# The role of endogenous Cellular Communication Network factor 5 (CCN5) expression on normal β-cells growth and insulin resistance caused by diet-induced obesity.

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# Dedication

I dedicate this endeavor to the loving memory of my father, who left us last December. He wasn't just a teacher by profession; he was my life teacher. His boundless passion for education and unshakable belief in my capabilities continue to inspire me. This thesis stands as a tribute to his enduring legacy.

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## Abstract

The role of endogenous Cellular Communication Network factor 5 (CCN5) expression on normal β-cells growth and insulin resistance caused by diet-induced obesity.

Metabolic syndrome and diabetes mellitus are increasing rapidly recently. Pancreas regeneration and replication of β-cell are some of the challenges that medical doctors and researchers face today. Cellular communication network factor 5 (CCN5) is a matricellular protein, the expression of which is under the regulation of Wnt signaling and IGF-1. Our previous characterization supports the notion that CCN5 promotes the proliferation and survival of pancreatic β-cells leading to metabolic benefits. To examine the role of endogenous CCN5 in metabolism and specifically in pancreatic islets and adipose tissues we generated total CCN5 knockout mice and fed them either with a normal-chow diet or high-fat diet (HFD - 60% kcal fat) for 27 weeks.

Contrary to previous findings, our CCN5 knockout mice, both male and female, exhibited no significant change in lean/fat mass, insulin sensitivity, nor glucose tolerance when fed a chow diet, despite ~50% reductions in β-cell area and serum insulin level at 9 mo. Upon high-fat diet feeding, CCN knockout mice gained similar amounts of weight as their wild-type counterparts (male 2.0 vs. 1.9-fold; female 2.7 vs. 2.4-fold) but demonstrated sexual dimorphic changes in insulin sensitivity. Interestingly, male knockout mice displayed significant improvements in insulin sensitivity and glucose tolerance, despite being equally obese as their wild-type controls. The smaller improvements in female knockout mice were not as significant. At the end of the 27 weeks of high-fat diet, the increased levels of insulin in response to obesity, were 30-60% lower in knockout mice (both male and female) than in wild-type counterparts, also supporting improved insulin sensitivity. On the other hand, the number of islets β-cells in the pancreas of

normal-chow diet-fed mice was significantly decreased in CCN5 gene-deficient mice indicating that CCN5 is required for β- cell growth. This was not true for the HFD group.

Taken together this thesis demonstrate that endogenous CCN5 expression is required for normal β cell growth but detrimental to insulin and glucose tolerance in response to diet-induced obesity. Its impact is dual-faceted, influencing both the specific functions of β-cells and the overall metabolic activities.

## Résumé

Le rôle de l'expression endogène du facteur 5 du réseau de communication cellulaiure (CCN5) sur la croissance normale des cellules β et la résistance à l'insuline provoquée par l'obésité induite par le régime alimentaire

Le syndrome métabolique et le diabète mellitus, connaissent actuellement une progression rapide. La régénération du pancréas et la réplication des cellules β constituent des défis auxquels les médicins et les chercheurs sont confrontés. CCN5 est une protéine matricellulaire sous la régulation de la signalisation Wnt et lIGF-1. Nos travaux précédents soutiement l'idée que le CCN5 est impliqué dans la prolifération et la survie des cellules pancréatiques β, ce qui conduit à des bénéfices métaboliques.

Pour examiner le rôle du CCN5 endogène dans le métabolisme spécifiquement dans les îlots pancréatiques et les tissus adipeux, nous avons généré des souris dépourvues de CCN5 et les avons nourries soit avec un régime alimentaire normal, soit avec un régime riche en graisses (HFD - 60% kcal fat) pendant 27 semaines.

Contrairement aux découvertes précédentes, nos souris dépourvues de CCN5, mâles et femelles, n'ont montré changement significatif en matière de masse maigre/grasse, de sensibilité à l'insuline, ni de tolérance au glucose lorsqu'elles étaient nourries avec un régime normal, malgré des réductions d'environ 50% de la zone des cellules β et du taux d'insuline sérique à 9 mois. Suite à un régime riche en graisses, les souris dépourvues de CCN ont pris une quantité de poids similaire aux souris sauvages (mâle 2,0 vs. 1,9 fois ; femelle 2,7 vs. 2,4 fois), mais ont démontré des modifications dimorphiques sexuelles en matière de sensibilité à l'insuline. Les souris mâles

