Role of Gremlin1 and Bone Morphogenic Protein 4 (BMP4) in metabolic diseases

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To my son, Danial

Never take advice from those who are not where you aspire to be in life. People will always reveal their true selves—just give them time.

Be wise enough to create your own opportunities; don't wait for them to come to you. The secret to happiness is freedom, and the secret to freedom is courage. In life, you must take action if you wish to experience true freedom.

Remember, challenges and conflicts will always be a part of life. You must learn to enjoy life even as you work through them. There is beauty in every experience; you just need to adjust your perspective. You can either cry in the storm or dance in the rain.

My dearest, the bird perched on a branch does not fear it breaking because its trust is not in the branch, but in the strength of its own wings. In the same way, place your faith in your own abilities, knowing that your true strength comes from within, not from the circumstances that surround you.

Danial, your love has given me the courage, motivation, and sense of purpose to grow and pursue my dreams. For this, I am eternally grateful. Being your mother fills me with immense pride, and your love is my greatest treasure.

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ABSTRACT

Gremlin1, a protein highly expressed in human adipocytes, plays a key role in the differentiation of precursor cells into white adipose tissue and is linked to insulin resistance in hypertrophic obesity. This study investigated Gremlin1 expression in patients with obesity, type 2 diabetes (T2D), and related metabolic disorders, as well as its effects on obesity and insulin sensitivity in a diet-induced obese mouse model. We further investigated BMP4 for its potential therapeutic effects in a diabetic mouse model.

We found elevated Gremlin1 mRNA levels in adipose tissue of first-degree relatives of T2D patients, correlated with body fat percentage and insulin resistance markers. Increased expression was also observed in the subcutaneous and visceral fat, and liver of T2D patients, particularly those with metabolic dysfunction-associated steatohepatitis (MASH). Additionally, Gremlin1 levels were higher in obese T2D patients compared to lean individuals.

In vitro, Gremlin1 was shown to disrupt insulin signaling in human hepatocytes, adipocytes, and muscle cells by inhibiting Akt phosphorylation. However, in vivo studies in mice revealed that neither increasing Gremlin1 expression nor administering Gremlin1 protein significantly affected metabolism.

BMP4 gene therapy in diabetic mice showed limited benefits, such as short-term weight gain and delayed insulin decline, but did not reverse diabetes or have lasting metabolic effects.

CONCLUSIONS: Gremlin1 is a secreted protein that antagonizes insulin signaling, particularly in adipose tissue and liver, and is associated with insulin resistance and metabolic dysfunction in humans. While it presents a potential therapeutic target for obesity-related conditions, its metabolic impact appears to be species-specific. BMP4 therapy showed initial promise in diabetic mice but requires further research for long-term effectiveness.

Keywords: Gremlin1, obesity, type 2 diabetes, metabolic dysfunction-associated fatty liver disease, MASLD, metabolic dysfunction-associated steatohepatitis, MASH, BMP4.

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SAMMANFATTNING PÅ SVENSKA

Sockersjuka drabbar ungefär en halv miljon personer i Sverige. Mörkertalet lär vara ännu högre och många vet inte ens om att de har sjukdomen. Vid sockersjuka, så kallad typ-2 diabetes (T2D), är sockerregleringen satt ur spel. Detta händer på grund av bristande insulinproduktion, eller dålig känslighet av kroppens vävnader tex. fettvävnad för insulin (kallad för insulinresistens). Efter vi ätit, bryts maten ner i molekyler (bl.a druvsocker) som tillsätts till blodet som sedan transporteras till hela kroppen. Insulin är hormonet som signalerar cellerna att ta upp druvsocker som sedermera omvandlas till energi inne i cellen.

Gremlin1 är ett protein som har en stor roll i hur våra kroppar lagrar fett. Detta protein fungerar som en sorts knapp som talar om för cellerna att bli fettceller, och det finns i höga nivåer hos människor som är överviktiga. Ju mer Gremlin1 det finns, desto mer påverkar det hur våra kroppar använder insulin.

I mina studier tittade jag på hur Gremlin1 beter sig hos människor med fetma och T2D, samt hos deras nära släktingar som kan vara i riskzonen för dessa tillstånd. Jag tittade också på vad som händer i levern, som kan bli belamrad av fett och inflammerad när kroppen inte hanterar fett och socker på ett korrekt sätt.

Vad jag hittade var ganska intressant: Hos personer som är i riskzonen för T2D var nivåerna av Gremlin1 högre och kopplade till hur mycket fett de hade och hur bra deras kroppar kunde använda insulin. Hos personer med T2D, speciellt de med en fettlever sjukdom, var nivåerna av Gremlin1 ännu högre.

Mina studier visade också att Gremlin1 kan blockera insulin från att göra sitt jobb i celler som är viktiga för att bearbeta socker, som leverceller, fettceller och muskelceller. Detta sker eftersom Gremlin1 stoppar ett nyckelprotein kallad Akt från att fungera korrekt, vilket är avgörande för att insulin ska kunna utföra sin funktion.

Dock, när jag letade efter dessa resultat hos möss, hittade jag något oväntat, Gremlin1 verkade inte ha samma påverkan på möss som det hade på människor. Och när jag försökte använda ett protein kallat BMP4, som är avsett att motverka Gremlin1, hjälpte det möss en aning till en början men löste inte problemet på lång sikt.

Budskapet är att Gremlin1 verkar vara en nyckelspelare i fetma och insulinresistens, och det kan vara ett mål för nya behandlingar. Men det finns fortfarande mycket att lära, speciellt eftersom möss och människor reagerar på olika sätt. Det är ett fascinerande forskningsområde som kan leda till bättre behandlingar av fetma, typ 2-diabetes och relaterade leversjukdomar i framtiden.

LIST OF PAPERS

This thesis is based on the following three papers.

Paper I. Shahram Hedjazifar, Roxana Khatib Shahidi, Ann Hammarstedt, Laurianne Bonnet, Christopher Church, Jeremie Boucher, Mathias Bluher and Ulf Smith. **The novel adipokine Gremlin1 antagonizes insulin action and is increased in type-2 diabetes and NAFLD/NASH.** *Diabetes* 2020, **69**(3); 331–41.

Paper II. Roxana Khatib Shahidi, Jenny M. Hoffman, Shahram Hedjazifar, Laurianne Bonnet, Ritesh K. Baboota, Stephanie Heasman, Christopher Church, Ivet Elias, Fatima Bosch, Jeremie Boucher, Ann Hammarstedt, Ulf Smith. **Adult mice are unresponsive to AAV8-Gremlin1 gene therapy targeting the liver.** *PLoS One* 2021, **16**(2); e0247300.

Paper III. Roxana Khatib Shahidi and Jeremie Boucher. **BMP4 gene therapy delays diabetes progression in the diabetic *db/db* mice.** [Manuscript].

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ABBREVIATIONS

AAV	Adeno-associated virus
Akt/PKB	Protein kinase B
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
BAT	Brown adipose tissue
BMI	Body mass index
BMP	Bone morphogenic protein
CD	Control diet
CVD	Cardiovascular disease
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
EPI	Epididymal
FDR	First-degree relative
FFA	Free fatty acid
GLUT	Glucose transporter
GTT	Glucose tolerance test
HFD	High-fat diet
IGF	Insulin-like growth factor
IHC	Immunohistochemistry

IL	Interleukin
IP	Intraperitoneal
IR	Insulin resistance
IRS	Insulin receptor substrate protein
ITT	Insulin tolerance test
MASLD	Metabolic dysfunction-associated steatotic liver disease
MASH	Metabolic dysfunction-associated steatohepatitis
MAPK	Mitogen-activated protein kinase
MSC	Mesenchymal stem cell
PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent protein kinase 1
PI3K	Phosphoinositide 3 kinase
PIP3	Phosphatidylinositol-3,4,5-triphosphate
PKB	Protein kinase B
PPAR	Peroxisome proliferator-activated receptor
PPAR	Peroxisome proliferator-activated receptor
PTT	Pyruvate tolerance test
RT-PCR	Real-time polymerase chain reaction
RNA	Ribonucleic acid
SAT	Subcutaneous adipose tissue
SEM	Standard error mean

SGLT	Sodium-glucose linked transporter
T2D	Type 2 diabetes
TAG	Triacylglycerol
TNF	Tumor necrosis factor
UCP1	Uncoupling protein 1
VEGF	Vascular endothelial growth factor
WAT	White adipose tissue
WT	Wild type
ZFP	Zinc-finger protein

1 INTRODUCTION

1.1 PREVALENCE OF OBESITY AND TYPE 2 DIABETES

Obesity is a chronic metabolic disease that is now a global pandemic of adults and children. Major factors include increased consumption of high-calorie foods and inactive lifestyles, in addition to genetic and environmental factors [1, 2]. The World Health Organization (WHO) defines obesity in adults as having a body mass index (BMI) of 30 or higher, and overweight as having a BMI of 25 or more [3].

In 2018, over one-third of the world population was categorized as overweight or obese [4], and at high risk for developing type 2 diabetes (T2D) [5, 6]. Moreover, overweight and obese individuals have a ten times greater relative risk of developing T2D than lean individuals [7]. The World Health Atlas (2022) predicts that every eighth person in the world will suffer from obesity by the end of the this decade [3].

T2D is reported to be the most critical global health threat of the twenty-first century and is the century's greatest challenge [8]. WHO defines T2D as a condition in which an individual either uses insulin, takes hypoglycemic agents, or has a fasting blood glucose level above 7.0 mmol/l [9]. Low- and middle-income countries face a heightened risk of developing T2D due to urbanization, nutritional transitions, and sedentary lifestyles [10].

This increasing prevalence requires new interventions to avoid reaching critical disease rates in the future. In 2020, global prevalence of T2D was projected to raise to 7079 individuals per 100000 by 2030 [11]. Fig. 1 shows the forecasted disease trend [11].

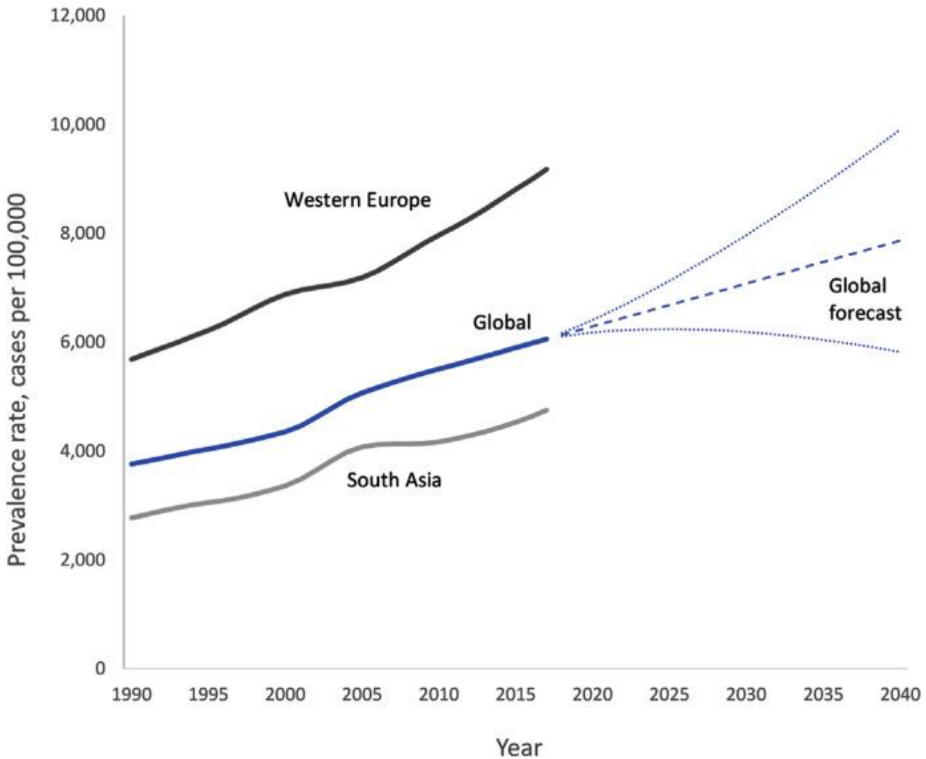

Figure 1. Trends in global prevalence of type 2 diabetes, Forecast estimates using SPSS Time Series Modeler (Ljung Box Q, p = 0.16). Dotted lines indicate upper and lower confidence limits. This figure is adopted from [11].

