LEW.1WR1 rats have altered hepatic metabolism during their type 1 diabetes susceptibility window

Madushika Wimalarathne
LEW.1WR1 RATS HAVE ALTERED HEPATIC METABOLISM DURING THEIR TYPE 1 DIABETES SUSCEPTIBILITY WINDOW

by

MADUSHIKA WIMALARANTHNE

A THESIS

Submitted in partial fulfillment of the requirements for the degree of Master of Science in The Department of Biological Sciences to The School of Graduate Studies of The University of Alabama in Huntsville

HUNTSVILLE, ALABAMA

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We, the undersigned members of the Graduate Faculty of the University of Alabama in Huntsville, certify that we have advised and/or supervised the candidate on the work described in this thesis. We further certify that we have reviewed the thesis manuscript and approve it in partial fulfillment of the requirements for the degree of Master of Science in Biology.

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ABSTRACT
The School of Graduate Studies
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Degree Master of Science College/Dept. Science/Biological Sciences
Name of Candidate Madushika Wimalarathne
Title LEW.1WR1 rats have altered hepatic metabolism during their type 1 diabetes susceptibility window.

LEW.1WR1 (1WR1) rats have increased type 1 diabetes (T1D) onset with the administration of polyinosinic-polycytidylic acid (PIC). The objective of this study was to compare the insulitis status and liver gene expression in both control and treated 1WR1 rats during disease induction with a T1D-resistant strain of rat, the LEW/SsNHsd (SsNHsd). Using immunohistochemistry we observed higher T cell infiltration in the 1WR1 treated rats compared to the control strain, the SsNHsd PIC-treated rat. Yet we confirmed that the 1WR1 rats were not undergoing significant insulitis at the time point we selected.

Interestingly the 1WR1 treated rat had significantly increased insulin levels compared to the SsNHsd rats, both groups of the 1WR1 rats also had increased liver FAT10 gene expression compared to the SsNHsd. We observed 1WR1 rats have increased expression of genes related to lipid catabolism and lower expression of glucose metabolism-related genes. In conclusion, 1WR1 rats have reduced insulin sensitivity in the context of increased inflammation due to PIC injection which increases beta-cell sensitivity to eventual T-cell mediated destruction.
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Chapter 1
Introduction

1.1 Type 1 Diabetes

There are major two types of Diabetes: 1) Type 1 diabetes and 2) Type 2 Diabetes. People with Type 1 diabetes cannot produce insulin. People with Type 2 diabetes can produce insulin but are unable to respond to insulin as they should in normal conditions. Both types of diabetes can lead to high blood glucose levels later in the disease.

When glucose is present in the blood, Insulin binds with the insulin receptors which are bound to cell membranes. Once, insulin binds to insulin receptors, tyrosine kinase activates. The cell signaling molecules like IRS.P13K, PDK1, AKT, AS160 and eventually lead to cell survival and proliferation. GLUT4 transports from cytoplasm to cell membrane. GLUT4 is an insulin regulated-glucose transporter primarily found in adipose tissue and skeletal muscles. Once GLUT4 transporters open, cells can uptake glucose. Glucose will be used in several ways in cells. Glucose can make ATP and pyruvate through glycolysis. Pyruvate can convert to lipids through lipogenesis. Glucose can store as glycogen through glycogenesis. If cells cannot uptake glucose, all these processes will be affected. Without insulin, Glucose cannot uptake by cells and cells starved with energy (Jennifer J et al., 2017).

1.1a What is the prevalence

Type 1 diabetes (T1D) is an autoimmune disease usually diagnosed in the early stage of life. TID patients cannot produce insulin in their body, so they depended on daily exogenous insulin treatments. It is an enormous burden to both patients and the healthcare system of any country. This disease accounts for the primary cause of mortality of its patients. (Tao B et al.,2010).
There are approximately 1.25 million type 1 diabetic patients in the United States. (National diabetes statistics report, 2017). It is expected that 5 million people will be diagnosed with type 1 diabetes in the next 5 years and 40,000 people will be diagnosed with type 1 diabetes every year. According to the National Diabetes statistic report, 20000 people under 20 years have type 1 diabetes. For the same age group, there was a 21% increment in T1D diagnoses in the United States from 2001 and 2009. Moreover, it expects that 600,000 people under the age of 20 will be diagnosed with this disease by 2050 with non-hispanic whites having the highest risk of being diagnosed. T1D isn’t just an epidemic in the United States. People living with T1D are prevalent all over the world. Out of 3.7 million people living with diabetes in the UK, 10% of them have Type 1, which is approximately 40,000 people. Twenty-nine thousand children living in the UK have type 1 diabetes (National diabetes statistics report, 2017). Over 300,000 Canadians live with Type 1 diabetes, of which 33,000 are children between the ages 5 and 18 (National diabetes statistics report, 2017). In the last few years, the rate of type 1 diabetes diagnoses has gone up in New Zealand by 12%. Approximately, over two million people are living with diabetes in Argentina, and 7 per 100,000 kids aged between 0-14 are usually diagnosed with TID. Fifty thousand children and adolescents below 20 years old are diagnosed with T1D in china. Over 100,000 children in India and approximately 35,000 children in Saudi Arabia are living with Type 1 diabetes. (National Diabetes Statistics Report, 2017). By looking through the above statistics, one can get a clear picture of the diabetes epidemic around the world.

1.1b. The associated cost worldwide

The medical expenditure of diabetic patients is 2.3 times higher than a typical average person. Insulin treatment is common practice in type 1 and type 2 diabetes, and the cost of insulin has increased from $100 to $200 per month to $400 to $500 per month, depending on the brand. (Spero
et al., 2016). The total cost for diabetes has risen from $245 billion to $327 billion, from 2012 to 2017. It is a 26% increment over the past 5 years. According to Economic Costs of Diabetes in the U.S. in 2017, increased financial burden, health resources and low productivity played a huge role in cost increment related to diabetes. When breaking down the cost for medical expenditure, 30% for hospital inpatient care, 30% for medications, 15% antidiabetic agents, 13% physical office visits were counted (Economic cost of Diabetes in USA, 2017, American diabetic association). Average medical expenses of diabetic patients is $16,752 per year which is 2.3 times higher than normal nondiabetic person’s medical expenditure (Economic cost of Diabetes in USA, 2017, American diabetic association).

The great effort has been made to prevent type 1 diabetes. Unfortunately, the exact etiology and pathogenesis of type 1 diabetes are still unknown.

1.1c How is T1D clinically identified

Several methods are used to identify type 1 diabetes in children. One of them is a simple blood sugar test, with initial screening tests where a higher than 200 milligrams per deciliter (mg/dl) in blood glucose level is considered as type 1 diabetes. The Glycated hemoglobin (A1C) test indicates the child's average blood sugar level over the past few months by looking through the percentage of blood sugar attached to hemoglobin. An AIC level above 6.5 is considered as diabetes. Another test is a fasting blood sugar test taken after the patient fasts for 24 hours. If fasting blood glucose level is higher than 126 mg/dl, the patient is considered to have type 1 diabetes. Additional tests for diabetes include checking antibodies related to type 1 diabetes and checking ketone bodies in urine.
Accurate and early diagnosis is playing a crucial role in the treatment of diabetes. However, not all type 1 diabetic patients show those symptoms. Patients with immunological, self-reactive antibodies have type 1A diabetes, and if specific pathogenicity is not apparent, they belong to type 1B (idiopathic) diabetes (Gianani R et al., 2010). Even at the onset of type 1 diabetes, 70% of islets contain no insulin. The symptoms appear after 90% of beta cells are lost. An individual who has had type 1 diabetes for five years, may only be left with insulin-deficient cells. The CD8+ T cells are the most prominent cells in type of 1 diabetes induction followed by macrophages (CD68+), CD4+ T cells, B lymphocytes (CD20+), and plasma cells (CD138+) (Willcox A et al., 2009). The patients have smaller pancreas compared to their age, lower BMI compared to normal people in their age (Atkinson MA et al., 2013).

Most of the individuals get symptoms of type 1 diabetes-like high blood glucose level and low insulin level, decades after initial induction (Bonifacio E et al., 2010). It takes time to see visible symptoms, and within that time, most of the islets get damaged and at the unrecovered stage. It is marked that C-peptides are decreasing two years before disease onset (Sosenko JM et al., 2010) and the glucose fluctuation at the beginning (Sosenko JM et al., 2010). It is not clear what is the critical mass value of β cells, to say as a victim of type 1 diabetes, when need to start exogenous insulin replacement. The disease onset can be varying from months to years, depending on genetic susceptibility to the disease, environmental factors, etc. The loss of β-cells may affect the performance of the remaining beta cells. Maintaining residual islets in function is challenging because it again depends on genetic factors, age, and how the disease management process works (Atkinson MA et al., 2013).
1.1d. Importance of studying T1D

Type 1 diabetes is also known as juvenile diabetes, which causes the destruction of insulin-producing beta cells in the pancreas. Type 1 diabetes (T1D) is a complex autoimmune disease, which needs constant medical care with risk reduction strategies and glycemic control. T1D is the most severe form of diabetes, requiring daily injections of insulin. Even with excellent glucose control, there is a risk of developing complications regarding diabetes. The high blood sugar level in type 1 diabetes causes kidney failure, stroke, eye problems, dental disease, nerve damage, depression, foot problems, etc. The exact causes for the T1D remain unclear. One key to the prevention of full-blown T1D is the identification of noninvasive, targetable signals that precede the onset of disease.

Diagnosing diabetes is not very accurate because of some reasons. One reason is that symptoms take longer to show up in adults than kids, mainly if doctors do not specialize in the condition. Another reason is that people diagnosed with type 1 diabetes have a healthy and lean weight which rules out with type 2 diabetes with overweight (Adults Can Get Type 1 Diabetes, Too, reviewed by Carol DE Sarkissian, 2018).

1.2 Viral infection and autoimmunity

1.2.a Disease Induction

As mentioned above, Type 1 diabetes (T1D) is an autoimmune disease characterized by immune cell-mediated destruction of pancreatic β cells (Atkinson MA et al., 2001). There are various factors that influence T1D including genetic factors, environmental factors like toxicants and viruses, etc. The initial step of induction of autoimmunity can be a virus infection (Bortell et al., 2012). The different strains of viruses can initiate different adverse effects on beta cells (Roivainen M et al., 2006). Viruses can unmask beta cells for recognition from CD8+ T cells. CD8+ and T
lymphocytes can rapidly kill beta cells by secreting IFN gamma like interferons and upregulating (MHC) (Foulis AK et al., 1988).

It is known that viruses are mimicking islet antigens and activating autoreactive T cells (Wucherpfennig KW et al., 2001). There are many cases that are related to virus infection and autoimmunity. The increase rate of autoimmunity is observed in many diabetic cases and has been associated with enteroviruses (Stene LC et al., 2010). Enterovirus can initiate islet autoimmunity and progress to the glycemic stage (Coppieeters KT et al., 2010). Furthermore, CVB RNA has been detected during the onset of type 1 diabetic patients (Andreoletti L et al., 1997). CVB virus was isolated from a child with diabetic ketoacidosis (Juhela S et al., 2000). By inoculating the homogenate of a patient's pancreas to a mouse, monkey, and human cells helped to isolate B4 diabetogenic version of Coxsackievirus (Yoon JW et al., 1979). Virus infections activate strong immune responses. CVB4 infection reportedly induces natural killer cells (NK) within the islets. Since virus infection can induce Type 1 diabetes (T1D), we used LEW.1WR1 rats as our rat model to virus mimic PIC induced T1D rat model.

LEW rats are only naturally occurring type 1 diabetes rat models that closely resemble human type 1 diabetes in terms of histology, pathogenesis, lack of sex basis, and MHC class II association (Wurst W et al., 1988). Type 1 diabetes is both autoimmune and inducible, which is common in inbred rat strains, like humans who express high-risk MHC II haplotype this is designated RT1B/Du. Among susceptible rat strains, the most suitable one is LEW.1WR1 strains. About 2.5% of LEW.1WR1 rats develop TID spontaneously and generally, during early reproductive years, both sexes are affected in the same way, and islets show insulitis (John P et al., 2005). There are several types of LEW rat models that are used for T1D studies. The Lewis-Insulin dependent
diabetes mellitus (LEW-iddm) rats can survive after becoming diabetic and can be used to find diabetic completions (Matthews CE et al., 2005).

The LEW.1WR1 rats can develop spontaneous T1D diabetes at a low rate (2.5%). But, with Poly IC induction, LEW.1WR1 rats can readily develop T1D at a high rate (98%).

The rats have MHC II major histocompatibility, class II ‘μ’ haplotype, which generally needs to become autoimmune diabetes in rats. PBS (Phosphate-buffered saline) dissolved Poly IC (1µg/g body weight) was intraperitoneally injected to 21-25 days old rats in both sexes on day-3, -2, -1 and with KRV on day 0. Diabetes was diagnosed by plasma blood glucose level(>250mg/dL) (Mordes J et al., 2009).