dépourvues de CCN5 ont montré des améliorations significatives en matière de sensibilité à l'insuline et de tolérance au glucose, malgré un niveau d'obésité équivalent aux sauvages. Les faibles améliorations chez les souris femelles dépourvues de CCN5 n'étaient pas aussi significatives. À la fin des 27 semaines de régime riche en graisses, les niveaux augmentés d'insuline en réponse à l'obésité étaient de 30 à 60% inférieurs chez les souris dépourvues de CCN5 (mâles et femelles) par rapport aux sauvages, confirmant une amélioration de la sensibilité à l'insuline. D'un autre part, le nombre de cellules β dans les îlots du pancréas des souris nourries avec un régime alimentaire normal était significativement réduit chez les souris dépourvues du gène CCN5, indiquant que le CCN5 est nécessaire pour la croissance des cellules β. Ce n'était pas le cas pour le groupe HFD.

En résumé, cette thèse montre que l'expression endogène du CCN5 est nécessaire pour une croissance normale des cellules β mais est néfaste pour la tolérance à l'insuline et au glucose en réponse à l'obésité induite par le régime alimentaire. Son impact est à double facette, influençant à la fois les fonctions spécifiques des cellules β et les activités métaboliques globales.

## Acknowledgments

First and foremost, I thank God, who made this beautiful journey possible.

The most cherished aspect of this journey has undoubtedly been the incredible individuals who have accompanied me. I feel fortunate to have been surrounded by exceptional people. Transitioning from a clinical background to molecular science was a challenging endeavor, and without their support, I could not have handled it.

A special mention must be made of my supervisor, Dr. Jun Liu. His unwavering support, belief in my potential, and instrumental guidance have shaped my path in ways I could never have imagined. His mentorship has been a beacon of inspiration, and I am profoundly grateful for his presence in my academic life.

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## Contributions of authors

This thesis adheres to McGill University's "Guidelines for Thesis Preparation" and follows the format of a "traditional monograph thesis." The contributions of each author are:

**Viktoria Xega**

- Authored the complete thesis, including all sections from the introduction to conclusions.
- Conducted all mice experiments, including ITT, GTT, sacrificing the mice, collecting blood, performing ELISA procedures, and providing tissue for immunohistochemistry, with assistance from Dr. Mahvash Zakikhani.
- Measured β cell percentage using Nikon software.

**Jun-Li Liu**

- Designed the study concept and provided mentorship, guidance, and insightful feedback throughout the project.
- Supported data interpretation and supervised research personnel and students.

**Mahvash Zakikhani**

- Contributed to the conception and design of the study.
- Developed and refined research methodologies.
- Analyzed and interpreted data.
- Provided mentorship, administrative, technical, and material support.

**Fazila Chouiali and team (Histology platform)**

- Performed immunohistochemistry images.

**Xiaohong Liu**

- Conducted EchoMRI procedures.

**Marzieh Eskandari Shahraki**

- Established initial genotyping protocols.

All work was conducted under the review and supervision of Dr. Jun Li Liu, who also provided consistent support and funding for the study.

## List of Abbreviations

- ACT: Adrenocorticoid Tumors
- AKT: Protein Kinase B
- AUC: Area Under the Curve
- BAT: Brown Adipose Tissue
- BMR: Basal Metabolic Rate
- BMPs: Bone Morphogenic Proteins
- C/EBP: CCAAT/Enhancer-Binding Protein
- CCN: Cellular Communication Network
- CCN5: Cellular Communication Network Factor 5
- CDK: Cyclin-Dependent Kinase
- CEF10: Chicken Embryo Fibroblast-Derived Protein 10
- CTGF: Connective Tissue Growth Factor
- CTGF-L: CTGF-Like
- CYR61: Cysteine-Rich Angiogenic Inducer 61
- DAPI Staining: 4',6-Diamidino-2-Phenylindole
- ECM: Extracellular Matrix
- EchoMRI: Echo Magnetic Resonance Imaging
- ER: Estrogen Receptor
- ER-α: Estrogen Receptor Alpha
- EREs: Estrogen Response Elements
- ERK: Extracellular Signal-Regulated Kinase
- ES:  Embryonic Stem cells

