.	Cocaine and Amphetamine Regulated Transcript (CART)	car	1	-1
17.	Cognitive Functions	cfn	1	-1
18.	Cortico-Releasing Hormone (CRH)	crh	-1	1
19.	Corticosteroids	cts	-1	1
20.	Cytokines	ctk	0	0
21.	Diplomat Behaviour	dip	1	-1
22.	Dopamine	dop	-1	1
23.	Endorphins	edp	-1	1
24.	Endothelin-1	et1	1	-1
25.	Epidermal Growth Factor (EGF)	egf	-1	1
26.	Erythropoeitin	epo	-1	1
27.	Exercise	exe	0	0
28.	Fertility	fty	-1	1
29.	Food Intake	fdi	1	-1
30.	Free Fatty Acids	ffa	1	-1
31.	γ-Aminobutyric acid (GABA) pancreas	gap	-1	1
32.	γ-Aminobutyric acid (GABA) brain	gab	0	0
33.	Gastrin	gst	1	-1
34.	Ghrelin	ghr	0	0
35.	Glucagon	glg	-1	1
36.	Glucagon-Like Peptide-1 (GLP-1)	glp	0	0
37.	Gluconeogenesis	gng	-1	1
38.	Glucose Transporter-1 (GLUT-1)	gt1	-1	1
39.	Gonadotropin-Releasing Hormone (GnRH)	grh	1	-1
40.	Growth Hormone	hgh	0	0
41.	Gut Motility	gmo	1	-1
42.	Histamine	hst	-1	1
43.	Inflammatory Response	inr	1	1
44.	Injury (Growth Factors)	inj	-1	1
45.	Insulin	ins	1	-1
46.	Insulin Action	ina	-1	1

(*Continued*)

Table 1. (*Continued*)

Serial Number	Signals/ Nodes	Three Letter Code	State in the insulin resistant attractor	State in the insulin sensitive attractor
47.	Insulin-like Growth Factor (IGF-1)	igf	-1	1
48.	Interleukin-6	il6	0	0
49.	Keto Acids	ktg	-1	1
50.	Klotho	klt	0	0
51.	Leptin	lep	1	-1
52.	Leptin Action	lpa	0	0
53.	Melatonin	mlt	0	0
54.	Muscle Strength/ Muscle Mass	msl	-1	1
55.	Myostatin	myo	1	-1
56.	Nerve Growth Factor (NGF)	ngf	-1	1
57.	Nitric Oxide	nox	1	-1
58.	Nociception	noc	1	-1
59.	Nor-epinephrine	nep	-1	1
60.	Oestrogen	otg	-1	1
61.	Osteocalcin	ocl	-1	1
62.	Oxytocin	oxy	-1	1
63.	Plasma Glucose	pgl	1	-1
64.	Secreted Frizzled-Related Protein 5 (SFRP-5)	sfr	1	-1
65.	Serotonin	ser	1	-1
66.	Sympathetic Stimulation	sys	0	0
67.	Testosterone	tet	-1	1
68.	Triglycerides	tri	1	-1
69.	Tumour necrosis factor-α (TNF-α)	tnf	1	-1
70.	Vasodilation	vdl	0	0
71.	Vitamin B12	v12	0	0
72.	Vitamin D3	vd3	0	0

https://doi.org/10.1371/journal.pone.0181536.t001

Sensitivity of the model to assumptions and contradictions

1. Assumptions. To test the sensitivity of bi-stability to the underlying assumptions of the model, we relaxed the assumptions one by one and in combinations to see whether bi-stability was an artefact caused by some of them.

When we changed the mode of signal additions from simple arithmetic addition to qualitative addition, i.e. when a given node received both non-zero up-regulation and non-zero down-regulation links, the net signal strength was treated as zero. When a node received only positive signals, the node was up-regulated and when it received only negative signals, it was down-regulated. This invariably resulted in to chaos with every node and no long term tendency towards being up-regulated or down-regulated. A null model with qualitative additions invariably gave chaos. Therefore this result appears to be more of a statistical generality than any specific character of this network. The qualitative addition never allowed a sustained departure from the zero state. In the context of T2DM, this would mean that a stable insulin resistant or diabetic state may never be obtained. In reality, long term stability of insulin resistant or diabetic state is common and reversal is difficult. The qualitative addition mode did not appear to represent a realistic picture. Thus, relaxing some of the assumptions did not affect bi-stability and relaxing certain others gave rise to unrealistic chaotic results. None of the assumptions gave rise to good homeostatic control where the system returned to its ground

state on its own. This demonstrated the robustness of bi-stability and the soundness of the set of assumptions used in the model.