Importantly, first-degree relatives (FDRs) of people with T2D have larger fat cells (hypertrophic expansion) in abdominal white subcutaneous adipose tissue (SAT) than those without a genetic predisposition for the disease [12] and the genetic predisposition is also associated with impaired insulin sensitivity, even in lean adults [13]. These findings establish a connection between genetic predisposition to T2D, dysfunctional fat leading to hypertrophic obesity, and ectopic fat accumulation [12, 14, 15].

There is abundant evidence that obesity is associated with diseases such as T2D, dyslipidemia and Metabolic dysfunction-associated steatotic liver disease (MASLD) [16-18]. Primary risk factors include insulin resistance,

elevated pro-inflammatory markers, and ectopic lipid accumulation in organs that do not naturally store fat [19, 20]. Resistance occurs when the organs targeted by insulin become unresponsive to it [21], and its effect on glucose uptake is compromised, as are its anti-lipolytic effects, resulting in chronic elevation of free fatty acids (FFAs) in the blood, and leading to ectopic fat accumulation [22]. Insulin resistance therefore appears to be the fundamental risk factor for developing obesity and T2D [23].

1.2 THE ROLE OF INSULIN IN GLUCOSE HOMEOSTASIS

The endocrine system consists of a network of glands that regulate physiology and behavior. Glands produce, store and release signaling molecules hormones into the circulation; they are the primary communicators between organs [24, 25]. The hormones insulin and glucagon, released from the beta and alpha cells of the pancreas, respectively, continuously regulate blood glucose levels [26-28].

The anabolic hormone, insulin, is important for promoting the synthesis of carbohydrates, fats and proteins [21, 29]. Insulin secretion is triggered by elevated blood glucose after eating, which results in uptake of glucose into peripheral tissues, and a reduction in circulating glucose [23, 30].

Insulin has varying effects on different tissues. In the liver, it promotes lipid and glycogen synthesis, and suppresses glucose production [23, 30]; in skeletal muscle, it facilitates the entry of glucose, via increased translocation of glucose transporters to the plasma membrane, and converts it to glycogen; in adipocytes, it stimulates glucose uptake and inhibits lipolysis, resulting in decreased blood glucose and the release of FFAs **(Fig. 2)** [29]. Insulin sensitivity in these organs is compromised in metabolic diseases and is

therefore a critical factor that connects obesity, T2D and MASLD and MASH [19, 20].

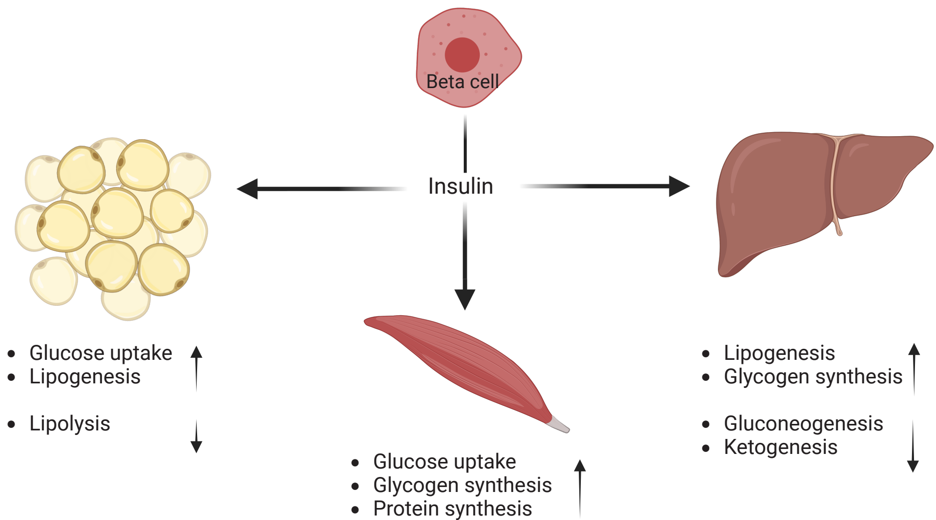

Figure2. Insulin, produced by beta cells of the pancreas, regulates glucose homeostasis in different targeted tissues. In adipocytes and muscles, insulin stimulates glucose transporter type 4 (GLUT4) translocation to the cell membrane and glucose uptake. In adipocytes and hepatocytes, it induces lipogenesis. In the liver, it increases glycogen synthesis while reducing gluconeogenesis and ketogenesis. In adipocytes, insulin reduces lipolysis. Draw with biorender.

1.3 HEPATIC FAT ACCUMULATION, NONALCOHOLIC FATTY LIVER DISEASE

Hepatocytes are important in lipid metabolism, maintaining a finely regulated balance between triglyceride synthesis and hydrolysis in the liver, which is crucial for hepatic lipid homeostasis [31, 32].

The intimate connection between adipose tissue and liver metabolism in humans arises from the fact that adipocytes provide more than two-thirds of

the fatty acids necessary for hepatic triglyceride synthesis [33]. When hepatocytes experience insulin resistance and hyperglycemia, there's an increased accumulation of specific lipids, notably diacylglycerol and ceramides [33, 34].

MASLD is a chronic liver ailment progresses alongside insulin resistance and is usually not detected until patients develop cirrhosis [35]. MASLD is characterized by increased liver fat content exceeding 5%, occurring without significant alcohol consumption or other secondary cause of steatosis [36]. MASLD affects around 25% of the global population. It has a spectrum of pathological severity, from simple steatosis to more severe liver damage and inflammation called MASH [37]. Although BMI may not be increased in MASLD, there may be other metabolically relevant complications of obesity, such as insulin resistance, high blood pressure, atherogenic dyslipidemia and hyperglycemia [34]. Studies show that people with T2D (or a genetic predisposition for it) are at higher risk of developing MASLD [36].

1.4 INSULIN SIGNALING AND GLUCOSE UPTAKE

Insulin receptors (IRs), part of the transmembrane tyrosine kinase protein family, are vital in cell metabolism. They comprise a tetrameric protein with two alpha and two beta subunits, and they operate as dimers, with four insulin-binding sites. Alpha subunits on the extracellular side of the cell membrane are similar to insulin-like growth factor-1 receptors [38], and are known to inhibit the tyrosine kinase activity of the beta subunits [39].

Insulin signaling begins when insulin binds to alpha subunits. This receptor activation triggers auto-phosphorylation of beta subunits and activates a cascade of phosphorylation events [39]. The IR substrate proteins (IRS1 and

IRS2) serve as docking sites, facilitating the recruitment and activation of phosphoinositide 3-kinase (PI3K) [21], which results in a surge in intracellular concentrations of the phospholipid, phosphatidylinositol-3,4,5-triphosphate (PIP3). Membrane–bound PIP3 activates phosphoinositide-dependent protein kinase 1 (PDK1), which in turn phosphorylates Akt/PKB (protein kinase B) at serin 473 (pSer473-AKT). When activated, pSer473-AKT causes phosphorylation of two Rab-GTPase activating proteins, AS160/Tbc1d4 at Thr-642 and TBC1D1 at Ser-237 and Thr-596 [40]. Phosphorylation of AS160 and TBC1D1 increases the translocation of glucose transporter-4 (GLUT4) to the extracellular membrane, and results in increased glucose uptake (Fig. 3) [39-41].

The interplay between insulin receptors and IRs also initiates the Ras mitogen-activated protein kinase (ras-MAPK) signaling cascade, which regulates cellular activities such as cell proliferation, cell differentiation and growth [42].

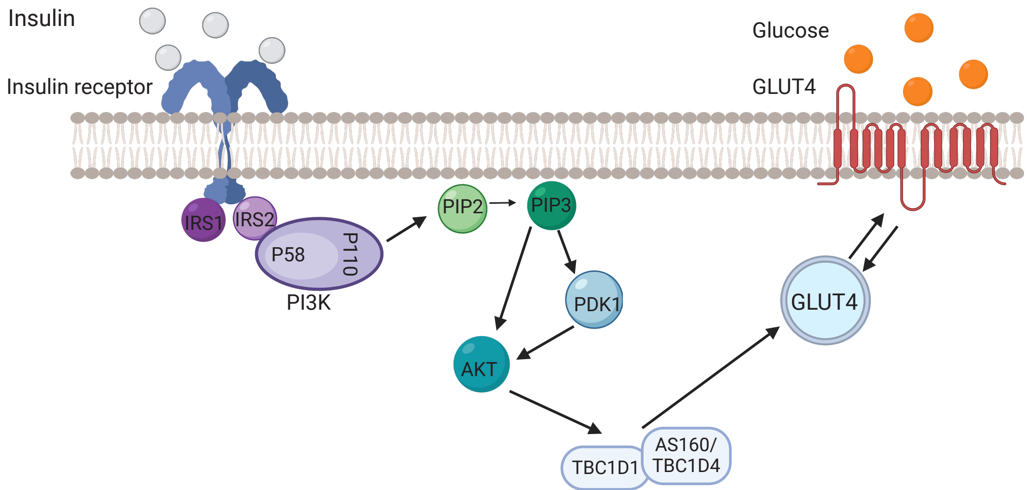

Figure3. Overview of the insulin signaling pathway involving insulin receptor substrates 1 and 2 (IRS1, IRS2), phosphoinositide 3-kinase (PI3K), phosphatidylinositol 4,5-bisphosphate (PIP2), phosphatidylinositol 3,4,5-triphosphate (PIP3), 3-phosphoinositide-dependent protein kinase-1 (PDK1), protein kinase B (PKB/AKT), TBC1 domain family member 1,4 (TBC1D1, AS160/TBC1D4) and glucose transporter 4 (GLUT4). [Draw with Biorender.]

