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Wirkung von ARA290 auf Körpergewicht und Blutglukose von adipösen Mäusen / Effects of ARA290 on body weight and blood glucose in obese mice

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Abstract

Due to increased calorie intake and decreased physical activity, Diabetes mellitus increasingly becomes a global burden. As numbers of patients are still rising, it is necessary to develop novel treatment options. In male obese mice, erythropoietin (EPO) has been identified as a potential mediator of improvements in blood glucose and insulin sensitivity, but clinical utility is limited by EPO´s erythropoietic activity, which causes an increase in the hematocrit. ARA290, a non-hematopoietic peptide developed from the structure of EPO, is believed to retain EPO´s potential for amelioration of obesity-associated metabolic disturbances without affecting the hematocrit. ARA290 selectively interacts with a receptor complex consisting of EPO-receptor (EpoR) and beta common receptor (βcR) (the latter a mediator of tissue protection). Several preceding studies reported glucose lowering action of ARA290 in laboratory rodents, but so far beneficial action has only been shown in conjunction with weight loss. The present study therefore aimed to sort out whether ARA290-induced lowering of blood glucose is secondary to weight loss or a direct, *i.e.* weight-independent, effect. C57BL/6J mice, which were obese due to high fat diet (HFD), received three intraperitoneal injections (i.p.) per week of the vehicle or of ARA290 over a period of 85 days (75 µg/kg ARA290 until day 27; 150 µg/kg thereafter). Basal blood glucose, glucose tolerance, plasma insulin, and other metabolic parameters were documented. No effects of ARA290 on blood glucose or body weight were detected in male obese mice, which is at variance to findings from previous studies. These results moderate hopes that ARA290 could be an effective anti-diabetic compound with potential in the treatment of hyperglycemia and insulin resistance (IR).

Zusammenfassung

Durch erhöhte Kalorienzufuhr und verminderte körperliche Aktivität wird Diabetes mellitus zunehmend zu einer globalen Belastung. Da die Zahl der Patienten weiterhin steigt, ist es notwendig, neue Behandlungsmöglichkeiten zu entwickeln. Bei männlichen adipösen Mäusen wurde Erythropoeitin (EPO) als potenzieller Mediator für Verbesserungen der Blutglukosekonzentration und der Insulinsensitivität identifiziert. Der klinische Nutzen ist jedoch durch die hämatopoetische Aktivität von EPO begrenzt, die einen Anstieg des Hämatokrits bewirkt. ARA290, ein nicht-hämatopoetisches Peptid, das aus der Struktur von EPO entwickelt wurde, soll das Potenzial von EPO zur Verbesserung von Adipositas-assoziierten Stoffwechselstörungen beibehalten, ohne den Hämatokrit zu beeinflussen. ARA290 interagiert selektiv mit einem Rezeptorkomplex, der aus dem EPO-Rezeptor und dem beta common Rezeptor (βcR) besteht (letzterer ein Mediator der Gewebeprotektion). Mehrere vorangegangene Studien berichteten über die glukosesenkende Wirkung von ARA290 bei Labornagern, aber bisher wurde eine positive Wirkung nur in Verbindung mit einer Gewichtsabnahme gezeigt. In der vorliegenden Studie sollte daher geklärt werden, ob die ARA290-induzierte Senkung der Blutglukosekonzentration sekundär der Gewichtsabnahme auftritt oder ein direkter, d.h. gewichtsunabhängiger Effekt ist. Männliche C57BL/6J-Mäuse, die aufgrund einer fettreichen Diät (HFD) adipös waren, erhielten drei intraperitoneale Injektionen (i.p.) pro Woche des Vehikels oder von ARA290 über einen Zeitraum von 85 Tagen (75 µg/kg ARA290 bis Tag 27; danach 150 µg/kg). Nüchternblutglukose, Glukosetoleranz, Plasma-Insulin und andere Stoffwechselparameter wurden dokumentiert. Bei männlichen adipösen Mäusen wurden keine Effekte von ARA290 auf die Blutglukose oder das Körpergewicht festgestellt, was im Widerspruch zu Ergebnissen aus früheren Studien steht. Diese Ergebnisse schwächen die Hoffnung, dass ARA290 ein wirksamer antidiabetischer Wirkstoff mit Potenzial zur Behandlung von Hyperglykämie und Insulinresistenz (IR) sein könnte.

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Abbreviations

ADA	American Diabetes Association
ATP	Adenosine triphosphate
βcR	Beta common receptor
CVD	Cardiovascular disease
EPO	Erythropoietin
EpoR	Erythropoietin receptor
FPG	Fasting plasma glucose
GCK	Glucokinase
GDM	Gestational diabetes mellitus
GLUT	Glucose transporter
HbA1c	Hemoglobin A1c
HFD	High fat diet
i.p.	Intraperitoneal
IPGTT	Intraperitoneal glucose tolerance test
IR	Insulin resistance
LADA	Latent autoimmune diabetes in adults
MODY	Maturity onset diabetes of the young
OGTT	Oral glucose tolerance test
PBS	Phosphat puffer saline
rHEPO	Recombinant human EPO
T1DM	Type 1 diabetes mellitus
T2DM	Type 2 diabetes mellitus

1. INTRODUCTION

1.1 Regulation of blood glucose

Carbohydrates, besides protein and fat, are one out of three main components of a normal diet and also represent an important source of energy. Carbohydrates can cover 60% and more of our daily energy demands. After a meal, the digestive system breaks down ingested carbohydrates into small molecules. Starch is the most common carbohydrate in our food, which is broken down to glucose in the intestine and then absorbed into the bloodstream. From there glucose has access to the organs and the different cell types of our body, where it is an indispensable fuel and a source of chemical energy (Holesh J. E. et al., 2021).

In the healthy state, the concentration of glucose in blood is kept within a narrow target range by a process, which is called ‚blood glucose homeostasis'. In humans, blood glucose levels should be between 72 to 108 mg/dL. Under starving and specific disease conditions, the concentration of blood glucose decreases to levels below the normal range, which is referred to as hypoglycemia (Mathew T. K. et al., 2021). The opposite would be hyperglycemia, which means blood glucose above the normal range and can result from pathological states like a deficiency in the glucose lowering hormone insulin or resistance to insulin. At the extremes, both conditions, hypoglycemia and hyperglycemia, can cause life threatening scenarios (Mouri M. et al., 2021).

Lowering of blood glucose is driven by the anabolic hormone insulin. Insulin is an endogenous storage signal, it is produced in the pancreatic beta cells and released in response to increased blood glucose. It stimulates many cells like, e.g., fat and muscle cells to take up glucose and to store it as glycogen. In hepatocytes, glucose uptake is insulin independent, but its storage as glycogen is stimulated by insulin, as it is also the case in fat and muscle. Cells need specific proteins, so called glucose transporters (GLUT), to take up glucose, whereby the subtype GLUT4 is responsible for insulin-stimulated glucose uptake (Bopp A., 2001).

After a longer fasting period, when blood glucose and, as a result, plasma insulin drop, low glucose is a signal for the liver to activate glycogenolysis (the breakdown of glycogen stores) and gluconeogenesis (the production of glucose from precursor molecules like amino acids

or lactate) so that glucose is released into the blood and made available to other organs. Under conditions of low circulating glucose, hepatic production and release of glucose is stimulated by glucagon, an insulin-antagonistic hormone produced in the alpha cells of the pancreatic islets of Langerhans (Robber H. et al., 1981).

1.2 Insulin

1.2.1 Insulin biosynthesis

Insulin is a peptide hormone produced by pancreatic beta cells. It consists of two polypeptide chains, the A-chain and the B-chain, which are linked by disulfide bonds. Initially, a single polypeptide called preproinsulin is translated and synthesized in the beta cell, which beside the insulin A- and B-chain contains the so-called C-peptide and a signal peptide. The signal peptide is cleaved after preproinsulin enters the endoplasmic reticulum. The residual peptide is referred to as proinsulin, which is then packed into the secretion vesicle. There the C-peptide, an amino acid sequence that connects the A- and the B-chain, is cleaved and the final insulin molecule is formed for release into the bloodstream. C-peptide is released at an equimolar rate with insulin, but it is of minor relevance to metabolic regulation (Müller-Esterl W. et al., 2011).

1.2.2 Insulin release and lowering of blood glucose

Circulating nutrients, in particular blood glucose, are important regulators of insulin secretion (Ritzel R. A. et al., 2003). In the insulin producing beta cells of the pancreatic islets of Langerhans, the signal of an elevated circulating glucose concentration is converted into amplification of insulin release in a multi-stage process (Müller-Esterl W. et al., 2011) (Figure 1). Glucose enters pancreatic beta cells through facilitated diffusion via GLUT2. Inside the beta cell, glucose is phosphorylated and converted to glucose-6-phosphate by the enzyme glucokinase. Via glycolysis and glucose oxidation the molecule is then converted to carbon dioxide and water, giving rise to the main source of chemical energy in our cells, adenosine triphosphate (ATP). Hence, increased glucose supply will result in increased ATP production and a rising ATP concentration within the cell. In consequence, ATP-sensitive potassium channels (K_{ATP} channels) in the cell membrane are closed, which leads to depolarization.

Depolarization in turn is the trigger for the opening of voltage-gated calcium channels, which results in a rise of the intracellular calcium concentration. This rise is amplified by opening of voltage-dependent calcium channels located on the endoplasmic reticulum, which releases additional calcium into the cytoplasm. The increase in cytosolic calcium finally triggers the release of insulin, which is stored in granules within intracellular vesicles, into the bloodstream. This process is referred to as exocytosis (Figure 1) (Müller-Esterl W. et al., 2011).

Once in the circulation, insulin binds to specific receptors located in the membrane of target cells, such as hepatocytes, adipocytes, and muscle cells. Insulin has a variety of effects that can be categorized as anabolic, which means that insulin promotes the storage of fuel molecules. Insulin stimulation promotes uptake and use of glucose by insulin-sensitive tissues like muscle and fat. To this end, insulin causes the translocation of GLUT4 glucose transporters into cell membrane of adipocytes and muscle cells. When blood glucose increases, more glucose can therefore be taken up into the cell via the GLUT4 transporter. Inside the cell, insulin provokes that more glucose is oxidized and more glucose is stored as glycogen, while glycogenolysis is inhibited (Müller-Esterl W. et al., 2011). The quantitatively most important tissues for insulin-stimulated glycogen storage are muscle and liver. Additionally, insulin turns off gluconeogenesis and, hence, the rate of glucose release from the liver. The shift of glucose into glycogen stores along with inhibition of hepatic glucose production finally results in the lowering of blood glucose. As an anabolic storage signal, insulin interferes not only with carbohydrate metabolism, but also with lipid and protein metabolism through enhancing lipogenesis in association with inhibition of lipolysis, as well as through promotion of protein synthesis in association with inhibition of proteolysis. As a result, fat and protein will be stored. Beside the immediate effects of insulin on cellular metabolism, which are mainly induced via activation or deactivation of relevant enzymes, insulin can also exert delayed effects via promoting the expression of insulin sensitive genes (Müller Esterl W. et al., 2001).