The LEW.1WR1 rats develop Type 1 diabetes spontaneously. The rats are very susceptible to develop Type 1 diabetes with polyinosinic:polycytidylic acid (PIC) followed by the KRV virus injection. The T1D rats are strongly associated with class II MHC haplotype. The MHC locus is designated to RT1. The RT1-B/DU and RT1-B/DU are homologous to human HLA-DQ (Ellerman KE et al., 2000). It was found that locus (Iddm37) locus was responsible for the virus induced type 1 diabetes in LEW.1WR1 rats (Blankenhorn E et al., 2009.)

1.2.b UBD/FAT 10 gene

In the rat, the MHC locus is designated RT1 and the permissive class II haplotype is designated RT1-B/DU 24. The RT1-B locus is homologous to human HLA-DQ and RT1-D to HLA-DR. The mutations in UBD gene is associated with susceptibility of virus triggered autoimmune T1D in LEW.1WR1 rats. The SINE (short interspersed element) is resistant to virus induced T1D and LEW.1WR1 rat is missing a 59 nucleotides insertion near the promoter of the UBD gene. When
the UBD gene in LEW.1WR1 rats was deleted, the results showed that T1D incidences were significantly reduced. In addition, susceptibility locus iddm37 and iddm14 near to MHC class II determined the susceptibility to virus induced T1D (Cort L et al., 2014).

The ubiquitin family has grown over the years and basically there are two classes of ubiquitin. The first class is ubiquitin binding proteins (UBPs) and second class is protein compromises UBD like proteins which include LC3, SUM (Marshall S et al., 2006), ISG15 (Hochstrasser M et al., 2009). Ubiquitin can influence protein function without proteolysis. Ubiquitin can regulate insulin homeostasis, insulin signaling pathway and β cell response to calcium (Rome S et al., 2001). ULM (Ubiquitin-like molecules) can regulate GLUT4 (Glucose transporter 4) degradation and regulate response to stored GLUT4 components in adipocytes and muscles (Liu LB et al., 2007).

FAT 10 is a pleiotropic UBL protein which has a role to play in cellular localization and fate of proteins (Bedford L et al., 2011). UBD (Ubiquitin D) or FAT 10 has 76 amino residues and makes peptide bonds with free C-terminal and glycine with alpha amino group of lysine at target end.

The experiments were done with FAT10 ko mice and looked at age related metabolic changes of these rats. The Glucose tolerance test and insulin tolerance test were performed during the experiment period. Those FAT10 ko mice lived longer than the control group and observed 50% less white adipose mass compared to control WT (Wild Type) rats. Elevated levels of triglyceride hydrolysis in adipocytes was observed. The longevity was inversely related to adiposity including yeasts, flies, worms, fish, and rodents (Fontana L et al., 2007). This susceptibility of this model was related to FAT10 overexpression, a ubiquitin-like protein that plays an unclear role in the regulation of lipid metabolism (Blankenhorn EP et al., 2009). FAT10ko (FAT10 knockout) mice have a low blood glucose level and insulin level compared to WT mice (Canaan A et al., 2013). The researchers were interested about the effects of a high fat diet in FAT10ko mice and if a high
fat diet will result in low adiposity and low glucose and insulin concentration in their blood. They found out that mice with the absence of FAT10 are not completely immune to becoming obese while on a high fat diet. This is drawn from the fact that Fat10Ko mice on a high fat diet only had a 16% decreased total adiposity while the lean FAT10 mice had a 50% decreased total adiposity (Defuria J et al., 2011).

1.2 c. The rationale of the selection of the LEW.1WR1 rat for this thesis

The animal model for each selected for diabetes depends on what aspects of disease is being studied. Furthermore, animal models play a vital role in the understanding of diabetes pathogenesis and functional and genetic characterization (Arndt T et al., 2013). There are rat models that varied from chemical ablation of beta cells to breeding rats that spontaneously develop autoimmune diabetes. The animals used for type 1 diabetes are highly inbred (Phillips B et al., 2011). These models include models of insulin resistance or models of beta-cell failure. On the other hand, T2D models are obese, reflecting the human obesity of type 2 diabetes. Most of these models have abnormalities of one or more genes causing glucose intolerance, insulin resistance, obesity leading to a high blood glucose level. These malfunctions cause insulin resistance, hyperglycemia, hyperlipidemia (Calcutt NA et al., 2009).
1.3 Other rodent models used in T1D

![Diagram of animal models of diabetes mellitus]

1.3.a NOD mice

Although almost all NOD mice of both sexes develop insulitis, only some of them develop overt diabetes, suggesting that several factors modify the process from insulitis to beta-cell destruction and the development of overt diabetes. NOD mice spontaneously develop hyperglycemia which leads to autoimmune diabetes: infiltration of mononuclear cells into the pancreatic islet (insulitis) is observed. Insulitis is not seen before three weeks of age but appears spontaneously at around four weeks of age. The frequency of insulitis reaches 70%-90% by three weeks of age, and almost mononuclear cells infiltrating the islets are mostly T cells (CD4+ and CD8+), but B cells, dendritic cells, and macrophages are also observed. Despite massive infiltration of mononuclear cells into the islets, beta-cells remain intact until 12-15 weeks of age, when destruction of beta-cells becomes aggressive and overt diabetes develops. NOD females are known to have a higher burden of islet infiltrates than males of the same
age, causing earlier onset of diabetes, correlating with a previous onset of overt diabetes (Pozzilli P et al., 1993).

After the onset overt diabetes, marked polyuria and polydipsia develop, and mice lose weight and die within one to two months unless treated with a daily injection of insulin (Makino., et al. 1980), as in the case of human T1D. The main problem with NOD mice is sex bias when comes to disease development.

1.3.b LEW.1AR1 rat

In LEW is 1AR1-insulin-dependent diabetes mellitus (LEW.1AR1-iddm) rat, is a recessive model. The genome-wide genome analysis using a (BN6LEW.1AR1-iddm) × LEW.1AR1-iddm] N2 (N2 BN) helped to discover three T1DM susceptibility loci in this rat model. Insulin-dependent diabetes mellitus 8 (iddm8) was identified as a site of mutation for disease susceptibility recently caused by the LEW.1AR1 strain (Weiss H. et al., 2008).

1.3.c Sprague-Dawley Rat

There is age dependent toxicity associated with STZ injection that makes the model unreliable and sex bias when it comes to disease induction. But the high triglyceride and cholesterol levels in blood, kidney damage, and dyslipidemia, common morbidities observed in rat model and diabetic patients (Gangula P et al., 2007).

1.3.d BB rats

BB rats are derived from Canadian colony of outbred Wistar rats in which spontaneous hyperglycemia and ketoacidosis occurred in 1970 (Mordes J et al., 2001) Affected animals were the founders for two colonies later used to establish all other BB rat colonies. The colony in
Worcester, Massachusetts has been inbred, and the spontaneously diabetic are formally “BBDP/Wor.” A second colony remains in Ottawa, Canada; the “BBdp”. Rats from this colony are outbred.

The natural course of insulitis in the spontaneously diabetic BB rat is different from that of the NOD mouse; in the rat, there is little or no persistent infiltration adjacent to the islets (‘peri-insulitis’) before progression to frank insulitis and overt diabetes. Insulitis in BB rats is morphologically similar to that observed in human TID and features a predominance of Th1-type lymphocytes (Hurbert K et al., 1996). After the onset of hyperglycemia, residual end-stage islets with few or no inflammatory cells are observed. These are small and comprise predominantly non-beta cells. Islet alpha, delta, and pancreatic polypeptide (PP) cell numbers and morphology appear to be preserved. There is no published data on islets ghrelin cells in the rat. Unless treated with insulin, hyperglycemic BB rats quickly develop fatal diabetic ketoacidosis. The working hypothesis of autoimmune diabetes was initially derived from studies in the BB/Wor rat (Mordes J et al., 1996). As depicted this hypothesis holds that diabetes in the rat results from an imbalance between beta cell-cytotoxic effector cells and regulatory cells that generally prevent disease. The hypothesis predicts the existence of at least two and perhaps three defects in all susceptible rat strains. The first defect is genetic, involves the class II\(\mu\) allele of MHC, and leads to the generation of autoreactive effector cells, not only in the BB rat but also in several other rat strains like LEW.1WR1. The second defect leads to amplification of the autoreactive population or to regulatory cells that generally prevent disease. The hypothesis predicts the existence of at least two and perhaps three defects in all susceptible rat strains. The defect can be genetic or acquired. In diabetes-prone BB rats, this defect is congenital lymphopenia, which can be overcome by the transfusion of CD4+ ART2+ Treg cells. In the case of KDP (Komeda diabetes-prone) BB which
is a sub strain of BB rat (Haruhiko Y et al.,1997), a loss of function mutation in the cblb gene appears to predispose to abnormal T-cell activation (Haruhiko Y et al., 2002). The KDP rat combines an MHC -dependent genetic predisposition with a “disequilibrating” genetic defect leading to autoreactive T cell activation.

The LEW.1AR1 (RT1-Aa, RT1-B/Du, and RT1-Cu) and LEW.1WR1 (RT1-Au, RT1-B/Du, and RT1-Ca) strains express also the MHC-II RT1-B/Du haplotype, and, conversely, the original LEW (RT1-AI, RT1-B/DI, and RT1-CI) strain cannot develop diabetes because of the missing RT1-B/Du haplotype (Al-Awar A et al.,2016).

The rat model, LEW.1WR1 (LEW) rat which has heritable T1D susceptibility genes. This model has a reproducible window of induction that can be studied in vivo. Various perturbations of the immune system can efficiently induce type 1 diabetes up to 100% of animals. Perturbants include regulated T-cell (Treg) depletion, innate immune activation with polyinosinic:polycytidylic acid (poly I:C), infection with Kilham rat virus(KRV) or rat cytomegalovirus. LEW.1WR1 rats develop diabetes at an increased rate (18%) after coxsackie B serotype 4(CVB4) infections, but only if pretreated with a low dose of poly IC daily for three days.

1.3.e LEW/SsNHsd rats

LEW/SsNHsd (LEWIS) have LEWIS.RT1.L-L haplotype while LEW.1WR1 rats have LEW.RT1.L-U Haplotype (Cort L et al., 2014). The LEW.1WR1 UBD promoter allele leads to higher inducible levels of T1D. UBD-deficient rats show substantially reduced diabetes after viral infection. LEWIS rats have low UBD mRNA expression and LEW.1WR1 rats have high UBD mRNA expression (Cort L et al., 2014). The Lewis rat is sensitive to the development of several autoimmune diseases, including adjuvant-induced arthritis (Brad B et al., 2011).
Overall, LEW.1WR1 rats have susceptibility genes: UBD, RT1-N1, susceptibility loci: Iddm37, Idm14. They have a low rate of spontaneous diabetes, not lymphopenic, both sexes are sensitive to polyinosinic-polycytidylic acid (Poly IC) and/or virus induced T1D. So, we decided to use LEW.1WR1 rats as our T1D rat model.

1.4 Metabolism in T1D

1.4. a Glucose and insulin homeostasis under normal conditions

There are two major types of diabetes, type 1 and type 2. People with type 1 diabetes cannot produce insulin. People with type 2 diabetes can produce insulin, but they have reduced responses to insulin. This thesis will focus on insulin regulation in type 1 diabetes.

Beta cells have a remarkable ability to store and secrete insulin with correct timing. Glucose is the primary driver of insulin release. To sense the nutritional state, beta cells are clustered together in islets (Schmitz O et al., 2008). Islets are connected through small blood vessels and receive ten times more blood when compared to the exocrine regions near the islets. Normal blood glucose levels range between 70-140 mg/dL. The glucose transporter 2 (GLUT2) in rodents β cells maintains blood glucose level, but notably GLUT1 in human β cells facilitates glucose entry only. The insulin secretion is regulated by K⁺ ATP dependent and K⁺ ATP independent pathways in β cells (Henquin J et al., 2011).

Oral ingestion of 75g of glucose can increase plasma insulin level by 20-30 pmol/L to 250-300 pmol/L within 30 minutes while the intake of the same amount of fat and protein can only increase insulin level up to 50-60 pmol/L in a human body (Chang TW et al., 1978). β-cells constitutively express GLUT2 which facilitates glucose diffusion. When glucose enters β cells, glucose is
phosphorylated using glucokinase, which is a hexokinase. Glucokinase can be found in mammalian cells, hepatic cells, beta cells, enterocytes and neurons (Suckale et al.,2008). Glucokinase has a lower affinity to glucose compared to other hexokinases. Glucokinase is inhibited by its product, glucose 6-phosphate. If glucose-6-phosphate accumulates in the cell, there is a feedback inhibition until cells consume all the glucose-6-phosphotases. Therefore, glucokinase is considered as a rate-limiting factor in β-cell glucose metabolism (Jakob S et al.,2008). The end point of glycolysis, pyruvate is converted to Acetyl-CoA and oxidized in the TCA cycle in the mitochondria of beta cells to produce ATP. Glucose catabolism (glycolysis, TCA cycle, and oxidative phosphorylation) are coupled with the K-ATP channel to regulate "ATP sensitive potassium channel-dependent insulin release." Increases in the ATP/ADP ratio closes the K-ATP channel and depolarizes the plasma membrane, causing the opening of voltage-dependent Ca++ channels. The influx of Ca++ activates exocytosis of insulin-containing granules.