- FABP4: Adipocyte-Type Fatty Acid Binding Protein
- FAK: Focal Adhesion Kinase
- FAHFAs: Branched Fatty Acid Esters of Hydroxy Fatty Acids
- GFAT: glutamine: fructose-6-phosphate amidotransferase
- GPCRs: G Protein-Coupled Receptors
- GTT: Glucose Tolerance Test
- HDL: High-Density Lipoproteins
- HFD: High-Fat Diet
- HBGF-0.8: Heparin-Binding Growth Factor 0.8
- HBP - Hexosamine Biosynthesis Pathway
- Hcs24: Hypertrophic Chondrocyte-Specific Protein 24
- IGF: Insulin-Like Growth Factor
- IGF-1: Insulin-Like Growth Factor 1
- IGFBP-rP4: Related Protein to IGFBP
- IGFBP9: Insulin-Like Growth Factor-Binding Protein 9
- IMPC: International Mouse Phenotyping Consortium
- IR: Insulin Resistance
- IRS: Insulin Receptor Substrate
- ITT: Insulin Tolerance Tests
- KO: Knockout
- LRP: Low-Density Lipoprotein-Related Protein
- MafA: V-Maf Musculoaponeurotic Fibrosarcoma Oncogene Homolog A

- MCP-1: Monocyte Chemoattractant Protein 1

- MetS: Metabolic Syndrome

- miRNAs: microRNAs

- mTORC1: Mechanistic Target of Rapamycin Complex 1

- MSCs: Mesenchymal Stem Cells

- NAFLD: Non-Alcoholic Fatty Liver Disease

- NCD: Normal-chow Diet

- Ngn3: Neurogenin 3

- Nkx6.1: NK6 Homeobox 1

- NOV: Nephroblastoma Overexpressed

- O-GlcNAcylation: post-translational modification involving the addition of a single N-acetylglucosamine in O-linkage to serine or threonine residues of proteins.

- PAI-1: Plasminogen Activating Factor 1

- PCR: Polymerase Chain Reaction

- PDGF: Platelet-Derived Growth Factor

- Pdx1: Pancreatic and Duodenal Homeobox 1

- PGR: Progesterone Receptor

- PGRMC1: Progesterone Receptor Membrane Component 1

- PPAR: Peroxisome Proliferator-Activated Receptor

- PPARs: Peroxisome Proliferator-Activated Receptors

- PKA: Protein Kinase A

- PKC: Protein Kinase C

- PI3K: Phosphoinositide 3-Kinase

- PCOS: Polycystic Ovary Syndrome
- PTP1B: Protein Tyrosine Phosphatase 1B
- RBP4: Retinol-Binding Protein 4
- SD: Standard Deviation
- SEM: Standard Error of the Mean
- SHBG: Sex Hormone-Binding Globulin
- SOCS: Suppressor of Cytokine Signaling
- T2D: Type 2 Diabetes Mellitus
- TGF-β: Transforming Growth Factor-B
- TG: Triglycerides
- UDP-GlcNAc - Uridine 5'-diphosphate N-acetylglucosamine
- VEGF: Vascular Endothelial Growth Factor
- VLDL: Very Low-Density Lipoprotein
- VSMC: Vascular Smooth Muscle Cells
- WAT: White Adipose Tissue
- Wnt: Wingless-Related Integration Site
- WT: Wild Type
- WISP-1: WNT1 Inducible Signaling Pathway Protein 1
- WISP-2: WNT1 Inducible Signaling Pathway Protein 2

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# Chapter 1. Introduction and Literature Review

## 1.1. Understanding Metabolic Syndrome (MetS)

### 1.1.1. Historical background of metabolic syndrome

Metabolic syndrome (MetS) also known by several other names including Syndrome X and Reaven syndrome, represents a constellation of physiological, biochemical, clinical, and metabolic factors that increase an individual's risk of cardiovascular disease and type 2 diabetes (T2D). Specifically, it refers to the coexisting factors such as abdominal obesity, elevated blood pressure, high glucose levels, insulin resistance (IR), atherogenic dyslipidemia (high serum triglycerides, and low high-density lipoprotein (HDL), a prothrombotic and pro-inflammatory state [1-7] .

The concept of MetS can be traced back to the 20th century. French physician Jean Vague first observed in the 1940s and 1950s that individuals with upper body obesity seemed to have an increased risk for cardiovascular diseases and diabetes [8,9]. These initial observations paved the way for a more structured understanding of this metabolic cluster.

The term "metabolic syndrome" wasn't coined until the 1970s, and the subsequent decades saw several major definitions proposed by various medical organizations. The World Health Organization (WHO) in 1998 was the first major international organization to formally define MetS [10]. Later, other notable definitions were provided by International Diabetes Federation (IDF)[11], and the National Cholesterol Education Program's Adult Treatment Panel III (NCEP ATP III)[12], each contributing significantly to the contemporary understanding and diagnosis of the syndrome.