2. Contradictions. For all the contradictory interactions, simulations were run using positive or negative links. The interesting and surprising finding was that having or not having the compensatory hyperinsulinemia link did not affect the bi-stability of the network or the signatures of the two attractors. Since some researchers have argued for compensatory insulin resistance in response to primary hyperinsulinemia [19], we reversed the causal arrow between insulin resistance and insulin levels which again did not affect bi-stability. Similarly, reversing between the assumptions that obesity causes insulin resistance or insulin resistance causes obesity, or deleting the obesity-insulin resistance link altogether, did not affect bi-stability or the attractor signatures except for the state of obesity (i.e. the node 'adipose tissue') itself. When insulin and insulin action together down-regulated glucose, bi-stability was unaltered but when insulin alone down-regulated glucose independent of insulin action, the system oscillated with large periodicity (up to 32 cycles) and there were multiple resultant states. Therefore inclusion of the insulin sensitivity-resistance axis was one of the critical conditions for the bi-stability of the system.

For 10 out of the 11 up versus down-regulation contradictions examined, the system still retained bi-stability with the up-regulation or down-regulation arrows. Eight out of the 10 contradicting interactions that retained bi-stability showed no effects on the attractor signatures although the basins of attractions altered marginally ($< 15\%$) in some of them. Two of the interactions brought about marginal changes in the attractor signatures. The only up versus down-regulation contradiction that affected bi-stability was when endothelial nitric oxide synthase (e-NOS) and neuronal nitric oxide synthase (n-NOS) action were considered a single node. Different studies have found either up-regulating [22,23] or down-regulating [24–27] action of NOS on aggression. Bi-stability was retained for the down-regulation link but not for the up-regulation link. After segregating the actions of e-NOS and n-NOS, bi-stability was retained. Since different studies report up or down regulating action of n-NOS on aggression, the model was run with either of the links at a time. With both types of links, bi-stability was maintained but the inclusion of n-NOS in the basin of attraction was affected.

Reactive oxygen species (ROS) is considered an important player in the pathophysiology of T2DM. During redundancy filtering, ROS was filtered out since it was tightly linked to inflammation and both shared identical incoming and outgoing links. But since ROS is believed to be an important player, we simulated keeping ROS as a separate node. This change again did not affect bi-stability and up-regulation of ROS led to insulin resistant state.

Glucagon has a direct up-regulation effect on insulin secretion [28], but through the agency of kisspeptin, it has a down-regulation effect [29], making the net effect zero. The signal between glucagon and insulin was therefore filtered out. However, since insulin and glucagon are believed to be central molecules to T2DM we operated the model with and without these links singly and in combination. The bi-stability remained robust to the inclusion or exclusion of these links. The effect of glucose on beta cell mass also has contradictory literature. Glucose is shown to stimulate proliferation of beta cells on the one hand [30] and on the other glucotoxicity is said to affect beta cell function [31]. Nevertheless the bi-stability of the model was not sensitive to either of the assumptions.

Sustained perturbations

We perturbed each node singularly, in a sustained manner, and observed the downstream effects. Sustained perturbation of the nodes in the network did not affect bi-stability. A fraction of these perturbations led to stable short repetitive oscillations in the states of some nodes. Out

of the 72 nodes 49 sustained perturbations gave identical results as respective point perturbations. Remaining 23 sustained perturbations showed some changes in the attractor signatures as compared to their respective point perturbations. Bi-stability was nevertheless maintained in all cases.

Combining sustained and point perturbations

With each of the sustained perturbations in the background, every other node was point perturbed one at a time and simulations were run for a minimum of 300 cycles. Out of the 72 sustained perturbations, 60 led to bi-stability although the signatures of the attractors changed occasionally. Eleven sustained up-regulations gave rise to a single insulin sensitive attractor and these were aggression, adiponectin, dopamine, ghrelin, growth hormone, insulin action, melatonin, muscle strength, oestrogen, osteocalcin, and testosterone. And sustained up-regulation of serotonin invariably led to the insulin resistant attractor. Sustained up-regulation of the 11 nodes or down-regulation of serotonin never allowed the system to become insulin resistant. Not only that, but aggression, dopamine, ghrelin, insulin action, muscle strength, oestrogen and osteocalcin were able to completely reverse the states leading to the insulin sensitive attractor if the simulations began from the insulin resistant attracter as the starting conditions.

Although with combinations of perturbations the signatures of attractors could change, there were significant associations between the states of several nodes. We clustered the nodes based on the distance between pairs of nodes defined as the number of times the states of the two nodes did not match across all possible combinations of perturbations. The 3 different clusters obtained were (see S1 and S2 Figs for details of cluster analysis):

1. *and*, *agr*, *ang*, *aox*, *bdn*, *btc*, *cck*, *cts*, *crh*, *dop*, *egf*, *edp*, *epo*, *fty*, *gap*, *glg*, *gng*, *gt1*, *hst*, *igf*, *inj*, *ina*, *ktg*, *msl*, *ngf*, *nep*, *otg*, *ost*, *ocl*, *oxy*, *bgl*, *tet*
2. *ata*, *adp*, *msh*, *car*, *chl*, *cfn*, *dip*, *et1*, *ffa*, *fdi*, *gst*, *grh*, *inr*, *ins*, *lep*, *myo*, *nox*, *pgl*, *sfr*, *ser*, *tnf*, *tri*, *gmo*, *noc*
3. *avp*, *bar*, *ctk*, *gab*, *ghr*, *hgh*, *il6*, *klt*, *lpa*, *mlt*, *sys*, *vdl*, *vd3*, *exe*, *glp*, *v12*