Insulin is initially in the form of proinsulin and stored in secretory granules. Proinsulin is converted to insulin by removing C-peptides by signal peptidases. Insulin gets released on-demand in a pulsatile flow. The synthesis of insulin is regulated at the transcriptional and translational level.

Pancreatic beta cells dysfunction plays a crucial role in type 1 diabetes as insulin secretion from beta cells is a critical regulator in glucose metabolism in other tissues. The beta cells respond to hormones like Gastrointestinal Insulinotropic Peptide (GIP), glucagon-like peptide (GLP-1) (Baggio, L et al.,2007). Insulin directly suppresses the secretion of Glucagon as do Amylin which co secreted with Insulin. But, GLP-1 inhibits the glucagon release from alpha cells (David S.et al.,2014). When fasting blood glucose levels rise modestly higher, it can lead to severe dysregulation of insulin secretion.
1.4.b Stages of beta cell destruction

The progression of diabetes can be viewed as 5 definitive stages according to Gordon C et al., 2004. These stages are characterized by beta cell mass, function, and phenotype. Stage 1 is the β cell compensation stage. β cells secrete more insulin to increase blood insulin levels and maintain normal blood glucose levels. The β cells are also infiltrated by macrophages and T cells. Stage 1 is characterized by hypersensitive glucose dependent insulin secretion and normal β cell mass. At this stage, β cells are extraordinarily efficient at storing and secretion of insulin to maintain normoglycemia. The stage 2 - stable adaptation stage starts with high blood glucose levels. Glucose dependent insulin secretion is decreased. β cell mass is decreasing. The beta cells can no longer compensate so normal blood glucose level cannot be maintained. β cells are dedifferentiating at this stage which is the loss of phenotypic beta cell response to increased blood glucose level. The fasting blood glucose level is varying from 89-130mg/dL (5.5-6 mmol/l). The stage 2 cannot no longer be considered as a compensated stage, because normal blood glucose level cannot be truly maintained. Stage 3 is a transient stage hallmarked by rapidly rising blood glucose level. The fasting blood glucose level rises from 130mg/dL-350mg/dL. Stage 4 is characterized by β cell decompensation and severe β cell dedifferentiation. Blood glucose levels are typically above 350 mg/dL with severe insulitis. The rapid destruction of beta cells in type 1 diabetic patients can lead to stage 5. The final stage, stage 5 is characterized by severe decompensation, severe beta cell destruction and ketosis.

Plasma insulin levels are clearly a function of β cells health. In the early stages of type 1 diabetes development, deficiency of β cell mass or function lead to changes in plasma insulin level. The
deficiency of insulin in blood results in hyperglycemia and eventual diabetic ketoacidosis.

The role of \( \beta \) cells in humans during diabetic induction is not very clear. Scientific advances have been restricted due to reduced access to human samples. Procedures that remove pancreas sections are extremely harmful to patients as the pancreas controls several endocrine and exocrine functions. The total output of insulin is totally dependent on the number of \( \beta \) cells (beta cell mass) and the ability of each \( \beta \) cell to release insulin (function). Most information about \( \beta \) cell mass and function prior to stage 3 is currently from Non-Obese Diabetic (NOD) mice. But the problem is, \( \beta \) cell mass loss of NOD mice models used in research experiments are vastly different from humans. That is a one of the reasons to use the LEW.1WR1 rat model, showing similarities in disease induction and progression to humans (discussed further in next section). There is limited information about beta cell function and mass in prediabetic human patients.

There are other methods like measuring C-peptide, counting unmethylated INS DNA, glucose tolerance test and first phase insulin response at an intravenous glucose stimulation test. Results from these tests contribute to current knowledge about \( \beta \) cell function during disease onset in humans. There is no glucose fluctuation until immediately before onset of diabetes due to the body’s tight regulation of blood glucose levels (Srikanta S et al., 1984). But researchers have observed losing first phase insulin release at 4-6 years before clinical onset of type 1 diabetes (Keskinen P et al., 2002). The \( \beta \) cell mass was not changed during the time, however, the functional impairment of beta cells during early on set is possible. When closer to hyperglycemia, decreased first phase insulin secretion and increased C-peptide levels were observed (Sosenko J et al., 2006). This might be due to dysfunction of \( \beta \) cells or death of \( \beta \)cells at onset of diabetes. Altogether, dysfunction of \( \beta \) cells and losing \( \beta \) cells at a slow rate at the beginning will lead to rapid destruction and functional abortion of \( \beta \) cells at later stages of progressing T1D, due to the activation of stress
responses genes, proapoptotic genes, and NO like compounds (Chen C et al., 2017). In addition to compromising β cells function and survival, cytokines can recruit macrophages and T cells into the islets, thus augmenting inflammation.

Viral infection is one of the causes of type 1 diabetes and it leads to β cell destruction. The islet resident dendritic cells can interact with a β antigen and bind with receptors in dendritic cells. These dendritic cells are caused to differentiate T-Helper cells (Th1) and activate B cells. The helper cells, also called as CD4 + cells can activate other immune cells by releasing cytokines. Th0 can secrete IL-2, IL-4 and interferon gamma (IFN-γ).

Those B cells can produce autoantibodies against β cells. Th1 can activate neutrophil, macrophages, and start inflammatory reactions. Th1 can activate ROS dependent β cell destruction by activating macrophages and neutrophil cells. Inflammatory cytokines like IL1B, TNFα, IFN can activate NFκβ and STAT-1, which eventually decrease PDX1 and GLUT1 expression, leading to reduced insulin production and secretion. The NFκβ induces endoplasmic reticulum stress and β cell destruction. So, releasing cytokines from β cells can induce apoptosis (Maria-Luisa et al., 2011).

Researchers observed that cytokines have significant impacts on beta cell populations due to direct cell to cell contact which can increase β cell damage in prediabetic stage. (Skowera A et al., 2008), (Brozzi F et al., 2015). Researchers have found that activation of NF-kB is a crucial step and causes NO production, which leads to β cell destruction. IL-1β, IFN-γ, and TNF-α are cumulatively enhancing the NO production by overexpressing NO synthase (Thomas HE et al., 2002). Transgenic mice that are overexpressing the NO synthase (iNOS) developed type 1 diabetes in their lifetime (Takamura T et al., 1998). And knockout iNOS mice did not develop type1 diabetes,
and \( \beta \) cells were protected (Flodstrom M et al., 1999). The INOS overexpressed cells can produce a large amount of NO, which can downregulate the transcription factors like PDX-1 and Isl-1, eventually leading to regulate \( \beta \)-cell dedifferentiation. The cytokine-dependent NF-\( \kappa \)B can upregulate the production of monocyte chemoattractant protein-1 (MCP-1) (Chen MC et al., 2001). The downregulated SERCA-2b (sarco endoplasmic reticulum Ca\( ^2+ \) ATPase type 2b) causes severe stress to the endoplasmic reticulum, leading to apoptosis of \( \beta \) cells (Cardozo AK et al., 2001). A member of MAPK (mitogen-activated protein kinase, c-Jun NH2-terminal kinase (JNK)) is activated by IL-1\( \beta \) causing beta-cell apoptosis (Ferdaoussi M et al., 2008). IFN-\( \gamma \) also cumulatively participates with IL-1\( \beta \) in this process (Eizirik DL et al., 2001).

The classically characterized T1D is the presence of antibodies and T-cells, which are reacting with islet antigens at new-onset adult (\( >16 \) years old) type 1 diabetic patients (Roberto M et al., 2007). Giving drugs that can suppress immune activity can slow down the \( \beta \)-cell apoptosis process, and histology analysis of pancreases shows that immunological activity in islets. T lymphocytes are considered as a primary mediator for T1D. Notably, the drugs against T lymphocytes have delayed the T1D progression (Roep BO et al., 2003). CD4+ and CD8+ are two types of T lymphocytes that are involved in T1D and the role of these two types of cells is controversial. It is suggested that CD4+ T cells contribute to proper homing and CD+8 T cells as effector cells (Thivolet C et al., 1991). CD4+ T lymphocytes are considered as T helper cells (Th0), and after encountering antibodies, they secrete IL-2, INF-\( \gamma \), and TNF-\( \alpha \) and Th2 cells secrete IL-4, IL-5, IL-10 and IL-13. Th1 cells can destroy beta cells by secreting INF-\( \gamma \). Th2 cells are also causing \( \beta \)-cell apoptosis through IL-10 (Heitmeier MR et al., 1997). Activated T cells and macrophages release several inflammatory cytokines like interferon-\( \gamma \) (IFN-\( \gamma \)), interleukin-1\( \beta \) (IL-1\( \beta \)) and tumor necrosis factor-\( \alpha \) (TNF-\( \alpha \)), which cause \( \beta \)-cell destruction (Thomas H et al., 2002). Pro-
inflammatory cytokines like interleukin-1beta (IL-1β) and gamma-interferon (γ-IFN) can activate signaling pathways, causing dysfunction of β-cells (Jason C et al., 2011). In vitro culture showed that cumulative effects of IL-1β or IL-1β+IFN-γ could cause β-cell destruction similar to what they observed in prediabetic patients (Hostens K et al., 1999).

Leptin has an anti-apoptotic effect on β cells that eventually reduce triglyceride accumulation in the liver. Leptin level typically increases with inflammation and infection. Moreover, exposure to external or internal stimuli like IL-1, TNF-α and LPS increases the circulating of leptin and leptin expression in adipose tissue. (Noriko I et al 2008). According to Morika et al., 2007, mice with a disrupted leptin receptor had severe glucose intolerance and low insulin secretion from beta cells. All these findings suggest that leptin has a protective effect on β cells. Resistin is usually expressed in mice and macrophages in humans (Tomaru T et al., 2009). An increased resistin level is associated with insulin resistance in humans and rodents. (Brown E et al., 2007).

1.4.c How beta cells impact metabolic tissues and hormones

Insulin is produced and secreted in pancreatic β cells, which are responsible for glucose influx. By suppressing the glucose production in the liver and stimulating glucose uptake in muscle and fat, insulin reduces blood glucose level. Skeletal muscle accounts for 60-70% of whole-body insulin stimulated glucose uptake. Insulin regulates muscle metabolism by promoting glucose uptake, glycogen synthesis and lipid utilization and storage. Insulin stimulates glucose uptake in skeletal muscle by translocating GLUT4 (Jingjing Z.et al., 2014). Insulin action in the liver leads to reduction of glycogen breakdown, glucose synthesis, increased glycogen production and enhanced lipid and glycogen storage. GLUT2 is expressed in the liver, in renal and intestinal absorptive cells unlike GLUT4, which primarily expresses in muscle and fat cells.
When there is an increase in blood glucose, pancreatic beta cells release insulin. Insulin binds with the insulin receptors and activates the tyrosine kinase domain initiating a cascade of signals. The cell signaling molecules like IRS, P13K, PDK1, AKT and AS160 are activated and eventually lead to cell survival and proliferation. The GLUT4 translocation is initiated by activating IRS/ - phosphatidylinositol (PI)-3 pathway. GLUT4 translocates from cytoplasm to cell membrane. Once GLUT4 transporters are open, cells can uptake glucose. Glucose will be used in several ways in cells. Glucose can make ATP and pyruvate through glycolysis. Pyruvate can convert to lipids through lipogenesis. Glucose can store as glycogen through glycogenesis. If cells cannot uptake glucose, all these processes will be affected. Without insulin, glucose cannot be up taken by cells and cells are starved from energy (Jennifer J et al., 2001)

1.4.c.1 Muscles

The lack of insulin in T1D causes poor protein, lipid and glucose metabolism. So, this may eventually cause both acute and chronic biochemical and anatomical problems in skeletal muscles. The muscle cramping is one of the common symptoms with Diabetes Mellitus. It can result from electrolyte imbalance, hypoglycemia, or neuropathies. Muscle fraction is rare and only seen in poorly controlled diabetic patients. Other than that, researchers observed contractile weaknesses, decreased oxidative activity, reduced muscle mass, muscle mitochondrial dysfunction and insulin resistance in T1D patients. With T1D, β cells destroy and reduce insulin signaling. Therefore, reduced amounts of insulin receptors decrease the activity of P13K. This may eventually cause impaired GLUT4 translocations in skeletal muscles. Animals and human models of T1D show changes in downstream signaling cascades, including protein metabolism, fatty acid utilization, cellular stress responses, glycolysis and nucleotide metabolism in skeletal muscles (Ohlendieck K et al., 2012).
1.4.c.2 Liver

The liver plays an important role in glucose metabolism because it stores glycogen and produces glucose through glycogenesis and gluconeogenesis. There are several hormones and cytokines that act on glucose homeostasis in the liver. Thirty to sixty percent of glucose uptake by the digestive tract, undergoes hepatic metabolism through storing glycogen and converting them into amino acids or fatty acids. Normal blood glucose levels are maintained by insulin which simulates glucose uptake by adipose tissues and skeletal muscles and inhibits hepatic release of glucose. When beta cells are not properly functioning, glucose uptake is reduced by adipose tissues and skeletal muscles and decreased inhibition of hepatic glucose output (Chi Zhang et al., 2012).