### 1.1.2. The risk factors and consequences of MetS

Dyslipidemia includes elevated very low-density lipoprotein (VLDL), triglycerides (TG) and decreased high-density lipoproteins (HDL). A prothrombotic profile suggests impairments in procoagulant factors, anti-fibrinolytic factors, platelet abnormalities and endothelial dysfunction. An inflammatory state is illustrated by increased circulating cytokines and acute-phase reactants [4-6]. The development of MetS involves a complex interplay of both genetic factors and lifestyle habits, all of which contribute to a state of insulin resistance and persistent, low-level inflammation.

Identifying and diagnosing this syndrome plays a crucial role in health care by targeting high-risk individuals for early intervention and management, thereby reducing the incidence of cardiovascular disease and diabetes [1,2].

### 1.1.3. The dynamic nature of adipose tissue and its role in MetS

Adipose tissue, more than just a reservoir for fat storage, is now understood as a complex endocrine organ central to the development of the metabolic syndrome [4,13,14]. This multifaceted organ orchestrates whole-body metabolism, modulates immune responses, and regulates hormones. Its remarkable plasticity enables quick expansion or reduction in response to nutrient shifts, a dynamism mainly attributed to adipocytes, which adapt in size and number to the body's metabolic condition [15].

#### *1.1.3.1. Different types of adipose tissue*

Adipose tissue is not a monolithic entity; rather, it comprises several distinct types with specific metabolic functions.

**White Adipose Tissue (WAT):** Predominantly involved in energy storage as triglycerides, WAT is found throughout the body in subcutaneous and visceral depots. It expands through cell

size increase (hypertrophy) during overnutrition. Excessive WAT expansion, especially in the visceral area, can lead to issues such as metabolic dysfunction, inflammation, and insulin resistance, key features of metabolic syndrome [15].

**Brown Adipose Tissue (BAT)**: Specialized in energy dissipation, BAT produces heat (thermogenesis) through a unique protein, UCP-1, found in its numerous mitochondria. BAT metabolism is regulated by environmental, nutritional, endocrine, and neural factors [16]. The most powerful and physiological stimulus to activate BAT is cold exposure or hormonal stimuli, like β-adrenergic agonists. [16-19]. It is prevalent in infants and exists in adults mainly in specific areas like the supraclavicular region. Human BAT shows molecular markers resembling murine beige fat more than classical brown fat [18,20-22]. Its activation can counteract the harmful effects of metabolic syndrome [15].

**Beige or Brite Adipocytes:** These unique cells are found within WAT and exhibit characteristics of both white and brown fat cells. They can switch between energy-storing and energy-burning phenotypes in response to stimuli like cold exposure or exercise [23-25]. Though similar to BAT, beige cells have distinct features and lower levels of certain BAT genes [22,23,26]. Their activation presents an exciting potential avenue for treating metabolic syndrome, as they can enhance energy expenditure and metabolic health without extensive WAT reduction [5,25.]

#### *1.1.3.2. Role of Adipose Tissue in MetS Development*

Metabolic syndrome is often marked by the uncontrolled growth of WAT, leading to issues like hypoxia, inflammation, and insulin resistance. This occurs when the growth of WAT exceeds its ability to develop new blood vessels (angiogenesis) [15]. Conversely, boosting the activity of Brown Adipose Tissue (BAT) or beige adipocytes could alleviate these problems. The

adaptability and diverse functions of adipose tissue thus play a vital role in metabolic regulation, highlighting its importance in understanding and potentially treating metabolic syndrome.

### 1.1.4. Adipokines: The regulators of metabolic balance

Adipose tissue secretes a myriad of hormones or "adipokines," which regulate interorgan communication. Notable adipokines such as leptin and adiponectin, play crucial roles in controlling hunger and promoting insulin sensitivity, respectively. However, adipokines can have paradoxical effects on metabolic health.

In the context of obesity, adiponectin secretion decreases, exacerbating insulin resistance; paradoxically leptin production increases, yet its satiety-inducing effect is blunted due to the onset of leptin resistance. These altered adipokine surroundings are a crucial contributor to the pathogenesis of metabolic syndrome [27].

Other adipokines secreted by adipose tissue are resistin, visfatin, monocyte chemoattractant protein 1 (MCP-1), retinol-binding protein 4 (RBP4), adipocyte-type fatty acid binding protein, (FABP4) plasminogen activating factor 1(PAI-1) [28,29].

New adipocyte hormones are revealing adipose tissue's intricacy and dynamics. FAHFAs (branched fatty acid esters of hydroxy fatty acids), may improve metabolic parameters in animal models by activating G-protein-coupled receptors GPR120 and GPR40. [30].

