Unpacking the Influence of Viral Infections on Diabetes Mellitus: Understanding Mechanisms and Intervention Implications

Student:
Arianna Afshar
Epidemiology Master Student

Examiner:
Dr. Charlotte Lewis
University of Washington

Second Reviewer:
Dr. Egil Fischer
Utrecht University

Student Number: 2902583

Word Count: 5597

Abstract

This grant proposal aims to elucidate mechanisms behind existing risk factors for diabetes mellitus, specifically viral infections. Diabetes mellitus is a chronic disease that is a leading cause of global morbidity and mortality and continues to rise, becoming an increasingly important global health issue. Diabetes is categorized into two different types: type 1 and type 2. Type 1 diabetes is an autoimmune disease that is often diagnosed early in life and is characterized by the complete destruction of ß-cells produced in the pancreas. Type 2 diabetes typically develops later in life and occurs due to both decreased insulin production and insulin resistance. Both types share the quality of not being able to sufficiently metabolize circulating blood glucose, resulting in hyperglycemia.

Despite current knowledge and evidence showing an association between viral infections and diabetes onset, the specific pathogenesis is still unknown. The primary objective of this study is to understand the mechanisms behind how viral infections impact the onset or progression of diabetes mellitus. Specifically, we will explore if viral infections accelerate the onset of diabetes, if it causes an increased progression of the disease, and if common antiviral treatments may work to delay progression of the disease.

To achieve these objectives, we propose using a mouse model to directly compare how introduction of a viral infection alters the disease trajectory. This study will characterize the complicated relationship between viral infections and diabetes mellitus, leading to new therapeutic approaches and public health strategies.

Key words: Diabetes mellites, Viral infections, Pathogenesis

Layman Summary

Everyone knows that food is the fuel your body needs to live. If you want to have energy to do things, your body needs an energy source, and it gets that from the foods you eat. One key component, glucose, is an essential building block of the carbohydrates we eat. Once ingested and absorbed into circulation, glucose enters cells where it is metabolized for energy. However, to metabolize glucose, a hormone called insulin, which is made by special cells in the pancreas called beta cells, is required. If your body does not have adequate insulin, or is unable to use insulin, it goes into a state of hyperglycemia – an overabundance of glucose accumulating in your blood, which is a feature common to both types of diabetes mellitus. The root cause of the adverse effects of diabetes mellitus are due to both excessive glucose in the circulation and an inability of glucose to enter cells where it will be converted to energy. A variety of risk factors for the development of diabetes mellitus (DM) have been investigated, one of which is viral infections. While viral infections have been established to increase risk for diabetes, the way that viral infections do this is unclear.

This proposed research aims to further our understanding of the role viral infections play in DM development. The primary objectives of this research study are: 1) looking at how quickly someone develops diabetes after contracting a viral infection; 2) determining whether the virus speeds up the progression of DM, and 3) examining how treating the viral infection with common prescribed medications may change the progression of the disease.

To do this, we propose to use an animal model consisting of mice that are already predisposed to developing diabetes. We will then divide the mice into different groups – some that will not have been infected with a virus, acting as a control group, and some that will develop a virus, acting as the experimental group. This allows us to directly compare the effect viral infections have on the magnitude and direction of the onset of diabetes. Both the experimental and control groups will be monitored over time for development of DM Additionally, we will provide antiviral treatment to the experimental group to see if such treatment alters the progression of DM in any way.

Learning more about the specific way that viral infections impact the onset or progression of diabetes will open doors for more research on personalized medical treatment and advancements in public health policy and clinical therapeutics. This proposal has significant scientific and societal implications, which could ultimately lead to improved preventative and treatment strategies for a disease that is a leading cause of global morbidity and mortality.

Table of Contents

Unpacking the Influence of Viral Infections on Diabetes Mellitus: Understanding Mechanisms and Intervention Implications

I. Introduction

I.I. Brief Overview of Diabetes Mellitus: Prevalence, Incidence, and Mechanistic Pathway

Diabetes mellitus is a chronic metabolic disease that is perpetually increasing in prevalence and thus is becoming a large contributor to public health concerns. In fact, it is now in the list of the top 10 causes of mortality globally (1). Diabetes mellitus (DM) has increased significantly in recent years. In 2017, the global DM prevalence was 476 million cases, with an incidence rate of 22.9 million, resulting in 1.37 million deaths. It is projected to increase to a prevalence of 570.9 million cases with an incidence rate of 26.6 million cases and 1.59 million deaths in 2025 (1,2).

DM is categorized as a metabolic disease. There are two forms of DM, type 1, and type 2 – both inducing hyperglycemia due to insulin deficiency and/or resistance. Type 1 diabetes (T1DM) results from autoimmune inflammatory response causing the destruction of pancreatic ß-cells. This in turn causes absolute insulin deficiency. Type 2 diabetes (T2DM) results from a pancreatic ß-cell deficiency, combined with an increase of insulin resistance (3,4). Unlike T1DM, T2DM is not an established autoimmune disorder, but recent research has identified that immune dysfunction may play a role in T2DM development. In both types, the body is unable to sufficiently use insulin, which is needed to metabolize glucose for energy.

Glucose is a single molecule building block of carbohydrates that are ingested as food and then broken down in the gut back to glucose molecules. Glucose is then absorbed and transported via the blood to energy-requiring organs. Glucose metabolism is the primary source of energy for humans and other mammals. For the body to use glucose, an important protein is needed – insulin (5). Insulin is a peptide hormone produced by ß-cells found in pancreatic islet cells. Insulin plays a fundamental role in the human body as it is responsible for maintaining appropriate homeostatic blood glucose levels (6,7). Insulin acts as a key to open the door for cells to uptake glucose to use for energy. When glucose enters the circulation, it signals the pancreas islet cells to produce and release insulin, known as glucose-stimulated insulin secretion (GSIS). When insulin is released, it goes to these energy-requiring cells, binds to an insulin receptor, and allows uptake of glucose into that cell (7,8). In DM, this process has gone awry. In T1DM, the body's immune system attacks and destroys the pancreatic islet ß-cells that are responsible for producing the body's insulin supply. This means that individuals with T1DM are not able to produce insulin and thus are not able to use any of the glucose in the body for energy unless exogenous insulin is provided. People with T2DM are still able to produce insulin, but the insulin receptors of the cells have become resistant to the insulin, meaning that insulin is less effective in allowing cellular uptake of glucose. Both T1DM and T2DM result in high levels of blood glucose known as hyperglycemia (3,5,7,9).