Validation of the network model

These clusters suggested a way of validating the model. We expected all the nodes in a cluster to be positively correlated to each other in real life data. Currently there are no studies that provide quantitative data on all the nodes together. However different studies have looked at different correlations. Of particular value are correlations between nodes that do not have a direct link between them but they lie in the same cluster in the above classification. Demonstrated correlations compatible with this expectations include myostatin to leptin [32], TNF-α to triglycerides, plasma glucose to cholesterol [33], vitamin D3 to vasodilation [34] and growth hormone to klotho [35]. We did not find any correlation in literature contrary to the model expectations.

A comparison with the classical theory

The classical theory of insulin resistance states that obesity leads to insulin resistance, insulin resistance tends to increase plasma glucose which stimulates increased insulin secretion. This increased insulin secretion brings glucose back to normal leading to an insulin resistant-hyperinsulinemic-normoglycemic stable state. Failure of compensatory hyperinsulinemia owing to

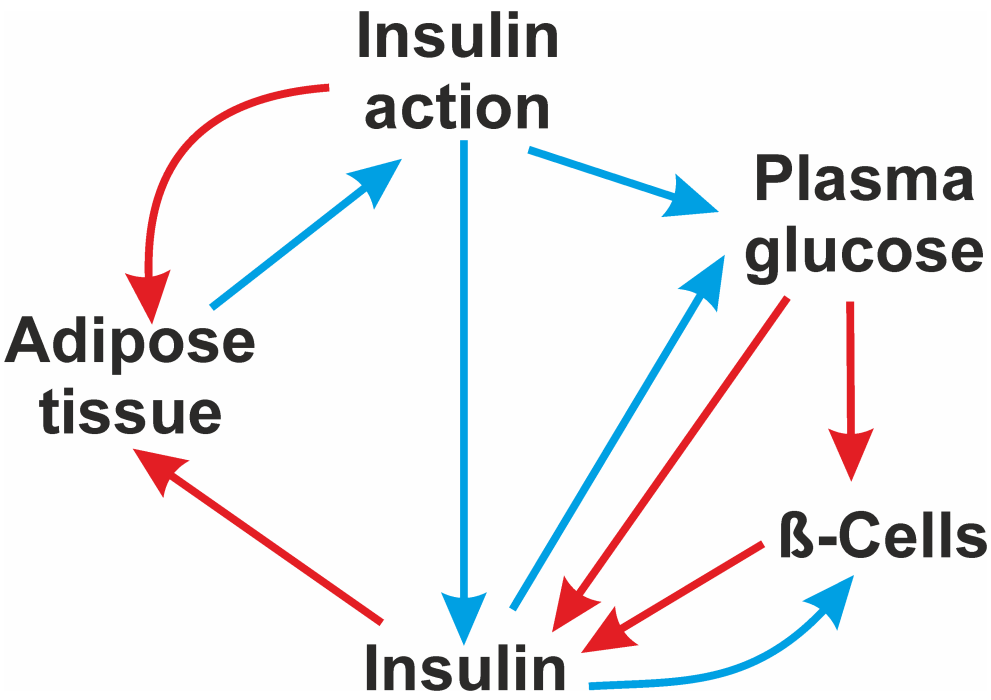

Fig 3. Classical model. Interactions among adipose tissue, insulin action, plasma glucose, plasma insulin and beta cell mass according to the classical theory are shown with red arrows indicating up-regulation links and cyan, down-regulation links.

https://doi.org/10.1371/journal.pone.0181536.g003

beta cell exhaustion or dysfunction results in to hyperglycemia. We included only adipose tissue, insulin, insulin action, beta cell mass and plasma glucose (Fig 3) as nodes in the model and included all known and classically believed links. In this classical model, we failed to see bi-stability under any condition. After any point perturbation in any of the five nodes, the system returned to the initial basal state in not more than 4–5 cycles or showed stable oscillations around the initial basal state. This is a typical behaviour of a homeostatic system. No point perturbation could change the basal state and lead to a stable insulin resistant state. Being a smaller and simpler system it is easier to visualize the reasons. For example, when we up-regulated adipose tissue mass, insulin resistance and subsequently plasma glucose increased. This increased insulin levels and subsequently glucose returned to normal. As glucose returned to normal, insulin could not remain elevated. Thus a normoglycemic-hyperinsulinemic state was not stable. Further in a state of high insulin resistance, the lipogenic action of insulin was suppressed and therefore adipose tissue was reduced. Reduction in adipose tissue normalized insulin resistance and thus the system was back to its starting state. Even if we assume that chronic overproduction of insulin affects beta cell function, beta cell mass remains in a homeostatic state since glucose is known to stimulate beta cell proliferation. Further, owing to the other homeostatic loops, both glucose and insulin return to normal thereby removing beta cell stress. Inclusion of glucotoxicity, that is, considering *pgl* to *btc* a negative regulator, did not drift the system away from homeostasis. Assuming beta cell loss as irreversible, that is, fixing *btc* state to -1 resulted into oscillation of insulin between zero and -1 states but glucose remained normal because of feedback loops operating through *adp* and *ina*. All the links in this small network made effective negative feedback loops and therefore the system failed to give a persistent insulin resistant state under any condition.