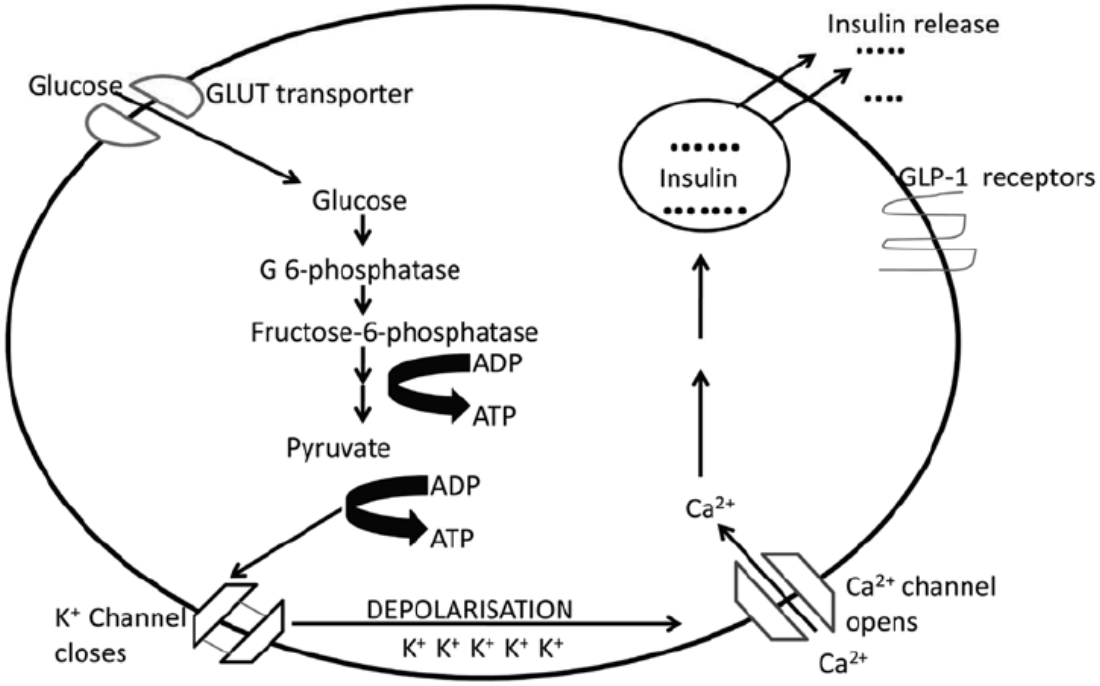
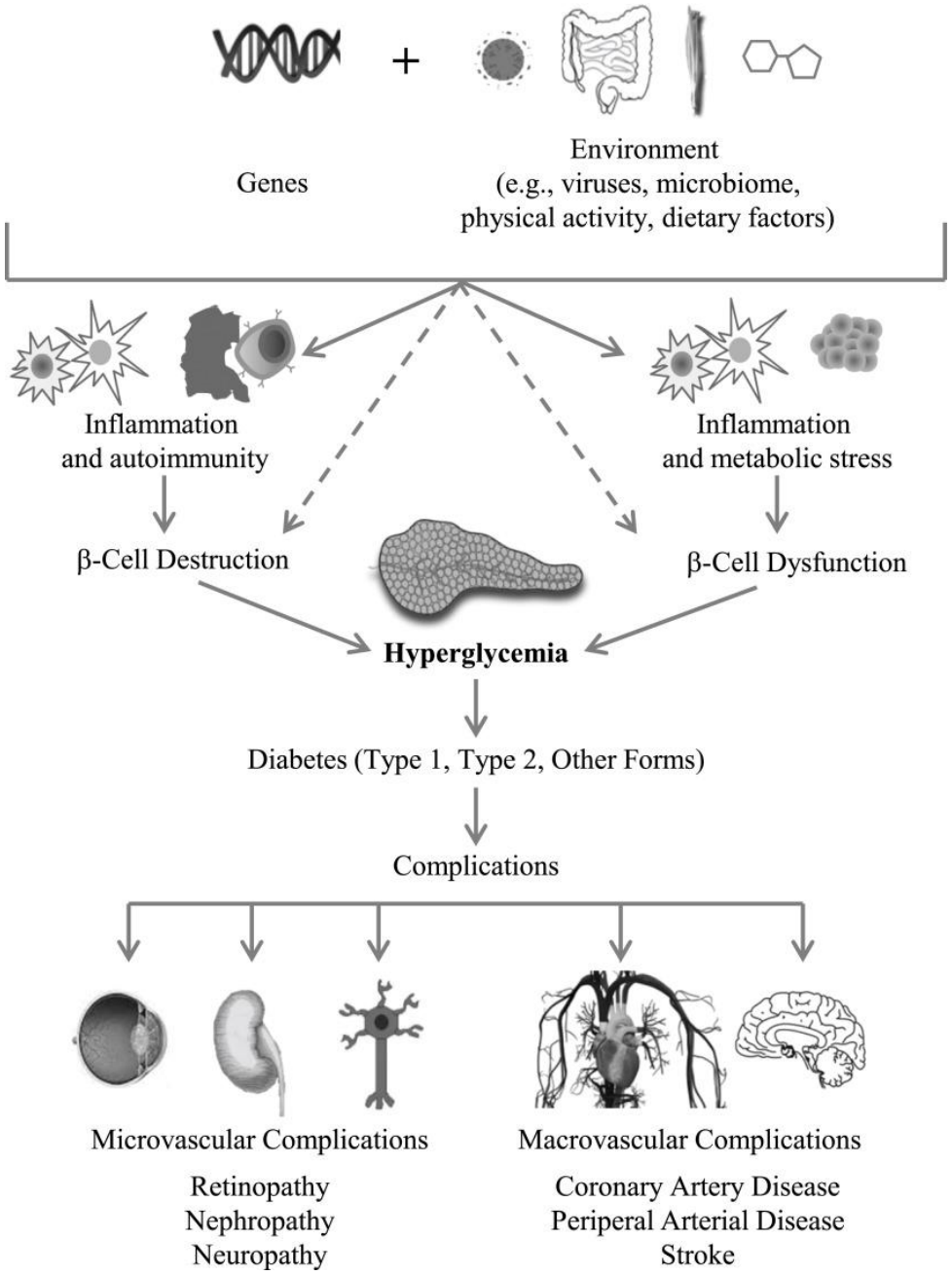


Figure 1: Mechanism of glucose-stimulated insulin secretion (from https://www.researchgate.net/figure/Steps-involved-in-glucose-stimulated-insulin-secretion-by-the-pancreatic-beta-cell_fig1_269984917)

1.3 Diabetes mellitus

Diabetes mellitus is a metabolic condition, which is defined by a pathologically high concentration of blood glucose (hyperglycemia), which in the long term damages a variety of different tissues (Figure 2). In most cases, diabetes is a lifelong disease and its prevalence by abnormalities in lipid and protein metabolism (Robber H. et. al., 1981). Proper therapy is essential not only to avoid acute life threatening events, but also to avoid late complications. Most patients are classified into one of two broad categories: Type 1 Diabetes Mellitus



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increases dramatically throughout the world. Important factors driving the diabetes epidemic are obesity and physical inactivity. Abnormalities in glucose metabolism are accompanied (T1DM) and Type 2 Diabetes Mellitus (T2DM). Apart from this, there are several less common forms like Gestational Diabetes Mellitus (GDM), Latent Autoimmune Diabetes in Adults (LADA) or Maturity Onset Diabetes of the Young (MODY) (Bopp A., 2001).

1.3.1 Types of Diabetes mellitus

Type 1 Diabetes Mellitus (T1DM)

T1DM occurs when pancreatic beta cells are destroyed by the immune system and the pancreas is no longer able to produce adequate amounts of insulin to regulate blood glucose levels (Eyth E. et al., 2021). Usually this form of diabetes is developed by children or adolescents. However, it is also possible to develop T1DM later in life. Young patients suffer from fast beta cell destruction accompanied by ketoacidosis, while adequate insulin secretion in adults might be maintained for many years (Solis-Herrera C. et al., 2018). To avoid complications resulting from hyperglycemia, patients need exogenous insulin therapy, using rapid- and long-acting insulin analogues, accompanied by stringent glycemic control (Subramanian S. et al., 2016).

T1DM can be categorized more specifically into type-1a- and type-1b-diabetes. In type-1a-diabetes, the body develops antibodies directed against the insulin-producing beta cells of the islets of Langerhans or against insulin itself (autoimmune disease). Following inflammatory reactions lead to further pancreatic destruction. Only when 80-90% of the beta cell mass is destroyed, a clinically relevant insulin deficiency is present (Bopp A. 2001, Hien P. et al., 2013, Schatz H. et al., 2014). Markers of type-1a-diabetes include antibodies to the islet cell (ICAs), to glutamic acid decarboxylase (GAD65), tyrosine phosphatases IA-2 and IA-2b, ZnT8, and insulin auto-antibodies (IAAs) (Solis-Herrera C. et al., 2018). Type-1a is caused by genetic predisposition, but environmental factors and viral infections are also likely to be contributing factors. Possible risk factors that might accelerate the loss of beta cells are virus infections, immune stimulators, breastfeeding less than 3 months and nutritional factors such as cow milk and vitamin D deficiency (Bopp A. 2001, Hien P. et al., 2013, Schatz H. et al., 2014).

Less common is type-1b, which is also known as idiopathic diabetes type 1 (unknown cause). Here, too, beta cells are destroyed and a subsequent insulin deficiency occurs (Hien P. et al., 2013).

Type 2 Diabetes Mellitus (T2DM)

T2DM describes a polygenic metabolic disorder (Hoffman L. S. et al., 2020), which is more

common than T1DM and can also be categorized in type-2a- (without obesity) and type-2b-diabetes (obese patients) (Hien P. et al., 2013).

Major causes of T2DM are obesity and physical inactivity. Additionally, genetic predisposition and lifestyle factors significantly promote the development. T2DM is characterized by insulin resistance (IR) and relative or absolute insulin deficiency leading to chronic hyperglycemia with a reduced life expectancy (Smushkin G. et al., 2010).

On the one hand insulin secretion is reduced due to beta cell dysfunction, which leads to high blood glucose levels. On the other hand, IR leads to increased glucose production in the liver and decreased glucose uptake in several tissues (Galicia-Garcia U. et al. 2020).

Peripheral target cells (e.g. muscle, liver and adipose tissue cells) are no longer able to sense insulin and so blood glucose levels rise, which means to the body that more insulin is needed. As a result of IR, increased insulin secretion (= compensatory hyperinsulinemia) will be promoted. Due to IR, beta cells are permanently challenged. They may initially be able to compensate IR through increased insulin release. This, in combination with continuing a bad lifestyle and progression of the disease, leads to down-regulation of insulin receptors and a relative insulin deficiency. Hence, IR can no longer be compensated. Consequences are rising blood glucose levels and an impaired glucose tolerance. This trend can be reversed in 75% of patients by increased physical activity and a healthy diet. If no countermeasures are taken, pancreatic beta cells will be increasingly destroyed and absolute insulin deficiency results (Hien P. et al., 2013).

<u>More than Type- 1 and 2 (LADA, MODY and GDM)</u>

While T1DM and T2DM represent the most common forms, other forms of diabetes, such as LADA, MODY or GDM occur less frequently.

LADA is a specific form of autoimmune diabetes and characterized by a slow reduction of beta cell function (Pieralice S. & Pozzilli P., 2018). Symptoms are similar to those of T1DM but only appear in adulthood. Due to slow progression of damage to pancreatic beta cells LADA might often be misdiagnosed for T2DM. Therefore, LADA patients respond to oral antidiabetics in the first 6 months, but at some point daily insulin therapy becomes necessary. As in T1DM, an absolute insulin deficiency occurs and autoantibodies against islet cell components can be detected in the serum (Rahmadi A. et al., 2019).

This special form, with features of T1DM as well as T2DM (Rajkumar V. et al., 2020), is characterized by low C-peptide concentrations. In contrast, patients with IR and classic

T2DM usually show high concentrations of C-peptide. About 50% of patients diagnosed with T2DM without obesity are actually LADA patients. Still LADA is not always associated with underweight, obesity can also occur with LADA (Rahmadi A. et al., 2019).