Treatment for DM varies depending on the type of DM. Because people with T1DM are not able to produce insulin, insulin therapy is required—i.e., exogenous insulin must be administered to the body by injection (4). T2DM occurs due to genetic and/or lifestyle factors; specifically, the most important risk factor for T2DM is a sedentary lifestyle. Because of this, treatment often starts with lifestyle changes to promote weight loss and increase activity, but medication and insulin are also used in the treatment of T2DM (3,9).

I.2. Multifactorial Disease – Current Understanding of Risk Factors of Diabetes Mellitus

The aetiology underlying T1DM and T2DM lies in genetic and environmental factors. The key feature of T1DM, is autoimmune destruction of pancreatic ß-cells by apoptosis (10). The primary risk factor for T1DM is genetic susceptibility. More precisely, genes produced by the major histocompatibility complex, also referred to as HLA (human leukocyte antigen) account for 40-50% of familial cases of T1DM. Among the various HLA alleles, the major genetic determinants of T1DM are polymorphism of class II HLA genes encoding DQ, DR and less so, DP. Class II HLA molecules are responsible for presenting exogenous antigens to T-cells (11,12). The specific pathophysiology of T1DM is incompletely understood, but researchers have been able to identify additional non-genetic risk factors contributing to its development through twin studies. Monozygotic twins are genetically identical, meaning that, if any phenotypic variations are observed between the twins, it is due to environmental influences (13). Twin studies shed light on epigenetic changes that influence the gene expression linked to the development or progression of DM (14). From these studies, it is observed that these monozygotic twins show a 30-50% concordance rate in T1DM (15), meaning that genetic susceptibility explains half or less of the incidence of T1DM and non-genetic or environmental factors, including dietary, infectious, and toxins (11,15–18).

For instance, in the case of T1DM, it has been hypothesized that consumption of cow milk at an early age can in part be responsible for the onset of T1DM. This is due to the cow milk-containing proteins that when ingested causes mucosal inflammation resulting in increased gut permeability. This permeability allows for an increase in food antigens to enter the gut and initiate autoimmune processes, which in turn can trigger pancreatic islet inflammation and ß-cell destruction (19,20). Another example of an environmental risk factor is the exposure to toxins. During the late 19th century, many countries experienced an exponential growth of industrialization during which human labor was replaced with mechanization in a process that released harmful pollutants and toxins into the environment (21). There was a rapid increase in the incidence of T1DM in the late 19th century compared to early part of the century, concurrent with accelerated industrialization, indicating toxic exposures are a potential T1DM risk factor (19).

Risk factors for T2DM include both genetic and non-genetic factors, but non-genetic factors have a more established role. Specifically, lifestyle factors such as obesity, smoking, alcohol use, and level of physical activity all contribute to the onset of T2DM (22,23). It has also been observed that certain ethnic populations have a greater risk of development of T2DM than others. Recent findings have shown that Native Americans have a fivefold increase in overall risk compared to the general population in the United States of experiencing T2DM in their lifetime (24).

One of the most studied potential triggers for T1DM is viral infections, of which the most studied are human enteroviruses (HEV). While the exact mechanism remains unclear, it appears that viral infections such as those from HEV induce an autoimmune response resulting in the destruction of islet cells thus leading to T1DM (19). The role of viral infection in the etiology of T1DM has been extensively studied, however more recently, viral infections have also been implicated in the pathogenesis of T2DM. The two types of DM—T1DM and T2DM—are distinct diseases, but they share common etiologic features including genetic predisposition interacting with non-genetic factors including viral infection, which together mediate detrimental immunologic effects that result in DM. In this way, both T1DM and T2DM involve interactions between the endocrine and immune systems.

I.3. Endocrine System and Immune Systems – Importance, Pathways, and Relations

The body is comprised of a series of physiologic systems that work together to allow for its functioning and overall health. The endocrine system and immune system both play integral roles in homeostasis in humans. The endocrine system is responsible for maintaining balance throughout the body. This means that the endocrine system works in part to communicate with the other organs in the body and make sure that the internal environment stays within healthy thresholds. One of the main functions of the endocrine system is to aid in the production and release of necessary hormones – hormone-mediated communication. The endocrine system responds to stimuli, which triggers the release of hormones into the blood stream to then go to a specific organ and cell (25). For example, when there is a heightened concentration of plasma glucose levels, the endocrine system responds to this stimulus by triggering the release of insulin, which then in turns allows for the uptake of glucose resulting in a lowered blood glucose level (25,26). This both keeps plasma glucose levels at a healthy level but also allows organs that are in need of energy to get that glucose and derive the necessary energy from it (3,5,8,25).

Another vital physiological system is the immune system. The immune system is an intricate network of components that work together to protect the body from any potentially harmful organisms. Pathogens are pervasive and people are almost always exposed to them daily, which is where the importance of the immune system lies. To maintain a healthy state, the body's defense system recognizes, fights, and protects itself via the immune system. Two main parts constitute immunity – the innate and adaptive responses (27). Both response types occur when exposed to a pathogen. The innate response is a natural response; it is a non-specific immunity that acts as the first line of defense against a pathogen. Its goal is to prevent initial invasion and replication of the pathogen. Innate responses do not change over time as they do not have immunologic memory (28,29). The adaptive response is a response that is continuously changing over time. The more exposures are experienced, the more the adaptive response learns and adapts to provide the most optimal response against the infection (27,29).

I.4 Role of Viral Infections in DM: Mechanistic Pathway in the Development or Progression of DM

Although the precise role that viral infections play in the mechanistic pathway of the onset of DM has not been established, different hypotheses have been formulated. The main difference between the hypotheses depends on the type of virus. Viruses that result in infection of the pancreatic ß-cells can lead to destruction, causing non-immune-mediated T1D, whereas others can cause inflammation, contributing to insulin resistance and therefor inducing the onset of T2DM (Table 1) (10, 17, 18, 30-44).