Applications of the network model

1. Is there any key node?. To check the sensitivity of the model to the nodes involved in the network and also to highlight the important nodes which when removed lead to the collapse of bi-stability, we deleted each node one at a time and observed the effect of perturbing every other node. A node under focus was frozen to the zero state all the time. This turned all the incoming as well as outgoing links from the node ineffective and thereby the node was cut-off from the rest of the network. This analysis also suggested whether tight homeostatic control over any node is sufficient for homeostasis of the entire system. We found that in 71 of the 72 deletions, there was no deviation from bi-stability. The system showed a deviation from bi-stability only when the node fertility (*fty*) was deleted. Deletion of *fty* led to multiple stable states; some being insulin sensitive and others being insulin resistant. Most of the correlates of insulin resistance remained similar except that high cholesterol was now associated with insulin sensitivity. To check whether any particular outgoing link of *fty* was responsible for this effect, we deleted each of them individually. None of the links made by *fty* when individually deleted affected the bi-stability. It seems to be a compound effect of the 3 links downstream to *fty* namely up-regulation of EGF, oestrogen and oxytocin. It is interesting to note that freezing glucose to the normal state did not ensure homeostasis of the entire network suggesting that glucose homeostasis is not central and critical to the behaviour of the network.

2. Is there a key node combination?. In addition to single node deletion, we deleted combinations of nodes by randomly freezing to zero 10% of the nodes at a time. Out of 1000 such simulations, bi-stability was conserved 81% of the times. Among the remaining 19%, there was complete loss of stability 1.1% of the times. Among the deleted combinations that led to loss of stability the nodes aggression, dopamine and fertility were overrepresented. Among the other non-bi-stability outcomes 2.2% was contributed by uni-stability where the states of the nodes were at and around the basal zero state indicating that the network was in a robust homeostatic state. Among the combinations of deletions that gave robust homeostasis adiponectin, cholesterol, fertility, histamine, insulin action, leptin and oxytocin were overrepresented suggesting that these nodes in combination are critical for bi-stable behaviour of the system. It is interesting to note that glucose did not appear in this list indicating that ensuring glucose homeostasis along with a few other key nodes does not assure homeostasis of the entire system. See S3 Table for the list of combinations of deletions that led to homeostatic uni-stability and complete loss of stability. In the remaining 15.7% cases tri, tetra or penta-stability was obtained in which some states were insulin sensitive and others resistant.

3. Is there a critical missing link?. We tested the robustness of the bi-stability of the model by random addition of a link between two randomly chosen nodes also. In 1,000 such random addition trials, bi-stability was not altered except for 8 specific link additions. In 6 out of the 8 there were 3 stable states instead of 2 and in only 2 cases there were multiple stable states. None of the additions resulted in chaos or homeostatic return to the starting state. This demonstrates further that the bi-stability is unlikely to be because of some critical missing link.

4. What makes the bi-stability robust?. Since there were only two resultant attractors in the baseline model, the nodes could be classified as the ones whose up-regulation led to the insulin sensitive attractor and the other whose up-regulation led to insulin resistant attractor. Notably, point up-regulation of 40 of the 72 nodes, led to a stable state in which they remained up-regulated. This is a positive feedback effect. Sixteen of the nodes resumed the zero state although they drove the system to one of the two stable states. The remaining 16 showed an overcompensation-like response, i.e. point up-regulation of these 16 nodes led to a state in which they were down-regulated. Overall the network had a preponderance of positive feedback circuits which explains the robust bi-stable behaviour of the system.