According to Bernarf T et al., 1990, the expression of liver/beta glucose transporter in hyperglycemic rats have reduced but in the liver, expression of same transporter is minimally changed over the experiment period. Not only that, hyperglycemia causes the liver to dysfunction and hepatic cell death via oxidative stress in T1D through Bcl2 proteins that act on mitochondria. Signals from mitochondria cause mitochondria dependent cell death in liver (Prasenjit M. et al., 2010).

1.5. What is known about the metabolic phenotype of the LEW.1WR1 rat

There are very few studies that have been done related to metabolic changes of 1WR1 rats. The 1WR1 rats have genetic predisposition to autoimmune destruction of beta cells and collagen-induced arthritis with high incidence in both genders. LEW.1WR1 rats also develop more severe and more human-like complications of diabetes when compared to other models such as NOD mice and Sprague Dawley like induced diabetes models.

These rat models can show insulitis even without Poly IC induction. According to John P. study, among the non-diabetic pancreases, 24 showed no pathology, but one male rat with +1 insulitis
and one female with +3 insulitis (low insulitis is +1 and high insulitis is +4). To quantify insulitis, pancreases were harvested and fixed in 10% formalin and scored by light microscopy on a scale of 0 to 4. But only 2% of 72 total rats developed diabetes spontaneously.

The diabetic symptoms of 1WR1 rats are weight loss, increased water consumption, urinary ketones and glycosuria. When animals were treated with insulin, they showed rapid improvement, such as, low blood glucose level, resolution of ketonuria and weight gain. The histology analysis of diabetic 1WR1 rats showed distorted small size islets and infiltration of lymphocyte (Mordes J et al., 2005). LEW.1WR1 rats are not lymphopenic. There was no evidence showing severe loss of CD4+ (T helper cells), CD8+ (Cytotoxic killer cells), or ART2+ cells, which is a characteristic of autoimmune diabetes in BBDR rats. They have normal immunophenotypes, but in barrier housing, only a small percentage produce ketosis prone diabetes mellitus on a consistent basis. Immunochemistry has confirmed that insulin containing cells are largely missing in both acute and chronic diabetic rats. These rats are susceptible to collagen induced arthritis. Rheumatoid arthritis is common in type 1 diabetic families (Mordes J et al., 2010). Elevated TCR-Cβ transcripts levels were observed 7-9 days after poly IC induction and a similar pattern was observed with insulin transcripts at the same time. Dr. Love-Rutledge has seen high blood insulin levels in LEW.1WR1 rats (Unpublished data).
LEW.1WR1 rats are carrying RT1u haplotype as BB rats (even though BB rats are lymphopenic) which makes them susceptible to develop T1D. Increased insulin in control rats suggests that this may not be the case, but that there is some other underlying metabolic aberration occurring in 1WR1 rats. When these animals are backcrossed with parental resistant strains with a different haplotype, the susceptibility is reduced to 25% (Cort L et al., 2014). RT1l is a diabetes resistant haplotype and RT1u is a diabetes susceptible haplotype. In a study of Wistar rats bred to have the RT1a and RT1u haplotype, Wistar Ottawa Karlsburg W (WOKW) rats developed impaired glucose tolerance and were hyper insulinemic but did not develop diabetes. So, the WOKW rat model is characterized with insulin resistance, hyperinsulinemia, obesity, impaired glucose tolerance, dyslipidemia, and hypertension. This rat group showed metabolic syndrome. WOKW rats are significantly heavier than control rat groups and both sexes showed similar tendencies toward body weights and BMI. Insulin level is significantly higher at the age of 10 weeks and serum triglyceride levels are significantly high in all age groups. Furthermore, serum cholesterol and LDL levels are low in both sexes of WOKW rats compared to controlled rat groups. These rats have a significantly higher adipose index number which determines obesity status. This shared haplotype suggests that there is an underlying metabolic mechanism that plays a role in developing type 1 diabetes in the 1WR1 rat. It is sometimes difficult to distinguish between T2D, T1D and metabolic syndrome susceptibility which depend on the different yet sometimes overlapping genetic backgrounds with a specific mixture of metabolic predispositions (Jens van den Brandt et
It has not been proven that the WOKW rat overexpresses UBD, an identified susceptibility gene found within the RT1u haplotype in the 1WR1 rat. However, this gene has been shown to have an impact on the metabolism of rodents.

1.5.a Lipid metabolism

UBD promotes adiposity, insulin resistance, and inflammation, and reduces fat oxidation, uncoupling, and AMPK activation in skeletal muscle. UBD deletion can expand lifespan of mice through increased phosphorylation of lipid droplets and reduced adipose mass, beta oxidation and mitochondrial respiration. When UBD was knocked out in mice, they had enhanced fatty acid oxidation, upregulation of lipid metabolism genes, and appeared leaner (Allon et al., 2012). Insulin inhibits breakdown of fat in adipose tissue by inhibiting the intracellular lipase that hydrolyzes triglycerides to release fatty acids. Decreased adiposity with UBD knockout reflects the contribution of over expressed UBD gene in lipid synthesis and lipolysis in diabetic induction.

The gene encoding Lipe (HSL) and PNPLA2 (ATGL) are major lipase genes in adipocytes, upregulated in UBD/FAT 10 knockout mice. And also, UBD knockout mice have significantly higher expression of fatty acid breakdown related genes (ACOX, ACOT1, ACOT3, PPARα). Lipolysis is associated with β-oxidation and recycling of triglycerides. So, genes indirectly related with β oxidation and triglyceride synthesis are upregulated. This data suggests increasing lipolysis in UBD knockout genes. So, we expect to observe UBD over expressed LEW.1WR1 rats that have altered lipid metabolism compared to our controlled rat group.

The LEW.1WR1 rats become diabetic 12 days after Poly IC injection. They start losing the ability to maintain their blood glucose level. Poly IC treated rats have increased membrane lipids and ceramides in rat islets due to ER stress (Nandan K et al., 2018).
1.5.b. Glucose metabolism

Insulin promotes glucose uptake and fatty acids uptake in adipocytes. Insulin tolerance tests were performed after 6 hours of starving in UBD knockout mice. The study has shown KO mice on normal diet have changes in body composition, energy usage and tissue metabolism. Those rats have reduced circulating insulin and glucose in metabolic tissues (Allon C et al., 2014). LEW.1WR1 rats have a high blood glucose level and reduced insulin sensitivity with T1D. Wistar Ottawa Karlsburg W rat strain shows hyperinsulinemia, obesity, insulin resistance and glucose tolerance (Amaya A. et al., 2009). Karlsburg W rats were at age 4, 6, 8, 10, 12, and 14 weeks and LEW.1WR1 rats we used for the study were 18 days old. So, LEW.1WR1 rats are younger in age and may have changes indicative of the early stages of WOKW metabolic dysfunction.

It is crucial to know exactly the typical features of the metabolic abnormalities in order to differentiate specific categories of metabolic phenotypes associated with T1D. These data suggest that phenotypic differences we observed with T1D induction are not completely driven by the immune response.

According to this data, there should be an underlying mechanism to develop T1D diabetes in 1WR1 rats other than directed by immune responses. Although the 1WR1 rat model that is commonly used in T1D studies is virus induced LEW.1WR1 rats, they can develop T1D spontaneously and the underlying metabolic mechanism has a role to play which was not clearly addressed before. We hypothesize that overexpressed UBD gene may alter the lipid, glucose and insulin mechanism which increases the susceptibility to spontaneously T1D. Identifying the
profound metabolic changes that occur in this population of people with T1D susceptibility can be used as potent biomarkers in future for early diagnoses.
Chapter 2
Methodology

Chemicals

10mL of endotoxin-free physiological water was added to 10mg of Polynosinic:polycytidylic acid (poly(I:C)) (HMW) Vacci grade InvoGen and made 1mg/mL Poly(I:C) Invivogen (San Diego, CA). In Vaccine Grade vial. Mixed the solution by pipetting up and down. The mixture was heated for 10 minutes at 65 - 70°C. Allowed to cool for 1 hour at room temperature to ensure proper annealing. All steps should be done under a biological safety cabinet to maintain sterility. Poly IC at a concentration of 1mg/ml stored at -20 (add units) until use. 10 mL of (PBS (10X), pH 7.4 (Gibco™)) were mixed with 9mL with Distilled water to make 1X PBS. 1x Phosphate Buffer Saline (PBS) from Thermo Fisher Scientific (Waltham, MA) was used for control rats and Poly IC dilutions prior to injecting treated rats.

Animals

LEW/SsNHsd (SsNHsd) and LEW.1WR1 (1WR1) (Envigo (Indianapolis, IN) and Biomere (Worcester, MA) rats were nearly 21-24 days old when we obtained them. At the beginning of study rats were 28 days old. Rats kept in UAH Vivarium were maintained with a 12-hour light-dark cycle, and protocols approved by UAH IACUC. Four rats kept in one cage, and distilled water and (invigo++ 18% protein rodent diet) supplied ad libitum. Rats of each strain were randomly separated into two groups, control and treated, 1WR1-Control, 1WR1-treated, SsNHsd -Control, and SsNHsd -Treated.

Poly IC injection

The body masses were measured before injection during the induction period. 1μg/g body mass Poly IC was injected to treated rats and with the corresponding volumes of PBS injected into
control rats, instead. Injections in the treated and control rats occurred every other day for six days. The rats were 4 weeks old at the time of harvesting.

Terminal blood glucose level measurement

The terminal fasting blood glucose level was measured after 6 and a half hours of fasting in rat metabolic cages. The terminal fasting blood glucose level was measured using Alpha Tracks 2 meter by Zeotis (Parsippany.NJ). Rats were anesthetized using isoflurane; the tail wiped with ethanol, and the dorsal vein was pricked using a 25G needle. We discarded the first blood drop.

Figure 3. Islet Size Comparison. This figure shows the representative images of insulin-stained islets at sizes extra-small (<10 mm), small (10-15 mm), medium (16-20 mm), and large (>20 mm). Magnification was at 2 x 500 μ.

Histology

Rats were anesthetized and exsanguinated by cardiac puncture. The pancreas was carefully dissected out without disrupting the integrity of the organ — the organs were placed inside a cassette. The cassettes were put into a beaker of 10% neutral buffered formalin (Fisher Scientific) to fix for approximately 24 hours at room temperature. After fixing, the organ was placed in 70% Ethanol (Thermo Scientific™) inside and mailed to Histowiz (Brooklyn, New York) for paraffin embedding, sectioning, staining, and digitizing of slides. The volume of fixative must be 20x over the sample volume, and the tissue must be immersed in the solution. The samples were sectioned (4 μm) and stained against CD68 (Monocytes and Macrophages, Dendritic cells), CD3 (T cells),
and insulin (beta cells). Insulin staining was done to determine the relative size of each islet. The digitized slides were scored based on appearance by Blinded scorers. Undergraduate scorers were recruited to score total islets on each slide and separated them according to size differences, Extra small, small, Medium, Large islets. The level of intensity of the CD3+ and CD68+ also scored in 10 representative islets in each slide. Islets scored on a scale from 1-4, and 1 indicates low infiltration, and 4 indicates the highest infiltration. The darker CD3+ stain indicates a higher number of T cells. The size chart used to score the size of each islet is depicted in Figure 4.