Table 1. Established viral infection risk factors in DM

VIRUS	MECHANISTIC PATHWAY	REFERENCE
ENTEROVIRUS	- Production of viral proteins that resemble self-antigens - Self-antigens activate the immune system - The immune system then attacks and destroys ß-cells - Low-grade inflammation	(30,31)
RUBELLA	- Production of islet antibodies - Destruction of islet cells	(32)
MUMPS	- Directly infect and destroy ß-cells	(33)
ROTAVIRUS	- Molecular mimicry - Immune system attacks. ß-cells	(34)
CYTOMEGALOVIRUS	- Directly infect and destroy ß-cells - Low-grade inflammation	(35,36)
COVID-19	- Severe immune response - Destruction of ß-cells	(37–40)
ENCEPHALOMYOCARDITIS	- Directly infect and destroy ß-cells	(41)
COXSACKIE B	- Systemic immune response - Destruction of ß-cells - Low-grade inflammation	(10,17–19,42)
HEPITITUS C	- Systemic immune response - Destruction of ß-cells	(43,44)

I.5 Current Knowledge and Gaps on Viral Infections and DM: Need for Further Investigation

As the incidence of diabetes continues to rise, it has been suggested that a wider range of factors than initially thought could be contributing to the onset on diabetes mellitus, including viral infections (18). Current predictions are centered around the impact viral infections have on autoimmunity which in turn initiates the onset of DM (17,18). Although a significant amount of research has been conducted looking at this effect, it is still unclear, and is a long way from determining causality. Many epidemiological studies have compared associations between certain viral infection outbreaks and incidence of DM but have not come to definitive results on the topic (17). For example, associations between enteroviruses and the onset of T1D have been seen, but the mechanistic pathway and quantified impact of this viral trigger has not been established (18). This gap in knowledge illustrates the crucial role this research study plays.

II. Study Aim:

The overall aim of this study is to elucidate the mechanistic pathway through which the established risk factors of viral infections have on the initiation and progression of DM.

Research objectives

II.1. To quantitively determine whether and to what extent viral infections induce onset of DM.

II.2 To what extent do viral infections (i.e., attenuate or increase) the progression of DM.

II.3 To investigate how common antiviral treatments impact the progression and severity of DM.

Hypotheses:

II.1 We hypothesize the that the experimental group will have a significantly shorter time to DM development relative to control animals.

II.2 We hypothesize that the viral infected group will have significantly lower circulating insulin levels indicating more rapid progression.

I.3 we hypothesize that the groups treated with anti-viral agents will have higher/lower ??circulating insulin levels relative to controls indicating slower progression.

III. Approach:

Study Design:

This study is designed as an experimental study. We propose using a mouse model with a set follow-up period to investigate the impact that viral infections have on the onset and progression of T1DM and T2DM. Three different experiments will be performed (Figure 1). Each experimental design consists of two groups – one representing T1DM, and the other T2DM. Both groups will have their own control and experimental sample populations. The experimental group is subdivided into 3 categories with each category looking at a different virus. This is done via the use of different mice that will be chemically infected. The three viruses that will be chemically inoculated are: Coxsackie B Virus, Rubella Virus, and Cytomegalovirus. These viral infections were chosen based on current scientific knowledge, as these are the three most scientifically supported established risk factors of DM (10, 18, 30, 31, 32, 35, 36)

For T1DM, we will use mice that are characterized as non-obese diabetic (NOD) mice. Furthermore, these mice are susceptible to spontaneous onset of T1DM, similar to human onset (45,46). These mice are inbred over many generations in a laboratory, allowing for the mice to be genetically predisposed to autoimmune ß-cell destruction inducing T1DM (46). For type 2 diabetes, we would use *db/db* mice: these mice have a mutation in an allele in the leptin receptor that is responsible for regulating appetite. This mutation causes these mice to have defective signaling, resulting in overeating. This overeating causes obesity, which is a prominent risk factor for T2DM, allowing for a comparative model to human T2DM (24,46,47). All designs will be conducted over a time period of 9 months, with measurements taken at 1-week intervals.

Study Population:

We will restrict the study sample to mice between the age of 3 to 4 weeks at study onset, prior to their development of DM (24,46,47). These mice will be all sourced from the same laboratory and have equal proportion of males and females. This study will consist of non-obese diabetic mice to mimic T1DM, and *db/db* mice to exemplify T2DM. Both groups are predisposed to DM, and within these groups, subgroups will consist of virally inoculated mice. Mice will be excluded if they already show blood glucose levels that indicate diabetic levels at the start of the study.

Methods and techniques:

Experiment 1 is looking at how different viral infections impact the onset of DM. All mice start without any indication of DM. The experimental groups are the groups that contain mice with the three listed viral infections of interest, then be inoculated with the specific virus. RT-PCR will be conducted to ensure successful infection (46). Baseline measurements including weight, blood glucose level, insulin level, behavioral observations, and physical activity level will be obtained. Then mice will be fed a standard diet and weekly blood glucose levels measured. Once the blood glucose level reaches 250 mg/dl, the mouse is considered to have DM, and DM-level blood glucose levels typically occur in NOD mice at 10-12 weeks and in db/db mice at 5-8 weeks of age. (47,58).

Once established to have DM, the mice will enter experiment 2, which will examine how different viral infections impact the progression or severity of the disease. At the start of this phase, all the mice in the experimental groups as well as the control groups will have newly established diabetes. Both control groups will remain non-infected (will they be kept separate to avoid getting infected from the experimental mice). Baseline data will be recorded, in addition to weekly follow-up time points. Progression of disease will be assessed by looking at the severity of the disease at each of the time points. Severity will be determined by different parameters such as the fasting blood glucose level, body mass index (BMI), insulin levels, immune response markers, eating habits, and activity levels (46,47). This experiment will follow the mice for 3 months, gathering data weekly (listed in Table 2).