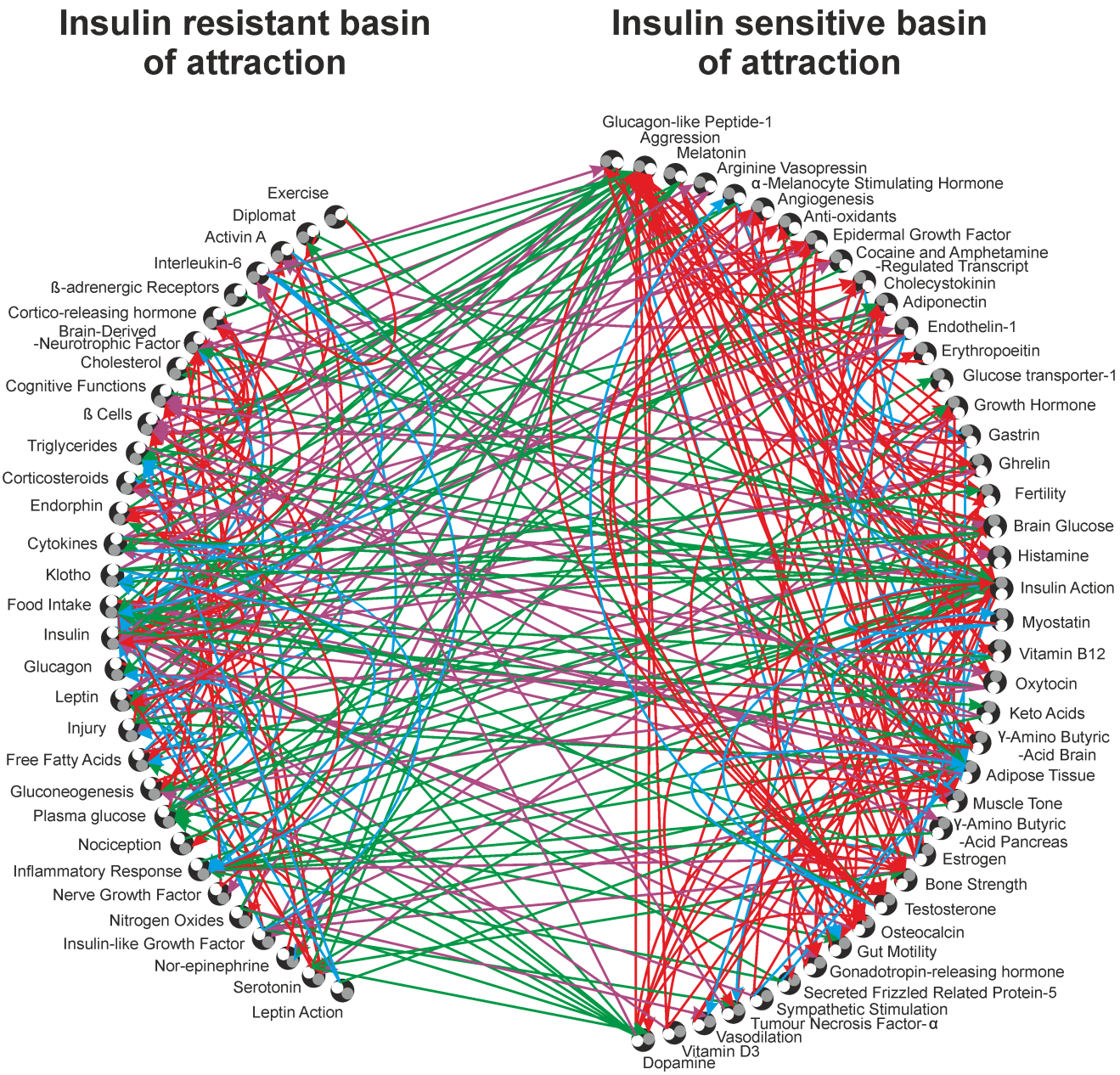

Fig 4. Basins of attraction. T2DM Signalling network segregated according to the point perturbations leading to the two attractors. The outbound (white circle) and inbound (grey circle) portals are shown for each node. Red arrows indicate intra-group up-regulation links; cyan, intra-group down-regulation links; purple, inter-groups up-regulation links; green, inter-groups down-regulation links.

https://doi.org/10.1371/journal.pone.0181536.g004

If the network is redrawn segregating the two groups of nodes (Fig 4), it can be appreciated that there are significantly more positive links within group as compared to between groups and there are significantly more negative links between groups as compared to within groups (chi square = 37.33619, df = 3, $p < 0.0001$). This makes the bi-stability and the dichotomous grouping of the nodes very robust. Within group positive and between groups negative links will stabilize and reinforce the attractors; whereas within group negative and between groups positive links will tend to destabilize the attractors. Since there were 216 stabilizing and 114