Maturity onset diabetes of the Young, also known as MODY, is an autosomal dominantly inherited form of diabetes. It is caused by heterozygous mutations in different genes, especially genes with a role in pancreatic beta cell development or insulin secretion (Hoffmann L. S. et al., 2020). Nowadays there are 14 different mutations known to cause MODY (Hoffmann L. S. et al., 2020). Mutations in the glucokinase (GCK) (MODY 2) and hepatocyte nuclear factor (HNF)1A/4A (MODY3 and MODY1) genes represent the most common causes for developing this non-insulin dependent form of diabetes. Most of them need different treatment (Anık A. et al., 2015). MODY in non-obese children, adolescents and young adults might often be misdiagnosed as T1DM or T2DM. In comparison to T1DM no pancreatic antibodies are formed and IR, which normally occurs in T2DM, is usually not developed at an early stage. To diagnose MODY, a genetic test is necessary (Hoffmann L. S. et al., 2020). Sulfonylureas are often considered as first line therapy (Naylor R. et al., 2018), even though insulin might be necessary later in life (Anık A. et al., 2015).

Another form of diabetes is gestational diabetes mellitus (GDM). GDM is a form of diabetes firstly developed during the second and third trimester of pregnancy. Hormonal changes, increased body weight, decreased physical activity and other risk factors similar to those of T2DM have been noted to promote the development of GDM. Also the course of the disease, characterized by increasing IR and decreasing beta cell compensation, is similar to the clinical outcome of T2DM (Quintanilla Rodriguez B. S. et al., 2020).

To diagnose GDM, an oral glucose tolerance test (OGTT) is performed. Initial therapy consists of a healthy diet and physical activity. In addition, tightly controlled blood glucose levels are of utmost importance to determine whether pharmacotherapy is necessary. If pharmacotherapy is necessary, treatment with insulin should be initiated. Insulin does not pass the placental barrier and is thus considered to be first line therapy (Kleinwechter H. et al., 2017). For the mother´s health and long term health of the baby it is important to diagnose GDM as early as possible. When left untreated, severe complications can occur (Plows J. F. et al., 2018). On the part of the mother there is an increased risk of high blood pressure, preeclampsia, preterm birth (Plows J. F. et al., 2018), increased birth weight of the fetus and the possibility of developing diabetes in later life, including a high probability of developing GDM again in future pregnancies (Kleinwechter H. et al., 2017). Maternal hyperglycemia (fetal hyperinsulinemia) can lead to increased insulin secretion and

consequently to hypoglycemia in the fetus (Plows J. F. et al., 2018). Macrosomia and an increase in abdominal circumference of the baby can occur and are caused by the storage of fat and glycogen (Kleinwechter H. et al., 2017). Later in life, babies of GDM pregnancies are suffering from a greater risk of developing obesity, T2DM, cardiovascular disease (CVD) and associated metabolic diseases (Plows J. F. et al., 2018).

1.3.2 Diagnosis of Diabetes mellitus

For a better outcome and to delay the progression of all forms of diabetes, early detection is important (Sinnott M. 2015). Special screenings for diabetes mellitus should be performed, wherever there is an increased risk of diabetes due to family history, comorbidities, if the plasma glucose is increased occasionally, or if typical symptoms of diabetes (weight loss, polyuria, polydipsia) appear (Abholz H. et al., 2013). The diagnosis is based on the determination of fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), and the performance of an OGTT (Wascher T., 2016).

Fasting plasma glucose (FPG)

FPG means glucose levels after fasting for at least 8h. FPG levels in healthy patients are defined as under 100 mg/dL. According to the American Diabetes Association (ADA) diabetes mellitus is diagnosed, when FPG-levels are greater than or equal to 126 mg/dL in two different tests (Gurung P. et al., 2020).

Oral glucose tolerance test (OGTT)

The OGTT is a blood test (Eyth E. et al., 2020) and gold standard for diagnosing diabetes (Phillips P., 2012). It gives an impression of how fast glucose is cleared from the blood (Eyth E. et al., 2020). To determine an impaired glucose metabolism, the test is performed in the fasted state (Eyth E. et al., 2020). After fasting overnight, a glucose formula is drunk (containing 75 g of glucose) and blood glucose levels are measured after 0 and 120 minutes (Phillips P., 2012).
During testing patients are advised to stay inactive. Collected samples are analyzed in the laboratory (Eyth E. et al., 2020).

Results of the OGTT are then interpreted as follows: Prediabetes is present, when FPG is between 110 and 124 mg/dl and blood glucose levels after 2 hours are between 141 and 198 mg/dl. Prediabetes is a condition, in which blood glucose levels are too high but not yet in the diabetic range (at this stage, diet and exercise likely have a positive effect). In this condition an increased macrovascular risk, but not yet an increased microvascular risk, is present. Above 126 mg/dl (FPG) and 200 mg/dl (2 hours blood glucose) diabetes is diagnosed and both macro- and microvascular risks are present (Phillips P., 2012).

Hemoglobin A1c (HbA1c)

HbA1c represents the amount of glycated hemoglobin in the blood and is a reliable biomarker for the prognosis, diagnosis, and management of diabetes. The nonenzymatic glycolysation of hemoglobin is a normal physiologic event, where glucose and the N-terminal end of the β-chain of hemoglobin form a Schiff base. It has been shown that HbA1c levels are directly proportional to the blood glucose levels over time and reflect an individual´s average blood glucose levels during the previous two to three months (Sherwani S. et al., 2016). To prevent complications Hb1Ac levels <7% are the aim of the therapy (Wascher T., 2016). In comparison to FPG or OGTT, HbA1c shows better reproducibility and allows measurement at any time of the day. Patients do not need to fast overnight and only one blood sample is required, making the procedure less time consuming than the OGTT (Florkowski C., 2013).

1.3.3 Late diabetic complications

On the long term, diabetes mellitus is associated with severe late complications, which adds to the medical challenge associated with the current diabetes epidemic. As bad as the situation and the world wide numbers already seem, this definitely can get worse. The International Diabetes Federation estimates an increase from 366 million to 552 million people suffering from diabetes by 2030. Increasing incidences of both types (T1DM, T2DM) hence go along with an elevation of severe acute and chronic complications.

Acute complications include severe hyperglycemia and ketoacidosis (Nickerson H. D. et al., 2012) and are often the result of a lack of insulin. Hypoglycemia, triggered by an overdose of insulin (T1DM or T2DM) or certain oral antidiabetic drugs (T2DM), is another most

dangerous acute complication and, if left untreated, can lead to convulsions, coma and death. Treatment of mild hypoglycemia (when the patient is awake and conscious) is treated by the administration of carbohydrates such as dextrose (Pieper D. et al., 2018). In severe hypoglycemia (loss of consciousness), either a 40% glucose solution must be administered i.v. or 1 mg glucagon i.m.. Glucagon is the antagonist of insulin and leads to a release of glucose from glycogen stores. Hence, there must be adequate glycogen reserves in the body for glucagon to be effective (Pieper D. et al., 2018).

Among late complications a distinction is made between complications involving large vessels (macroangiopathy) and small vessels (microangiopathy) (Nickerson H. D. et al., 2012), also referred to as macrovascular and microvascular complications. Coronary heart disease, cerebrovascular disease and peripheral arterial disease belong to the group of macrovascular complications, whereas nephropathy, neuropathy, diabetic foot syndrome and diabetic eye disease (retinopathy) are microvascular complications. The risk of long-term microvascular problems decreases in response to a stringent glycemic control, whereas benefit for macrovascular diseases is less well documented (Diem P., 2020).

1.3.4 Treatment of Diabetes mellitus

Treatment of Type 1 Diabetes Mellitus

Patients suffering from T1DM rely on lifelong exogenous insulin administration. Due to complete beta cell destruction exogenous insulin replacement therapy represents the cornerstone of management of T1DM (Pathak V., et. al., 2019) in order to prevent life-threatening hyperglycemia (Subramanian S. et al., 2016).

Exogenous insulin as therapy was first described by Banting and Best in 1921. At the beginning, crude extracts of animal pancreas were used but promptly replaced because of problems associated with immune responses and the pharmacokinetics of insulin, and thus problems in achieving long-term glycemic control (Pathak V. et. al., 2019).

Use of synthetic human insulin was preferable and meanwhile numerous different forms of humanized insulin analogues have been developed, which show different pharmacokinetic profiles. Nowadays a variety of rapid- and long-acting insulin analogues are available (Table

1) and, together with stringent glycemic control, used for the treatment of T1DM (Pathak V. et. al., 2019). The treatment consists of multiple daily injections of basal (injections given at set times of the day; Janež, A. et al., 2020) and prandial (injections given at mealtimes; Janež, A. et al., 2020) insulin or continuous subcutaneous insulin infusion via pumps (Pathak V. et al. 2019).

Type	Brand names	Time action profile	Dose
Rapid acting	Insulin aspart (Novorapid, Fiasp), insulin lispro (Humalog), insulin glulisine (Apidra)	Usually 4-20 min after s.c. injection with peak at 20-30 min	Three times a day up to 15 min before food intake
Short acting	Actrapid (Novo Nordisk), Humulin S (Lilly), Insuman Rapid (Aventis)	Begins from 30 min after s.c. injection with peak action reaching 2-4 h	Three times a day, 30 min before food intake
Long acting	Levemir (Novo Nordisk), ABASAGLAR (Lilly), Lantus (Aventis), Toujeo (Aventis), Tresiba (Novo Nordisk)	Beyond 24 h and up to 36 h	Once daily s.c., usually at the same time everyday with minimum 8 h interval between consecutive doses
Intermediate acting	Insulatard (Novo Nordisk), Insuman Basal (Aventis)	Peak onset from 4-6 h, with duration of action until 14-16 h	Once or twice daily s.c.

Abbreviation: s.c., subcutaneous.

Table 1: Various types of insulin available/published in the National Health Service formulary of the USA. (Table from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6501476/)

Newer strategies to restore euglycemia in patients with T1DM include the use of incretin-mimetics or SGLT-2-inhibitors in addition to insulin injections. Pancreas and islet cell transplantation are novel approaches but still far from being established as standard treatments (Pathak V. et al. 2019).

Treatment of Type 2 Diabetes Mellitus

In order to enhance the quality of life and life expectancy of T2DM diagnosed patients, therapy options are more versatile than in T1DM. At the very late stage of the disease, insulin treatment can become as inevitable in T2DM as in T1DM. At the earlier stages, diet and physical activity represent two utmost important treatment approaches for T2DM (Blaslov K. et al., 2018).

If changes in lifestyle are not sufficient or not sufficiently adopted by the patient, a number of oral antidiabetic drugs is available for the treatment of T2DM. One of the oldest drugs used in the therapy of T2DM is metformin and it still remains first-line therapy for most patients (Marín-Peñalver, J. J. et al.,2016). In the past decades the pharmacological approach brought about a wide spectrum of further oral antidiabetic drugs (Blaslov K. et al., 2018). Oral antidiabetic drugs include agents that promote insulin secretion (e.g. sulfonylureas, GLP-1 analogues) and others that increase insulin sensitivity (thiazolidinediones), or cause glucose to be excreted in the urine (gliflozins) (Brunmair et al., 2004).

Still there are treatment goals that are not achieved (Blaslov K. et al., 2018) and mechanisms of action of some antidiabetic drugs that are not fully understood. The best example of the latter is metformin, which is in use since decades without any clarification of its molecular mechanism of antidiabetic action (Brunmair et al., 2004). In order to find additional novel treatment options for T2DM and/or its late complications ARA290, a small molecule derived from the structure of erythropoietin (EPO), is under evaluation (Collino M. et al., 2014, Liu Y. et al., 2015). The present study makes a contribution to elucidating the glucose lowering mode of action of ARA290.