1.5 GLUCOSE TRANSPORTERS

Glucose is an essential metabolic substrate in all mammalian cells. Two primary types of glucose transporters regulate glucose uptake: sodium–glucose linked transporters (SGLT) and solute carrier glucose transporters (GLUTs) [43-45]. SGLTs are mainly found on the luminal surfaces of cells lining the small intestine and the renal tubules of the kidneys. In the small intestine, they move glucose against the gradient, ensuring cell uptake. In the kidneys, they are involved in the reabsorption of glucose from the glomerular filtrate. They co-transport glucose and sodium ions in the same direction, which does not directly consume energy as adenosine triphosphate (ATP) but relies on sodium concentration gradients established by sodium–potassium ATPase [44].

Cell membrane-bound GLUTs comprise twelve membrane-spanning regions with intracellular amino and carboxyl terminals, and facilitate glucose transport via diffusion, commonly acting as uniport transporters. [46].

Three categories of glucose transporters exist [46]. GLUT4, classified under Class I, acts as an insulin-responsive transporter for glucose and is distributed in the heart, skeletal muscle, adipose tissue, and brain. Initially housed in cell cytoplasm within vesicles, it undergoes translocation to the plasma membrane under insulin influence [45].This insulin-driven process leads to a substantial boost in glucose transport, typically by 10 to 20 times [47]. Skeletal muscle accounts for up to 75% of insulin-driven glucose uptake, facilitated by GLUT4 [46]. Insulin quickly promotes the translocation of GLUT4 and glucose transport in fat and muscle cells. Signals originating from occupied insulin receptors trigger downstream changes in serine/threonine kinases rapidly, ultimately resulting in GLUT4 delivery and accumulation at the plasma membrane. Kinetic studies have identified distinct phases in insulin stimulation. Initially, there is a quick GLUT4 delivery elevation to the cell membrane from a subcellular reservoir, followed by a sustained level of stimulation where GLUT4 recycles through various subcellular locations [48]. This overview, Figure 3, delves into the phases of insulin stimulation concerning GLUT4 translocation and discusses the molecules currently implicated in activating these trafficking steps.

1.6 ADIPOSE TISSUE

1.6.1 ADIPOSE TISSUES CLASSIFICATION AND FUNCTION

Adipocytes fall into three distinct types – white, brown and beige – depending on their morphology, their capacity for lipid storage, and their ability to convert calories into heat due to increases the number of mitochondria and UCP1 [49].

White adipose tissue (WAT) is the most abundant store of fat [50]. WAT is identified by its white or yellow appearance, with lower vascularization and innervation compared to brown tissue. Fat cells in WAT typically range in size from 20-200 um and are unilocular, meaning they contain a single lipid vacuole. Within this vacuole, lipids are stored to be utilized during periods of energy demand. Approximately 90 to 99% of the lipids contained within the white adipocyte lipid vacuole are stored for energy use[49] . It is subdivided according to body location into (a) intra-abdominal or visceral adipose tissue surrounds the organs and is in the peritoneal cavity [51], and (b) SAT, located just beneath the skin [51]. SAT is a primary storage site for excess energy, in the form of triacylglycerol (TAGs) within lipid droplets. When the body requires energy, lipolysis occurs and FFAs are released into the bloodstream [49, 52].

Brown adipose tissue (BAT) is characterized as a multilocular cell containing several small lipid droplets and abundance of mitochondria characterized by dense ridges. These features contribute to the tissue's brown coloration, along with its dense vascularization [53]. These cells have a polygonal shape and measure 15 to 50 um [54]. BAT is found in specific anatomical regions, well-characterized in rodents, where it plays a crucial role in helping them survive cold temperatures [53]. Although BAT was once thought to be exclusive to hibernating and small mammals and present only in human infants, recent

research has revealed that adult humans also possess functional and inducible BAT [55]. Using an advanced imaging technique, fluorodeoxyglucose positron emission tomography combined with X-ray computed tomography, researchers have identified metabolically active BAT in adult humans, comprising about 1% to 2% of total fat stores. These BAT depots are primarily located in the cervical, supraclavicular, interscapular, axillary, paravertebral, mediastinal, and upper abdominal regions [55].

The primary role of BAT is to disperse energy in the form of heat via thermogenesis process. The abundance of mitochondria and elevated levels of UCP1 are crucial elements in the thermogenic mechanism within BAT [56]. The expression of UCP1 enables BAT to dissipate the electrochemical gradient typically utilized for ATP synthesis, thus generating heat via detaching oxidative phosphorylation. This function of BAT relies on the expression of UCP1, regulated by the sympathetic nervous system through noradrenaline signaling, which is triggered by cold exposure, thyroid hormone, adrenergic stimulation, β3-agonists and retinoids [56, 57]. BAT offers improved whole-body energy expenditure, as well as protecting against diet-induced obesity in mature lean mice [58, 59].

Adipose tissue demonstrates remarkable adaptability, able to undergo transformations triggered by environmental or dietary factors. One potential method to enhance the population of UCP1-rich cells within adipose tissue involves converting white (pre)adipocytes into brown-like fat cells. This process, termed "browning" of adipocytes, refers to the reversible transdifferentiating of the adipose organ [60]. They have a smaller capacity for fat storage and an increased number of mitochondria than WAT, therefore a greater thermogenic ability which can burn extra energy in the form of heat

production [50, 61]. The presence of BAT and beige adipocytes differs based on factors such as sex, age, ambient temperature, and metabolic status [57, 61].

1.6.2 ADIPOSE TISSUE IN OBESE AND TYPE 2 DIABETES

Historically, the main measure of metabolic health was BMI. However, about 30% of obese people have a normal metabolic profile [62], and a similar percentage of lean people have signs of metabolic problems and decreased insulin sensitivity [63]. Increased BMI is therefore not an adequate measure of the risk for decreased insulin sensitivity and may not be reliable as a single marker for assessing T2D risk [63]. Other factors, such as existing fat functional capacity (whether it operates as intended or not), are crucial to the potential for developing insulin resistance and T2D [64].

Obesity and T2D are multifactorial and heterogeneous diseases. Over 50 years ago, a positive correlation was found between visceral fat (VF) distribution and energy metabolism in individuals with T2D and their FDRs, suggesting another factor to consider [65, 66]. People who are genetically predisposed for abdominal obesity have twice the risk of developing T2D for any BMI value [67]. Previous studies revealed the importance of adipose tissue in relation to impaired insulin sensitivity, reporting that weight gain in insulin-sensitive individuals leads to adipocyte remodeling, and associated insulin resistance. Adipocyte cell size increases (hypertrophic expansion) in insulin resistance (Fig. 4), suggesting that adipocyte enlargement may predict T2D more accurately than BMI. It’s important to note that while a higher BMI is a risk factor for insulin resistance and T2D, individuals with elevated visceral fat are at an even greater risk of developing T2D, regardless of their BMI. This highlights the critical role of fat distribution in disease risk [68, 69]. The

adaptive changes of adipocytes, including cell size and location of fat accumulation (visceral fat), are a risk indicator of metabolic complications such as T2D [70].

When the storage capacity of SAT is reached (the primary storage site for energy in the form of lipid droplets), in conditions like obesity, excess lipid accumulates in visceral and ectopic sites [69, 71]. Visceral fat is directly linked to the liver via the portal vein, so the extra fat drains into hepatocytes, and accumulates in the liver [69]. Note that visceral fat obesity correlates positively with insulin resistance, but there is no correlation between SAT adipocyte cell size and insulin sensitivity [69, 72].

1.6.3 WHITE ADIPOSE TISSUE AS AN ENDOCRINE ORGAN

As well as storing fat, adipose tissue is a dynamic endocrine organ that actively secretes signaling molecules called adipokines. These have diverse roles in both local and systemic signaling pathways. The key adipokines are leptin, adiponectin, tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), and insulin-like growth factor 1 (IGF-1) [25].

Leptin, the first adipokine to be identified, acts primarily on the central nervous system, to reduce appetite and consequently limit food intake [25, 73]. Circulating leptin levels are elevated in obese individuals and correlate directly with body fat mass [73]. Serum levels of leptin correlates positively with fasting insulin and glucose levels and a marker of insulin resistance in human (HOMA-IR) [74].

Adiponectin is primarily secreted by mature adipocytes. It binds to adiponectin receptors and triggers a series of tissue-specific signaling pathways; it activates p-38 MAPK, phosphorylates adenosine monophosphate (AMPK), and enhances peroxisome proliferator-activated receptor alpha (PPAR-α) ligand activity. In muscle, it promotes fatty acid oxidation and suppresses hepatocyte glucose production, thus stabilizing glucose homeostasis. Adiponectin levels correlate inversely with fat mass, and it is a key indicator of insulin sensitivity [25, 73]. In obese mice, overexpression results in beneficial growth of SAT, mitigating the diabetic phenotype and leading to reductions in systemic inflammation [71].

TNF-α is implicated in initiating insulin resistance in obesity through its effect on insulin receptors. TNF-α inhibits human pre-adipocytes differentiation leading to an impaired adipogenesis in obesity [75]. IL-6 is highly secreted by SAT and is further increased due to hypertrophic expansion of SAT and elevated serum levels of IL-6 is associated with insulin resistance [22]. Furthermore, the hypertrophic expansion of adipocytes, increased secretion of IL-6 and TNF-α, disrupts the regulation of lipolysis, leading to heightened FFA release, diminished peripheral glucose uptake, and increased hepatic glucose production [22, 52, 76]. Elevated systemic levels of FFAs impair glucose uptake from the bloodstream, resulting in ectopic fat storage [22](Fig 4).

VEGF promotes angiogenesis, in the context of adipose tissue, VEGF facilitates the expansion of fat cells by ensuring an adequate blood supply to support their growth. IGF-1 was initially discovered due to its capacity to replicate the metabolic effects of insulin. This adipokine mediates growth hormone effects, promoting cell proliferation in various tissues including adipose tissue expansion in obesity [25]. Reduced plasma level of IGF-1 is associated hepatic steatosis [77].

1.6.4 WHITE ADIPOSE TISSUE AND INFLAMMATION

Many adipokines function as immunoregulatory proteins. When secreted into the circulation they induce the local secretion of pro-inflammatory cytokines, notably TNF-α and IL-6. Elevated serum levels of these adipokines subsequently lead to the recruitment of pro-inflammatory immune cells [78, 79].

Obesity, hypertrophic expansion of the adipocytes is marked by an influx of macrophages into the adipose tissue leading to a chronic inflammation. These macrophages primarily arise from localized macrophage proliferation in WAT during the initial stages of obesity, a response to heightened in situ macrophage proliferation [80].

Macrophages are white blood cells recognized for their phagocytic capabilities and their role in tissue remodeling during an innate immune response [81]. Pro-inflammatory stimuli, such as TNF-α, can activate M1 macrophages which is a pro-inflammatory type of macrophages [82]. One distinctive feature of inflammation in adipose tissue is the build-up of M1 macrophages, which has been linked to the onset of metabolic diseases and T2D [73, 78, 83, 84].

The secretion of IL-6 and TNF-α culminates in the accumulation of macrophages in adipose tissue, promoting local insulin resistance [22, 85]. These processes collectively contribute to systemic insulin resistance and glucose intolerance, hallmark features of obesity and T2D [14]. As illustrated in Fig 4, hypertrophic expansion of adipose tissue alters adipokine secretion, reduces lipolysis and insulin-stimulated glucose uptake. The increased cell volume triggers pro-inflammatory responses and fibrosis and leads to dysfunctional WAT, so that lipids accumulate in ectopic tissues (liver and muscle) and induce peripheral insulin resistance [22].