Quantitative Polymerase Chain Reaction

Approximately 0.15g piece of liver was placed into a 1.5mL centrifuge tube and weighed. Immediately add 1 mL of TriZOL was added to the tube, flash frozen in liquid nitrogen and stored at -80°C until processed. On the day of processing, liver sample is transferred onto a piece of folded aluminum foil, flash-frozen in liquid nitrogen, crushed with a pestle and transferred into (V-Bottom ScrewTop Tube 500 µL matrix tube (Thermo Fisher Scientific). 1mL of TriZOL was transferred into matrix tubes. The samples were homogenized with Super FastPrep-2 (Thermo Fisher Scientific) at 6 settings for 40 seconds then immediately placed on ice for 5 minutes. Supernatant was removed and placed into the original centrifuge tube. We then added 0.2mL of chloroform to the tube vortex for 15 seconds and then incubated for 2 minutes at room temperature. The tube was centrifuged for 15 minutes at 12,100 x G and 4°C Upon completion, the upper phase transferred to a new 1.5mL tube 0.5 mL of isopropanol added to precipitate the mRNA. This solution was vortexed at speed 8 for 5 seconds and then incubated at room temperature for 10 minutes. The centrifuge tube was then spun at 12,100 x G at 4°C for 10 min to pellet the RNA. The liquid was carefully removed, leaving behind only the white pellet. The pellet was washed
with 75% ethanol, vortexed, and centrifuged to remove the supernatant. The pellet was air dried using a Savant Vac with low temperature for 5 minutes to remove the traces of ethanol. The dried sample was dissolved with 25 microliters of Nuclease free water and placed in a 55°C heat block to rehydrate for 10 minutes. The sample was cooled immediately by placing on ice for 15 secs. Then the quantity and quality of RNA was measured using Thermo Scientific™ NanoDrop™ One Microvolume UV-Vis Spectrophotometer. The ratio of absorbance at 260 nm and 280 nm was used to assess the purity of DNA and RNA. The ratio of ~2.0 generally accepted as “pure” for RNA. If the ratio is appreciably lower in either case, it may indicate the presence of protein, phenol, or other contaminants that absorb strongly at or near 280 nm. We stored the samples at -80°C until cDNA conversion.

cDNA amplification

The 2X reverse Transcriptase Master Mix was Prepared from the High Capacity cDNA Reverse Transcriptase Kit from Applied Biosystems following the manufacturer’s instructions.

Preparing the cDNA RT reactions: Nucleic Acid (ng/uL) of each sample was known. Since it’s too concentrated (above 5000), samples diluted by a 1:100 ratio using nuclease-free water. The value you obtained was divided by 500 and found the volume of RNA needed for 50µl reaction volume. The rest of it is nuclease-free water.

Each cap was Labeled of a MicroAmp 8-Tube strip 0.1mL with the sample number. Pipetted the calculated volume of nuclease-free water and mRNA into the designated well. Next pipetted the correct amount of 2XRTmaster mix into each tube close it, and the final volume of each tube should be 50 microliters.

Performed reverse transcription in SimpliAmp Thermal Cycler:
Program conditions for each step as follows:

a. Step 1: 25 C for 10 minutes

b. Step 2: 37 C for 120 minutes

c. Step 3: 85 C for 5 minutes

d. Step 4: 4 C for infinity

Place the tubes in a thermal cycler securely and start the run. Once the run finished samples were stored at 4 C.

Preparation of Primers

The NCBI Primer Blast tool was used and selected the gene of interest from the drop-down list. Typed the gene wanted to make primer and searched for that for Rattus norvegicus". Then from all the hits mRNA sequence for the selected gene should be selected. cDNA was selected without introns. To make PCR primers, we need the whole sequence. The NCBI Primer Blast was selected. The mRNA sequence or accession number of gene interest was copied to the primer window. So you can change the size of primers, Melting temperature. Minimum and Maximum temperature should be selected 2 degrees around optimum temperature. The organism should always be “Rattus norvegicus”. Then clicked the primer blast button. The software runs for a few minutes and shows a graphical presentation of primers and where it binds to genes. Scrolled down and each primer pair shown with the length of primer, melting temperature, GC% content. The primers were selected by specificity and checked whether they can bind to another site of gene. Then the selected primer sequences were sent to Invitrogen to make DNA oligos, primers for PCR. The first primer set was selected and sent to Invitrogen to create primers. The primers were centrifuged to ensure
all the DNA at the bottom of the tube before opening. In the tube, there is nmol amount primer shown. For example, if a forward primer has a 320nmol amount and added 320 µL of RNAse water. Water was added to make a 100 µM solution. 10 µL aliquot was taken and added to 90 µL of nuclease-free water to prepare a 10µM solution for qPCR analysis.

The primer pairs we used for analysis were followed.

Table 1. Table of Primer

<table>
<thead>
<tr>
<th>Gene Name</th>
<th>Forward 5’-3’</th>
<th>Reverse 5’-3’</th>
</tr>
</thead>
<tbody>
<tr>
<td>RPL32</td>
<td>AAACTGGCGGAAACCAGAG</td>
<td>GCAATCTCAGCACAGTAAGATT</td>
</tr>
<tr>
<td>ATGL</td>
<td>CGGCATTTTCAGACAACCTGCCACT</td>
<td>GCAGGTTGAATTGGATGCTGGTGT</td>
</tr>
<tr>
<td>SCD-1</td>
<td>ACATTCAATCTCGGGAGAACA</td>
<td>CCATGCAGTCGATGAAGAAC</td>
</tr>
<tr>
<td>DGAT-2</td>
<td>CGCCCTCTTCATCTGGTTTAT</td>
<td>GCTCACAGCCTCTCTGATTT</td>
</tr>
<tr>
<td>DGAT-1</td>
<td>GTAAGTGTTGAGTGGTGTATG</td>
<td>GGCTTCATGGAGTTCTGGATAG</td>
</tr>
<tr>
<td>GLUT-2</td>
<td>GGTGAGTGGGTTGCTCATAAA</td>
<td>CACCAGTCACCTTCTGGGATAA</td>
</tr>
<tr>
<td>SREBP-1c</td>
<td>GAGACTGGAGACAGGGTAAATG</td>
<td>GCCTCAATCTGACCTCCTTATC</td>
</tr>
<tr>
<td>ChREBP</td>
<td>GGGACAGCCATCAGCTATTT</td>
<td>GGTTCACAAGCTCCAGTATTT</td>
</tr>
<tr>
<td>P53</td>
<td>CCCTCTTACCCTACCTCCTATCT</td>
<td>GGAGCTGAAGCTCTGGTTATAG</td>
</tr>
<tr>
<td>UBD</td>
<td>CTTTCTCACTCGGCTCTTG</td>
<td>GAGACCTGTGGTTGGGACCT</td>
</tr>
<tr>
<td>ACSL-1</td>
<td>CAAGGGTGCTTCAGCCTACCA</td>
<td>ATCCAACAGCCATCGCCTCA</td>
</tr>
<tr>
<td>PCK-1</td>
<td>GTCCCCCTTGTTACGGAAGC</td>
<td>GGCCTCTGATCTCATGGCAG</td>
</tr>
<tr>
<td>Gene</td>
<td>Forward Primer</td>
<td>Reverse Primer</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>G6PC</td>
<td>GTGGGCAATTGTTTGCTGGT</td>
<td>GACTCTCCCCTTGCCGTTTT</td>
</tr>
<tr>
<td>PFKB3</td>
<td>CCACCAAAAAGCCTCGCATC</td>
<td>TCCCTAGCAAAGGTTGTCCG</td>
</tr>
<tr>
<td>PNPLA2</td>
<td>GCCACAGTACACAGGGATAAA</td>
<td>GAGTTTCGGATGGAGAATGT</td>
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<tr>
<td>CPT-1</td>
<td>CCACGAAGCCCTCAAACAGA</td>
<td>CACACCCACCACCACGATAA</td>
</tr>
<tr>
<td>IFN-b</td>
<td>ACTACAAGCAGCTCCAGTTC</td>
<td>TGAGGTTGAGCCTTCCATTTC</td>
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Table 2. Power Up Sybr green Master mix

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>PowerUpTM SYBR™ Green Master Mix (2X)</td>
<td>10 μL</td>
</tr>
<tr>
<td>Forward Primers (use 300–800 nM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Reverse Primers (use 300–800 nM)</td>
<td>1 μL</td>
</tr>
<tr>
<td>Nuclease-Free Water</td>
<td>7 μL</td>
</tr>
<tr>
<td>Total</td>
<td>19 μL</td>
</tr>
</tbody>
</table>

The PowerUp Sybr Green Master Mix (Applied BioSystems) was used for a 20 μM total volume reaction. The reactions were prepared according to the manufacturer protocol Scale, the components according to the number of reactions, and included 10% overage.

Components of the sybr green mix were mixed thoroughly, then centrifuged briefly to spin down all the content and eliminate any air bubbles. The 19 μL of total volume added to each well and 1 μL of DNA template from each sample was added separately to each well to reduce pipetting error. There were 3 replicates per each sample. In the end, a total volume of 20 μL was transferred to each reaction well of the optical plate. The plate was sealed and covered with an optical adhesive cover. The plate was centrifuged for 1 min at 1000 rpm (112 G) to remove any air bubbles — the
reaction plate placed in the Quant Studio (TM) 3 Real Time qPCR was performed on the reaction plate using the following cycling model.

Table 3. Standard cycling mode (primer Tm <60°C)

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature</th>
<th>Duration</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDG activation</td>
<td>50°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Dual-Lock™ DNA Polymerase</td>
<td>95°C</td>
<td>2 minutes</td>
<td>Hold</td>
</tr>
<tr>
<td>Denature</td>
<td>95°C</td>
<td>15 seconds</td>
<td>40</td>
</tr>
<tr>
<td>Anneal</td>
<td>55–60°C</td>
<td>15 seconds</td>
<td></td>
</tr>
<tr>
<td>Extend</td>
<td>72°C</td>
<td>1 minute</td>
<td></td>
</tr>
</tbody>
</table>

Table 4. Dissociation curve conditions were listed below.

<table>
<thead>
<tr>
<th>Step</th>
<th>Ramp rate</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.6°C/second</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
<tr>
<td>2</td>
<td>1.6°C/second</td>
<td>60°C</td>
<td>1 minute</td>
</tr>
<tr>
<td>3</td>
<td>0.15°C/second</td>
<td>95°C</td>
<td>15 seconds</td>
</tr>
</tbody>
</table>

The Quant Studio 3 instrument was set to perform above steps. The non-specific amplifications were checked using a dissociation curve.

Upon completion of qPCR, relative gene expression was calculated using the ΔΔ^Ct^ method, which relates the PCR signal of the target transcript in a treatment group to that of another sample such as an untreated control. The 2(ΔΔ^Ct^) method is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments.

Gene expression was normalized to the RPL32 gene, the LEW/SsNHsd Control group as the calibrator or untreated control. Samples were run with RNAse free water instead of template DNA to see if there any impurities that give signals. Each sample Ct mean was calculated, and standard
deviations calculated for each mean Ct value. Each sample run in triplicate and data was normalized to RPL32 and control LEW/SsNHsd group. When you evaluate fold change; the unequal PCR efficiencies decrease in the accuracy of the calculated fold change. Those samples were repeated to improve standard deviations. Mean Ct values and standard deviations used in the $\Delta\Delta^{\text{Ct}}$ calculations to test to compare intergroup differences.

Terminal blood sampling and analysis

Blood collected in a 15 ml conical tube and 4mL heparinized vacutainer. Nearly 10mL of blood from heart puncture was taken and marked rat number and date. The conical tube samples time was recorded, and the blood clotted on a rack for 15 minutes exactly, and then centrifuged for 10 minutes 1000xg at 4ºC. Samples were removed and serum was separated from red blood cells with a micropipette and put in pre-labeled Eppendorf tubes. The rat plasma separated by centrifuge the blood at 1000 x g for 10 in at 4ºC and stored in -80ºC. Serum samples were used for Insulin assay, Triglyceride, Cholesterol, metabolomics, cytokine, and chemokine assays.

Chemokine and cytokine multiplex array.

Serum samples were prepared and sent to eve technologies, Eppendorf were labeled as L1, L2, and L3. L1 for Metabolic Array, R1 for Chemokine array. The 0.5 – 0.65mL snap-cap microcentrifuge tubes used for the metabolic and chemokine array as preferred by Eve Technologies. 0.1 µl of aliquot for metabolic panel and chemokines were prepared as follows. For the Metabolic array, 1µL of DPP-IV (Thermo Scientific) 1µL of aprotinin inhibitors (Thermo Scientific) was directly added to 50µL of serum. This was mixed and added to the final safeloc Eppendorf tube. For Chemokine array, two-fold dilution was done by adding 1X PBS (PBS ((10X), pH 7.4 (Gibco™)), 100µL of PBS was added to 100µL of serum for chemokine array then
80 µL were transferred to a labeled safe lock Eppendorf tube and temporarily frozen in -20°C. Samples were moved to and stored in -80 degrees until processing. The metabolic hormone, cytokines, and cytokines level were measured by Eve Technologies Calgary, Canada. Blood hormone and cytokine levels are analyzed using Rat Hormone 10-Plex Discovery assay and Rat Cytokines 27-plex Discovery assay (Eve Technology, Canada). Results of the following biomarkers were given, Eotaxin, G-CSF, GM-CSF, IFN gamma, IL-1alpha, IL-1beta, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-9, IL-10, IL-12 (p40), IL-12 (p70), IL-13, IL-15, IL-17A, IP-10, KC, LIF, LIX, MCP-1, M-CSF, MIG, MIP-1alpha, MIP-1beta, MIP-2, RANTES, TNF alpha, LIX. One-way ANOVA followed by Turkey multiple comparison tests were performed using GraphPad Prism 7.04 (La Jolla, CA) to compare each rat strain and treatment group. Mean, Standard deviation, and the number of samples for each group used to calculate and see the difference is significant (p <0.005) for each gene measured.