1.4 Erythropoietin and ARA290

1.4.1 Erythropoietin (EPO)

The hormone erythropoietin (EPO) is a glycoprotein containing 165 amino acids, which form four α helices. It belongs to the type 1 cytokine superfamily (Peng B. et. al, 2020). In adults, EPO is mainly produced in the kidneys but also other organs, such as liver (high production in newborns) and lungs (Remmele W.,1963). Its receptor (Erythropoietin receptor -EpoR) is also expressed in different cells and organs such as bone marrow, spleen, kidney, adipocytes, macrophages, neurons, endothelial cells, and cardiac cells (Liu Y. et al., 2015). The acidic glycoprotein EPO is the fundamental regulator of erythropoiesis. When erythrocyte levels decline and hypoxia is detected by renal tubular interstitial cells, EPO will be secreted into the circulation. In bone marrow it binds the homodimeric EpoR and stimulates erythropoiesis (Peng B. et al, 2020).

EPO is nowadays widely used in the clinic. One of the most common indications for EPO treatment is renal anemia, which is caused by chronic kidney failure. The kidneys are no longer able to produce sufficient amounts of EPO and thus erythropoiesis decreases. Other fields of application for EPO are tumor anemia, premature anemia and anemia in HIV disease, but EPO is also used to stimulate erythropoiesis in autologous blood donation (Remmele W.,1963).

More recently, attention has focused on a receptor heterocomplex consisting of the EpoR and the beta common receptor (βcR or CD131), which is also activated by EPO and which is known to mediate tissue protection without inducing erythropoiesis (Brines M. et al., 2006). Under physical or metabolic stress, many tissues produce EPO to attenuate pro-inflammatory agents and promote downregulation of apoptosis (Peng B. et al., 2020).

Current studies also show that EPO is involved in the regulation of body weight, fat mass and glucose metabolism. For example, overexpression of EPO in the skeletal muscle of mice resulted in reduced body weight and reduced fat mass. Normalization of plasma insulin concentration and glucose tolerance in mice fed a high-fat diet were also shown. These observations suggest that the non-hematopoietic effects of EPO may extend to the regulation of fat and glucose metabolism (Foskett et al., 2011). The clinical exploitation of the non-hematopoietic benefits of EPO is complicated by serious side effects related to increases in red cell mass (hematocrit). To avoid such unwanted increases in red cell mass (hematocrit), as well as other serious side effects, chemically modified EPO can be used (Peng B. et al., 2020).

1.4.2 EPO in obesity and diabetes

Obesity is a worldwide growing health epidemic that goes along with a maturation of precursor cells into new, additional fat cells (adipocyte hyperplasia) as well as with an increase in the diameter of the individual fat cell (adipocyte hypertrophy). A relation between obesity-induced inflammation and obesity-related IR was established, involving pro-inflammatory cytokines (e.g., TNFα) and cells like adipose tissue macrophages (ATMs), which could be a cornerstone in the development of metabolic dysfunction (Liu Y. et al., 2015).

EPO deficiency can be found in patients with diabetes (Craig K. J. et al., 2005; McGill J.B. and Bell D. S., 2006; Thomas M. C., 2006). Under treatment with EPO, studies showed less differentiation of preadipocytes into adipocytes and reduced lipopolysaccharide (LPS)-induced expression of TNFα in macrophages (Liu Y. et al., 2015). Furthermore, EPO treatment was shown to be associated with decreased IR, amelioration of deranged glucose metabolism, and lowering chronic inflammation (Collino M. et al., 2014). Hence, the idea was raised to search for a molecule that triggers the same beneficial effects on anti-inflammatory mechanisms as EPO, but without stimulation of hematopoiesis and with reduced adverse effects.

1.4.3 ARA290

Aiming to elicit tissue-protective effects without stimulating hematopoiesis ARA290, an EPO-derived helix B-surface peptide (Figure 3), was designed to selectively bind to the heteromeric receptor composed of EpoR and βcR, but not to the erythropoietic EpoR homodimer (Collino M. et al., 2014; Liu Y. et al., 2015). Due to its peptide structure, the plasma half-life of ARA290 is limited to several minutes, but by activating related signal pathways the protective effects can last for hours to days in animals and humans. Very few studies have reported information about potential side effects and the long-term effects are not fully investigated (Liu Y. et al., 2015).

ARA290 is a non-erythropoietic peptide derived from the tertiary structure of EPO (Swartjes M. et al, 2011). ARA290 was developed by Araim Pharmaceuticals, NY, and is also referred to as Cibinetide (O'Leary O. E. et al, 2019; Yao M. et al., 2020) or pHBSP (Seeger N. et al, 2011; Liu Y. et al., 2015). The molecule contains eleven amino acids and was designed to mediate tissue protection without limiting side effects related to the original structure and the hematopoietic potential of EPO. Previous clinical studies showed that many cells produce hypoglycosylated EPO in situ in order to activate healing processes (Brines M. et al, 2014). These effects are not mediated by the homodimer of EpoR, but by a heterocomplex composed of EpoR and βcR (Collino M. et al, 2014), which is also known as tissue-protective receptor (TPR) or innate repair receptor (IRR) (Peng B. et. al, 2020). Although recombinant human EPO (rhEPO) improved for example the diabetic state in several animal models, its use is limited by serious side effects resulting from the stimulation of hematopoiesis, causing

predisposition to thrombosis (Brines M. et al, 2014) and other cardiovascular side effects (Peng B. et al, 2020). In recent years, there was an increasing interest in ARA290's neuroprotective and anti-inflammatory effects, without stimulating hematopoiesis (Chen H. et al, 2014).

Also there is published data from preclinical research as well as from phase 1 and 2 studies suggesting that ARA290 might be useful for the treatment of diabetic nephropathies, non-alcoholic steatohepatitis, cardiovascular disorders, diabetic macular edema, diabetic neuropathies, neuropathic pain, and rheumatoid arthritis (Springer 2020).

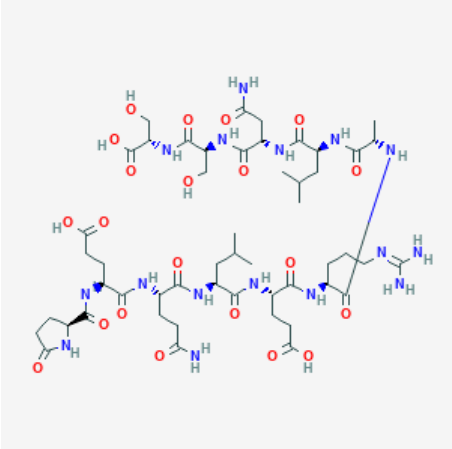

Figure 3: Chemical structure of ARA290 (= Cibinetide, pHBSP) (National Center for Biotechnology Information (2021). PubChem Compound Summary for CID 91810664, Cibinetide. Retrieved March 4, 2021 from https://pubchem.ncbi.nlm.nih.gov/compound/Cibinetide.)

2. AIM OF THESIS

In recent years, increasing prevalence and incidence of Diabetes Mellitus became a global burden and numbers are still rising This master thesis investigates a compound, which is in development as a potential new anti diabetic drug, ARA290. The aim of this work is a critical analysis of the effects of ARA290 on body weight and blood glucose in male mice. Glucose lowering effects of ARA290 in laboratory rodents have so far been described only in conjunction with weight loss. Hence, the primary question to be answered here was:

Is the blood glucose lowering effect of ARA290 in mice a consequence of weight loss or does ARA290 have a direct, *i.e*. weight-independent, effect on blood glucose?

A result showing that ARA290 lowers blood glucose independently of body weight would suggest the existence of an interesting and previously unknown glucose lowering mechanism via activation of the presumed target of ARA290, the hybrid receptor of EpoR and βcR. This would also support the idea that EPO itself executes its glucose lowering effects via this hybrid receptor.

A result indicating that ARA290-induced glucose lowering is entirely secondary to reduced weight would on the other side moderate hopes in the utility of ARA290 as a glucose lowering agent, because weight loss (or impaired weight gain) in mice is a rather unspecific indicator of discomfort or impaired health.

3. MATERIALS AND METHODS

3.1 Laboratory animals

All mice used for the experiment were male. C57BL/6J mice were purchased from the breeding facilities of the Medical University of Vienna in Himberg, Austria. In total 39 mice were delivered on 03.02.2020 and 10.02.2020. At the time of delivery, mice were approximately eight weeks old. Mice were maintained in small groups in plastic cages with a lattice cover, in a room with constant room temperature and under an artificial day-night circle of 12 hours light and 12 hours dark.

Two days after delivery, mice were switched to a high fat diet with 60% of the calories as fat (HFD; diet # D12492 from Research Diets, New Brunswick, USA). Where not mentioned otherwise, mice always had access to tap water and HFD ad libitum.

The development of metabolic derangement was essential for my experiment, because I wanted to investigate the potential improvement under treatment with ARA290. Male C57BL/6J mice were selected for the study, because it is known from previous reports that male mice of the C57BL/6J strain readily gain fat mass and body weight on HFD and that they develop metabolic derangements typically associated with overweight and obesity. This has been demonstrated, e.g., by Montgomery et al. (2013) in a study showing the effect of HFD in comparison to low fat diet on metabolic parameters in five different strains of mice (C57BL/6J, 129X1/SvJ, BALB/c, DBA/2, and FVB/N). They found that HFD caused a significantly accelerated gain of body fat in all mice, and that all strains, except BALB/c, showed significant deterioration of glucose tolerance, albeit to a variable extent. Gollisch et al. (2009) likewise observed the development of overweight-associated disease in HFD-fed rodents, which showed dramatic increases in the total adipose tissue mass in association with impaired oral glucose tolerance.

3.2 Test procedure and protocols

3.2.1 Intervention and test groups

About two months after delivery and, hence, of HFD feeding, treatment with ARA290 was started. All mice remained on HFD during the entire course of the further experiment. To individualize mice housed in the same cages, their tails were colored with a marker. Body weight, 4 h fasted blood glucose, and body composition were determined. Based on that results, mice were split into three groups matched for weight, glycemia, and relative fat mass. One group (n=17) was treated with regular injections of ARA290. The other two groups were control groups, which were injected with the vehicle only (PBS, phosphate buffered saline). One of the control groups would always have access to HFD ad libitum ("Ad Libitum" group; n=10). Since ARA290 has previously been reported to reduce weight gain (Collino M. et al., 2014), the second control group was meant to be fed restrictedly, receiving only one portion of food daily in order to always match the weight curve of the ARA290-treated mice as closely as possible ("Restricted" group; n=12). The use of two separate control groups was to dissect direct actions of ARA290 from indirect actions, as may occur as a secondary consequence of reduced weight gain.

ARA290 or the plain vehicle were injected intraperitoneally (i.p.), which means injection of the solution into the peritoneal cavity, a technique rarely used in humans but standard in laboratory rodents (Figure 4). Three injections per week (Monday – Wednesday - Friday) were given over a period of three months. Until day 27, the dose of ARA290 per injection was 75 µg/kg body weight, from day 29 until the end of the study the dose was doubled to 150 µg/kg body weight. The injection volume always was 3 µl/g body weight for both, ARA290 and plain PBS. The vehicle was injected to induce comparable injection stress also in the control groups. The solution containing ARA290 was stored frozen at -20°C until shortly before use, PBS was stored at 4°C. Injections were done with 1 ml syringes (1 ml insulin safety syringe, 29Gx1/2" Magellan Covidien, Dublin, OH, USA).

Figure 4: The intraperitoneal injection technique applied to a mouse.

3.2.2 Measurements

Body weight and amount of food intake were determined at least once per week. Fasting blood glucose was measured 5 days prior to the start of the experiment and on days 7, 17, and 62 days after starting the injections. On day 60, plasma was collected for determination of insulin concentration and calculation of the HOMA index. On day 76, hemoglobin content, oxygen saturation, and hematocrit were determined. A glucose tolerance test followed on day 83. Also body composition was documented 2 days prior to the start of the experiment, as well as on day 74. On day 85 of the experiment the mice were killed and terminal samples of plasma and various tissues were collected.