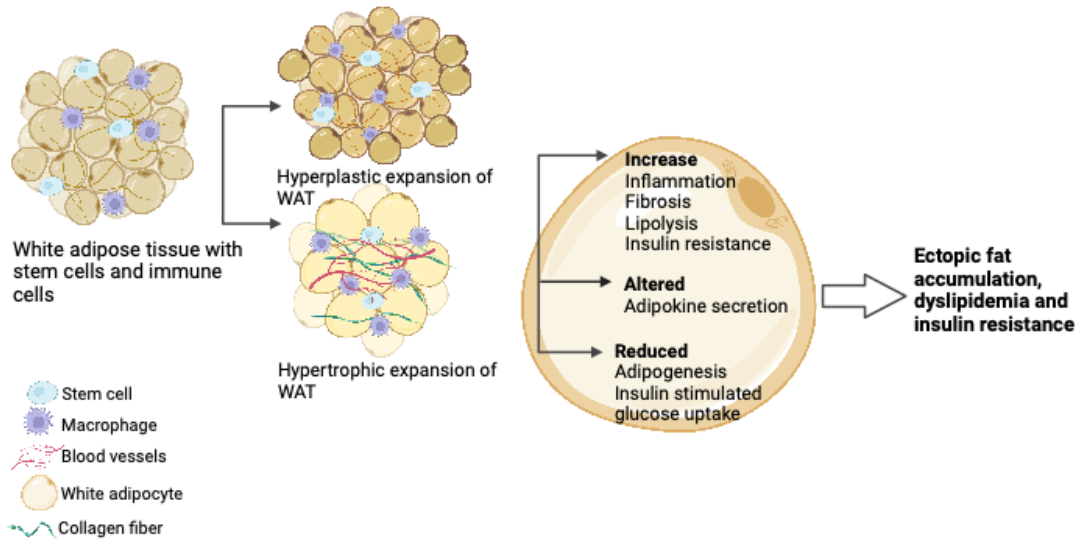

Figure 4. White adipose tissue (WAT), showing hyperplastic (cell number) and hypertrophic (cell volume) adipocyte expansion, and the consequences on white adipocyte function. The hypertrophic expansion alters adipokine secretion, reduces lipolysis and insulin-stimulated glucose uptake. The increased cell volume triggers pro-inflammatory responses and fibrosis and leads to dysfunctional WAT, so that lipids accumulate in ectopic tissues (liver and muscle) and induce peripheral insulin resistance. Adapted from [86]. Illustrated using Biorender.

1.7 BONE MORPHOGENIC PROTEINS

BMPs are part of the transforming growth factor (TGF)-beta superfamily. They are so named because of their capacity to trigger ectopic bone formation, but they also have important roles in regulating cell differentiation, organogenesis, and developmental morphogenesis. Their regulation is vital for developmental processes and preventing disease [87, 88]. BMP4 and BMP7 are expressed in various tissues and are fundamental for adipogenesis. BMP4 and BMP7 are

distributed widely, including the lungs, liver, kidneys, brain, spinal cord, heart, skeletal muscles, spleen, thymus, prostate and pancreas [88].

1.7.1 BONE MORPHOGENIC PROTEIN SIGNALING

BMPs have important roles in cellular processes such as cell differentiation, proliferation, and apoptosis, and in tissue homeostasis [87]. BMP receptors comprise a heterodimeric protein with two type-1 and two type-2 subunits [88]. BMP signaling encompasses both Smad-dependent (canonical) and Smad-independent pathways [87, 88].

In the Smad-dependent BMP signaling pathway, BMP dimers bind to type-2 subunits of BMP receptors on cell surfaces, resulting in phosphorylation of type-2 receptor subunits. Then receptor-regulated Smads (R-Smad1,5,8) undergo phosphorylation. Co-mediator Smad4 (co-Smad4) then associates with phosphorylated Smad1,5,8 and moves into the cell nucleus (Fig. 5), facilitated by nuclear import signals from the Smad proteins. Once inside the nucleus, the complex modulates the expression of various genes that are critical for the downstream effects of the BMP4 pathway[87, 89].

In the Smad-independent BMP-signaling pathway (Fig. 5), various signaling cascades are activated. In the MAPK pathway the ligand attaches to BMP receptor 1a (BMPR1a), and this initiates p38 MAPK signaling, which governs processes such as cell proliferation, differentiation, survival, and migration [87, 88]. P38MAPK also regulates mitochondrial biogenesis and insulin-dependent glucose uptake by stimulation of protein kinase B (Akt), which is crucial for regulating cell survival, growth, and metabolism [90].

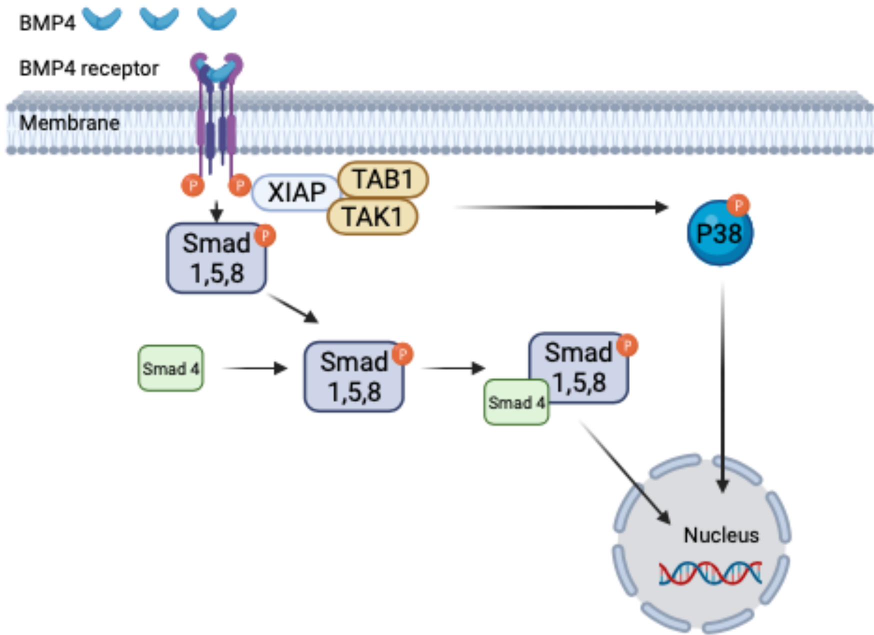

Figure 5. Overview of the canonical BMP4 Smad-dependent and Smad-independent pathways. Components include Smad 1,5,8; co-Smad 4; X-linked inhibitor of apoptosis protein; TGF-β activated protein (TAB1); TGF-β activated kinase; and mitogen-activated protein kinase (P38). Adapted from [89].Illustrated using Biorender.

1.7.2 THE ROLE OF BONE MORPHOGENIC PROTEINS IN ADIPOSE TISSUE

BMP4, and 7 are critical in white and brown adipogenesis [15, 91, 92]. These BMPs play an essential role in pre-adipocyte cell's commitment towards the adipose lineage, as well as in the development and health of adipose tissue [93]. BMP4 is required for commitment of precursor cells into white adipose linage therefore, required for WAT expansion [94]. BMP4 expression and secretion persist throughout white adipocyte differentiation [91]. The sustained BMP4 expression promotes a beige phenotype in white adipocytes leading to an increased thermogenic activation [95]. BMP7 induces commitment in

precursor cells into brow adipose tissue [92] and its gene expression level remains undetected after differentiation in human SAT [94]. Therefore, BMP7 is not a focal point of this thesis.

Moreover, hypertrophic adipocytes exhibit increased BMP4 expression and BMP4 mRNA levels correlate positively with isolated human adipocyte cell size from SAT[94]. However, the beneficial effect of BMP4 is undermined by increased levels of its inherent antagonists, such as Chordin-like-1 (CHRDL1), Noggin, and Gremlin1 [94]. Specifically, CHRDL1 enhances human mesenchymal stem cell proliferation and acts as an inhibitor for BMP2, 4, and 7 [96]. Noggin, another BMP antagonist, exerts its inhibitory effect by binding to the BMP receptor and is significantly increased in diet-induced obesity mice model [58].

Gremlin1, the centerpiece of this thesis, is a well-researched intra- and extracellular BMP4 antagonist [97]. It has distinct roles in organogenesis and fibrosis [98, 99]. These BMP4 adversaries hinder BMP4 signaling by interfering with the action of ligands and receptors [100].

In contrast, levels of BMP4 antagonists also rise during the hypertrophic expansion of adipocytes. This increase prevents cells from capitalizing on BMP4's beneficial effects. For example, mRNA expressions of BMP4 inhibitors, such as CHRDL1 and Gremlin1, correlate positively with adipocyte cell size [94].

1.8 GREMLIN1, BONE MORPHOGENIC PROTEIN ANTAGONIST

Gremlin1 is expressed in various tissues, including fat. It disrupts BMP signaling by binding to the BMP ligand and inhibits the canonical BMP signaling pathway by preventing phosphorylation of Smad1,5,8 and BMP gene expression [99, 101].

Gremlin1 has multiple actions, both cell- and context-dependent. For example, developmental defects of the kidneys and limbs in Gremlin1 knockout mice result in neonatal death [102]. It is highly expressed in pancreatic fibrosis, and heterozygous knockouts have about a 30% reduction in pancreatic fibrosis [103]. The mechanisms by which Gremlin1 induce fibrosis relates to the formation of myofibroblasts in different tissues [99]; high levels are associated with fibrotic diseases of the liver, lungs, eyes and kidneys, as well as pulmonary hypertension [97, 99].

In genetically engineered mice that overexpress Gremlin1, bone density diminishes, increasing the risk of spontaneous fractures [104]. Overexpression also impedes cell differentiation and is involved in the progression of certain carcinomas [105]. In the kidney, increased expression in the tubular epithelial compartment influences the fibrotic response to renal injury in mice [106]. There is also evidence that it advances diabetic kidney fibrosis by modulating NOTCH signaling [107].

Gremlin1 is the smallest BMP antagonist and is a soluble secreted DAN protein (DAN refers to *d*ifferential screening-selected gene *a*berrative in *n*euroblastoma). It displays most homology within its central cysteine-rich domain, while its termini have low conservation [108], and it has three different splicing patterns with a molecular weight of about 20 kDa. However,

its weight is variable (24–25 KDa) due to post-translational modifications, glycosylation of its *N*-terminal, and phosphorylation [108, 109].

1.9 GREMLIN1 IN OBESITY AND INSULIN RESISTANCE

Gremlin1 has a critical a role in obesity-related complications and glucose metabolism. Expression is increased in response to elevated blood glucose levels. The significant positive correlation between Gremlin1 expression and adipocyte cell size has been found in biopsy samples from people with hypertrophic obesity [110]. The degree of insulin resistance correlates positively with adipocyte cell size [91], raising the risk of insulin resistance and T2D [91].

In adipose tissue, Gremlin1 interferes with adipocyte commitment and differentiation induced by BMP4, and levels positively correlate with BMI [94].