Other lipid adipocyte hormones such as dihydroxy-9Z-octadecenoic acids (diHOMEs) have been linked with increased fatty acid uptake and enhanced cold tolerance, signifying their likely role in energy homeostasis [30]. Adipocytes also secrete microRNAs (miRNAs), a class of non-coding RNAs, that could have significant roles in intertissue communication, influencing metabolic balance and contributing to disease pathogenesis [30].

### 1.1.5. Targeting adipose tissue in metabolic syndrome: opportunities and challenges

Given the central role of adipose tissue in metabolic syndrome, it presents as a potential therapeutic target. Opportunities abound, including the development of mimics or antagonists of adipose hormones, and utilizing adipose-derived exosomal miRNAs for diagnostic and therapeutic strategies. Furthermore, understanding the heterogeneity of adipose tissue, such as differentiating white, brown, and beige fat, as well as within WAT itself, may open new avenues for drug development that can modify adipose tissue distribution or induce a shift from a metabolically unhealthy subtype to a healthier one [30].

### 1.1.6. Importance of Insulin Resistance in MetS.

#### *1.1.6.1 Definition and impact of insulin resistance.*

Insulin resistance (IR) is a critical pathophysiological condition in which the body's tissues, especially adipose tissue, liver, and skeletal muscle, fail to respond effectively to insulin. This condition is a hallmark metabolic syndrome and type 2 diabetes mellitus (T2D) [31-34]. Moreover, emerging research suggests that IR may play a role in the pathogenesis of various other health conditions, including neurodegenerative diseases like Alzheimer's [35,36], certain types of cancer [37], polycystic ovary syndrome (PCOS)[38-41], and non-alcoholic fatty liver disease (NAFLD)[33,34,42,43]

At a molecular level, insulin resistance primarily results from impairments in insulin signaling pathways. Insulin binds to its receptor, leading to the activation of insulin receptor substrates (IRS), which in turn activate the PI3K-AKT pathway. This pathway is vital for the translocation of glucose transporter type 4 (GLUT4) to the cell surface, promoting glucose uptake into cells [44]. In the context of insulin resistance, these signalling pathways are disrupted, reducing GLUT4 translocation and impairing glucose uptake.

***1.1.6.2. Molecular mechanism and factors contributing to insulin resistance.***

IR is a multifaceted physiological anomaly with diverse origins:

*Inflammatory response*: Persistent inflammation, often associated with obesity, interferes with insulin's regulatory pathways due to pro-inflammatory cytokines like TNF-α and IL-6 [45,46].

*Oxidative imbalance:* Disproportionate production of reactive oxygen species compared to the body's antioxidant mechanisms can compromise insulin signaling components [47,48].

*Endoplasmic Reticulum disturbances*: Disruptions within the endoplasmic reticulum can negatively affect insulin signaling, primarily due to the activation of specific stress-induced pathways, notably the unfolded protein response (UPR) [49].

*Lipid misplacement*: The ectopic deposition of lipids in organs not physiologically designed for fat storage, such as the liver, heart, and skeletal muscle, can lead to the generation of deleterious metabolites. These metabolites can impair insulin signaling pathways, thereby contributing to insulin resistence [50-55]. Intriguingly, this pathological process can manifest even in the absence of increased visceral adiposity, highlighting the complex interplay between lipid metabolism and endocrine function [50,56].

*Challenges with Fatty Acids*: Elevated plasma-free fatty acids can be a precursor to insulin resistance, a trait often seen in conditions like obesity and type 2 diabetes mellitus [56,57].

*Selective Insulin Resistance:* Some insulin pathways in hyperinsulinemia are highly responsive to insulin. This is called selective insulin resistance. For instance, in the hepatic insulin resistance scenarios, insulin fails to suppress hepatic glucose production but stimulates lipogenesis, which results in hyperglycemia, hyperlipidemia, and hepatic steatosis [56,58].

*The glucose-fatty acid (Randle) cycle considerations:* This postulates that lipid-mediated insulin resistance in muscles, marked by decreased glucose absorption, is due to restricted glucose utilization under insulin's influence [56,59].