Enzyme-Linked Immuno Sorbent Assay

Mercordia Ultrasensitive Rat Insulin ELISA kit used and 96 well plate was used, and each sample and calibrators have duplicates. Duplicating allows you to calculate and assess inter-assay variability, or the reproducibility of results. All reagents and samples must be brought to room temperature before starting the experiment. A calibration curve prepared for every assay. Enzyme conjugate 1 X solution and samples and wash buffer prepared. The buffer solution was made by adding 1000ml distilled water. 25 µl each of calibrators, samples, controls, pipetted out for each well. 100µl of enzyme conjugate 1x solution into each well. The plate incubated on a plate shaker (Corning-LSE) for 2 hours at room temperature. The plate was washed 6 times with 700 µl of 1X wash buffer solution per and discarded the reaction volume by inverting the microplate by over a sink. Nearly 350 µL of wash buffer was added to each right tap firmly several times to remove any
excess liquid and repeated 5 times. Then 200µl of substrate TMB was added to each well and incubated for 15 minutes at room temperature, then 50µl of stop solution was added. The plate has been placed on a shaker for nearly 5 seconds for mixing. The optical density measured at 450nm, and results were calculated using GraphPad Prism 7.04 (La Jolla, CA).

Triglyceride Assay

Triglyceride Colorimetric Assay (Cayman. 10010303) was done using rat liver samples and plasma samples. Plasma samples were an aliquot from the previously described preparation.

Liver preparation

Approximately 320 mg of liver samples were used for the assay. The liver samples were homogenized using Homogenizer Super FastPrep-2 (Thermo Fisher Scientific).in 2 ml of the diluted NP40 Substitute Assay Reagent samples were centrifuged at 10,000 x g for 10 minutes at 4° C. Once it ends transfer the entire supernatant into another tube and stored at -80 ºC. The supernatant used for the assay. 1:10 ratio diluted with NP40 Substitute Assay Reagent used for the assay.

10 µl of each standard added to the designated wells on the plate. 10 µl of each sample in duplicate added to each designated well. 150 µl of enzyme mixture added to each well. The plate was covered using Parafilm and shaken for a few seconds using a shaker (Fisher Scientific), then incubated for 15 minutes at room temperature. The absorbance was measured at 530-550 nm using a plate reader (Molecular Devices, Spectra Max).

Cholesterol Assay
Cholesterol Fluorometric Assay (Cayman 10007640) was done using serum. The standard was made up of 10 mM cholesterol (5-Golestan-3β-ol in ethanol. The reagent is ready to use for the preparation of the diluted cholesterol standards. 50μL of sample is serum used for each well, and 2 replicates were taken for each sample to obtain reproducible results. The first two rows allocated for duplicates of standards 20 μl of cholesterol assay standard (Item No. 10008053) diluted with 980 μl of diluted assay buffer. The eight clean glass test tubes were taken and mark them A-H. The amount of cholesterol standard and assay buffer to each tube was added, as described in the table below.

Table 5. Amount of cholesterol standard and assay buffer

<table>
<thead>
<tr>
<th>Tube</th>
<th>200 μM Cholesterol Standard(μl)</th>
<th>Assay Buffer(μl)</th>
<th>Final Concentration (μM cholesterol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>10000</td>
<td>0</td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>990</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>20</td>
<td>980</td>
<td>4</td>
</tr>
<tr>
<td>D</td>
<td>30</td>
<td>970</td>
<td>6</td>
</tr>
<tr>
<td>E</td>
<td>40</td>
<td>960</td>
<td>8</td>
</tr>
<tr>
<td>F</td>
<td>60</td>
<td>940</td>
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</tr>
<tr>
<td>G</td>
<td>80</td>
<td>920</td>
<td>16</td>
</tr>
<tr>
<td>H</td>
<td>100</td>
<td>900</td>
<td>20</td>
</tr>
</tbody>
</table>

The assay cocktail was prepared using assay buffer (4.745 ml), cholesterol detector (150 μl), HRP (50 μl), cholesterol oxidase (50 μl), and cholesterol esterase (5 μl) Covered the plate with the plate cover provided. The plate allowed to sit for 30 min at 37°C in an incubator (Fisher Scientific) and measured the fluorescence of excitation wavelength at 530nm, 535 nm, and 540nm.
Statistical Analysis

For qPCR analysis, triglycerides and cholesterol assays if the standard deviation of triplicates standard deviation above 0.25 sample replicates neglected. One-way ANOVA followed by Turkey multiple comparison tests performed using GraphPad Prism 7.04 (La Jolla, CA) to compare each rat strain and treatment group. Mean, standard deviation, and the number of samples for each group used to calculate and see the difference is significant (p < 0.005) for each gene measured.

Results

The masses of each rat measured during the experiment period. During 6 days of the experiment period, we didn’t observe any significant variation of bodyweight of our LEW.1WR1 rats that were kept only for 6 days. We have observed increasing body weights of all rat groups with time.

Figure 4. Body mass changes over the induction period.
No differences in body mass during the treatment period. Data are expressed as the mean with SD, n=8 in LEW/SsNHsd control, LEW/SsNHsd treated, LEW.1WR1Control, and LEW.1WR1treated rat groups.

Although there are no differences in mass, control LEW.1WR1 rats have higher terminal fasting blood glucose levels compared to LEW/SsNHsd rats (Figure 2). At this time point, the rats were not hyperglycemic but LEW.1WR1 rats had blood glucose level nearing 150mg/dL. If those rats were hyperglycemic, they would have more than 200mg/dL (Pournaghi P et al., 2012.).

![Figure 5. Terminal blood glucose level of four different rat groups.](image)

One-way ANOVA was performed. Different letters represent significant differences between each group. Similar letters represent that groups are not significantly different to each other. Terminal blood glucose level was measured in mg/dL. LEW.1WR1 Control rats have significantly higher terminal fasting blood glucose when compared to LEW/SsNHsd rats. Data are expressed as the mean with SD. n=8, group different letters represent p<0.05.
TNF-1a level is significantly low in LEW.1WR1 rats compared to LEW/SsNHsd rats. TNF-a levels were elevated in both treated rat groups compared to their control rat group. Fold change of TNF-a is significantly elevated with LEW.1WR1Treated rats compared to control LEW.1WR1 rats and LEW/SsNHsd rats. TNF a level is trending upward with PIC treatment in both strains.

Figure 6. Rat cytokine TNF-a level in serum multiplex data.

LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 Control, LEW.1WR1 Treated rat groups respectively. n=8,8,7,8. b Fold change of TNF-a in LEW.1WR1rats. Different letters represent significance among groups (p<0.05).
Figure 7a. Terminal proinflammatory, anti-inflammatory cytokine levels of 4 different rat groups LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 Control, LEW.1WR1 Treated rat groups respectively. IL-12 (n=5,7,4,3), IL-18 (n=7,7,4,6), IL-17 (n=8,8,6,7) and IL-4(n=8,8,7,7) ,IL-10(n= 8,8,3,4) The Different letters represent significant differences between groups. (p<0.05).

Figure 7b. Fold changes of proinflammatory cytokines. Fold change was only calculated in groups n ≤7 samples.Fold change was calculated in 4 different rat groups starting from LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 Control, LEW.1WR1 Treated rat groups respectively.
Both Treated rat groups have elevated pro inflammatory cytokine level compared to control groups respectively. There is significant difference between two rat strains and LEW.1WR1 rats have significantly low pro inflammatory cytokine levels compared to SsNHsd groups. Fold changes are determined due to strong strain effect which covered the effect of the treatment. IL-17 and IL-12 fold changes are elevated in both treated rat groups compared to control rat groups. IL-4 level is elevated in PIC treated SsNHsd rats compared to SsNHsd control rats while IL-4 level is decreased in PIC treated LEW.1WR1 rats compared to LEW.1WR1 control rats. IL-18 fold changes cannot be performed due to less number of samples.

Then we assessed hormones in the blood. PYY, PP, LEPTIN, GLP-1 (Glucagon-like peptide-1), Amylin expression significantly increased in LEW.1WR1 treated rats compared to 1WR1 control rats. LEW/SsNHsd treated rats have lower expression of amylin compared to SsNHsd control rats. GIP (Gastric inhibitory polypeptide) expression increased in 1WR1 rats. Ghrelin levels increased in both treated rat groups compared to their related control rat groups. GIP (Gastric inhibitory polypeptide) level increased in the LEW.1WR1 treated group compared to LEW.1WR1 rats and both LEW/SsNHsd rat groups. GLP-1(Glucagon-like peptide-1) hormone level increased in LEW.1WR1. There is no significant difference between each rat group. Leptin is no significant difference between each group. PP level significantly differences among two rat groups. PYY level is not significantly different among rat groups. But LEW.1WR1 treated rats showed increased PYY level compared to LEW.1WR1 control rats.
Figure 8. Rat Metabolic Hormone Level Multiplex data.

Starting from top Amylin- (n=7,8,2,4 ) were presented for Ghrelin(n=6,6,5,7), GIP-Gastric inhibitory polypeptide (n=7,7,8,8) GLP-1-Glucagon-like peptide-1(n=8,8,8,8), Glucagon (n=8,8,7,8), Leptin(n=8,8,8,8), PP-Pancreatic polypeptide (n=8,8,7,8), PYY-Peptide YY (n=8,8,7,8) LEW/SsHNd Control, LEW/SsHNd Treated, LEW.1WR1 Control ,LEW.1WR1 Treated groups. One way ANOVA with multiple comparisons were done. Error bars represent standard deviation p<0.05.
Fold change of GIP, GLP-1, PP, Glucagon, Leptin and PYY in LEW/SsNHsd and LEW.1WR1 control and treated rats. Fold change was done with number samples ≤ 7 groups.

GIP, GLP, Leptin levels are increased in IWR1 Treated rats compared to IWR1 Control rats. Glucagon, PP, PYY levels are decreased in LEW.1WR1 treated rats compared to control rats. PP, GIP, Glucagon levels decreased in LEW/SsNHsd treated rats compared to LEW/SsNHsd control rats. PYY and GLP-1 levels are barely changed with Poly IC treatment in LEW/SsNHsd rats. Leptin level increased in LEW/SsNHsd treated rats compared to LEW/SsNHsd control rats.

Because of huge strain differences, PIC effect was masked. We performed fold changes by normalizing data to each control strain. We observed that GIP level is significantly increased in LEW.1WR1 treated rats compared to control rats. GIP level is trending downward in LEW/SsNHsd rats with PIC treatment while GIP level is significantly upward in LAW.WR1
treated rats compared to control LEW.1WR1 rats. Glucagon level is not significantly different among each group but, trending downward with PIC treatment in both treated groups. PYY level is significantly increased in LEW.1WR1 treated rats compared to other groups.

Figure 9a. Histology of the CD3\(^+\) stained pancreases.

Treatment rats show increased T cell staining. (A) to the top left is the LEW.1WR1-Control, (B) to the top right is LEW.1WR1-Treated, (C) to the bottom left is LEW/SsNHsd-Control, and (D) to the bottom right is the LEW/SsNHsd-Treated. Islets were circled. II.) Average CD3\(^+\) infiltration scores. Data are expressed as mean with SD P<0.005 n=4,8,8,8 for in LEW.1WR1 Control, LEW.1WR1 Treated, LEW/SsNHsd Control LEW/SsNHsd Treated groups respectively. GLP-1 level is significantly increased in LEW.1WR1 treated rats compared to LEW/SsNHsd treated rats.
In figure 3 that 1WR1 rats have blood glucose levels high as 150mg/dL, which is higher than SsNHsd rats, but it is below hyperglycemic levels. The CD3 (cluster of differentiation 3) T cell co-receptor helps to activate both the cytotoxic T cell (CD8+ naive T cells) and also T helper cells (CD4+ naive T cells). Both treated rat groups have high CD 3 cells compared to their control rat groups. SsNHsd treated rats have significantly high; average CD3 infiltration score compared to LEW/SsNHsd control rat groups. Then LEW.1WR1 Treated rat group has a significantly high; average CD3 infiltration score compared to control LEW.1WR1 rats. But LEW/SsNHsd treated and LEW.1WR1 control rat group infiltration scores are not significant.LEW.1WR1 rats have significantly high infiltration scores compared to SsNHsd rats suggesting poly IC induction causes to activate inflammation signals in both rat groups. By looking at CD3 stain and blind scoring, it seems like that 1WR1 Rats have infiltration scores in 2-3 range, slightly less than what we expected.
Figure 9b. Histology of the CD68+ stained pancreases.