By antagonizing BMP4, Gremlin1 inhibits adipocyte differentiation and thus affects the equilibrium between pre-adipocyte and differentiated fat cells. Its mRNA levels correlate negatively with Ppar-g transcription activation in differentiated adipocytes [94].

The browning effect of sustained BMP4 expression on white adipocytes is hindered by Gremlin1. Suppressing Gremlin1 expression in human pre-adipocytes leads to increased UCP1, as well as mitochondrial cell numbers [94], Indeed, the adipose tissue of obese individuals shows enhanced BMP4 expression, although BMP4 signaling is diminished due to the overexpression of BMP4 inhibitors [94].

Obese mice have decreased levels of Gremlin1, but higher levels of other BMP4 antagonists such as Noggin in hypertrophic SAT. Noggin expression is also associated with markers of insulin resistance, such as body weight and SAT adipocyte size [58]. Human fat shows no clear differences in Noggin expression, and levels do not correlate with cell size or BMI, thus there is disparity in BMP4 inhibitor expression patterns and their roles in adipocytes between humans and mice [58, 94].

2 AIMS

There were multiple aims of this study, which comprised human studies with large well-characterized control and disease cohorts, and mouse models of diet-induced obesity and diabetes, comprising different age groups and stages of disease, as well as murine cell lines.

- Our aim was to elucidate the role of Gremlin1 in human obesity, T2D, and MASLD/MASH, in terms of insulin sensitivity and fibrosis in insulin-targeted tissues. We explore the Gremlin1 mRNA expression levels in subcutaneous and visceral fat, liver and its circulating levels in several cohorts' diagnosis for MASLD/MASH. Additionally, to study the potential effect of increased Gremlin1 protein on insulin sensitivity in insulin targeted cells we used human white adipocyte, human skeletal muscle cells (hSMC) and human IP-hepatocytes.
- We also aimed to investigate metabolic changes and insulin sensitivity by increasing circulating Gremlin1 in diet-induced obese mice and its potential mechanism of action using in vivo mouse model and *in vitro* murine cell lines.
- Additionally, aimed to evaluate the potential effect of prolonged BMP4 overexpression on diabetes development and insulin sensitivity in young pre-diabetic leptin-receptor-1 deficient *db/db* mice, and the efficacy of gene therapy on adult diabetic *db/db* mice.

3 METHODOLOGICAL CONSIDERATION

3.1 ETHICAL STATEMENT

The human studies were carried out in accordance with the Declaration of Helsinki and approved by the Ethical Committee of the University of Gothenburg and the ethics committee of the University of Leipzig. All patients provided written informed consent before participating. The mouse studies received prior approval from the local Ethics Committee for Animal Study at the Administrative Court of Appeals in Gothenburg and followed their guidelines.

3.2 HUMAN MATERIAL

The human material used in this study comes from well-characterized cohorts, as detailed in the Method section of Paper I (Appendix 1). All human materials, including tissue biopsies, blood samples, and cells, are valuable and require meticulous handling. However, due to practical constraints, sample sizes are often limited, which can affect the statistical power of the analysis.

Cohort FDR/Control: 34 nonobese individuals were examined: 17 participants with at least one known first-degree relative (FDR) with Type 2 Diabetes (T2D), and 17 individuals without any known genetic predisposition for T2D, defined as having no family history (control subjects). This cohort patients were matched for: BMI and age. Insulin resistance was assessed using fasting plasma insulin and glucose levels to calculate the HOMA of insulin resistance (HOMA-IR) index using the formula: HOMA-IR = (fasting plasma glucose × fasting plasma insulin) / 22.5. Local subcutaneous adipose tissue

samples were collected from the lower abdominal wall, as previously described [94].

Cohort ND/D: In a cross-sectional investigation, we examined Gremlin1 mRNA levels in paired samples of visceral/omental and abdominal subcutaneous adipose tissue (n = 233; BMI ≥ 30 kg/m2). Among these participants, 105 individuals exhibited normal glucose levels, while 128 had Type 2 Diabetes (T2D).

Cohort GIR: Within this subset of 93 individuals (BMI ranging from 24 to 37 kg/m2) with normal glucose tolerance (NGT), the expression of adipose tissue Gremlin1 mRNA was assessed in relation to the glucose infusion rate (GIR) during euglycemic-hyperinsulinemic clamps, following previously documented protocols [62].

Cohort ND/D/MASLD: This cohort comprised 52 obese individuals exhibiting a broad spectrum of liver fat content, with some diagnosed with Type 2 Diabetes (T2D) (n = 28; BMI ranging from 34 to 65.8 kg/m2), while others were without T2D (n = 23; BMI ranging from 34 to 65.9 kg/m2). Gremlin1 mRNA levels were assessed in both paired adipose tissue and liver samples. Metabolic parameters were measured using baseline blood samples collected between 8 and 10 A.M. after an overnight fast and analyzed using previously established methods [62].

Cohort Nob/obND/obD: Serum levels of Gremlin 1 were assessed in a total of 45 individuals. Among them, 30 had normal glucose tolerance (NGT), with a Body Mass Index (BMI) of less than 25 kg/m2 (n = 15) and 30 kg/m2 or greater (n = 15), while 15 individuals had known Type 2 Diabetes (T2D).

Other cohorts: During laparoscopic abdominal surgery, paired samples of subcutaneous adipose tissue, omental visceral adipose tissue, and liver were

obtained following previously established procedures [62]. The adipose tissue samples were promptly frozen in liquid nitrogen and stored at -80°C to prevent protein changes for investigations that were not conducted directly after sample collection.

3.3 DETERMINATION OF INSULIN RESISTANCE

3.3.1 HYPERINSULINEMIC-EUGLYCEMIC CLAMP

Intraperitoneal glucose and insulin tolerance tests reveal overall body glucose tolerance and insulin sensitivity, but measuring insulin sensitivity in insulin-targeted tissues can be more informative, as insulin action can vary in different insulin-targeted tissues. We chose to use a hyperinsulinemic–euglycemic clamp for examining *in vivo* insulin sensitivity in human tissue in paper I, cohort GIR [62] and in paper III, the mouse model of diabetes (leptin-receptor 1 deficient *db/db*).

The gold standard method for the measurement of peripheral IR is the euglycemic–hyperinsulinemic clamp (EHC), a technique that was first proposed by DeFronzo et al. in 1979 [111]. In Human, during the test, insulin is infused at a physiological rate, usually 40 mU/min/m^2 but higher doses also have been tested such as 50, 60, 80, 100, 120, or 160 mU/min/m^2. [112]

Because insulin's main effect is to decrease glucose concentrations by stopping endogenous glucose production (EGP) (mainly hepatic) and stimulating peripheral glucose disposal, glucose (or dextrose 20%) is infused at variable infusion rates, glucose infusion rates (GIR). This maintains plasma glucose concentration at constant levels throughout the study (around 5.5mmoL/L or 100 mg/dL). Thus, when glucose concentration is constant, the rate of glucose

appearance (Ra = EGP + GIR) equals the rate of glucose disappearance (Rd). These insulin infusion rates lead to insulin concentrations that almost completely suppresses EGP, thus GIR is used as an estimate of glucose appearance where GIR is the glucose infusion rate expressed in mg/kg/min [112].

We also used the hyperinsulinemia–euglycemic clamp in paper III for treatment cohort. The insulin clamp enable us administration of isotopic 2[^{14}C] deoxyglucose to assess tissue-specific glucose uptake [113]. It involves fasting mice for 5 hours, where t = 0 minutes refers to the end of the fast and the beginning of the insulin and glucose infusion (i.e. the clamp period). A typical priming dose is 1 µCi. We prepared a 0.05 µCi/µl [3-^{3}H] glucose solution in non-heparinized saline, and loaded it into a 1-ml syringe, which was secured in an infusion pump. The priming dose was a 1-minute infusion of 20 µl/min. Then 0.05 µCi/min (1 µl/min) was infused for 90 minutes (the equilibration period) [113]. More details are provided in the Method section of paper III (Appendix 3).

3.3.2 INSULIN-STIMULATED GLUCOSE UPTAKE IN CELL CULTURE

In Paper I we studied insulin-induced glucose uptake in human primary adipocytes. Cell cultures were used because [^{3}H] 2-deoxy-D-glucose ([^{3}H]2dG) is phosphorylated by hexokinase to 2-DG-6P, and cannot be metabolized further, allowing quantification of glucose imported into cells, thus indicating insulin-stimulated glucose uptake [114].

3.4 IN VITRO AND CELL MODEL SPECIFICATIONS

Culturing metabolic cells allows specific targets to be examined at a cellular level, rather than systemically. Isolated cells from tissue biopsies, cell lines and primary cells are widely used in metabolic studies. However, isolating specific cells and primary stellate cells requires human donor tissue. Primary cells are preferred due to their close similarity to mortal cells, although the data must be interpreted carefully. Further, inherited DNA variations among donors must be considered.

In contrast, immortalized cell lines are commercially available and are used in experiments that require repetition. Human primary adipocytes and primary skeletal muscle cells were collected by syringe biopsies from the donors in Paper I. In addition, immortalized human hepatocytes and a hepatocellular carcinoma cell line were used for the *in vitro* studies. Murine cell lines including 3T3/L1 and C2C12 were use in Paper II. The details of each cell model are given below.

Immortalized human hepatocyte: A cell line established from the liver cells of a 59-year-old man with colon cancer metastasis.

Hepatocellular carcinoma cell line: An immortalized human cell line produced from the liver of a 15-year-old male with a well-differentiated hepatocellular carcinoma. It has been used to study a range of liver disorders.

Human Primary Skeletal Stem Cells: Human myoblasts, differentiated into myotubes, with multinuclear morphology under light microscopy. The differentiation process is sensitive and takes at least 14 days.

3T3/L1: Mouse myoblast L1 cells, with a fibroblast morphology, that can be differentiated into mature adipocytes.

C2C12: Mouse myoblast cells with a relatively short differentiation period, that can speed up experiments and allow more straightforward repetition.

3.5 IN VIVO ANIMAL MODELS

Here is great value in using *in vivo* animal models to study human disease because they allow evaluations of hypotheses in more complex situations than *in vitro* studies. Despite the need to consider moral and ethical issues, animal models, especially rodents, facilitate simpler, quicker, and more repeatable studies.

We decided to use mice in our studies because they share large amounts of DNA with humans and have physiological similarities, despite differences in size and metabolic rates. They are practical to maintain in cages, and their dietary needs and living conditions are easily met. Female mice produce multiple litters each year, providing a constant supply of pure-bred mice and allowing observations at different developmental stages and across generations. However, we used male mice only, because they have a more robust altered metabolic phenotype. There is evidence that mouse metabolic processes are sex-dependent, thus in using males only we eliminated sex-related differences.

3.6 ANIMAL HOUSING AND SAMPLE COLLECTION

To evaluate metabolic changes, body weights were taken weekly. The 12/12-hour light–dark cycle was maintained, and free access given to water and food. The light–dark cycle was carefully maintained to avoid stress-induced

metabolic changes, because environmental stress can alter glucose homeostasis due to elevated adrenalin and noradrenalin. Furthermore, all metabolic tests in a study must be performed at a particular time point in the cycle. Mice demonstrate similar variations in glucose and metabolic hormones during 24 hours [115].