*Hexosamine biosynthesis pathway (HBP)*: has been proposed as a significant contributor to insulin resistance [56,60]. While the glucose-fatty acid cycle doesn't fully account for lipid-induced insulin resistance, elevated muscular acetyl-CoA and citrate levels, in tandem with increased fat oxidation, hint at another regulatory pathway for glucose transport [60]. In the HBP, a small fraction of fructose-6-phosphate, derived from glucose-6-phosphate, undergoes conversion to glucosamine-6-phosphate, facilitated by the enzyme glutamine: fructose-6-phosphate amidotransferase (GFAT) [60]. This molecule is further transformed into Uridine 5'-diphosphate N-acetylglucosamine (UDP-GlcNAc), which plays a role in the glycosylation of lipids and proteins [60]. Notably, O-GlcNAcylation (post-translational modification involving the addition of a single N-acetylglucosamine in O-linkage to serine or threonine residues of proteins) process, can influence proteins by altering gene expressions or enzyme activities [61]. Past studies have linked glucosamine-induced insulin resistance, elevated O-GlcNAc levels in diabetic conditions, and the overexpression of related enzymes to whole-body insulin resistance [62-64]. However, the exact molecular mechanisms of HBP in insulin resistance remain elusive, with O-GlcNAcylation being the most plausible mechanism, especially given its potential competition with phosphorylation sites that regulate protein activity [65-67]. Despite the accumulating evidence, the role of HBP in insulin resistance is still debated, with some studies showing contrasting results in in vivo and in vitro settings [68,69]. Comprehensive research using in vivo systems is essential to ascertain HBP's role and its mechanisms in insulin resistance [56].

#### *1.1.6.3. Clinical manifestations and therapeutic strategies in insulin resistance*

The clinical manifestation of insulin resistance includes hyperinsulinemia, impaired glucose tolerance, hyperglycemia, and dyslipidemia.

Therapeutic strategies aim at improving insulin sensitivity and typically involve lifestyle modifications such as diet, exercise, and weight loss. Pharmacological interventions include metformin, thiazolidinediones, and GLP-1 receptor agonists [70].

While our understanding of insulin resistance has significantly increased, more research is needed to fully understand its complex mechanisms and develop effective preventive and therapeutic strategies.

### 1.1.7. Genetic and epigenetic factors and MetS:

#### *1.1.7.1. Role of genetics in MetS*

A deeper understanding of the genetic architecture of MetS is crucial for better prevention, diagnosis, and treatment. For instance, a recent study identified several gene variants linked to lipid metabolism and obesity that contribute to the development and severity of MetS [2]. As part of future strategies, polygenic risk scores could be employed to identify high-risk groups, allowing for earlier intervention and prevention [2].

In particular, the differential gene expression of G protein-coupled receptors (GPCRs) has been linked to MetS. One example is GPR21, which plays a role in body weight, insulin sensitivity, and glucose tolerance, with its expression changing based on environmental and genetic etiologies of MetS [2]. Similarly, GPR82, another orphan receptor, is also implicated in metabolism and shows varied expression and effects depending on the MetS model [2]

#### *1.1.7.2. Understanding the epigenetics of MetS*

In recent years, the epigenetic impact on the development of MetS has gained considerable attention. Studies have shown that parental MetS could modify disease risk in offspring through the transfer of epigenetic alterations, reprogramming hepatic lipid metabolism and contributing to non-alcoholic fatty liver disease (NAFLD) development [2]. Understanding these parental epigenetic effects could offer new therapeutic targets to prevent disease progression.

## 1.2. Pancreas

### 1.2.1. Anatomy

Nestled within the uppermost section of our abdomen, the pancreas occupies a crucial space in the retroperitoneal domain. Its intricate design in humans comprises three segments: a pronounced head close to the duodenum, a body, and a tail that's near the spleen. Contrastingly, mammals like canines and rodents showcase a pancreas that's less differentiated, appearing almost as an unstructured tissue along the upper intestinal lining.

### 1.2.2. Physiology

The pancreas, in its essence, performs a dual role: aiding digestion (via its exocrine capacity) and supervising blood glucose equilibrium (through its endocrine mechanism).

#### *1.2.2.1. Exocrine pancreas*

Occupying the bulk of the pancreatic tissue, the exocrine pancreas is pivotal to the digestive process. Constituting more than 95% of the pancreas's total mass, it is primarily composed of acinar cells, ductal networks, and a comprehensive system of connective tissue, blood vessels, and nerves [71]. The primary role of the exocrine pancreas is to synthesize and dispatch digestive enzymes to the duodenum, thereby facilitating the breakdown of ingested nutrients, ensuring they're primed for assimilation within the body.

### *1.2.2.2. Endocrine pancreas*

On the flip side, the pancreatic islets, representing the organ's endocrine section, are pivotal for hormone synthesis and release, albeit they account for just 1–2% of the pancreas's total mass. Key hormones emanating from this segment include insulin, glucagon, somatostatin, pancreatic polypeptide (PP), and ghrelin [72] These hormones don't just regulate blood glucose; their influence extends to various nutritional cellular processes, from the absorption kinetics of nutrients to their subsequent storage or metabolism. A compromised function of the endocrine pancreas, or an aberrant tissue response to the hormones it secretes, can precipitate significant metabolic imbalances, including diabetes mellitus.