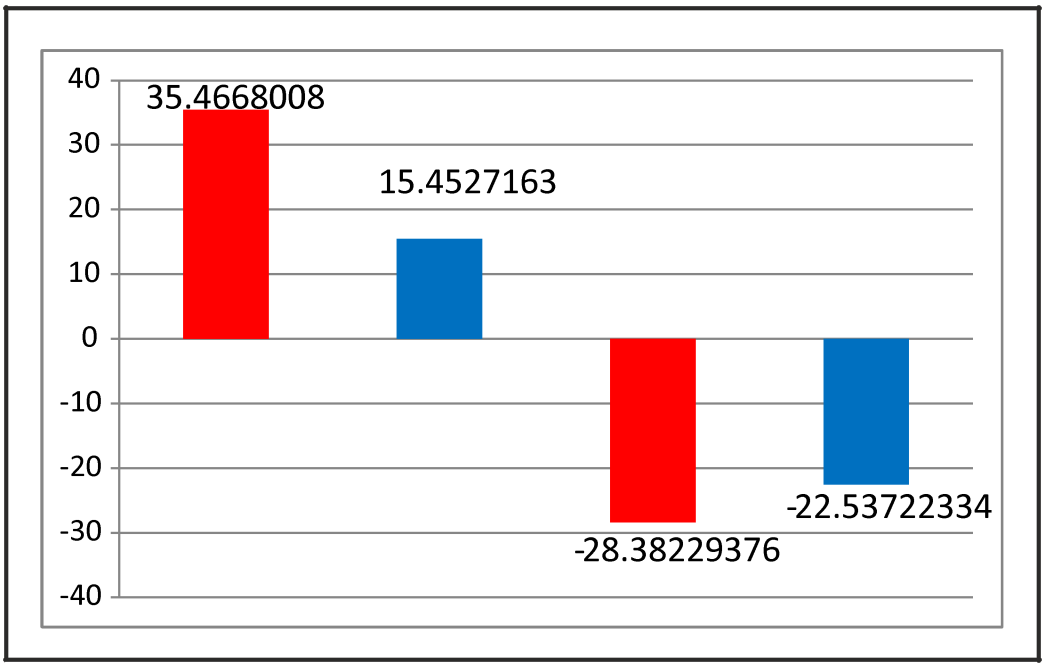

Fig 5. Link statistics. The bars represent the deviation from the expected number of links per cluster, the expected being calculated assuming independence. First two columns show the stabilizing links and the next two columns show the destabilizing links for the two clusters. The red and blue bars represent the insulin sensitive and the insulin resistant basins of attraction, respectively.

https://doi.org/10.1371/journal.pone.0181536.g005

destabilizing links, there is no wonder that the two attractors were highly stable and not sensitive to changing a few nodes or links (Fig 5).

5. Towards robust targets for treatment of T2DM. The combined perturbation simulation results give us possible new insights into long term effectiveness of a treatment. The critical question here is if a treatment target is sustainably locked into a desired state, how the network behaves in presence and absence of other perturbations. An ideal treatment target could be one which when locked should keep the system in an insulin sensitive state irrespective of any other perturbations. The different approaches currently targeted for treatment are suppression of liver gluconeogenesis, restoration of beta cell mass, incretin action, enhancement of insulin production, insulin supplementation, reduction in obesity, reduction in plasma free fatty acid levels, normalizing plasma glucose, reducing oxidative stress and exercise. None of these treatments was able to ensure an insulin sensitive state by sustained perturbation. The states were rather decided by the accompanying point perturbations. Thus none of these treatments were able to reverse the diabetic state in the long run although transient suppression of plasma glucose could be obtained with many of them. One major line of attempted treatment is to improve the beta cell function or introduce a new population of healthy beta cells. The critical underlying questions are whether beta cell regeneration in T2DM is reversible and whether improving beta cell function can reverse T2DM. When we operated the model assuming beta cell dysfunction to be reversible, in the insulin resistant attractor, the state -1 remained stable and up-regulating the state of beta cells, transiently (point perturbation) or sustainably, did not bring the system back to the insulin sensitive state. This suggests a possible solution to the beta cell paradox, that is, why beta cell dysfunction appears to be irreversible in T2DM when the cells have good regeneration capacity. In the model, other signals coming from the network kept beta cell function down-regulated. Alternatively, we assumed

beta cell dysfunction to be irreversible, that is, when beta cells achieved a state of -1, it was retained -1 through all further cycles. Even under this assumption, bi-stability was attained and the composition of the attractors was substantially the same.

In contrast, there were 11 nodes namely aggression (*agr*), testosterone (*tet*), dopamine (*dop*), oestrogen (*otg*), osteocalcin (*ocl*), melatonin (*mlt*), ghrelin (*ghr*), muscle strength (*msl*), adiponectin (*and*), insulin action (*ina*) and growth hormone (*hgh*) which when sustainably up-regulated, ensured insulin sensitivity. All these nodes connect to insulin sensitivity by multiple pathways with positive regulator pathways far outnumbering negative regulatory pathways (Table 2). For example, aggression links directly and indirectly to the first tier players from Fig 1 through EGF [6,36,37], IGF-1 [38,39], dopamine [40,41], muscle mass [42], bone strength [43], adiponectin [44,45], testosterone [46,47] and other intermediates. Similar role is shown to be played by oestrogen in females [47,48]. Osteocalcin, a marker of bone formation [49], also increases insulin sensitivity in humans [50]. Melatonin is also known to enhance insulin sensitivity [51], and also aggression [52]. Thus most of the above mentioned nodes that could ensure insulin sensitive state were closely related to aggression and aggression may hold the key to an insulin sensitive state as suggested by Belsare et al.[53], Watve [2] and Watve and Yajnik [54].