Currently point there is low grade CD68+ staining in the islets of the treated rats. (A) to the top left is the LEW.1WR1-Control, (B) to the top right is LEW.1WR1-Treated, (C) to the bottom left is LEW/SsNHsd-Control, and (D) to the bottom right is the LEW/SsNHsd-Treated. Islets were circled. II.) Average infiltration of CD68+ cell scores. Data are expressed as mean with SD P<0.005 n=4,8,8,8 for LEW.1WR1 Control, LEW.1WR1 Treated, LEW/SsNHsd Control LEW/SsNHsd Treated groups respectively.

CD68 is a protein highly expressed by cells in the monocyte lineage, by circulating macrophages, and by tissue macrophages again giving the signal of started inflammation. LEW/SsNHsd control is a significantly low infiltration score compared to LEW.1WR1 treated rats. Again, that LEW.1WR1 rats have high infiltration scores compared to LEW/SsNHsd rats.LEW.1WR1 rats have a high infiltration score even without giving Poly IC control. Even looking at the islets, it
observed that 1WR1 rats have small islets compared to SsNHsd rats. When looking at the CD68 stain, the values range between 1-1.5 slightly less than CD3.

Figure 9 c. Histology of the insulin stained pancreases.

The LEW.1WR1 rats showed a significantly lower number of total islets. (A) to the top left is the LEW.1WR1-Control, (B) to the top right is LEW.1WR1-Treated, (C) to the bottom left is LEW/SsNHsd-Control, and (D) to the bottom right is the LEW/SsNHsd-Treated. (Red stained clusters of cells are islets). II)A-E Semiquantitative analysis of islets and size. Data are expressed
as mean with SD. P<0.005 represents n=4,8,8,8 for in LEW.1WR1 Control, LEW.1WR1 Treated, LEW/SsNHsd Control LEW/SsNHsd Treated groups respectively.

In figure 9 insulin-stained islets separated by extra small, small, medium, large size. When looking at blind scoring data, 1WR1 rats have a significantly lower number of extra small and small islets compared to control groups. 1WR1 has an overall lower number of islets compared to control groups.

The results we got support the previous studies by showing the infiltration of CD3 increases infiltration of CD68. The staining of CD68 cells supports that insulitis has an essential role to play in insulitis (Willcox A et al., 2009). The results also verified the time point we choose that rats are not in full-blown diabetic, but they are growing toward that point as is growing infiltration of immune cells. (Magnuson AM et al., 2015). We have observed that at 12 days, a significant number of rats have infiltration 3-4 levels of both CD3 and CD68 in their islets. Most of the stained cells located in the periphery. On day 6, the rats were normoglycemic; by looking at CD3 stain and blind scoring, it seems like that 1WR1 Rats have infiltration scores in 2-3 range, slightly less as we expected. We don’t want them to be diabetic. When looking at the CD 68+ stained cells, the values changed between 1-1.5 slightly lesser than CD3+ scores. The scores for treated animals are higher compared to control groups. This result is the same for both rat groups. In figure 9 LEW/SsNHsd treated rats have increased number of islets compared to control LEW/SsNHsd rats. Even though, LEW.1WR1treated rats have decreased number of islets compared to control LEW.1WR1 rats. It seems like that LEW/SsNHsd rats have compensatory mechanism to save islets and 1WR1 rats do not.
Figure 10. Serum insulin level from multiplex data for 4 rat groups.

LEW.1WR1 rats have significantly high insulin concentration compared to LEW/SsNHsd control, LEW/SsNHsd rats. Error bars are SD of n=8, 5, 5, & 3 respectively and dark square for LEW/SsNHsd rats and dark circle for LEW.1WR1. Error bars reflect standard deviation P<0.05.

1WR1 rats Blood insulin levels are significantly higher compared to SsNHsd rats. But SsNHsd rats’ insulin levels are below the range and cannot be detected by multiplex. C-peptide produces when proinsulin is cleaved to produce insulin. Assessing the C-peptide data blood insulin should be a measurable. But insulin level for most of SsNHsd rats are below the range of Multiplex we used.
Figure 11. C-peptide level for 4 rat groups.

LEW/SsNHsd treated rats have significantly low C-peptide concentration compared to LEW/SsNHsd control, LEW.1WR1 Control, LEW.1WR1 Treated rats. n=8, 8, 8, & 8 for LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 Control, LEW.1WR1 Treated groups respectively. Error bars reflect standard deviation. P<0.005.

We have observed that C-peptide levels are above 300pg/mL which is measurable for all groups. C-peptide level is significantly decreased in LEW/SsNHsd treated rats compared to control LEW/SsNHsd rats and LEW.1WR1 rats.
Figure 12. Triglyceride concentration in serum for 4 rat groups.

LEW/SsNHsd treated rats have significantly high triglyceride concentration compare to LEW/SsNHsd control, LEW.1WR1 Control, LEW.1WR1 Treated rats. n=8, 8, 8, & 8 for LEW/SsNHsd Control, LEW/SsNHsd Treated groups, LEW.1WR1 Control, LEW.1WR1 Treated groups respectively. Error bars reflect standard deviation. P<0.005.

Cholesterol concentration of liver for 4 rat groups. n=8, 8, 8, & 8 for groups, LEW/SsNHsd Control, LEW/SsNHsd Treat, LEW.1WR1 Control, LEW.1WR1 Treated respectively. Error bars reflect standard deviation. P<0.005. LEW/SsNHsd Treated rats have significantly high Cholesterol concentration compared to LEW/SsNHsd control, LEW.1WR1 Treated, LEW.1WR1 control rats.
Figure 12. Triglyceride concentration of liver for 4 rat groups.

LEW/SsNHsd control rats have significantly low compared to LEW/SsHNsd treated, LEW.1WR1 Treated rats. n=8, 8, 8, & 8 for LEW/SsNHsd Control LEW/SsNHsd Treated groups, LEW.1WR1 Control, LEW.1WR1 Treated, respectively. Error bars reflect standard deviation. P<0.005

Since1WR1 rats are known to express the FAT 10 gene in response to TNFα. The liver is one of the organs that overexpress the FAT 10 gene and a significant regulator of metabolism. So, we looked at significant differences in lipid metabolism, which is regulated by insulin and altered when FAT 10 knock out.
Figure 14. Gene expression analysis of *UBD* and *p53* in liver.

LEW.1WR1 Control rats have significantly higher expression of UBD and P53 in liver compared to LEW/SsNHsd rats. Gene expression analysis of UBD and p53 in the liver. n=8, 8, 7, & 7 for Ubd and n=8, 8, 8, & 5 for p53 in LEW.1WR1 Control, LEW.1WR1 Treated, LEW/SsNHsd Control LEW/SsNHsd Treated groups respectively. Error bars reflect standard deviation. P<0.005.

Figure 15. Insulin induce Relative gene expression of liver genes

*FOXO1* involved in cholesterol synthesis and SREBP-1(Sterol regulatory element-binding transcription factor 1), SCD-1(Searoyl-CoA desaturase-1), was tested in LEW/SsNHsd
Control, LEW/SsNHsd Treated, EWLEW.1WR1 control, LEW.1WR1 Treated rats. FOXO1 expression was significantly high in LEW.1WR1 treated rats. Error bars reflect standard deviation. P<0.005.

Figure 16. Relative gene expression of lipolytic liver genes

Adipose triglyceride lipase (ATGL), Acyl-CoA Synthetase Long Chain Family Member 1 (ACSL1), and Patatin Like Phospholipase Domain Containing 2 (PNPLA2), CPT-1 (Carnitine palmitoyltransferase 1) involved in was tested in LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 control, LEW.1WR1 Treated rats.
Figure 17. Relative gene expression of lipogenic liver genes

FASN (Fatty acid synthase), SCD-1 (Stearoyl-CoA desaturase-1), DGAT1 (Diacylglycerol O-Acyltransferase 1), DGAT2 (Diacylglycerol O-Acyltransferase 2), were tested in LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 control, LEW.1WR1 Treated rats. Error bars reflect standard deviation. P<0.005.

LEW.1WR1 control rats have significantly increased DGAT1, DGAT2 compared to SsNHsd control and SsNHsd treated rats. On the other hand, SCD-1, FASN are significantly reduced in both control and treated 1WR1 rats compared to SsNHsd rats. SCD-1 significantly decreased SsNHsd treated rats compared to Control rats. DGAT1, DGAT2 expression levels are significantly increased in LEW.1WR1 control rats compared to LEW.1WR1 treated rats.
Figure 19. Relative gene expression of gluconeogenic/ glycogenolysis liver genes

GLUT2 (Glucose transporter 2), G6PC (Glucose-6-Phosphatase Catalytic Subunit), GAPDH (Glyceraldehyde 3-phosphate dehydrogenase), PCK-1 (Phosphoenolpyruvate carboxykinase 1) were tested in LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 control, LEW.1WR1 Treated rats. Error bars reflect standard deviation. P<0.005.

G6P levels are significantly increased in LEW.1WR1 rats compared to LEW/SsNHsd rats. On the other hand, GAPDH and GLUT2 expression levels of LEW.1WR1 rats are significantly lower compared to SsNHsd rats. PCK-1 expression level is increased in LEW/SsNHsd treated rats.
compared to LEW/SsNHsd rats. On the other hand, LEW.1WR1 treated rats have significantly lower gene expression compared to LEW.1WR1 control rats.

Figure 20. Relative gene expression of glycolytic liver genes

Carbohydrate responsive element binding Protein (ChREBP-1), (Phosphofructo-2-Kinase/Fructose-2,6-Bisphosphatase 3 (PFKFB3) were tested in LEW/SsNHsd Control, LEW/SsNHsd Treated, EWLEW.1WR1 control, LEW.1WR1 Treated rats. Error bars reflect standard deviation. P<0.005.

Glycolytic gene PFK3b, ChREBP-1 expression are significantly increased in LEW/SsNHsd treated rats compared to all the other groups. PFK3b, ChREBP-1 expressions are significantly lower in LEW.1WR1 treated rats compared to LEW.1WR1 control rats.
Figure 21. Relative Gene expression of inflammation liver genes IFNbeta

IFNbeta involved in immune response was tested in LEW/SsNHsd Control, LEW/SsNHsd Treated, LEW.1WR1 control, LEW.1WR1 Treated rats. Error bars reflect standard deviation. P<0.005.

IFN beta gene expression level is significantly increased in LEW.WR1 treated rats. IFN beta gene expressions are lower in LEW/SsNHsd control, LEW/SsNHsd treated, LEW.1WR1 control rats.
Discussion

It is known that environmental factors are associated with Type 1 diabetes (Atkinson, et al., 2014). The LEW.1WR1 rat model is triggered by Poly IC leading to the development of Type 1 diabetes. The susceptibility of this model has been shown to be related to the Type 1 Interferon receptor as well as an over expression of FAT10, but to date there have been no studies to understand the condition of the islets prior to insulitis or the metabolic condition of the rats during the time window we have chosen (Natasha Q et al., 2017). According to Atkinson et al., glucose fluctuation can be observed in humans at the onset of diabetes, yet it is not clear how it correlates to metabolism prior to autoimmunity. At the onset of diabetes, there is a phase denoted the “Honeymoon phase” (Partial remission phase) where insulin requirement is low (<174 pmol/L) (Bernard P et al., 1985) This phase is not clearly characterized since biopsy for human patients at this stage is detrimental to many processes as the pancreas has both endocrine and exocrine functions. We characterized the Day 6 time point in the 1WR1 rat to analyze the model at a time point preceding significant insulitis.

There were no significant weight differences during the induction period across the four groups (Figure 1). LEW/SsNHsd rats maintain (90mg/dL±) terminal fasting blood glucose level while, 1WR1 treated rats are maintaining a 150 mg/dL± (Figure 2) Diabetic rats have severely increased fasting blood glucose levels above 250 mg/dL which called “hyperglycemic” neither strain had hyperglycemia. (Anne J et al., 2005). GLUT2 is glucose transporter isoform 2, Glut2 is predominantly expressed in the liver and pancreas, suppression of Glut2 expression revealed the reduce uptake of glucose in liver (Thorens B et al., 2015) in LEW.1WR1 rats compared to SsNHsd rats. The treatment effect is not very visible at this stage.
Next, we assessed the stage of insulitis. For this we had blinded scorers grade the infiltration of the islet for CD3\(^+\) and CD68\(^+\) stained cells levels to evaluate T cell or macrophage, dendritic cell and monocyte infiltration respectively. When LEW.1AR1/Ztm-iddm rats were transitioning from normoglycemia to hyperglycemia at an early stage of infiltration, macrophages were predominant at infiltration of islets. 60-70\% of β cell loss was linked to T cell infiltration (Anne J et al.,2005). In the beginning of islets infiltration, immune cell infiltration is limited to the peripheral region of islets. As the disease progresses, the whole islet is infiltrated. Immune cells circulate in the blood and enter the pancreas through ducts, capillaries, and blood vessels. Most of the macrophages and T cells in all four rat groups are located in the ducts and not in the core of the islets at this time point. It has been previously reported that infiltration of CD3\(^+\) cells tend to precede the increase in CD68\(^+\) cells, LEW.1WR1 treated rats at this time point have the average CD3\(^+\) staining of a level of 3 while CD68\(^+\) cells have the average staining of a level of 1.5 and are not heavily infiltrating islets at the Day 6 time point. (Martha CT et al.,2016), (Mordes J et al., 2005). There are small amounts of T Cells and Macrophages present even in the control islets and pancreases as immune cells general survey organs to detect invasion.