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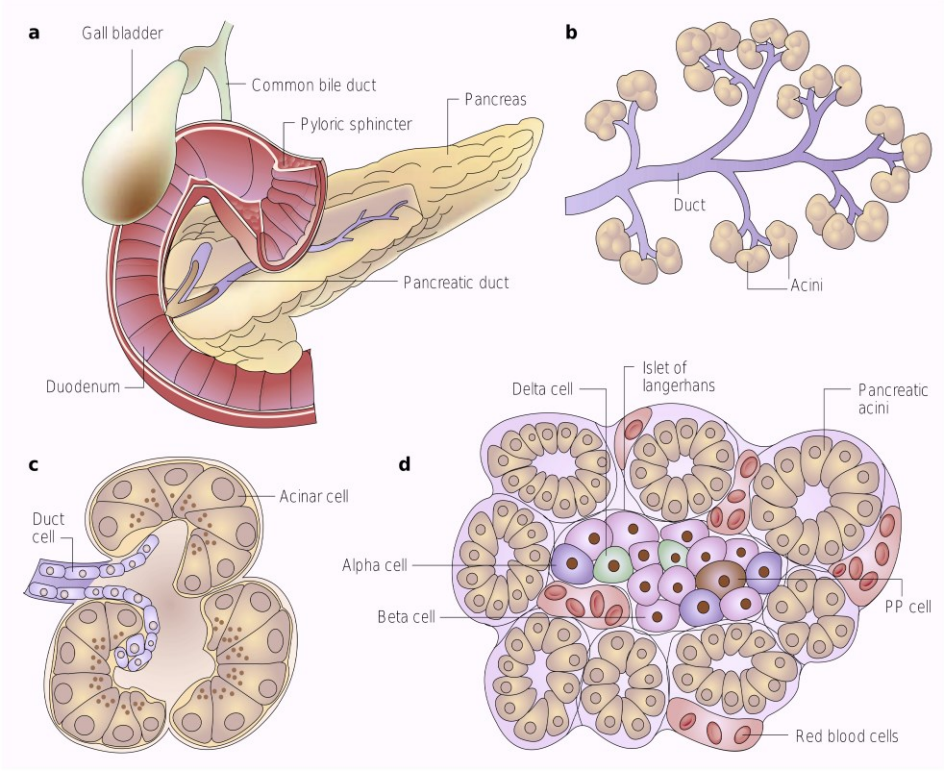


*Figure 1. The anatomy of a) the whole pancreas, b) the exocrine pancreas, c) a single acinus, d) a pancreatic islet embedded in exocrine tissue.*

*Figure adapted from Bardeesy &DePinho. 2002* [73]

#### 1.2.2.2.1 Pancreatic islet cell types

The pancreatic islets are intricate clusters of cells, each demonstrating specialized functionality by producing distinct hormones.

**Alpha cells** which constitute between 15–20% of the total islet cells [72] synthesize glucagon. Recognized as a catabolic hormone, glucagon predominantly targets hepatic and select renal cells. Its primary function is to elevate blood glucose levels by mobilizing glucose, fatty acids, and amino acids [74-76]

The most predominant cell type within the pancreatic islets are the **Beta cells** [72]. These cells are multifunctional, responsible for the synthesis of several substances including C peptide, proinsulin, insulin, amylin (IAPP) [77,78], Urocortin 3 (UCN3) [79], and γ-aminobutyric acid (GABA) [80]. Of these, insulin is paramount, serving as the pancreas's main hormonal output and playing an instrumental role in maintaining glucose equilibrium. The details of insulin biosynthesis, secretion, signalling and action are in section 3 of this chapter.

**Delta cells** that comprise 3-10% of the islet [72] produce somatostatin (SST), an inhibitory hormone released by multiple organs. The secretion of SST is directly influenced by extracellular glucose concentrations, interestingly requiring higher glucose levels than insulin for its activation [72,78,81,82]

**Gamma cells** also known as PP cells, make up about 1% of the islet's cellular composition [72]. Their primary function is to produce the pancreatic polypeptide (PP). The secretion of PP is invigorated by food ingestion and seems to play a role in appetite reduction, potentially through its interaction with the hypothalamus [83,84]

**Epsilon cells** which are sparse and represent merely 1% of the islet[72] produce ghrelin, known as the “hunger hormone”. Ghrelin, colloquially termed the "hunger hormone", activates the

pituitary somatotrophs and hypothalamus GHRH, leading to the secretion of growth hormone. The etymology of "ghrelin" traces back to the Proto-Indo-European root "ghre", synonymous with growth [85]. Ghrelin also modulates appetite, energy equilibrium, gastric motility, and acid release, primarily through its action on the neurons in the hypothalamic arcuate nucleus [86-88]

In summary, these distinct cell types within the pancreatic islets collaborate to form an intricate nutrient-detecting network, crucial for energy balance. Their combined hormonal output operates under the fine-tuned regulation of both paracrine and autocrine feedback loops, necessitating synergistic interactions with the nervous and digestive systems [89-91].