Experiment 3 will assess the impact that common anti-viral treatments on the progression of DM. These treatments include Enviroxime, Nitazoxanide, and ganciclovir (Table 3) (59,60,61). All the mice in this phase will have DM and will have been infected with one of the three viruses. In both experimental subgroups, the mice will be given the common anti-viral treatment for that specific virus at the start of this phase (Table 3). There will now be 6 different groups of mice within each of the two genetic variants—the NOD and the db/db: three groups—with each group infected by one virus--will receive the commonly prescribed antiviral medication, given orally and dosed by body weight. The other three groups, with each group infected by one virus, will be controls and will receive placebo. Similarly, to experiment 2, these mice will also be examined weekly for progression of the disease for a time frame of 3 months (Figure 1).

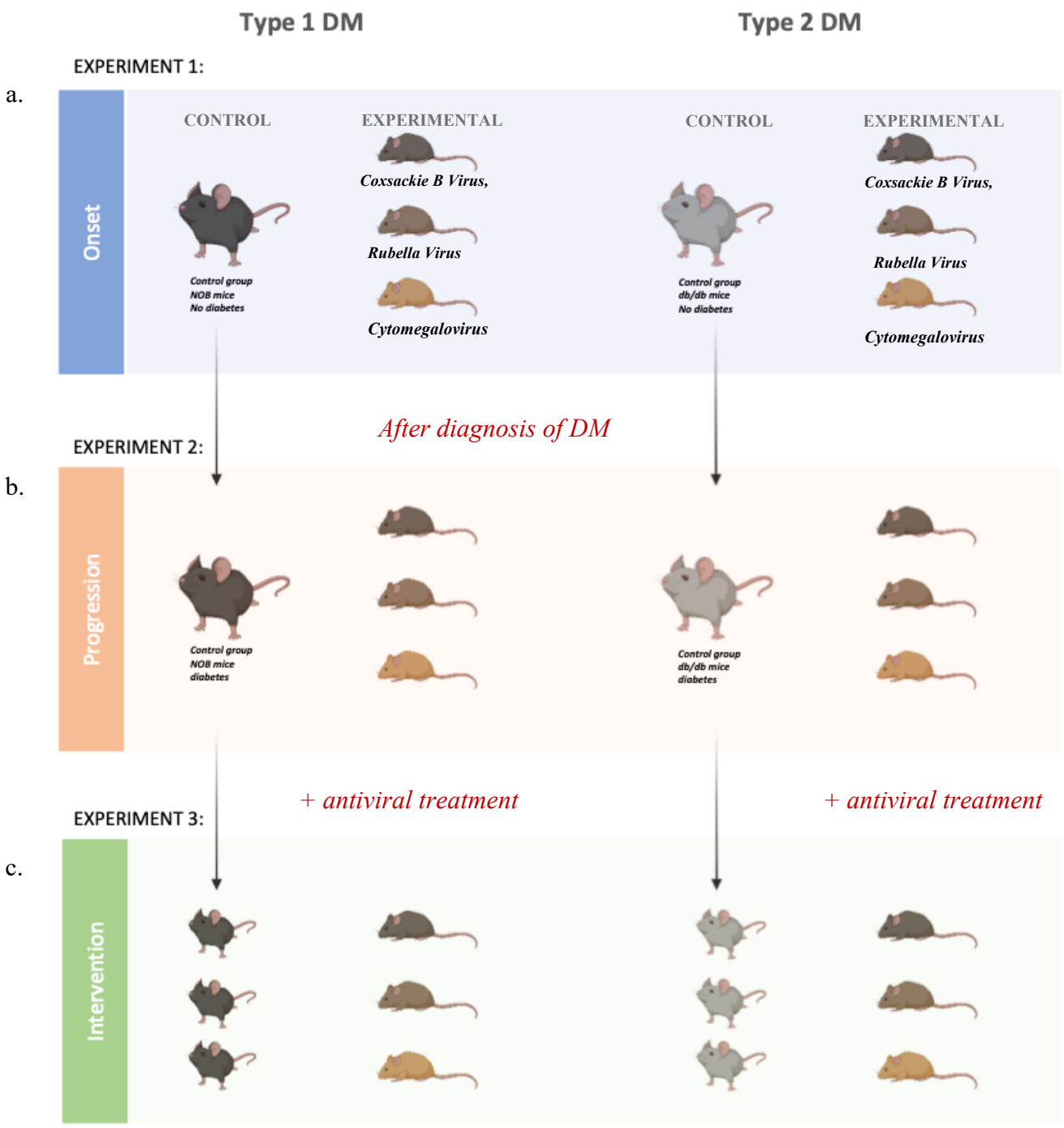

Fig. 1 Schematic overview of experimental design using a mouse model

[a.] Experiment 1: All mice start off without diabetes. Experimental groups are virally inoculated. Once the mice are diagnosed with DM, time of diagnosis is recorded, and they are moved on to be used in experiment 2.

[b.] Experiment 2: All mice start with recent DM diagnosis and a viral infection. Progression and severity are assessed over time.

[c.] Experiment 3: All mice start with DM and a viral infection. Experimental groups receive common antiviral treatment and progression, and severity of DM is assessed over time.

Sample Size Calculation:

The sample size will be calculated based on time-to-event, with the event being development of T1DM in the NOD mice or T2DM in db/db mice. Time-to-event data will be analyzed using survival analysis, and thus the sample size will be determined according to this framework. Because not all mice will experience diabetes and those that do will vary in the timing of DM development or if the mice experience other severe outcomes like death, there will be censoring involved

A precise sample size calculation will be conducted with a statistician after preliminary data collection. A statistical power of 80% and a significance of 5% will be assumed for the calculation. Given the complexity of the design, expertise from a statistician is required.

Because this is an animal model, our goal is to determine an appropriate sample size that is adequately powered and that allows use of the smallest number of animals possible, given ethical considerations around animal research (48). This will be done by combining all the control groups and experimental groups across the three experiments. Rather than using different samples of mice per experiment, efficiently combining all the experiments into different stages of the mice's lifespan will conserve the necessary sample size, adhering to ethical guidelines.

Variable definition and measurements:

Measurements will be taken from all mice that are a part of the study. When the study begins, all the data recorded will be considered at time = 0 (T_0) and will act as the starting point.