3.3 Measurement methods and analytics

3.3.1 Body weight and food intake

At least once a week body weight and food intake were determined and documented using a precision scale from Mettler Toledo (Columbus, OH, USA). To determine food intake, HFD was weighed, before it was put into the cages. After approximately one week it was weighed

again, the difference indicating the amount of food consumed per cage. Hence, food intake couldn't be calculated individually for each mouse, but only cage-wise.

For the determination of body weight, mice were put into a small box, which was placed on the scale. To account for the movements of the mice during weighing, the scale was used in the ‚dynamic weighing' mode, which provided the average weight over a period of three seconds.

3.3.2 Body composition

To determine lean and fat mass of the mice the Body Composition Analyzer EMR-188 was used (EchoMRI, Houston, TX, USA). For the measurement each mouse was locked in the measurement chamber (a tube inserted into the analyzer) and the machine provided numbers for fat mass and lean mass within a minute. Body composition was determined two days before starting the injections as well after 74 days under treatment.

3.3.3 Fasting blood glucose

Food was withdrawn 4 h before the measurement of basal blood glucose, while drinking water remained always available. Measurements of fasting glycemia were done 5 days before as well as 7, 17, and 62 days after starting the treatment, and it was always 48 hours after the last injection of either ARA290 or the vehicle. Blood glucose was measured using the portable glucometer „StatStrip Xpress" (Nova Biomedical, Waltham, MA, USA) as adapted by the company DSI (Data Sciences International, St. Paul, MN, USA) specifically for glucose measurements in small laboratory animals. The device requires only 1.2 µl of blood to determine the concentration of blood glucose. For the measurement, capillary blood collected from the mice tails was used. After a strip was placed in the glucometer, the tip of the tail was pricked with a needle. The tail was then gently pressed to obtain a small drop of blood, which was taken up with the test strip. The glucometer delivered the result a few seconds later.

Since this was of particular relevance for the present study, it should be noted that the employed glucometer has previously been shown to be hematocrit insensitive. Many other

glucometer devices are known to underestimate the blood glucose concentration in the presence of high hematocrit levels. Several studies have compared the StatStrip device employed in the present master thesis to a number of other glucometers and they consistently found superiority of StatStrip to all others with regard to the hematocrit insensitivity of glucose measurements. Unimpaired and reliable results were obtained with the StatStrip device at hematocrit levels up to 70% (Karon B. S. et al., 2008; Lyon M. E. et al., 2009; Vanavanan S. et al., 2010).

3.3.4. Intraperitoneal glucose tolerance test (IPGTT)

The glucose tolerance test was performed in mice fasted for 8 h and 48 h after the last injection of ARA290 or vehicle. Basal blood glucose was measured, before the mice were intraperitoneally injected with glucose solution (33% w/v, custom-made by gespag, Bad Ischl Pharmacy, Austria; 1.5 g/kg body weight in a volume of 4.5 µl/kg body weight). This was followed by further measurements of blood glucose 20, 40, 60, 90 and 120 min after injection of the glucose solution. For these measurements, it was sufficient to massage the tails gently to collect blood, repeated pricking of the tail was not necessary.

3.3.5 Hematocrit

The hematocrit is the volume percentage of red blood cells in blood, which also affects the viscosity of blood (Mondal H. & Budh D. P., 2020). As for other measurements, hematocrit was measured 48 h after the last injection, but without fasting the mice. Fifty µl of blood was collected from the tip of the tail into a heparin coated capillary (Minicaps, Hirschmann laboratory equipment GmbH & Co. KG, Eberstadt, Germany). Hemoglobin content and oxygen saturation were measured, and hematocrit was calculated by a blood analyzer (ABL 800 FLEX, Drott Medizintechnik GmbH, Wiener Neudorf, Austria). To reduce stress caused by sampling a large amount of blood from a single mouse, blood from two individuals was pooled for these particular measurements.

3.3.6 Plasma insulin and HOMA-Index

Immediately after basal glycemia was determined along the procedure describe above, a further 50 µl of capillary blood were collected into an EDTA-coated tube (Microvette CB 300 K2E, Sarstedt, Nümbrecht, Germany). After centrifugation, the plasma was collected and stored at -80°C. Later, plasma insulin was determined by means of an ELISA test (Ultrasensitive Mouse Insulin ELISA, Mercodia, Uppsala, Sweden).

Based on the concentrations of blood glucose and plasma insulin, the HOMA index (= Homeostasis Model Assessment) was calculated according to the formula glucose (mg / dl) * insulin (µU / ml) / 405. Provided that there is no insulin deficiency, the HOMA index can be used as a readout to estimate the insulin sensitivity.

3.3.7 Terminal collection of plasma and tissue samples

On the last day of the experiment mice were killed for the collection of plasma samples and of specimens of various tissues. Again, this was 48 h after the last treatment with ARA290 or saline and after fasting for 2-4 h. Mice were placed in a plastic box and anesthetized with inhalative isoflurane. As soon as they were under deep anesthesia, they were transferred to the section table still inhaling isoflurane from a small plastic tube placed over their nose and mouth. Chest and abdomen were opened using a small scissor, and heart puncture with a syringe prepared with EDTA was performed. The collected blood volume of 500-900 µl was immediately cooled to 4°C and centrifuged for the collection of plasma within 30 min. Plasma was then stored at -80°C.

Immediately after the heart puncture specimens of liver and gastrocnemius muscle, which are important organs for glucose homeostasis, were prepared. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C to be available for later examination.

4. RESULTS

4.1 Body weight and food intake

At the beginning of the experiment, body weight of the mice was approximately 40 g. Each of the three groups to be compared (Restricted, Ad Libitum, and ARA290) showed a steady and continuous increase in body weight over the course of the 85 days of experimentation, so that at no time there was a statistically significant difference (Figure 5).

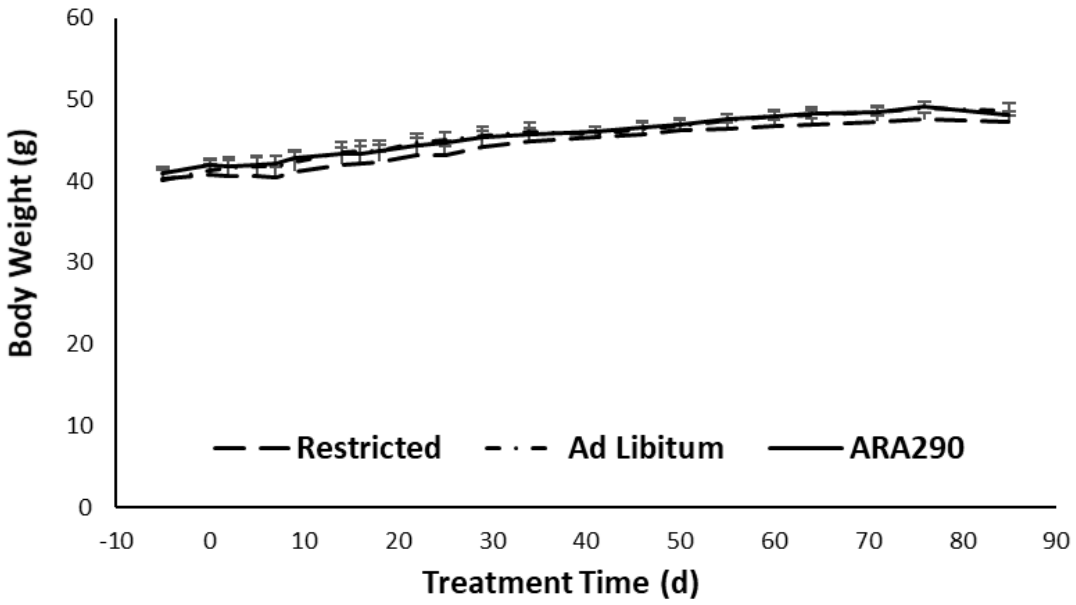

Figure 5: Effect of regular treatment with ARA290 over a period of 85 days (3 intraperitoneal injections of 75 µg/kg per week) on body weight of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of the vehicle). Means ± SE; no significant difference between the groups.

The group meant for restricted feeding started off with a weight of 40.2 ± 1.3 g, which increased to a final weight of 47.2 ± 0.8 g after 85 days of vehicle treatment. The Ad Libitum group showed very similar results with an increase from 40.1 ± 1.3 g to 48.6 ± 0.9 g, and so did the mice treated with ARA290 exhibiting an increase from 41.0 ± 0.8 g to 48.1 ± 0.5 g (Figure 5). Accordingly, individual weight gain during treatment was similar in the three

groups with +6.4 ± 0.6 g and +7.4 ± 0.9 g for the two control groups (Restricted and Ad Libitum) versus +6.1 ± 0.6 g for the ARA290-treated mice (n.s.; Figure 6).

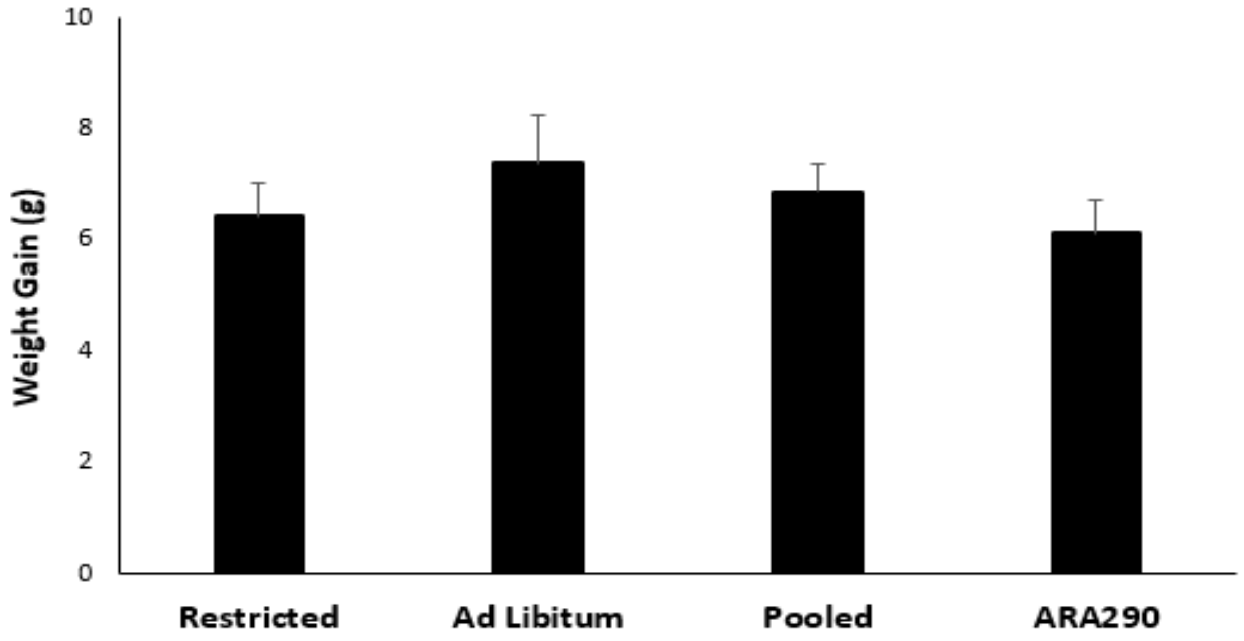

Figure 6: Effect of regular treatment with ARA290 over a period of 85 days (3 intraperitoneal injections of 75 µg/kg per week) on weight gain of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

ARA290 did not significantly affect body weight and the treated mice showed the same weight curve as controls with free access to food. It was not necessary to restrict feeding of the nominally “Restricted” control group, which had initially been meant for such an intervention. In other words, there was no difference in the treatment of the two control groups, both of which had free access to food. As will be documented below, there were no significant differences in the results obtained from the two control groups. Hence, all below graphs will depict the results from the two controls groups separately (to document the absence of differences), as well as the pooled results from these two groups.