We further examined how much time did each of the potential candidate nodes took for a reversal from insulin resistant to sensitive state. In this race, oestrogen was the fastest actor which made the transition in 3 cycles followed by ghrelin (4), aggression (5), dopamine (7), muscle strength (24) and osteocalcin (59). If serotonin was down-regulated for at least 10 cycles, it also pushed the system from insulin resistant to insulin sensitive state. Applying a combination of interventions could reduce the number of cycles required for transition from insulin resistant to sensitive state. A minimum of 3 nodes were required to be simultaneously up-regulated for bringing up the transition in one or two cycles. Eleven three-membered combinations containing *agr* along with two other from *dop*, *tet*, *ghr*, *mlt*, *msl*, *otg*, and *hgh*; *dop* and *otg* with either *tet* or *hgh* could change the attractor from insulin resistant to the insulin

Table 2. Number of pathways from the novel target to insulin action.

Novel Target	Total Pathways	Positive / Negative ratio
and	49	3.090909091
agr	140	1.955555556
dop	167	2.1
ghr	154	1.375
hgh	107	2.225806452
ina	49	1.705882353
mlt	138	1.976744186
msl	41	3
otg	110	2.678571429
ocl	68	1.56
tet	135	1.62
ser	99	0.446153846

All pathways that link the 12 promising nodes to insulin sensitivity were mapped and listed. The 11 nodes whose up-regulation increases insulin sensitivity, have a greater proportion of positive regulator pathways. Serotonin, whose down-regulation increases insulin sensitivity, had a greater proportion of negative regulator pathways. The 11 target to insulin action.l target to insulin action. The reference for each link and pathways far outnumbering negative r.

https://doi.org/10.1371/journal.pone.0181536.t002

sensitive state in a single cycle. Down-regulation of *ser* in combination with up-regulation of *agr* and either *dop*, *tet* or *ghr* could give the same effect. Once the system attained the insulin sensitive state by any of the above combinations of interventions, it could sustain itself against any point perturbations even when the interventions were withdrawn.

When these interventions were applied assuming beta cell degeneration to be irreversible, up-regulation of *agr*, *dop*, *otg*, *ocl* and *ina*; and down-regulation of ser could still lead to the insulin sensitive state. When these interventions were applied when both beta cell and insulin levels were kept fixed at -1, the results were identical. Thus the question whether beta cell degeneration is reversible or irreversible did not seem to be central to the reversal of an insulin resistant state to a sensitive one.

Discussion

Despite the limitation of the model owing to its qualitative nature, the results are realistic in multiple ways. Running the model under different sets of assumptions, accommodating contradictory empirical results and the sensitivity analysis demonstrates that the model is robust and the results are not the artefactual outcome of any particular assumption. The model was able to predict the clinically observed correlates of insulin resistance accurately. It also made correct correlational predictions between pairs of variables that did not have a direct causal connection. The classically perceived treatments targeting liver glucose production, insulin sensitivity, insulin secretion including incretin action and beta cell function failed to bring about a transition in the steady state in the model although they could temporarily improve glucose control. This matches with the clinical observations that all these lines of treatments have largely failed to cure diabetes or even control hyperglycemia in the long run [55]. Many large scale clinical trials have revealed that normalizing blood glucose is not effective in avoiding diabetic complications [4]. This finding is compatible with the model. Further, the model demonstrates that it might be impossible, in principle, to prevent diabetic complications by a sole focus on normalizing glucose. The ineffectiveness of aggressive glucose normalization trials may not be because of failure to appropriately regulate glucose. Even if glucose is regulated without hypoglycemic and other undesirable events, the complications may not be arrested since normalization of glucose alone does not reverse the network state.

The model also accounts for foetal programming. If we consider the all zero baseline state of the system as a foetal condition, certain stimuli faced in embryonic or early life can drive the system to one of the two states which are difficult to reverse. This may account for developmental origins of adulthood disease (DOHAD) [56] or predictive adaptive response [57]. Since the model is based entirely on experimental data and it appropriately accounts for many realistic phenomena, the unexpected outcomes of the model need to be considered seriously as new possibilities. Empirical work in this direction is needed to test whether they work in reality.

Limitations of the model mainly come from 3 of its attributes that some of links might yet have to be discovered, the experiments from which data are taken are carried out on different model systems and that the model is discrete. Nevertheless, many predictions of the model matched with observed data suggesting thereby that the network model works reasonably well despite the limitations. This suggests that the novel and unexpected predictions of the model need to be tested empirically.

The model essentially demonstrates that the pathophysiology of type 2 diabetes is orders of magnitude more complex than the classical picture of insulin resistance and relative insulin deficiency causing hyperglycemia. Insulin and glucose have been the two molecules central to classical thinking but apart from the burden of history, there are no other grounds to treat insulin and glucose to be more important in T2DM than any other nodes of the network. The

behaviour of the system is decided more by the network structure than by one or a few key molecules. In a network structure, it is possible to reach all nodes by starting from any random node. Therefore, although we started assembling the network from insulin and glucose, it does not mean the network is gluco-insulino-centric.