Table 2. Variable Definitions

Variable	**Definition**	**Measurement**
Blood Glucose Levels	Circulating blood glucose after an 8-hour fasting period	Diagnosis of DM threshold: 250 mg/dL
Insulin Level	Circulating insulin levels, can be used to monitor progression of DM	µU/mL
Glucose tolerance test	Assesses how quickly the mouse is able to clear glucose from blood after glucose administration	Repeated measurements in 15 minute intervals post glucose administration for 2 hours mg/dL
Viral load	Quantification of viral load in mouse to confirm successful infection	Copies/mL
Behavior	Eating, drinking, and sleeping habits	Eating and drinking = count data Sleeping = Time (Minutes)
Physical activity level	Monitoring time spent on running wheel in a 24-hour period	Time = minutes
Genetic background	NOD mouse representing T1DM *db/db* mouse representing T2DM	NOD: 0 *db/db*: 1

Table 3. Virus' of Interest and Their Associated Treatment

Viral Infection	**Common anti-viral treatment**
Coxsackie B Virus,	Enviroxime
Rubella Virus	Nitazoxanide
Cytomegalovirus	Ganciclovir

Baseline data:

Age, mother (if siblings are used), weight, and calculated BMI will be taken as a first step to create a baseline for all the mice. Blood tests including fasting blood glucose levels, insulin levels, and a lipid profile will be obtained from each mouse. Physical activity level will be assessed by recording time spent on running wheel in the span of a day. The data will be taken at the same time every week to ensure consistency since glucose and insulin levels greatly fluctuate depending on timing and food intake.

Table 4. Data collection table at baseline, and weekly follow-up time points

VARIABLE	MEASUREMENT AT T_{X*}
AGE	
SEX	
MOTHER	
WEIGHT	
BMI	
BLOOD GLUCOSE LEVELS	
INSULIN LEVEL	
GLUCOSE TOLERANCE TEST	
VIRAL LOAD	
BEHAVIOR	
PHYSICAL ACTIVITY LEVEL	
GENETIC BACKGROUND	

$x*$ = 0, week 1, week 2, week 3, etc.

Measures of association

Experiment 1:

Because the outcome is the onset of diabetes, this is considered a time-to-event outcome. After follow-up time is complete, a Cox regression model will be run. The time until the mouse developed diabetes will be used or will be considered censored if the mouse did not develop diabetes in the given time frame. After confirming the Cox regression assumptions have been met, statistical software will be used to fit the regression model. The control group will be used as the reference group for each of the virally induced experimental groups. From there, hazard ratios – the instantaneous risk of onset of diabetes -- will be calculated per group.

Experiment 2:

To look at the effect that viruses have on the progression of diabetes, a linear regression model will be used. Progression will be assessed by measured circulating insulin levels (indicator of DM severity) a continuous outcome variable-- of the different groups. Using the control as the reference group, the strength and direction will be compared within the different viral groups.

Experiment 3:

To look at the effect that anti-viral treatment has on the progression of diabetes, a linear regression model will be used with the same dependent variable—circulating insulin level—as in Experiment 2. Three separate models for the three different viruses being investigated will be conducted. The control group receiving placebo will be used as the reference. Changes in magnitude and direction will be evident per virus.

Time Management:

This study is broken down into 6 stages. The first stage consists of doing an extensive literature review on the topic. Since viral infections are already an established risk factor of DM, understanding the current knowledge and gaps on the topic is essential. From there, the hypotheses will be adjusted if needed. Due to the use of a mouse model, approval from an ethics board is required. While waiting for approval, techniques that will be used for data collection will be practiced, ensuring efficient use of time. Once approved, preliminary data will be collected, and the current protocol will be adjusted if necessary. During this stage, we will test the feasibility of the experimental design and identify and correct any issues that arise before starting the main experiment. Additionally, doses, timing, and measurements will be assessed and adjusted. Stage 3 is when the main experiment and data collection will take place. The updated protocol will be implemented, and data collection from the mice will be gathered weekly. Laboratory mice typically have a lifespan of 1-2 years, but the duration of this experimental stage of this study will be 9 months (41). It is likely that the duration of this stage will be less depending on how having both a viral infection and DM will decrease their lifespan. Once data are collected, stage 5 will begin where a statistical software package such as SPSS or RStudio will be used to conduct the described data analyses. During this stage, the research team will expand to include statisticians to ensure accuracy. Once analyses are complete, the final stage, stage 6 is the time allotted for preparation and submission of the manuscript. In total, it is estimated that this study will take approximately 24 months.

Project Timeline:

Table 3. Proposed schedule and allotted time for study duration

Stage	Timeline	Aim

1	Months 1-2	- Review existing literature
		  o Identify what is already known
		  o Establish gaps in knowledge
		- Establish Hypothesis

2	Month 3-4	- Learn study techniques and adjust study proposal if needed - Submit application for ethics board approval to mouse model
3	Month 5-6	- Conduct preliminary experiments - Adjust protocols if necessary
4	Months 7-16	- Conduct main experiments - Collect data weekly from mouse models
5	Months 17-20	- Thorough statistical analysis on the collected data
6	Months 21-24	- Final manuscript of protocol, findings, and clinical implications - Submit manuscript for publication

IV. Feasibility and Risk Assessment:

The proposed research aims to investigate the impact viral infections have on the onset and progression of DM. To do this, three different viral infections are used, and the experiment is executed using a mouse model. The overall feasibility of this study is significant and supported by previous studies that have successfully utilized this method to investigate the association of interest (10,34,41). To execute this study, a laboratory containing the necessary tools and resources to do such experiments is essential.

Despite the successful existing scientific literature on this topic, there is still a potential for risks. Some of the main risks being the ones associated with the use of mouse models. Animal models, and specifically mouse models have been widely used in biomedical research and has become an indispensable tool for novel scientific

discoveries (49). The results of mouse studies are extrapolated to humans, bringing forth issues on translation and comparability. Mice and humans are different in terms of their genetics, physiology, anatomy, lifespan, and disease presentation. In turn, differences like the immune system, manifestation, and progression of diseases like DM may significantly differ between mice and humans causing potential limitations. If there are large differences between the immune response to a viral infection between mice and humans, the applicability of the results can be compromised (50).