Food intake was determined cage-wise and calculated per mouse per day. During the first week of treatment, average food consumption per day was 2.06 g for the Restricted group, 2.28 g for the Ad Libitum group and 2.12 g for the ARA290 group. Over the entire experimental period of 85 days, average daily intake was 2.23 g (Restricted), 2.28 g (Ad Libitum), and 2.31 g (ARA290) per mouse. Cage numbers (n) were not sufficient for robust statistical comparison, but the outcome did not hint at relevant differences in appetite and food intake.

4.2 Body composition

To determine lean and fat mass of the mice body composition measurements were carried out using an Echo MRI device 2 days before the experiment started, as well as on day 74 of treatment. Based on the results obtained at the start of the experiment, the groups were matched.

Two days before the treatment started, lean mass was 23.3 ± 0.5 g in the Restricted group, 23.6 ± 0.4g in the Ad Libitum group, and 23.6 ± 0.3 g in the ARA290 group. After similar growth during the subsequent 76 days, values were 26.7 ± 0.6 g in the Restricted group, 26.8 ± 0.4 g in the Ad Libitum group, and 26.8 ± 0.4 g in the ARA290 group (Figure 7A). For fat mass, the Restricted group started with 16.3 ± 0.8 g, the Ad Libitum group with 16.6 ± 1.2 g, and the ARA290 group with 17.0 ± 0.7 g. Fat mass increased almost equally in all three groups until day 74 (Figure 7B). Final values were 20.4 ± 0.5 g in the Restricted group, 20.7 ± 0.5 g in the Ad Libitum group, and 20.9 ± 0.4 g in the ARA290 group. For lean and fat mass, there were no significant differences between the groups.

Based on the results obtained before the experiment, the groups were matched for % body fat, which was 40.9 ± 1.1 % in the Restricted group, 40.8 ± 1.9 % in the Ad Libitum group, and 41.5 ± 1.2 % in the ARA290 group. When body composition was again determined on day 74, there was still no significant difference between the groups. Relative body fat had modestly increased over the treatment period, but the increases were almost equal in the three groups. Percentage of fat was 43.4 ± 0.9% in the Restricted group, 43.6 ± 0.6% in the Ad Libitum group, and 43.9 ± 0.7% in the ARA290 group (Figure 7C).

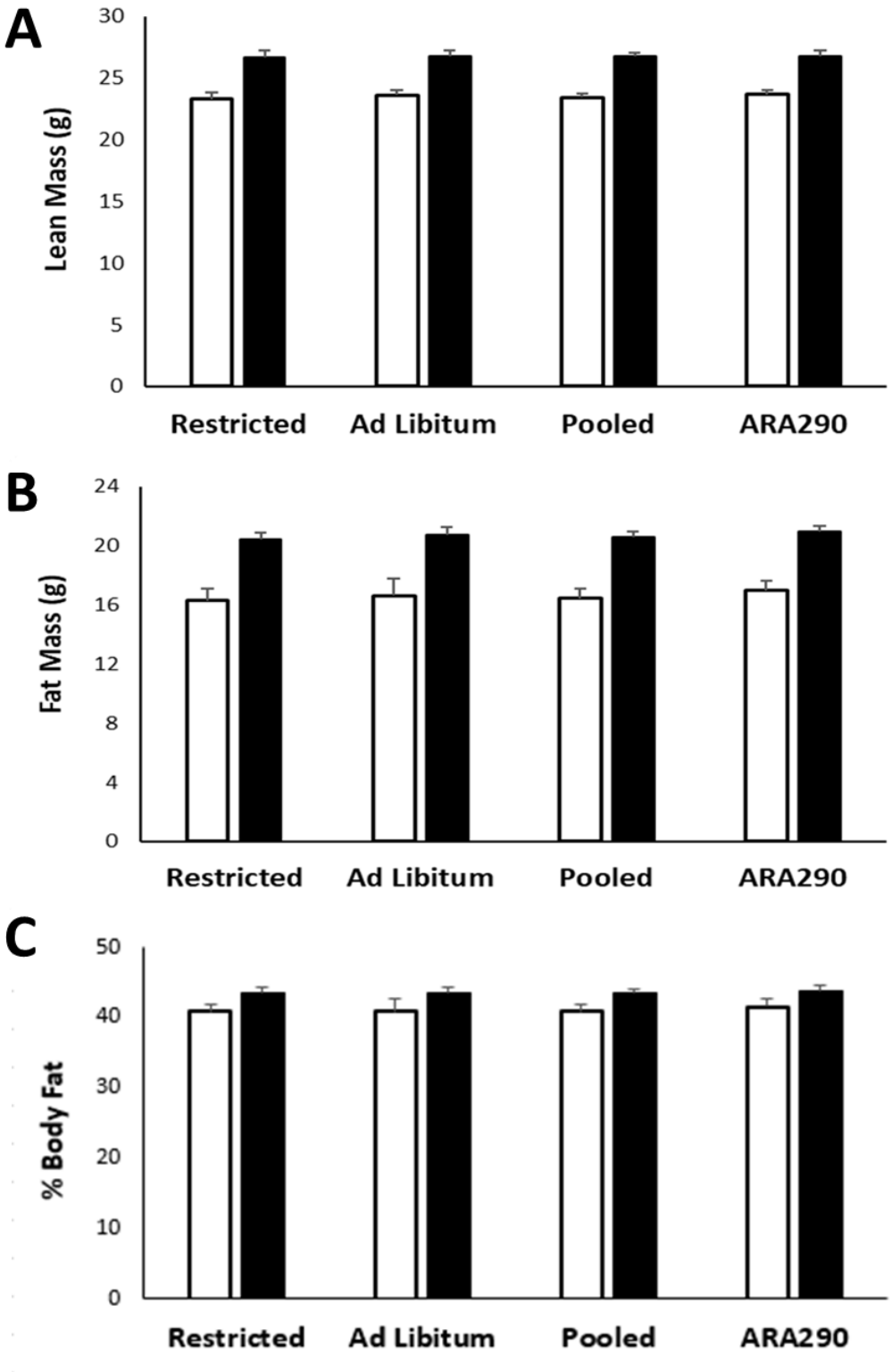

Figure 7: Effect of regular treatment with ARA290 over a period of 74 days (3 intraperitoneal injections of 75 µg/kg per week) on lean mass (**A**), fat mass (**B**), and % body fat (**C**) of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

4.3 Fasting blood glucose

In total, the blood glucose of the 4 h fasted mice was determined four times. The first measurement was carried out five days before the experiment started. Based on these results, the mice were allocated to their groups, which were matched for blood glucose (Restricted, Ad Libitum and ARA290).

Further measurements followed on days 7 (Restricted 168.9 ± 6.7 mg/dl, Ad Libitum 175.1 ± 10.3 mg/dl, ARA290 176.4 ± 5.9 mg/dl), 27 (Restricted 167.2 ± 6.8 mg/dl, Ad Libitum 184.5 ± 11.0 mg/dl, ARA290 183.4 ± 7.7 mg/dl) and 62 (Restricted 172.4 ± 5.0 mg/dl, Ad Libitum 181.6 ± 8.1 mg/dl, ARA290 167.6 ± 3.9 mg/dl). All groups showed similar results on all measurement days and there were no significant differences between the groups. Hence, ARA290 had no effect on the fasting blood glucose of the mice (Figure 8).

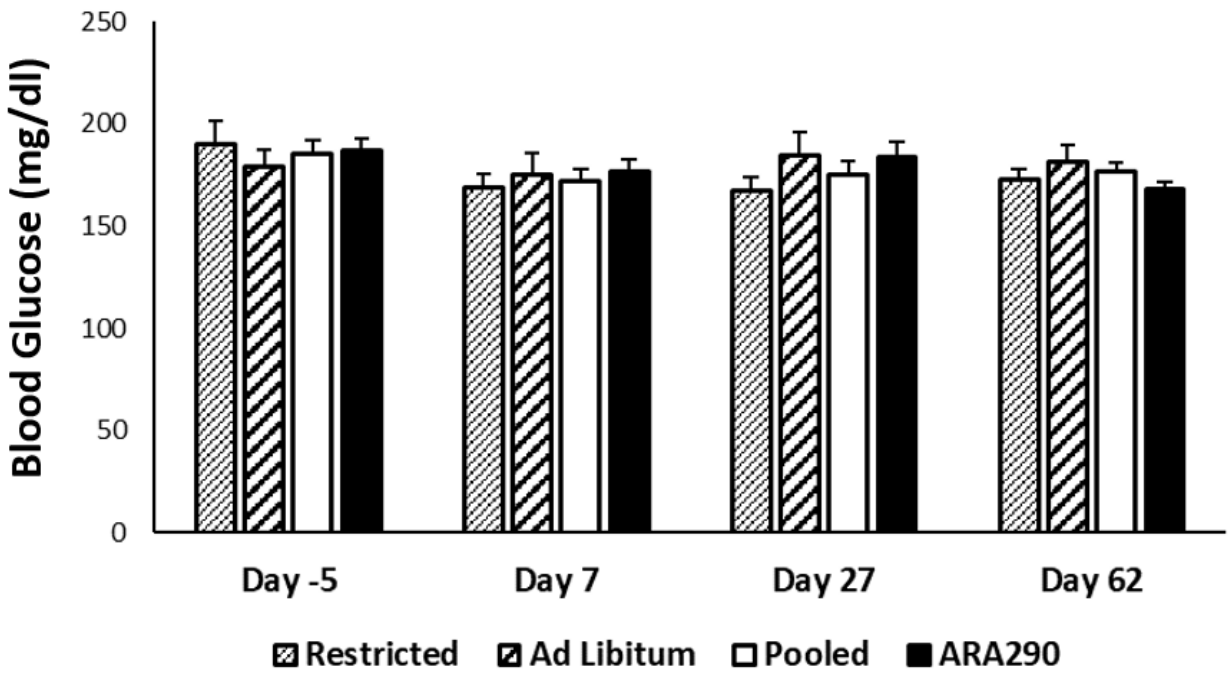

Figure 8: Effect of regular treatment with ARA290 over a period of 62 days (3 intraperitoneal injections of 75 µg/kg per week) on fasting blood glucose of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

4.4 Intraperitoneal glucose tolerance test (IPGTT)

After 8 h fasting and 48 h after the last ARA290 injection a glucose tolerance test was performed on day 83. First, the fasting blood glucose levels were determined and then, after injection of a 33% glucose solution into the peritoneum, blood glucose levels were determined again after 20, 40, 60, 90 and 120 minutes. Before start of the test, the fasting blood glucose levels of the ARA290 group were not significantly lower than those of the controls. Most notably, after 20 minutes, all three groups showed a steep increase in blood glucose levels, where the highest mean was recorded in the ARA290 group (450.5 mg/dl) (Figure 9).

In the further course of the glucose tolerance test, the blood glucose levels of the animals that had been treated with ARA290 were similar to those seen in control animals. After 120 minutes none of the three groups reached initial values. The mean changes of blood glucose levels of the ARA290 group were from 164.4 mg/ dl to 237.9 mg/ dl after 2h. Thus, the IPGTT (similar to the results of the fasting blood glucose) revealed that ARA290 had no blood glucose lowering effect and did not accelerate glucose clearance in the mice.