Serum IL-17 levels are increased in both treated rats. CD 3\(^+\) levels are also increased in both PIC rat groups. CD3 stain is staining every kind of T cell in pancreases including Th17 T Cells. IL-17 plays a crucial role in various inflammatory responses and autoimmune diseases. Recent studies have shown increased IL-17 in STZ (streptozotocin) induced diabetic animal models and non-obese diabetic model (NOD) mice. Even though we observed increased T Cells in pancreases with PIC treatment, those T Cells have not invaded the islets yet. Most of the stain patches are located in ducts near to islets.
Ryan A et al., 2014 observed a significant increase in infiltration of the islet by T cells on day 7 in Poly IC induced LEW.1WR1 rats. The scores of CD3+ and CD68+ together combined with the fasting blood glucoses reflect a low overall insulitis score which indicates that the rats do not have full blown diabetes at the time point we selected.

These animals also have a difference in blood insulin concentration independent of PIC injection; 1WR1 rats have high insulin levels compared to LEW/SsNHsd rats. Which suggests that the islets are either overproducing insulin or that there may be reduced insulin efficiency suggesting two rat strains may have different metabolic phenotypes. Previous findings showed that insulin transcripts in the pancreas of the 1WR1 rat are increased at day 7 during induction, but this paper did not compare these levels to control 1WR1 rats (James E et. al., 2014).

The result of the insulin stained sections was unexpected since 1WR1 rats have a lower number of small, extra small and total islets and compared to SsNHsd rats (Figure 8) yet they have significantly increased insulin levels (Figure 8). The results from CD3 and CD68 staining shows that rats do not have significant infiltration yet the insulin staining in the control 1WR1 rat pancreas suggests that there may be fewer islets in the beginning. Having fewer islets can reduce the ability of the rat to maintain a honeymoon phase as significant beta cell death would have a much larger impact on residual beta cell mass. It is observed that LEW/SsNHsd treated rats have high numbers of large and medium and islets while LEW.1WR1 treated rats have decreased numbers of islets compared to both LEW/SsNHsd rat groups and LEW.1WR1 control rats. We hypothesize that SsNHsd treated rats compensate for beta cell destruction by proliferating and 1WR1 treated rats cannot compensate for the beta cell destruction since they have a small number of islets at the beginning. Ki67 like staining has to be done to confirm the differences in proliferation rate. The
insulin levels, c-peptide levels and β cell staining also support that the rats from two rat groups have not developed full blown diabetes at the time point we choose.

The data from serum chemokine and cytokine array suggests that 1WR1 rats have different inflammatory responses than the SsNHsd rats. According to Natasha Qaisar et al., 2017 interferon receptor mediated signaling pathway (IFN pathway) and type 1 Interferon plays a major role in virus induced type 1 diabetes in LEW.1WR1 rats. Disrupting IFN pathway or IFN production can delay or prevent development of type 1 diabetes in LEW.1WR1 rats. But, IFN-γ values in both strains at this time point are below the detectable level of the assay. It is unclear at this time point how the IFN pathway is involved during PIC induction. So, as a future study we are hoping to look at IFN a and IFN beta expression levels in splenocytes. IL-1 β and TNFα crucially determines the fate of β cells through recruiting, activating and proliferating immune cells (Fernanda O et al., 2010). It is known that proinflammatory cytokines play a role in apoptosis of β cells (Thomas HE., et al.,2013) (Anne J. et al 2005). We observed that TNF α increased in both rat groups with Poly IC induction. IL-1β is trending higher but IL-1β levels in most samples were not detectable because the lifetime of IL-1β is around 4 hrs and we kept them for more than 7 hrs to do post injections analysis. We observed no significant difference in proinflammatory cytokines like IL-18 and IL-12 in LEW.1WR1 rats at 6-day time point.

On the other hand, anti-inflammatory signals like IL-4 are lower with PIC treatment in LEW.1WR1 rats while SsNHsd treated rats have increased IL-4 levels. IL-4 participates in lipid metabolism and diabetic susceptibility. IL-4 improves insulin sensitivity and glucose tolerance while inhibiting lipid accumulation (Ming-Yuh S et al., 2019). LEW.1WR1 rats have increased blood glucose levels and are less insulin sensitive according to our data. SsNHsd rats are normoglycemic and maintained normal blood insulin levels during the duration of the experiment.
Increased IL-4 level in SsNHsd rats correlates with improved insulin sensitivity, glucose tolerance and lipid accumulation in the liver. TNF and IFN β are major effectors of immune responses which eventually leads to production of pro/anti-inflammatory signals like IL-6 and IL-10. IL-6-IL-10 levels are not significantly different with treatment but with strain. But we observed elevated levels of IL-6, IL-10 cytokine levels in serum with PIC treatment as sign of inflammation.

Increased insulin and high terminal blood glucose levels in the control 1WR1 rat suggest that LEW.1WR1 rats have aberrant glucose and insulin homeostasis. We have observed a phenotypic strain difference in blood glucose and insulin as well as low numbers of islets in 1WR1 rats. It is clear that there is a definitive correlation between hormones and blood glucose levels, insulin sensitivities and other possible effects related to TID susceptibility.

Other serum hormone levels were also changed with treatment and or difference between rat strain. GLP-1, leptin, amylin, ghrelin is positively correlated with the serum cholesterol. Leptin is inversely related with Triglycerides. Poly IC treated SsNHsd rats have significantly high levels compared to SsNHsd rats, LEW.1WR1 rat groups possibly depositing cholesterol in the liver. On the other hand, Leptin helps to maintain insulin level in blood, and it is observed that 1WR1 rats have reduced level of leptin with Poly IC induction.

PP, PYY, and ghrelin are hormones all produced in the digestive system. These hormones are called hunger hormones and the level of these hunger hormones decreased in LEW.1WR1 rats. Ghrelin plays a role in glucose metabolism, food intake, and insulin metabolism. Ghrelin inhibits insulin release in order to prevent cell death in the pancreatic β cell line. It is observed that both Poly IC induced rat groups have high ghrelin levels suggesting trying to prevent dying of β cells (Andralojc K et al.,2014). PYY is strongly correlated with decreased sensitivity insulin (Viardot
A et al., 2008). PYY level is also increased in treated 1WR1 rats which means LEW.1WR1 rats are losing insulin sensitivity.

GLP-1 is known to induce the insulin secretion of β cells through ion channels. GLP-1 increased with 1WR1 rats with Poly IC treatment compared to 1WR1 control rat group. Since Treated LEW.1WR1 rats have high insulin level again send us to the same direction that increase GLP-1 is correlated with high insulin level. But is not really changed with SsNHsd rats which is again suggests that two rat groups are regulating different pathways in response to Poly IC induction. (Patrick E et al., 2002).

SsNHsd rats have increased serum cholesterol and triglyceride levels compared to 1WR1 rats. On the other hand, 1WR1 rats have lower serum triglyceride and serum cholesterol levels with higher triglyceride levels in the liver. LEW/SsNHsd rats/It seems like LEW.1WR1 rats start storing triglyceride in the liver and probably start using them as energy sources. So, we decided to look at lipolysis and lipogenesis gene expression in the liver. LEW.1WR1 rats over expressed fat10 gene which makes them susceptible to develop T1D. The liver is one of the organs that will over express fat10 gene. Overall, the fat10 gene role is not entirely understood. fat10 knockout mice have less adiposity and extended life span by 20% compared to their C57B6 mice (Canaan A et al., 2006). Those mice used fat as fuel for metabolism, reduced blood insulin levels and glucose levels and increased insulin sensitivity were observed (Canaan A et al., 2014). Conversely, fat10 overexpression in 1WR1 rats can be responsible for higher terminal fasting blood insulin and glucose levels.
TNF-α plays a role in stimulating lipolysis and increasing lipolytic rate. This could be due to TNF-α activating extracellular signal-related kinase, ERK 1 and ERK2 (Ryden L et al. 2002) and downregulating lipid droplet-associated protein perilipin (PLIN) which we did not measure. Erk1/2 has implicated in several diseases and chronic inflammations and plin2 promotes lipid accumulation in liver. TNF-α levels are increased in LEW.1WR1 and LEW/SsNHsd treated rats’ serum potentially leading to increased lipolysis. TNF-α levels correlate with increased lipolytic gene expression in pnpla2, atgl, acsl-1 in treated rat groups compared to respective control groups. Overall SsNHsd rats have lower expression of lipolytic genes and fatty acid oxidation genes compared to LEW.1WR1rats. Both LEW.1WR1 rat groups showed significantly high liver gene expression of cpt-1, a gene related to fatty acid oxidation.

LEW.1WR1 rats have increased lipogenic gene expression in the liver including SREBP-1. The transcription factors SREBP-1 and PPARα regulate lipid storage in the body (Jump DB et al., 2011). Insulin induces the sterol regulatory element binding protein-1 (SREBP-1) nuclear abundance through several mechanisms like transcription of the SREBP-1 gene and stabilizing SREBP-1 mRNA (Jump DB et al.,2008). SREBP-1 stimulates synthesis of fatty acids in the liver (Ichiro S et al., 1999). SREBP-1 gene expression level is increased twice as much as control LEW.1WR1 rats in treated LEW.1WR1 suggesting that 1WR1 treated rats have high fatty acid deposition in the liver with PIC treatment. (Renate S et al., 2019). diglyceride acyltransferase 1 and 2 (DGAT1 and DGAT2) catalyzes the formation of triglycerides from diacylglycerol and Acyl-CoA (Chi-Lian et al., 2008) which is significantly increased in LEW.1WR1 rats compared to SsNHsd rats. Stearoyl-CoA Desaturase-1(scd-1) catalyzes the rate-limiting reaction of monounsaturated fatty acid (MUFA) synthesis. (Sudha B. et al., 2006) Loss of scd-1 improves insulin sensitivity in lean mice (Flowers J. et al., 2007) and significantly low expression of scd-1
and in LEW.1WR1 rats compared to SSNHsd rats correlates with reduced insulin sensitivity. Both rat groups treated with poly IC had reduced expression of SCD1 and this could be related to ER stress responses as the islets in LEW.1WR1 rats at day 8 have been observed to have increased phosphatidylcholine, a marker of ER expansion in response to poly IC treatment.

Both control and treated LEW.1WR1 rats have high Glucose 6 Phosphatase (g6pc) and FOXO-1 gene expression and reduced PCK-1 expression compared to SSNHsd rat strain. G6pc converts Glucose 6 phosphate to glucose as the final step of gluconeogenesis while pck-1 is the second step of glucose synthesis from pyruvate. Both of these enzymes are regulatory steps in the pathway, but glucose 6 phosphate can be synthesized through other pathways like the pentose phosphate pathway. Foxo1 regulates gluconeogenesis in the liver by increasing expression of gluconeogenic genes. PGC-1α can robustly activate G6pc and pck-1. However, FOXO1 markedly diminished the ability of PGC-1α to activate pck-1 and G6pc genes. Hepatic gluconeogenesis is absolutely necessary during prolonged fasting and we have fasted our rats for 7 hours which probably causes an increased G6pc and FOXO-1 expression in LEW.1WR1 rats.

ChREBP-1(Carbohydrate Responsible binding protein -1) plays a role in glycolysis, is significantly lower in both control and treated LEW.1WR1 rats compared to SSNHsd rats. On The other hand, SSNHsd rats have high expression of ChREBP-1 and increased the expression with PIC treatment. SSNHsd rats have high gene expression related to glycolysis compared to SSNHsd rats and PIC treatment can elevate the effect at this stage. p53 downregulates glycolysis in liver. We have observed overexpressed p53 rats causing down regulation of Pck-1 (Zhang P.et al., 2014) in LEW.1WR1 rats. Leptin stimulates Pfk3 expression which also leads to glycolysis, in hepatocyte cells (Jonathan D. et al., 2018). We have observed decreased leptin levels and Pfk3 in LEW.1WR1 treated rats compared to control LEW.1WR1 rats suggesting down regulated
glycolysis in the liver. On the other hand, treated SsNHsd rats have significantly high P53, \textit{Pfk3} gene expression and leptin level compared to SsNHsd control rats which suggests increased glycolysis in the liver with PIC treatment.

**Conclusion**

According to the data we gathered that LEW.1WR1 rats are losing insulin sensitivity, \( \beta \) cells in the pancreas increase insulin production. The reduced insulin sensitivity makes LEW.1WR1 rats to increase triglyceride usage and production in the liver. And LEW.1WR1rats have reduced glycolysis and increased gluconeogenesis in the liver.
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