An alternative approach to study this association can be done using in vitro stem cells. These stem cells, especially induced pluripotent stem cells (iPSCs), are human cells that can produce pancreatic ß-cells, which are the main cell linked to diabetes (DM) (51). Then, these cells can be exposed to a specific virus to evaluate if the infection has any impact on cell survival or function. Although using human cells makes this method favorable, it lacks the systemic complexity and physiological significance of animal models, especially when researching a multifactorial disease like DM. This in vitro method would be most beneficial to use in conjunction with a mouse model to get a comprehensive understanding.

Ultimately, this proposed research is both feasible and significant despite potential risks. These risks will be managed and mitigated in a cautiously-derived study design in order to provide valuable insight into the role viral infections have on the onset and progression of DM.

V. Scientific impact:

Research regarding the topic of diabetes mellitus has been a prime focus in the science community in recent years. Uncovering unknown mechanisms regarding the effect viral infections have on the onset and progression of DM is just the start. It will have profound scientific implications for both short-range and long-range perspectives (52). Results from this study will lead to further research, expanding on how viral infections other than the ones studied impact DM, and also how other forms of non-viral diseases, such as hypertension can have similar adverse health outcomes. Additionally, the results will spark interest into looking deeper into how viral infections impact other chronic diseases as well, such as Alzheimer's disease. Further research into specific mechanisms that these viral infections effect will lead to further efforts into developing more effective antiviral treatment that will benefit more people.

VI. Societal Impact:

Due to the increasing global burden of diabetes mellitus, understanding ways in which the possible onset or progression of the disease can be prevented would have significant societal implications. These implications include disease prevention, management, as well as advancing public health policies regarding diabetes mellitus. DM is a continuously growing concern worldwide and is associated with severe health complications if not treated properly. These health complications from either type of DM include cardiovascular disease, liver and kidney damage, nerve health, and a decline in mental health. If not treated properly, likelihood of these adverse health outcomes increases tremendously and are able to cause permanent damage to the individual (53). Treatments for DM vary depending on the type of the disease and progression or severity, but the most common treatments include exogenous administration of insulin, diet, and adjustment of lifestyle factors (3,16,22).

Because diabetic patients have a constant need for blood sugar regulation, they greatly rely on the availability and accessibility of insulin (6). Insulin is not always available or accessible to everyone that needs it. In fact, one in two people who need insulin do not have access to it (54). This points to the importance of preventing DM and gaining this deeper understanding of established triggers of DM, such as viral infections. Preventative measures can be put into place, delaying onset of the disease, and allowing substantial individual and societal cost savings.

This new knowledge of risk factors will aid public health officers in creating and implementing policies regarding the association between viral infections and DM. For instance, policies can be implemented that prioritize viral infection prevention methods in high-risk areas and populations. Methods such as increased access to antiviral treatments, spreading awareness of viral prevention, as well as increased vaccination funding can be put into action (52).

A more immediate impact from this understanding is found in personalized treatment methods for those who have DM. By knowing whether and by what degree a viral infection exacerbates DM, medical providers can employ personalized treatment plans of those affected. Adjusting treatment plans to avoid any potential interaction between a viral infection and DM will reduce the likelihood of any further diabetes-related complications.

Overall, this deeper understanding of the impact viral infections have on the onset or progression of DM will produce countless societal implications, both short-and long- term.

VII. Ethical Considerations:

This research requires the intentional induction of viral infections with the expectation of observing the onset of diabetes. Because of this, the study method cannot be conducted using human patients as participants. Intentionally exposing someone to a known pathogen would be unethical.

The use of mouse models has been a recurring method in biomedical research. Despite all the benefits it brings, the ethical aspects behind using animal models still needs to be considered. One of the main concerns using this method for research purposes lies in a concept known as "the 3 Rs: Replacement, Reduction, and Refinement" (55). This principle stresses replacing animal models with a different method whenever possible, reducing the number of animals used, and ensuring measures are taken to minimize animal suffering as much as possible (55). Due to this study being centered around the onset and progression of DM, both human subjects and in vitro cell culture would not be options. In vitro cell culture would not allow us to observe changes in animal behavior. This study will adhere to the necessary guidelines regarding the use of animal models.

When using animal models, it is important to use the smallest feasible sample size as possible to avoid exposing too many mice to harmful health conditions, while at the same time, making sure to use an adequate number to ensure sufficient power to the statistical analyses. If too few are used, and precise and meaningful results are not achieved, the use of the animals that were used would be unjustified (55,56). This study requires exposure to harmful conditions like DM and viral infections, so too small of a sample resulting in statistically insignificant results would not justify the ethical implications of subjecting the mice to such adverse conditions (56).

In summary, ethical considerations when using a mouse model to investigate the impact of viral infections on onset and progression of DM will be of the utmost importance. These considerations will be followed with care, ensuring the pursuit of scientific advancement will not overshadow the welfare of the mice. Full transparency of the methods, treatments, and care of the animals will be recorded.

References:

1. Lin X, Xu Y, Pan X, Xu J, Ding Y, Sun X, et al. Global, regional, and national burden and trend of diabetes in 195 countries and territories: an analysis from 1990 to 2025. Sci Rep. 2020 Dec 1;10(1).
2. global incidence.
3. Egan Se AM, Dinneen F. What is diabetes? [Internet]. Available from: http://www.who.int/
4. Ikegami H, Noso S, Babaya N, Kawabata Y. Genetics and pathogenesis of type 1 diabetes: prospects for prevention and intervention. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.2040-1124.2011.00176.x,
5. Paris Hantzidiamantis AJ, Lappin Affiliations SL. Physiology, Glucose. 2023.
6. Wilcox G. Insulin and Insulin Resistance. Vol. 26, Clin Biochem Rev. 2005.
7. Jensen M V, Joseph JW, Ronnebaum SM, Burgess SC, Sherry AD, Newgard CB, et al. Metabolic cycling in control of glucose-stimulated insulin secretion. Am J Physiol Endocrinol Metab [Internet]. 2008;295:1287–97. Available from: http://www.ajpendo.org
8. Komatsu M, Takei M, Ishii H, Sato Y. Glucose-stimulated insulin secretion: A newer perspective. Available from: https://onlinelibrary.wiley.com/doi/10.1111/jdi.12094
9. Rother KI. Diabetes Treatment-Bridging the Divide.
10. De Beeck AO, Eizirik DL. Viral infections in type 1 diabetes mellitus-why the β cells? Vol. 12, Nature Reviews Endocrinology. Nature Publishing Group; 2016. p. 263–73.
11. Pociot F, Lernmark Å. Type 1 diabetes 1 Genetic risk factors for type 1 diabetes [Internet]. Vol. 387, www.thelancet.com. 2016. Available from: http://www.diabetesgenes.
12. Matthew Giwa A, Ahmed R, Omidian Z, Majety N, Ege Karakus K, Omer SM, et al. Current understandings of the pathogenesis of type 1 diabetes: Genetics to environment. Available from: https://dx.doi.org/10.4239/wjd.v11.i1.13
13. Hannon E, Knox O, Sugden K, Burrage J, Wong CCY, Belsky DW, et al. Characterizing genetic and environmental influences on variable DNA methylation using monozygotic and dizygotic twins. PLoS Genet. 2018 Aug 1;14(8).
14. Ma LL, Wu YL, Dong Y, Tanaka Y, Zhang W. Twin-Studies on the Role of Non-Genetic Factors in T1D Pathogenesis. 2017.
15. Lloyd RE, Tamhankar M, And˚andåke Lernmark A. Downloaded from www.annualreviews.org Access provided by 185. Annu Rev Med 2022 [Internet]. 2021;73:218. Available from: https://doi.org/10.1146/annurev-med-042320-
16. Kaprio J, Tuomilehto J, Koskenvuo M, Romanov K, Reunanen A, Eriksson J, et al. Concordance for Type 1 (insulin-dependent) and Type 2 (non-insulin-dependent) diabetes mellitus in a population-based cohort of twins in Finland. 1992.
17. Viruses and type 1 diabetes: ignorance acquires a better vocabulary | Enhanced Reader.
18. Van Der Werf N, Kroese FGM, Rozing J, Hillebrands JL. Viral infections as potential triggers of type 1 diabetes. Diabetes Metab Res Rev [Internet]. 2007;23:169–83. Available from: www.interscience.wiley.com
19. Butalia S, Kaplan GG, Khokhar B, Rabi DM. Environmental Risk Factors and Type 1 Diabetes: Past, Present, and Future. Vol. 40, Canadian Journal of Diabetes. Elsevier B.V.; 2016. p. 586–93.
20. Vaarala O, Atkinson MA, Neu J. The “perfect storm” for type 1 diabetes: The complex interplay between intestinal microbiota, gut permeability, and mucosal immunity. Vol. 57, Diabetes. 2008. p. 2555–62.
21. Szreter S. Industrialization and health. Br Med Bull [Internet]. 2004;69:75–86. Available from: https://academic.oup.com/bmb/article/69/1/75/523332
22. Chatterjee S, Khunti K, Davies MJ. Type 2 diabetes. Vol. 389, The Lancet. Lancet Publishing Group; 2017. p. 2239–51.
23. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Kaabi J Al. Epidemiology of Type 2 diabetes - Global burden of disease and forecasted trends. J Epidemiol Glob Health. 2020 Mar 1;10(1):107–11.
24. Haffner SM. O R I G I N A L Epidemiology of Type 2 Diabetes: Risk Factors [Internet]. Available from: http://diabetesjournals.org/care/article-pdf/21/Supplement_3/C3/448166/21-3-c3.pdf
25. Hiller-Sturmhöfel S, Bartke A. The Endocrine System An Overview.
26. Komatsu M, Takei M, Ishii H, Sato Y. Glucose-stimulated insulin secretion: A newer perspective. Vol. 4, Journal of Diabetes Investigation. 2013. p. 511–6.
27. Parkin J, Cohen B. An overview of the immune system. Vol. 357, Lancet. Elsevier B.V.; 2001. p. 1777–89.
28. Koyama S, Ishii KJ, Coban C, Akira S. Innate immune response to viral infection. Vol. 43, Cytokine. 2008. p. 336–41.
29. Eter P, Elves JD, Oitt VMR. T HE I MMUNE S YSTEM First of Two Parts. Vol. 343. 2000.