The mice were housed in a controlled humidity and temperature (21 °C). Because male mice are territorial, sometimes exhibiting severe aggression that compromises their welfare (and potentially study outcomes), we reduced behavior-based variability by ensuring each cage contained the same number of mice in both control and treatment groups. We placed four mice per cage and monitored their behavior carefully.

Different mouse strains vary greatly in glucose homeostasis and gene predispositions that can alter metabolism. therefore, it was crucial to use just one strain in our study. The C57BL/6 mouse is the most widely strain in metabolic disease studies.

Water intake was monitored every two days, and body weight was measured weekly throughout the experimental period. HbA1C levels were assessed regularly using HbA1C, and 4-hour fasting blood glucose and insulin levels were recorded every two weeks throughout the study period.

At termination, blood was collected by heart puncture and serum was stored immediately at -80℃. Tissues were weighed and collected for morphological analysis, or snap frozen in liquid nitrogen and stored at -80℃ for analysis of protein and gene expression.

3.6.1 AAV8 VECTOR-MEDIATED GENE EXPRESSION

We aimed to increase Gremlin1 expression in C57BL/6 mice, and BMP4 in *db/db* mice by targeting the liver. To achieve stable long-time increased expression of the gene, we used adeno-associated viral vector serotype 8 (AAV8), which encodes a codon-optimized murine Gremlin1/BMP4 cDNA sequence under the control of the human alpha 1-antitrypsin (hAAT1) promoter by triple transfection of HEK293 cells. Optimized cesium chloride gradient-based purification was used to produce high-purity batches of the vector (Fatima Bosch, Spain) [58, 59, 116].

In Paper II, C57BL/6 mice (Taconic, Hudson, NY, USA) received tail-vein injections (IV) of AAV8-Gremlin1 at 12 weeks of age and were studied at 30 weeks.

In Paper III, we used male homozygous *db/db* BKS.Cg-Dock7m+/+Leprdb/J mice (Charles River, Italy). It is known that *db/db* mice develop T2D at 4–8 weeks, thus we used two approaches: we administered IV *AAV8-BMP4* to (a) The treatment cohort at age 12 weeks. In this cohort, mice were studied for 10 weeks. (b) The prevention cohort (containing 2 sub-cohorts) received the AAV8-BMP4/null at the age of 6-7 weeks and mice were monitored for 6 weeks post vector administration.

A non-coding plasmid carrying the hAAT1 promoter was used in the control cohorts to produce empty vector.

Overexpression of the gene of interest (Gremlin1/BMP4) was expected to reach the desired point at 2 weeks post injection [117-119].

3.6.2 ANIMAL MODEL DIETS

In Paper II, after 12 weeks of a control chow diet (10 kcal% fat; Research Diets, New Brunswick, NJ, USA), male C57BL6/N mice were fed: (a) 45 kcal% high-fat diet (HFD) for AAV8-Gremlin1 analysis until termination at week 30 and (b) 60 kcal% HFD for IP rec-Gremlin1 analysis for 26 weeks, and thereafter received IP injections of rec-Gremlin1 (0.8µg/g; R&D Systems, Minneapolis, USA) or an equal volume of 2xDPBS in 1mM EDTA at pH 6.8 twice per week for a total of 5 weeks.

In Paper III, *db/db* mice were fed a control chow diet (10 kcal% fat, Research Diets, New Brunswick, NJ, USA) throughout the study.

3.6.3 METABOLIC TESTS

We challenged the mice with three metabolic tests to monitor glucose and insulin homeostasis. Although IP injections must be performed carefully to avoid the needle passing into the gut or stomach lumen [115], administering glucose orally is difficult and is stressful for the mice. Note that that plasma glucose following oral administration is significantly lower than when administered IP.

3.6.3.1 GLUCOSE TOLERANCE TEST

GTTs were performed to produce a “tolerance curve” for evaluating potential physiological changes due to glucose tolerance. Mice were fasted for 4 hours in the morning of the experiment and received an IP bolus of glucose around 12 noon. The sudden increase in blood glucose triggers insulin secretion, which reduces the extra circulating glucose. In healthy mice, blood glucose peaks 15 minutes after IP injection, then decreases due to uptake by muscle, fat and liver. We took plasma samples over time to allow us to calculate the area under the

curve (AUC) for insulin levels. In the obese mice, reduced insulin sensitivity was expected due to obesity complications [120].

3.6.3.2 INSULIN TOLERANCE TEST

An IP bolus dose of insulin is administrated. We measured glucose clearance rate to monitor general insulin sensitivity. This test reveals the sensitivity of insulin-targeted tissue, whereby a rapid decrease in blood glucose indicates whole-body insulin sensitivity [120].

3.6.3.3 PYRUVATE TOLERANCE TEST

We evaluated hepatic gluconeogenesis by measuring changes in blood glucose after an IP bolus dose of pyruvate. Pyruvate can be used by many other tissues, which can affect glucose concentration [121].

3.7 HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Tissues were also prepared for visual examination of the differences between treatment and control groups. Various staining methods were used to determine the effects of Gremlin1 on liver, WAT and BAT tissue structures, lipid accumulation, inflammation, and fibrosis. Standard staining with hematoxylin and eosin (H&E) was used to study general structures; a diazo-based fat-soluble dye (Oil Red O) was used for staining the lipid droplets and collagen was stained with Picrosirius Red.

Immunohistochemistry (IHC) was used for the paraffin-embedded tissue. Cell nuclei were colored using DAPI (4′,6-diamidino-2-phenylindole). This blue-fluorescent DNA stain exhibits an approximately 20-fold enhancement of fluorescence upon binding to AT adenine-thymine rich regions of dsDNA. Primary antibodies were used to find the protein of interest, detectable by fluorescent microscopy.

3.8 mRNA ANALYSIS AND Q-PCR

The first step for investigating tissue gene expression was by determining total RNA. Homogenized tissue was used for RNA extraction, and complementary DNA (cDNA) was made using Real-Time qPCR. The cycles of PCR heating and cooling melted the DNA, and the region of interest was replicated using commercially available primers to selectively amplify the unique sequence of the gene of interest. DNA polymerase makes double-strands by adding nucleotides.

We used TaqMan-based detection, which involves a probe containing fluorescent and quencher. An intact probe does not fluoresce. DNA polymerase separates quencher from the fluorescent, and the resulting signal can be measured to indicate the amount of PCR product, thus quantifying the gene of interest. We used the ribosomal housekeeping gene to normalize our data when evaluating changes in gene expression in the liver, WAT and BAT for markers of inflammation and fibrosis.

Gene expression of ribosomal housekeeping gene (18S RNA) was used to normalize the data.

3.9 PROTEIN EXTRACTION AND WESTERN BLOT ANALYSIS

To avoid protein degradation and phosphatase activity, a lysate from homogenized tissue was collected by a lysis buffer. Gel electrophoresis was used to separate proteins based on size. A rapid and reversible staining method (Ponceau) was performed to detect protein bands on Western blot membranes. To prevent non-specific antibody binding, the membrane was incubated with a blocking solution. Common blocking agents include non-fat dry milk or bovine plasma albumin (BSA). A synthetic or animal-derived antibody is used

as the primary antibody to detect a specific protein. Then a secondary enzyme conjugated antibody that recognized the primary antibody was used. The presence of the target protein is visualized by adding a substrate for the enzyme or by detecting the fluorescence emitted by the labeled secondary antibody. The signal is typically captured by a digital imaging system. The housekeeping protein was used to normalize data as described in the method section of each paper. This technique is used in all three papers.

3.10 ENZYME-LINKED IMMUNOSORBENT ASSAY

ELISA tests quantify protein levels by binding protein to a primary antibody. A microplate is coated with a capture antibody that is specific to the target antigen. Next, a secondary enzyme-conjugated antibody that recognizes the primary protein is applied. The conjugated enzyme commonly loaded are horseradish peroxidase (HRP) or alkaline phosphatase (AP). A substrate specific to the enzyme conjugated to the detection antibody is added to the plate. The enzyme catalyzes a reaction with the substrate, producing a detectable signal, such as a color change. The density of this color is measured to indicate the amount of enzyme and therefore the protein. This technique has been used in all three papers.

3.11 STATISTICS

Statistical analysis was used to evaluate (reject) the null hypotheses, specifically to reveal a lack of meaningful differences in data. We used inferential and hypothesis testing, with a two-group, two-tailed Student-test or Mann-Whitney non-parametric U-test to compare between groups, and analysis of variance (ANOVA) for multiple group comparisons. Spearman's non-parametric correlation coefficient was used to measure dependence

between variables. Our analyses allowed us to reject the null hypothesis, namely that two populations are equal.

In the human experiments, we calculated P-values using linear regression analysis adjusted for age, gender, and BMI. P-values were considered significant for values of <0.05, indicating a 95% chance that a null hypothesis is false; smaller values (e.g. <0.01, <0.001) provide stronger evidence for rejecting the null hypothesis.

All data were presented as means ± standard error of the mean (SEM).

4 RESULTS

4.1 THE ADIPOKINE GREMLIN1 ANTAGONISES INSULIN ACTION AND IS INCREASED IN TYPE 2 DIABETES AND MASLD/MASH

The human studies investigated Gremlin1 gene and protein expression in the circulation and specific tissues across specific cohorts: people without diabetes; patients with T2D, their FDRs, and patients diagnosed with MASLD or MASH, as well as the effect of Gremlin1 on insulin signaling in primary insulin-responsive tissues (adipocytes, skeletal muscles, and the liver) *in vitro*.

Regardless of the degree of obesity, Gremlin1 mRNA expression in adipose tissue (particularly in visceral fat) and circulating levels of Gremlin1 protein were significantly increased in T2D patients compared to those with normal glucose tolerance (NGT). Gremlin1 mRNA expression was also significantly higher in non-obese individuals with impaired insulin sensitivity and in FDRs.

Notably, Gremlin1 levels correlated with fasting plasma insulin, HbA1C and HOMA-IR (a marker of insulin resistance). Our *in vitro* analysis revealed that secreted and recombinant Gremlin1 protein impaired insulin signaling in human primary adipocytes, human skeletal muscle cells (SMCs) and liver cells.

These findings suggested that Gremlin1 protein directly impairs insulin sensitivity *in vitro* and that insulin-stimulated glucose uptake can be restored by anti-Gremlin1 antibody in human primary adipocytes and liver cells.

Importantly, we also found that Gremlin1 mRNA expression in adipose tissue and the liver, as well as circulating levels of Gremlin1, correlated with several markers of MASLD/MASH.

We attributed the modulation of insulin sensitivity to the secreted form of Gremlin1; the truncated protein did not inhibit insulin signaling. This may be because Gremlin1 exerts its inhibitory effect on insulin signaling via an important phosphatase, protein-tyrosine phosphatase-1B (PTP1B), which is implicated in the progression of insulin resistance and MASLD/MASH.