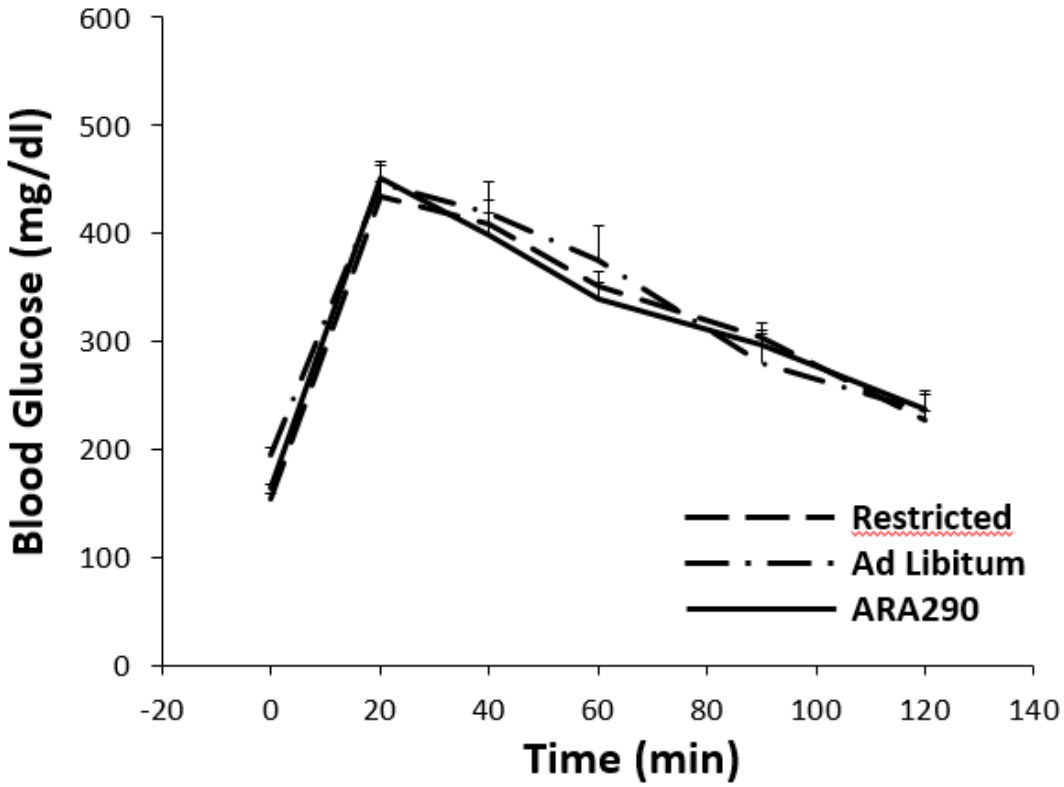

Figure 9: Effect of regular treatment with ARA290 over a period of 83 days (3 intraperitoneal injections of 75 µg/kg per week) on the glucose tolerance of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). Means ± SE; no significant difference between the groups.

Correspondingly, the comparison of the total areas under the glucose curves (AUC-tot) showed no statistically significant difference between the male ARA290 mice and the vehicle groups (Figure 10).

Also the incremental area under the glucose curve (AUC-inc) was not significantly lower in the ARA290 group than in the control groups (Figure 11).

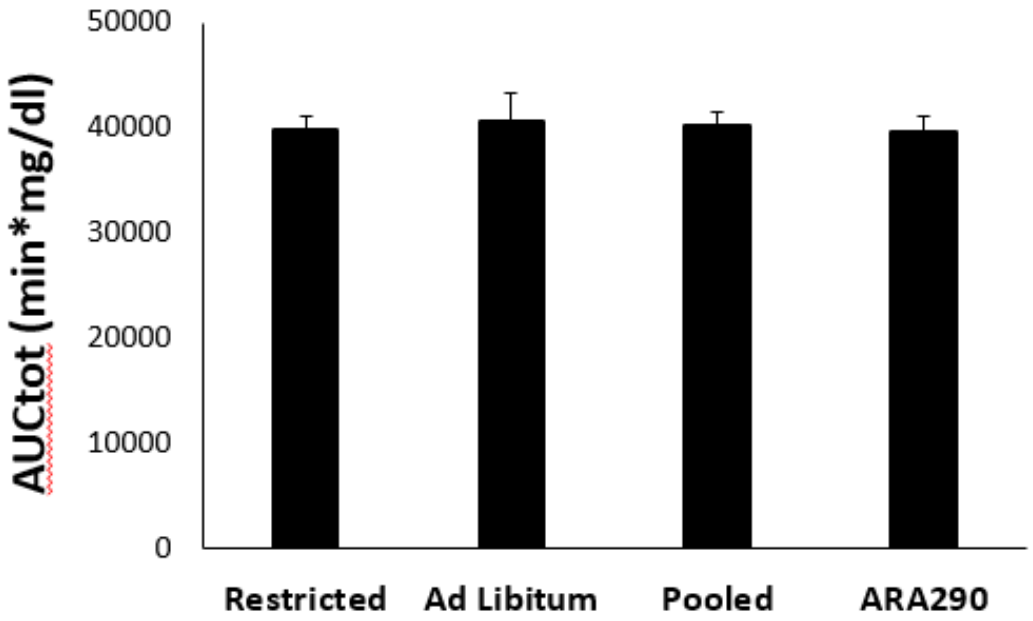

Figure 10: Effect of regular treatment with ARA290 (3 intraperitoneal injections of 75 µg/kg per week) on the total area under the glucose curve (AUC-tot) over a period of t = 120 min in the intraperitoneal glucose tolerance test of obese male mice. Means ± SE; no significant difference between the groups.

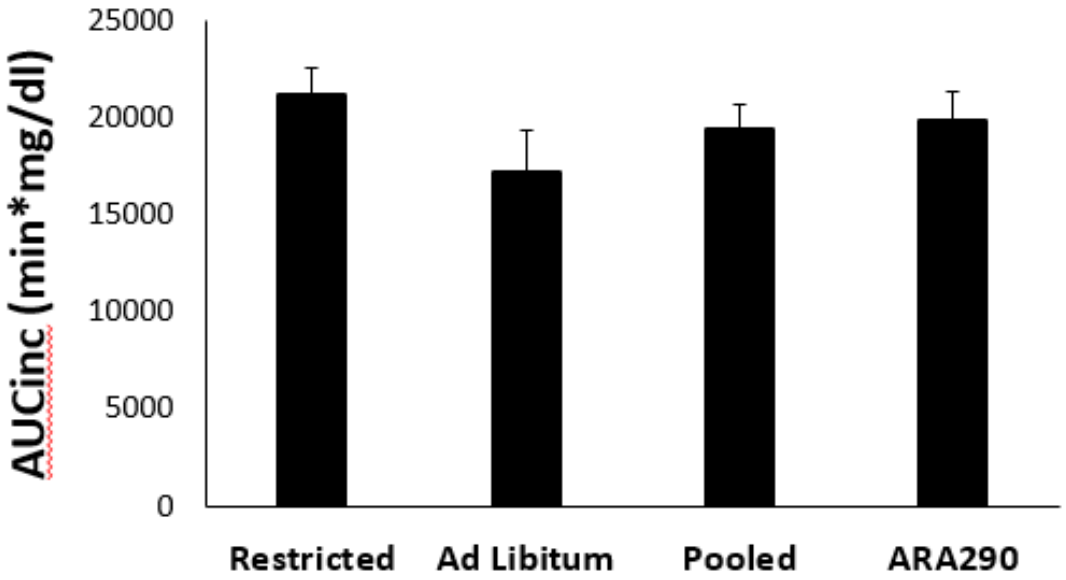

Figure 11: Effect of regular treatment with ARA290 (3 intraperitoneal injections of 75 µg/kg per week) on the incremental area under the glucose curve (AUC-inc) over a period of t = 120 min in the intraperitoneal glucose tolerance test of obese male mice. Means ± SE; no significant difference between the groups.

4.5 Hematocrit

On day 76, the hematocrit of the controls (pooled data from mice belonging to the Restricted and Ad Libitum groups) was 43.8 ± 1.0 % and the hematocrit of the ARA290-treated mice was 43.3 ± 0.7 %. There was no statistically significant difference between the controls and the ARA290 group (Figure 12).

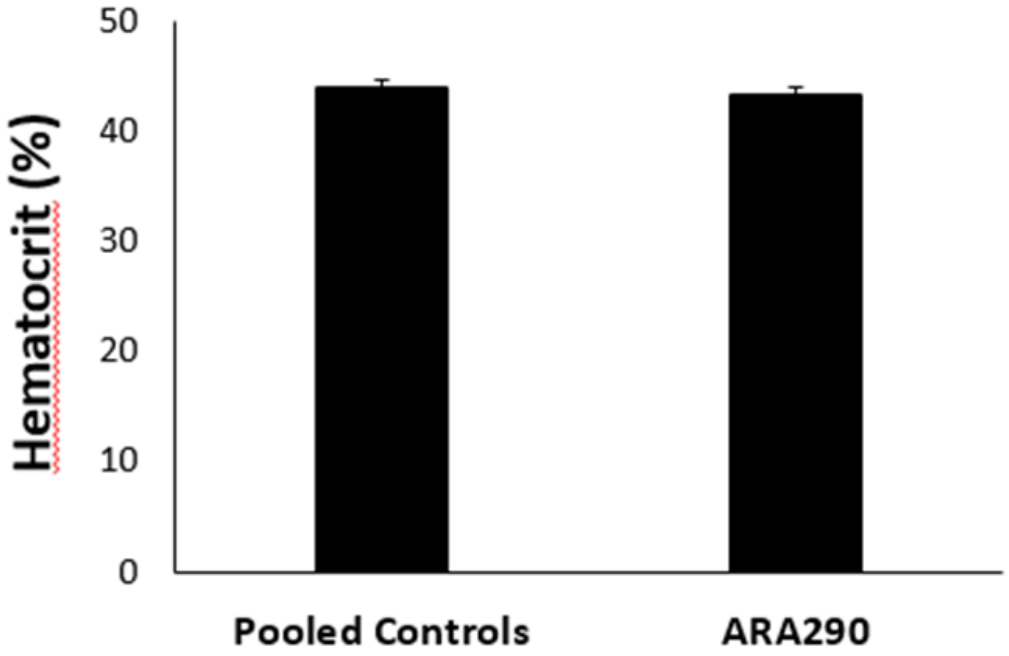

Figure 12: Effect of regular treatment with ARA290 over a period of 76 days (3 intraperitoneal injections of 75 µg/kg per week) on the hematocrit of obese male mice. “Pooled” refers to pooled data from the two control groups (“Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). Means ± SE; no significant difference between the groups.

4.6 Plasmainsulin and HOMA-Index

Immediately after determining the basal glycemia on day 60 (see Figure 13), plasma was collected and plasma insulin was later determined by ELISA. The plasma insulin values determined in all three groups did not show a significant difference (Figure 14).
The HOMA index is used to determine insulin sensitivity. For its calculation one needs the basal glycemia and the insulin concentration in the plasma. Again, no significant difference was observed between the groups (Figure 15).