30. Richardson SJ, Morgan NG. Enteroviral infections in the pathogenesis of type 1 diabetes: new insights for therapeutic intervention. Vol. 43, Current Opinion in Pharmacology. Elsevier Ltd; 2018. p. 11–9.
31. Rodriguez-Calvo T, Von Herrath MG. Enterovirus infection and type 1 diabetes: Closing in on a link? Vol. 64, Diabetes. 2015.
32. Banerjee RR, Cyphert HA, Walker EM, Chakravarthy H, Peiris H, Gu X, et al. Gestational diabetes mellitus from inactivation of prolactin receptor and MafB in islet β-cells. Diabetes. 2016;65(8).
33. Zhao Z, Jiang X, Kang C, Xiao Y, Hou C, Yu J, et al. Blockade of the T cell immunoglobulin and mucin domain protein 3 pathway exacerbates sepsis-induced immune deviation and immunosuppression. Clin Exp Immunol. 2014 Nov 1;178(2):279–91.
34. Honeyman MC, Coulson BS, Stone NL, Gellert SA, Goldwater PN, Steele CE, et al. Association between rotavirus infection and pancreatic islet autoimmunity in children at risk of developing type 1 diabetes. Diabetes. 2000;49(8).
35. Osame K, Takahashi Y, Takasawa H, Watanabe S, Kishimoto M, Yasuda K, et al. Rapid-onset type 1 diabetes associated with cytomegalovirus infection and islet autoantibody synthesis. Internal Medicine. 2007;46(12).
36. Pak CY, Mcarthur RG, Eun HM, Yoon JW. ASSOCIATION OF CYTOMEGALOVIRUS INFECTION WITH AUTOIMMUNE TYPE 1 DIABETES. The Lancet. 1988;332(8601).
37. COVID-19 infection may cause ketosis and ketoacidosis | Enhanced Reader.
38. New-onset diabetes in “long COVID.” 2021; Available from: https://onlinelibrary.wiley.com/doi/10.1111/1753-0407.13187
39. Boddu SK, Aurangabadkar G, Kuchay MS. New onset diabetes, type 1 diabetes and COVID-19. Vol. 14, Diabetes and Metabolic Syndrome: Clinical Research and Reviews. Elsevier Ltd; 2020. p. 2211–7.
40. Papadokostaki E, Tentolouris N, Liberopoulos E. COVID-19 and diabetes: What does the clinician need to know? Vol. 14, Primary Care Diabetes. 2020.
41. Yoon JW, Morishima T, Mcclintock PR, Austin M, Notkins AL. Virus-Induced Diabetes Mellitus: Mengovirus Infects Pancreatic Beta Cells in Strains of Mice Resistant to the Diabetogenic Effect of Encephalomyocarditis Virus [Internet]. Vol. 50, JOURNAL OF VIROLOGY. 1984. Available from: https://journals.asm.org/journal/jvi
42. Ashton MP, Eugster A, Walther D, Daehling N, Riethausen S, Kuehn D, et al. Incomplete immune response to coxsackie B viruses associates with early autoimmunity against insulin. Sci Rep. 2016;6.
43. Ribaldone DG, Sacco M, Saracco GM. The effect of viral clearance achieved by direct-acting antiviral agents on hepatitis c virus positive patients with type 2 diabetes mellitus: A word of caution after the initial enthusiasm. Vol. 9, Journal of Clinical Medicine. 2020.
44. Adinolfi LE, Rinaldi L, Marrone A, Giordano M. The effect of sustained virological response by direct-acting antivirals on insulin resistance and diabetes mellitus in patients with chronic hepatitis C. Vol. 16, Expert Review of Anti-Infective Therapy. 2018.
45. (No Title). 2005; Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1464-5491.2005.01499.x
46. King AJ. The use of animal models in diabetes research Keywords type 1 diabetes; type 2 diabetes; animal models. 2012; Available from: http://onlinelibrary.wiley.com/journal/
47. Sharma K, McCue P, Dunn SR. Diabetic kidney disease in the db/db mouse. 2003; Available from: http://www.ajprenal.org
48. Charan J, Kantharia ND. How to calculate sample size in animal studies? J Pharmacol Pharmacother [Internet]. Available from: http://www.3rs-reduction.co.uk/html/6__power_and_sample_size.html,http://www.acuc.berkeley.edu/,http://www.bu.edu/orccommittees/iacuc/policies-and-guidelines/sample-size-calculations/,http://www.ucd.ie/researchethics/etc.
49. Nagaya M, Hasegawa K, Uchikura A, Nakano K, Watanabe M, Umeyama K, et al. World Journal of Diabetes Feasibility of large experimental animal models in testing novel therapeutic strategies for diabetes Conflict-of-interest statement. World J Diabetes [Internet]. 2021;12(4):306–30. Available from: https://www.f6publishing.com
50. Chen YG, Mathews CE, Driver JP. The role of NOD mice in type 1 diabetes research: Lessons from the past and recommendations for the future. Front Endocrinol (Lausanne). 2018 Feb 23;9(FEB).
51. Hossain MK, Dayem AA, Han J, Saha SK, Yang GM, Choi HY, et al. Recent advances in disease modeling and drug discovery for diabetes mellitus using induced pluripotent stem cells. Vol. 17, International Journal of Molecular Sciences. MDPI AG; 2016.
52. Hayden MR. Type 2 diabetes mellitus increases the risk of late-Onset Alzheimer’s disease: Ultrastructural remodeling of the neurovascular unit and diabetic gliopathy. Brain Sci. 2019;9(10).

53. Ahamed BS, Arya MS, Sangeetha SKB, Auxilia Osvin N V. Diabetes Mellitus Disease Prediction and Type Classification Involving Predictive Modeling Using Machine Learning Techniques and Classifiers. Genet Res (Camb). 2022;2022.
54. Insulin prices, availability and affordability in 13 low-income and middle-income countries | Enhanced Reader.
55. Cleary SJ, Pitchford SC, Richard |, Amison T, Carrington | Robert, Lorena | C, et al. Animal models of mechanisms of SARS-CoV-2 infection and COVID-19 pathology. 2020; Available from: http://onlinelibrary.wiley.com/doi/10.1111/bph.v177.21/issuetoc
56. Moore RJ, Stanley D. Experimental design considerations in microbiota/inflammation studies. Vol. 5, Clinical and Translational Immunology. John Wiley and Sons Inc; 2016.
57. Korstanje, R., Ryan, J. L., Savage, H. S., Lyons, B. L., Kane, K. G., & Sukoff Rizzo, S. J. (2017). Continuous glucose monitoring in female nod mice reveals daily rhythms and a negative correlation with body temperature. *Endocrinology*, *158*(9), 2707–2712. https://doi.org/10.1210/en.2017-00266
58. Bogdanov, P., Corraliza, L., A. Villena, J., Carvalho, A. R., Garcia-Arumí, J., Ramos, D., Ruberte, J., Simó, R., & Hernández, C. (2014). The DB/DB Mouse: A useful model for the study of diabetic retinal neurodegeneration. *PLoS ONE*, *9*(5). https://doi.org/10.1371/journal.pone.0097302
59. professional CC medical. Cytomegalovirus (CMV) infection: Causes & symptoms [Internet]. [cited 2023 Jul 12]. Available from: https://my.clevelandclinic.org/health/diseases/21166-cytomegalovirus#:~:text=A%20provider%20can%20treat%20CMV,born%20with%20symptoms%20of%20CMV.
60. Perelygina L, Hautala T, Seppänen M, Adebayo A, Sullivan KE, Icenogle J. Inhibition of rubella virus replication by the broad-spectrum drug nitazoxanide in cell culture and in a patient with a primary immune deficiency. Antiviral Research. 2017;147:58–66. doi:10.1016/j.antiviral.2017.09.019
61. Honkimaa A, Sioofy-Khojine A-B, Oikarinen S, Bertin A, Hober D, Hyöty H. Eradication of persistent coxsackievirus B infection from a pancreatic cell line with clinically used antiviral drugs. Journal of Clinical Virology. 2020;128:104334. doi:10.1016/j.jcv.2020.104334