To summarize, our results confirmed Gremlin1 as an antagonist of insulin signaling in human adipocytes, skeletal muscle cells and liver cells, and showed that Gremlin1 levels are significantly increased in people with T2D and MASLD/MASH who have similar BMIs. Levels correlated with markers of insulin resistance and MASLD/MASH.

4.2 ADULT MICE ARE UNRESPONSIVE TO AAV8-GREMLIN1 GENE THERAPY TARGETING THE LIVER

We examined the effect of Gremlin1 on insulin sensitivity in adult mice through a dual approach.

First, we aimed to elevate circulating Gremlin1 levels using liver-targeted *AAV8-Gremlin1* gene therapy to enhance long-term Gremlin1 expression in the liver. We then validated our findings by administering rec-Gremlin1 protein via IP injection to diet-induced obese mice and further verified the results using in vitro cell models (3T3-L1 and C2C12 lines).

Note that the *AAV8-Gremlin1* group did not demonstrate any significant deviations in metabolism, compared with the control group, especially in terms of insulin sensitivity and glucose tolerance.

Overexpression of Gremlin1 protein in hepatocytes was confirmed by immunohistochemical staining. Despite this increased expression, however, the protein was not cleaved or secreted into the circulation.

IP injection of rec-Gremlin1 had no detectable effect on either body weight or whole-body insulin sensitivity, yet the GTT showed enhanced early insulin secretion. On closer analysis, we noted a decline in browning marker UCP1 levels in both WAT and BAT. However, this alteration was not sufficient to cause any noticeable phenotypic changes.

We sought to validate our findings by *in vitro* analysis of mouse cells. In this way we highlighted the inhibitory role of rec-Gremlin1 on BMP4 signaling in mice, particularly via the Smad-dependent signaling pathway. Despite the

inhibitory effect of Gremlin1 on Smad signaling, we were unable to identify any significant effects on insulin signaling.

In summary, Gremlin1 accumulated intracellularly and was not cleaved or secreted when it was overexpressed by gene therapy in mouse liver. Further, no phenotypic changes were observed. IP injections did not affect body weight or whole-body insulin sensitivity, but they did influence glucose tolerance and early insulin secretion. Incubating murine cells with Gremlin1 protein *in vitro* validated its inhibitory effect on BMP4 signaling but did not reveal an effect on insulin signaling.

4.3 BMP4 GENE THERAPY DELAYS DIABETES PROGRESSION IN THE DIABETIC DB/DB MOUSE MODEL

To use a model that is relevant to human T2D, we studied the leptin-receptor1 deficient (*db/db*) mouse model [122]. AAV8-BMP4 gene therapy enabled us to prolong BMP4 expression through the liver [58, 59].

12-week-old diabetic adult mice received AAV8-BMP4 or empty vector through tail vein injection and were observed for 6 weeks. We found no changes in water intake, HbA1C or blood fasting glucose levels, however there was weight gain for 2 weeks post injection, and a significant delay in the decline in blood fasting insulin levels.

We also used *AAV8-BMP4* gene therapy in two cohorts of young mice, aged 6 weeks and 7 weeks (at an early stage of disease progression) to evaluate the efficacy of BMP4 for preventing the onset of T2D. These mice were obese by the age of 6-7 weeks and already had high fasting blood insulin levels, and diabetes manifested in the following weeks. We observed a rise in blood glucose levels that peaked at around 4 weeks post injection. Interestingly, at 2 weeks post injection, the decline in blood fasting insulin levels was slower than that of the control group ($p<0.05$). In addition, BMP4 significantly reduced water intake in the BMP4 treated mice. However, at 5 weeks post injection, no differences were found in fasting insulin levels or insulin resistance compared to controls. We observed a significant decrease in circulating TAG and β-hydroxybutyrate in BMP4 treated mice, and subsequent gene expression analysis of SAT revealed a significant decrease in inflammatory marker MCP1 and an increase in browning marker UCP1.

In summary, *AAV8-BMP4* gene therapy did not reverse T2D in adult diabetic *db/db* mice. It had no effect on water intake, fasting blood glucose or HbA1C, however it was associated with a delay in fasting insulin reduction, and a short period of weight gain 2 weeks post injection. In young pre-diabetic mice, the treatment significantly reduced water intake, delayed insulin declines and mitigated diabetic cachexia. Early BMP4 treatment in young mice significantly reduced plasma TAGs and β-hydroxybutyrate, decreased mRNA expression of MCP1, and increased SAT UCP1.

5 DISCUSSION

The escalating prevalence of obesity and its related complications has reached epidemic proportions, imposing a significant economic strain on society and profoundly affecting the quality of life for those affected. Consequently, there is a pressing need for enhanced understanding to facilitate the development of novel therapies. This thesis delves into the impact of increased Gremlin1 expression on insulin sensitivity, both in vivo and in vitro. Additionally, we explore the effects of a Gremlin1 antagonist, BMP4, in a diabetic mouse model, and the comprehensive findings are discussed below.

5.1 GREMLIN1 ROLE IN INSULIN RESISTANCE AND MASLD/MASH

As previously demonstrated by our research group, Gremlin1 is a hormone secreted by adipocytes, and its expression is notably higher in hypertrophic adipocyte cells [94]. Gremlin1 serves as an antagonist of BMP4, thereby impeding the commitment of adipocyte precursor cells to the adipose lineage. Impaired adipogenesis has been observed in individuals with genetic markers associated with insulin resistance [123]. We investigated Gremlin1 plasma levels in humans and its mRNA expression in subcutaneous and visceral adipose tissue, liver and skeletal muscle in the context of obesity and its complications, namely T2D, MASLD and MASH.

We have reported Gremlin1 mRNA expression is elevated in adipose tissue, particularly in visceral fat of type 2 diabetic patients compared to normal glucose tolerance (NGT) patients regardless of degree of obesity. Our result shows Gremlin1 mRNA expression in adipose tissue correlates with several measure of insulin resistance such as fasting plasma insulin, HOMA-IR and HbA1C.

Hypertrophic expansion of adipocytes is linked to reduced insulin sensitivity and other obesity-related complications [68, 69]. Moreover, genetic predisposition of T2D have been reported as a risk factor of developing T2D [13, 124] while our findings do not establish a direct connection, we note that the elevated Gremlin1 mRNA levels in lean FDR and the relationship between hypertrophic adipocyte expansion and insulin resistance in FDR [124] suggest a significant role for Gremlin1 in insulin resistance and obesity-related complications.

Previous studies reported the link between visceral fat obesity and increased risk of insulin resistance and cardiometabolic disorders ([62, 68, 125]. In addition, circulating levels of Gremlin1 positively correlates with BMI [94]. Our result shows Gremlin1 mRNA expression in adipose tissue correlates with several measure of insulin resistance such as fasting plasma insulin, HOMA-IR and HbA1C.

Our human study revealed a significant positive correlation between Gremlin1 expression and degree of insulin resistance in both non-obese and obese individuals suggest that endogenous Gremlin1 can interfere with normal insulin sensitivity. Our in vitro results also show that Gremlin1 protein impairs insulin signaling directly in major insulin target cells in human (adipocyte, skeletal muscle and the liver). This inhibitory effect of cell-secreted or recombinant Gremlin1 and the sensitizing effect of anti-Gremlin1 on insulin-induced glucose uptake, support that Gremlin1 is an important endogenous regulator of target cell insulin signaling at least in adipose cells and liver cells where Gremlin1 is highly expressed and secreted in human.

Given the promising outcome of our clinical and in vitro human studies we aimed to evaluate Gremlin1 effect on insulin sensitivity in different tissues in vivo we used diet-induced obese mice. In our first approach, we utilized *AAV8-*

Gremlin1 gene therapy to increase long-term Gremlin1 expression and secretion in diet-induced obese mice. However, this endeavor encountered an unexpected challenge. Even though our vector was designed with the complete sequence — inclusive of the peptide essential for Gremlin1 secretion —this protein was accumulated intracellularly and was not secreted into the circulation. Consequently, we did not observe any apparent metabolic phenotypes in our in vivo model. A critical limitation in this context was the reliability of the Gremlin1 assay, which posed difficulties in accurately measuring small fluctuations in Gremlin1 levels, further complicating our interpretation.

As a complementary approach, we administered rec-Gremlin1 through weekly intraperitoneal (IP) injections. Intraperitoneal injections did produce some phenotypic consequences and similar procedures with Gremlin2 injection have also been reported to induce peripheral effects [126].

However, our findings from IP-injected rec-Gremlin1 mice did not reveal significant alterations in glucose tolerance or body weight in obese mice. Yet, a noteworthy trend emerged – a potential decrease in UCP1 protein expression in adipocytes within the Gremlin1-treated cohort. While this trend was not significant enough to induce a full phenotype, it did raise intriguing questions about Gremlin1's influence on UCP1 expression. This effect might stem from Gremlin1's inhibitory impact on BMP4 signaling, but we needed to delve further into the mechanisms to understand this phenomenon fully.

To gain better control over Gremlin1 concentration and exposure duration, we conducted in vitro experiments using mice adipocyte and muscle cell lines. Surprisingly, despite successfully inhibiting BMP4 canonical signaling (Smad-dependent pathway), we could not replicate the reduction in insulin signaling observed in human cells. This discrepancy between mouse and human cellular

responses underscored the need for caution when translating findings between species.

Furthermore, we noted a significant difference between our mouse model and humans regarding BMP4 inhibition. In obese mice, noggin, another BMP4 inhibitor, was found at high levels, in contrast to Gremlin1. This observation raised the intriguing possibility that noggin might induce similar complications to those reported in humans treated with Gremlin1. However, it's essential to clarify that our study was not designed to explore these potential effects comprehensively.

Our investigation using a mouse model of obesity highlighted the striking disparities between mouse and human physiology in response to elevated Gremlin1 levels. Given these differential outcomes, we caution against direct translation of Gremlin1 effects between mouse models and humans. Our study has shed light on the complexity of Gremlin1's actions and emphasized the need for further research to unravel its precise mechanisms and implications.

On the other hand, our study on human samples showed elevated serum levels of Gremlin1 in obese, T2D and MASLD/MASH in support that other tissues also can become targets of circulating Gremlin1 secreted by the adipose and other tissues. In this study, we also found that the insulin-sensitizing effect of anti-Gremlin1 is negatively correlated with initial degree of cellular insulin responsiveness. It should also be noted that Gremlin1 is particularly high in visceral adipose tissue and, thus, can be expected to have close crosstalk with the liver and its insulin sensitivity/response.

In our in vitro human model of adipocytes, hepatocytes, and skeletal muscle cells, we found that Gremlin1 recombinant protein antagonized insulin signaling. Importantly, we demonstrated that only secreted Gremlin1 had this

inhibitory effect on insulin signaling. Despite similar expression levels in the liver and adipose tissue, it is plausible that the substantial adipose depot in our patients results in adipose tissue being the primary source of circulating Gremlin1 protein. Additionally, we showed that the inhibitory effect of Gremlin1 on insulin-stimulated glucose uptake could be reversed by anti-Gremlin1. Thus, in line with our clinical data, we posit that Gremlin1 directly impacts insulin sensitivity.