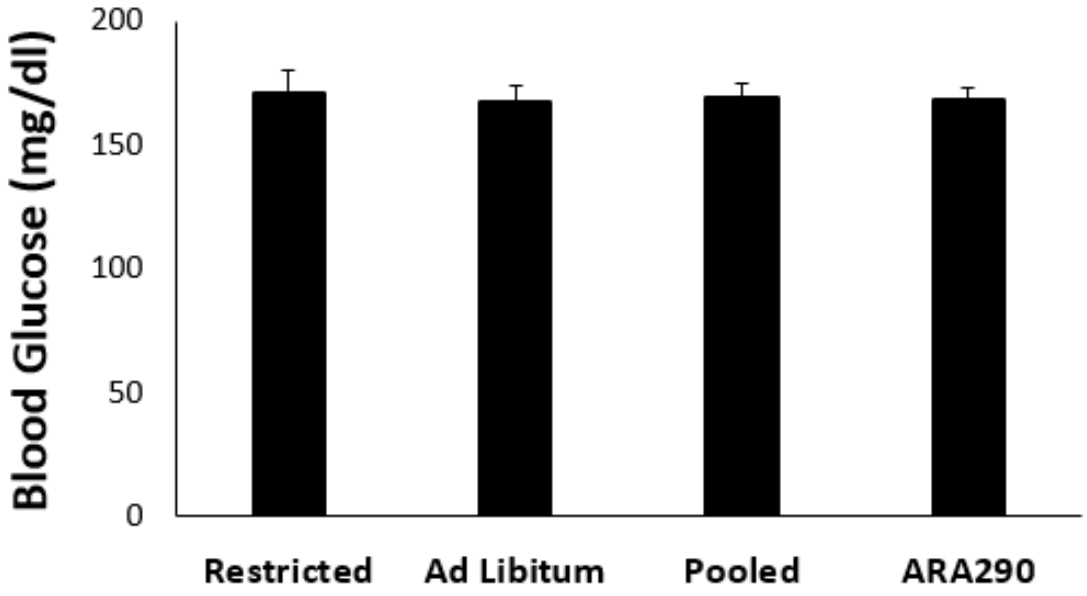

Figure 13: Effect of regular treatment with ARA290 over a period of 60 days (3 intraperitoneal injections of 75 µg/kg per week) on blood glucose of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

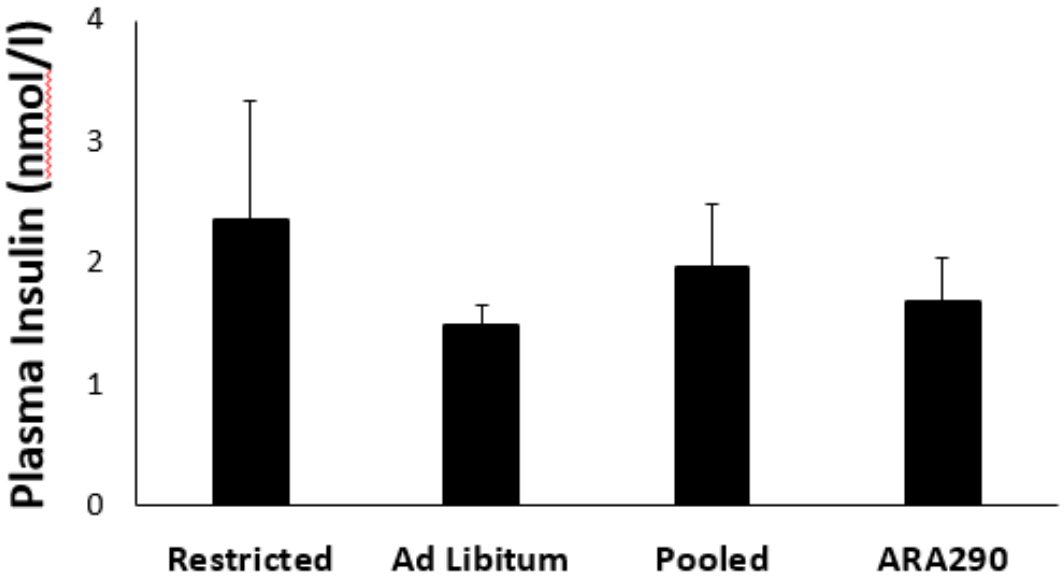

Figure 14: Effect of regular treatment with ARA290 over a period of 60 days (3 intraperitoneal injections of 75 µg/kg per week) on plasma insulin of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

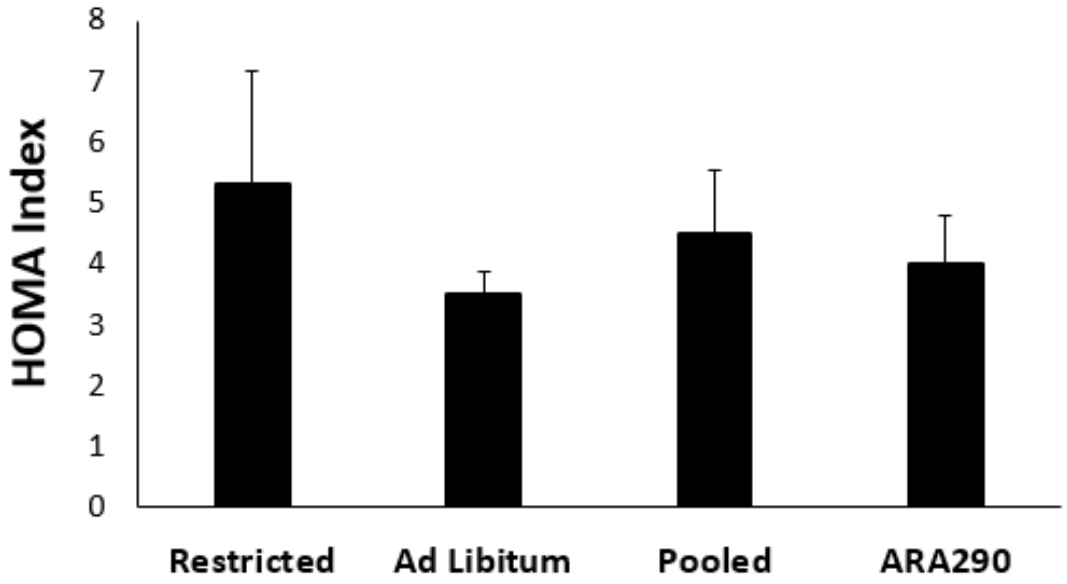

Figure 15: Effect of regular treatment with ARA290 over a period of 60 days (3 intraperitoneal injections of 75 µg/kg per week) on the HOMA-Index of obese male mice. “Restricted” and “Ad Libitum” refers to two control groups, which received the same treatment (injections of vehicle). “Pooled” refers to pooled data from the two control groups. Means ± SE; no significant difference between the groups.

5. DISCUSSION

Diabetes has become a global epidemic and numbers of afflicted patients are still rising. In the past years several different studies reported EPO deficiency in patients suffering from diabetes (Craig K. J. et . al., 2005; McGill J. B. and Bell D. S., 2006; Thomas M. C., 2006), as well as a relation between obesity-induced inflammation and obesity-related IR (Liu Y. et al., 2015). Since EPO´s potential to treat diabetes is limited by severe side effects including increases in hematocrit, blood pressure and thrombosis, recent studies have directed growing attention to the non-erythropoietic molecule ARA290 (also referred to as pHBSP) (Collino M. et. al., 2014; Peng B. et. al.; 2020, Yao M. et. al., 2020). The present study was designed to determine the effects of ARA290 on body weight and blood glucose in male obese mice.

In one of the previous studies by Collino M. et al. (2014) mice were fed with HFD (60% fat content) and treated with ARA290 or vehicle, as it was also the case in the present work. In Collino's study ARA290 had a clear blunting effect on gain of body weight and fat mass in male mice. In the results, the authors described: ‚*After 22 weeks of feeding, mice of the HFHS group had greater body and adipose tissue (epididymal fat) weights than control diet-fed animals, which were significantly reduced by pHBSP treatment.*' and ‚*In contrast, pHBSP administration exerted significant insulin- and glucose-lowering ability.*' (HFHS = high fat high sucrose, pHBSP = ARA290).

In his results the author described that after 22 weeks mice fed with a HFHS diet had greater body and adipose tissue (epididymal fat) weights in comparison to control diet-fed mice, which showed notably reduced adipose tissue in response to treatment with ARA290. Furthermore, Collino reported a significant insulin- and glucose-lowering ability of ARA290.

Another study by Liu Y. et al. (2015) also reported dose-dependently reduced body weight in response to treatment with ARA290 in mice with HFD-induced obesity. In addition, decreased food intake and decreased fat mass were observed. Different doses of ARA290 (120, 90, 60 and 30 µg/kg) were administered every other day from the beginning of HFD administration. In this study, ARA290 dose-dependently reduced body weight gain in mice with HFD-induced obesity, while food intake was also decreased. Furthermore, ARA290 decreased fat mass.

My findings, as described in the results, did not support the above mentioned previous outcomes from other laboratories. Significant differences were not found, neither in food intake nor in body weight. Treated mice showed the same weight curve as control animals with free access to food over the entire period of 85 days. Also, fat mass increased almost equally in all three groups.

A possible explanation for the differences in outcome of the studies might be that Collino et al. (2014), administrated the EPO-mimetic subcutaneously, while our mice were injected i.p. Another source of potential differences are the employed doses. While I started with a dose of 75 µg/kg i.p. three times per week, Liu Y. et al. (2015) described a dose-dependent effect ranging from 30 to 120 µg/kg every other day. In order to make sure that the absence of an effect on weight gain was not due to low dosing, I doubled the dose to 150 µg/kg after approximately one month. However, the outcomes remained the same. Also, the duration of the treatments differed between my and the previous studies, but I continued with the treatment for 85 days, which is not shorter than in the other studies, and I still did not observe any significant difference between the groups of mice.

The primary and specific aim of this master thesis was to find out, whether a blood glucose lowering effect can still be observed, when the effects of ARA290 on weight are balanced by restricted feeding of the controls, in other words, whether blood glucose can be lowered weight-independently via the hybrid-receptor targeted by this drug.

My findings did not confirm those from Muller C. et al. (2015), who had described significantly lower glucose AUCs in an IPGTT in mice subject to ARA290 treatment compared to placebo treatment after 2 and 4 weeks. The glucose curves as well as the areas under the glucose curves (AUC-tot and AUC-inc) of the IPGTT in the present study did not show any statistically significant difference between the male ARA290-treated mice and the vehicle groups. This rather unexpected finding may also relate to the different forms of application. As in the study by Collino M. et al. (2014), Muller C. et al. (2015) also injected the EPO-mimetic subcutaneously. Another possibly relevant difference to be mentioned is that Muller C. et al. (2015) did not use obese mice but Goto-Kakizaki rats in their study.

Collino M. et al. (2014) described a significant glucose-lowering effect of ARA290, while my results did not suggest anything alike. In the course of the IPGTT, ARA290-treated mice and controls showed almost identical blood glucose curves and no statistically significant difference was observed. Also fasting blood glucose levels, which were measured at several occasions, did not reveal differences between the groups. However, there were similarities in terms of blood glucose levels to a study by Muller C. et al. (2015) who wrote: ‚*ARA290 treatment had no effect on fasting plasma glucose levels in GK rats*' and ‚*..suggesting that altered extrahepatic and hepatic insulin sensitivity do not contribute to the improvement in plasma glucose levels*.'

Additionally, this work provides knowledge about the effect of ARA290 on the hematocrit. As mentioned earlier, the prolonged use of rhEPO for the treatment of diabetes is limited by an increase in red blood cells (hematocrit) (Liu Y. et al., 2015; Collino M. et al., 2014; Muller C. et al., 2015). Ad variance to differences found with respect to glycemia, here my results confirmed those of others. Neither Collino et al. (2014) nor Lui Y. et al. (2015) reported an increase in the hematocrit in ARA290-treated rodents. These results agree with our findings, since there was no statistically significant difference observed in the hematocrit levels of the mice. Hence, ARA290 should not raise the risk of thrombosis or other similar adverse effects related to an increased hematocrit.

Alltogether, my findings thus did not support the outcome of many recently published studies, since I was unable to show a blood glucose lowering effect of ARA290. Additionally, all of the previously described effects of ARA290 on body weight and fat mass were not found either.

6. CONCLUSION

In this investigation, the aim was to assess the effects of ARA290 on body weight and blood glucose in male mice. This study did not confirm earlier findings and, hence, several questions still remain to be answered. Further research is required to better understand the effectiveness and safety of ARA290.

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