Because of the anastomoses of the network, the function lost by deleting a link can be compensated by alternative paths. Since the number of links stabilizing the attractors far outnumber the ones destabilizing it, a few missing links are unlikely to alter the behaviour of the network. This may explain why knockouts such as MIRKO, or insulin suppressing agents failed to increase fasting glucose in experiments [13,58]. It is possible in a network that one or a few nodes play a central role, but if this is true, it should have been detected by systematic deletion of nodes that we performed. The system was generally robust in this analysis and the only node whose deletion or freezing made any changes in the behaviour of the system was not related to energy homeostasis but to fertility and behaviour. This might be surprising for the classical theory of T2DM but is expected by some of the upcoming evolutionary hypotheses for the origin of T2DM [54,59]. Unless a single node or single link makes a critical difference, a disorder is unlikely to originate in a single gene defect. Therefore it is no wonder then that genome wide association studies are able to explain not more than 2% of obesity [60] and10% insulin resistance [61] at a population level.

The multiply reinforced alternative stable states suggest that there could have been strong selective forces to stabilize both the states under different contexts. Some of the evolutionary hypotheses argue that insulin resistance is not an inevitable result of obesity but is a contextually adaptive state selected to face certain environments or to support certain coping strategies [2,59]. Bi-stability indicates an adaptive and evolved insulin resistant state rather than a pathological deviation from a homeostatic system [62].

Clinically the first important realization of the study is that a large number of signals can potentially influence insulin sensitivity and the current emphasis on obesity alone is perhaps overplayed and unwarranted. The means of transiting from the insulin resistant attractor to the insulin sensitive one revealed by the model are substantially different from the traditional line of thinking in clinical practice or in drug discovery. The model shows that none of the current lines of treatment are able to make this transit. Instead the model suggests some non-conventional lines of treatment. Of particular interest is the role of exercise. Sustained physical activity alone did not have effects comparable to aggression in the model. Physical activity has been classically considered to affect energy balance and reduce adiposity. Physical aggression on the other hand has many other direct endocrine effects [53] and this effectively assured insulin sensitivity in the model. This raises the possibility that exercises work more effectively through the behavioural neuro-endocrine pathways rather than through calorie consumption. In reality, many types of exercises have some or the other behavioural components and thereby stimulate the neuro-endocrine pathways [63–67] in addition to burning calories. A testable prediction of the model is that different exercises can be expected to have different endobolic effects even if the caloric requirement is matched [53,68].

We can no more view complex disorders by piecemeal and expect to treat the disorder effectively. The behaviour of a network can be substantially different from the behaviour of smaller pieces of the network. The model suggests molecular targets such as adiponectin, growth hormone, melatonin and testosterone for prevention of T2DM; and dopamine, ghrelin, oestrogen and osteocalcin for prevention as well as treatment of T2DM. But since all these molecules are behaviourally regulated, it is likely that behavioural intervention may have a better promise. It is quite likely that a paradigm shift is awaiting round the corner in the field and we need to be open to this possibility.

Supporting information

S1 Fig. Frequency distribution of distances of pairs of nodes. We clustered the nodes based on Simple Matching Coefficient (SMC) between pairs of nodes defined as the number of times the states of the two nodes matched across all possible combinations of perturbations. This led to a SMC matrix of 71 X 70 nodes to which the basic set of 71 point perturbations and 71 singular sustained perturbations were added to make the total 5112. All the scores were normalized by this total number 5112. Hence, every possible pair of nodes had a score from zero to one. To view this scoring as a distance between the two nodes under consideration, we subtracted that number from one. Hence, the pairs of nodes having a score nearer to zero mean that the nodes in the pair are strongly correlated and hence closer to each other and the pairs having a score of one denotes the longest possible distance and thereby no correlation between the nodes in that pair. These scores were used to construct a frequency distribution. Since the histogram shows two distinct peaks, it indicates clear clustering. The two peaks in the frequency distribution of pair-wise distances correspond to the intra-group distance and the inter-group distance respectively. We considered the first dip, i.e. 0.4 in the histogram as a threshold and listed all the pairs which had a distance less than that threshold. Clustering was made by associations starting with the first pair till the list was exhausted. In this way, 3 different clusters were obtained.
(TIF)

S2 Fig. Dendrogram generated by DendroUPGMA. To compare the method of clustering with a known method of clustering, we used DendroUPGMA (http://genomes.urv.cat/UPGMA/), open source online software to cluster the nodes in our network and plot a dendrogram. The software uses UPGMA (Unweighted Pair Group Method with Arithmetic mean) for clustering. We used the input data type as similarity matrix and fed in the 71 X 71 matrix with the original scores out of 5112 for each pair of nodes. Clusters identified by both the clustering protocols were identical.
(TIF)

S1 Table. Model organism used in each reference.
(DOCX)

S2 Table. Nodes and links with references.
(DOCX)

S3 Table. List of deletions of the 10% of the nodes that led to uni-stability and complete loss of stability.
(DOCX)

S1 Text. Merger and exclusion of links according to criteria defined in the text.
(DOCX)

Acknowledgments

We would like to thank Pramod Patil, Manawa Diwekar-Joshi and Poortata Lalwani for commenting on an earlier draft of the manuscript. We would also like to thank Sutirth Dey, Pranay Goel and Anu Raghunathan for their valuable input to the developing concept.

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