Furthermore, we noted higher Gremlin1 expression in visceral fat compared to subcutaneous adipose tissue. It is worth noting that visceral fat is connected to the liver through the portal vein [127], implying that high levels of secreted Gremlin1 can affect the liver, as demonstrated by our findings, leading to impaired insulin sensitivity in hepatocytes and the development of MASLD/MASH.

Our data indicate that Gremlin1 inhibits Smad-dependent BMP4 signaling; however, the exact mechanism by which Gremlin1 affects insulin signaling remains unclear. We also found that Gremlin1 may exert its inhibitory action on insulin signaling through protein-tyrosine phosphatase 1B (PTP1B), an important phosphatase that has been implicated in progression of insulin resistance and MASLD/MASH. Thus, Gremlin1 is an attractive target for treatment of T2D and its complications.

Additionally, our study emphasized a potential link between elevated Gremlin1 levels in circulation and liver damage. However, *AAV8-Gremlin1* or IP-injection of tis rec-protein in our diet-induced obese mice did not reveal significant differences in inflammation or fibrosis in the liver. This discrepancy could be attributed to the unsuccessful Gremlin1 secretion in *AAV8-Gremlin1* treated mice and possibly low concentration of Gremlin1 in circulation in IP-injected cohort.

5.2 BMP4 GENE THERAPY, A POTENTIAL APPROACH AGAINST TYPE 2 DIABETES

Furthermore, we shifted the focus of our study from Gremlin1 to BMP4, which is antagonized by Gremlin1. Elevated BMP4 circulating levels promote WAT expansion and increase the beige/browning of white adipose cells [58, 128]. In this research, we extended our prior investigation, which demonstrated the potential of BMP4 gene therapy in promoting browning of WAT and enhancing metabolism in lean mice [59]. Our objective was to assess its effectiveness in a leptin receptor-deficient mouse model susceptible to obesity and diabetes [122]. Utilizing a dual-strategy approach involving both preventive, with young prediabetic mice, and therapeutic interventions, with adult diabetic mice, using AAV8-BMP4, we observed promising outcomes in key metabolic parameters.

The impact of BMP4 treatment on adult db/db mice revealed a postponement in insulin decline two weeks after vector administration. Nevertheless, the efficacy of BMP4 treatment was more pronounced when applied to young prediabetic db/db mice. In the preventive cohorts, we noted a significant decrease in water intake and a delay in insulin decline. Although BMP4 treatment induced temporary metabolic improvements, suggesting enhanced beta-cell functionality [129] and delayed diabetes-related cachexia [130], it did not completely halt the progression of obesity and diabetes.

BMP4's critical role in the differentiation and thermogenic function of adipocytes is coupled with its regulatory effects on systemic glucose balance and insulin responsiveness. Despite the observed changes in metabolic parameters, these did not translate into long-term improvements, suggesting a complex relationship between BMP4 therapy and the disease progression in the context of obesity and hepatic metabolic dysfunction [131].

In obesity, disrupted fatty acid metabolism leads to increased triglycerides and potentially nonalcoholic fatty liver disease (MASLD) [131, 132]. Elevated systemic FFA levels further hinder glucose absorption and amplify ectopic fat storage, subsequently increasing liver very-low-density lipoprotein-triglyceride secretion [52, 76].

The decline in triglycerides with BMP4 treatment suggests it may enhance insulin's anti-lipolytic effects. Elevated BMP4 in MASLD, possibly a feedback response, along with its demonstrated ability to reduce liver triglycerides by boosting lipid metabolism and inhibiting the mTORC1 pathway, highlights its therapeutic potential despite the risk of ketogenic dysfunction and disease advancement [133]. The liver's ketogenic function, essential for removing excess lipids from hepatocytes, is further relevant to our findings. Early up-regulation of beta-hydroxybutyrate levels may signify an increased compensatory hepatic response to fat accumulation stress. Previous studies show that as illness progresses to metabolic dysfunction-associated steatohepatitis (MASH), this ketogenic function is compromised [134, 135].

While some studies emphasize its role in inhibiting glucose-stimulated insulin secretion, others highlight its influence on β-cell differentiation and growth [136, 137]. Our study consistently supports the idea of a complex interplay between BMP4 gene therapy, and the metabolic pathways involved in diabetes and obesity. The treatment cohort of adult db/db mice illustrates this complexity, with initial resistance to diabetes-associated weight loss post-BMP4 treatment indicating the therapy's potential in metabolic regulation. However, the transient nature of these benefits, along with the eventual recurrence of diabetic symptoms and the inability to sustain improved insulin levels, suggests a limitation in its therapeutic capacity. The advanced stage of

disease in these mice may indicate a potential critical window for BMP4 intervention that, once missed, cannot be compensated for later.

Furthermore, the prevention cohorts provided insights into BMP4’s role in early-stage metabolic modulation. The significant delay in insulin level decline and the temporary stabilization of glucose control in these younger mice emphasize BMP4’s promise as a preventive measure. However, the variability in results between the first and second prevention cohorts underscores the importance of timing and disease stage in BMP4’s efficacy. The discrepancy in basal glucose levels prior to treatment initiation in these two cohorts raises questions about the optimal conditions and stages at which BMP4 therapy would be most effective.

Our study's findings on BMP4 gene therapy's impact on diabetic progression provide a new perspective and offer insights into the complexities of metabolic regulation, extending previous research on lean mice to pre-diabetic and diabetic contexts [58, 59, 94]. Our observations align with past findings yet reveal critical differences in the sustainability of BMP4's effects, possibly due to the inherent metabolic distinctions between lean and obese conditions [59].

In our results, the considerable reduction in water consumption post-BMP4 therapy alludes to diminished glucose excretion, suggesting enhanced glucose management. However, the eventual plateauing of benefits and the alignment of metabolic profiles with control groups indicate that BMP4’s impact might be contingent on a fine balance of metabolic states and external factors. Regarding diabetes-related complications, BMP4 and its related proteins, like BMP2 and BMP7, have been spotlighted for their regenerative and antifibrotic properties, especially in the context of diabetic kidney disease. Moreover, these proteins exhibit pronounced anti-inflammatory and anti-atherogenic effects [131, 138, 139].

6 CONCLUSION

This thesis has explored the complex role of Gremlin1 in insulin resistance, metabolic dysfunctions, and its potential as a therapeutic target, alongside the therapeutic implications of BMP4, which is antagonized by Gremlin1. The findings presented highlight the complex and multifaceted nature of these molecular pathways in the context of obesity, type 2 diabetes (T2D), and associated metabolic disorders.

Gremlin1, a protein secreted by adipocytes, has been identified as a significant player in the development of insulin resistance. Elevated Gremlin1 levels in visceral adipose tissue, particularly in individuals with T2D, underscore its role in increasing insulin insensitivity and contributing to the pathogenesis of metabolic diseases. The correlation between Gremlin1 expression and insulin resistance parameters, such as fasting plasma insulin and HOMA-IR, indicates its potential as a biomarker for metabolic dysfunction. Furthermore, the in vitro studies with human adipocytes, hepatocytes, and skeletal muscle cells have demonstrated that Gremlin1 impairs insulin signaling, reinforcing the hypothesis that it plays a critical role in the regulation of glucose metabolism.

Despite promising in vitro results, the translation of these findings to in vivo models presented challenges. The lack of secretion of Gremlin1 in the *AAV8-Gremlin1* gene therapy model, and the modest phenotypic effects observed with rec-Gremlin1 injections, suggest species-specific differences in Gremlin1 function that complicate the extrapolation of results from mouse models to humans. These discrepancies highlight the necessity for caution in the application of animal model data to human contexts and underscore the complexity of Gremlin1's role in metabolic regulation.

On the other hand, the study's exploration of BMP4 as a therapeutic avenue offers intriguing possibilities. BMP4 gene therapy, particularly in the context of obesity and T2D, showed potential in modulating metabolic parameters. The findings indicate that BMP4 can influence the browning of white adipose tissue and improve insulin sensitivity, although these effects were more pronounced in early disease stages. The temporary metabolic improvements observed in db/db mice suggest that BMP4 may have a role in delaying disease progression, although it falls short of providing a long-term cure. The study's results imply that the timing of BMP4 intervention is critical, with earlier administration in prediabetic stages showing more substantial benefits.

In conclusion, this thesis provides significant insights into the roles of Gremlin1 and BMP4 in metabolic diseases. The data suggest that while Gremlin1 contributes to insulin resistance and metabolic dysfunctions, BMP4 holds promise as a therapeutic agent, particularly when administered at early stages of disease. However, the complexities of these molecular pathways, as evidenced by species-specific differences and the temporal nature of therapeutic effects, underscore the need for further research. A deeper understanding of these pathways could lead to more effective interventions for metabolic disorders, ultimately improving clinical outcomes for patients with obesity and T2D.

7 FUTURE PERSPECTIVES

Insulin resistance is an important determinant of human disease including T2D and associated complications of CVD and kidney disease. It is of high importance to disclose molecular mechanisms regulating insulin signaling and target tissue sensitivity. It is also important to study progression from MASLD to MASH. Studying Gremlin1 and characterizing its effects may give valuable insights into mechanisms and novel therapeutic targets.

A key question for future work is to study effects of Gremlin1 on BMP4-dependent and independent actions. A major question is if Gremlin1 as a secreted and circulating protein targets human endothelial cells. BMP4 is a proangiogenic molecule, also secreted by the adipose tissue, and angiogenesis is critical for adipose tissue growth.

In addition, it is interesting to examine RNA Seq in cells expressing full-length or truncated Gremlin1 protein to identify potential signaling pathways.

To delve deeper into the BMP4 potential therapeutic effect on diabetes progression, future research should therefore prioritize efforts to extend the duration of BMP4's metabolic benefits, delve into its mechanisms in diverse models, and conduct human trials to validate its efficacy across varied populations. The identification of biomarkers for optimal BMP4 therapy timing could also prove pivotal, opening avenues for personalized treatments in the management of metabolic diseases.

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9 APPENDIXES

Appendix 1 - paper I

The Novel Adipokine Gremlin 1 Antagonizes Insulin Action and Is Increased in Type 2 Diabetes and NAFLD/NASH.
Shahram Hedjazifar, Roxana Khatib Shahidi, Ann Hammarstedt, Laurianne Bonnet, Christopher Church, Jeremie Boucher, Mathias Bluher and Ulf Smith.
Diabetes, 2020. **69**(3): p. 331-341.

Appendix 2 -paper II

Adult mice are unresponsive to AAV8-Gremlin1 gene therapy targeting the liver.
Roxana Khatib Shahidi, Jenny M. Hoffman, Shahram Hedjazifar, Laurianne Bonnet, Ritesh K. Baboota, Stephanie Heasman, Christopher Church, Ivet Elias, Fatima Bosch, Jeremie Boucher, Ann Hammarstedt, Ulf Smith.
PLoS One, 2021. **16**(2): p. e0247300.

Appendix 3- paper III

BMP4 gene therapy delays diabetes progression in the diabetic db/db mouse model.

Roxana Khatib Shahidi, Louise Mannerås Holm, Tobias Kroon, Laurianne Bonnet, Daniel Nilsson, Andrea Ahnmark, Ivet Elias, Fatima Bosch, Ulf Smith and Jeremie Boucher. working draft of Manuscript.

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