While the cellular makeup of islets remains relatively consistent across species, including humans, rats, and mice, the spatial organization diverges. Rodent islets typically display a core of β-cells, encircled by other cell types. In contrast, human islets manifest a more intertwined arrangement of α- and β-cells. [92,93]

*Table 1.Cell types and their secretory products in adult human pancreatic islet of Langerhans*

| Cell types | Percentage of volume | Secretory products | Reference |
|---|---|---|---|
| α, cell | 15-20 | Glucagon, proglucagon | [72,74-76] |
| β cell | 65-80 | C peptide, proinsulin, insulin, IAPP, UCN3, GABA | [72,77-80,88] |
| δ cell | 3-10 | Somatostatin- 14 | [72,74,78,81] |
| ε cell | 1 | Ghrelin | [72,86,87] |
| γ (PP) cell | 3-5 | Pancreatic peptide | [72,83,84] |

## 1.3. Insulin

### 1.3.1. Insulin biosynthesis

Housed within the short arm of human chromosome 11, the genetic blueprint for insulin production is dictated by a unique ensemble of β-cell-specific transcription regulators. Transcription in the endoplasmic reticulum, specifically the rough variant (RER), results in a primary peptide compound denominated as preproinsulin, displaying a molecular mass of 11.5 kDa. Post-transcriptional enzymatic action inside the microsomal compartment swiftly modifies this initial molecule into a derivative known as proinsulin, displaying a reduced molecular mass of roughly 9 kDa. This proinsulin is then funneled through the cellular sorting machinery to the Golgi complex. It becomes ensconced in vesicular structures that initially sport a clathrin layer. As these vesicles evolve, the clathrin detaches, allowing prohormone convertases to sever proinsulin into insulin and its companion molecule, C-peptide. The fully mature vesicle features a balanced molecular assortment of insulin and C-peptide, with marginal remnants of proinsulin or its semi-processed derivatives[94,95].

Exogenous glucose is taken up by glucose transporter 2 (GLUT2) and undergoes glycolysis inside the cell. Elevated adenosine triphosphate (ATP) levels increase the ATP/ADP ratio, which in turn leads to the closure of ATP-sensitive K+-channels. The subsequent membrane depolarization opens voltage-dependent Ca2+-channels in causing an increase in intracellular calcium levels, which eventually trigger insulin secretion following vesicle fusion with the membrane[72].

### 1.3.2. Insulin secretion

Under physiologically normal conditions, the adult human pancreas secretes approximately 30 insulin units into the portal bloodstream each day. This secretion occurs in discreet pulsatile waves, each with an approximate duration of five minutes. Insulin discharge operates under a biphasic paradigm influenced by a multitude of modulators directly or indirectly affiliated with glucose metabolism.

The first secretion burst is prompted by a swift uptick in glucose concentrations. If the glucose elevation persists, the release of insulin gradually declines and then starts to rise once again to a stable level (the second phase) [96,97].

Basal or 'background' insulin emission persists in the absence of external inducements. Sensory capabilities of the β-cells allow for the direct detection of glucose concentration alterations. Sub-threshold glucose levels—usually under 80 to 100 mg/dL (4.4-5.6 mmol/L)—are generally ineffectual in eliciting insulin release. Concurrently, auxiliary physiological modulators are generally quiescent unless activated by glucose concentrations within the stimulatory ambit [98]. Glucose is the primary modulator of insulin secretion and changes in blood glucose levels can be directly “sensed” by β cells [99,100]

Upon entering β-cells via glucose-specific transporter proteins (GLUTs), glucose undergoes enzymatic degradation mediated by glucokinase. This catalytic activity escalates the intracellular ATP: ADP ratio, culminating in the deactivation of potassium channels sensitive to ATP levels. This provokes a membrane depolarization, enabling calcium ions to flood the cell through voltage-dependent calcium channels (VDCCs), ultimately catalyzing the fusion of insulin-loaded vesicles with the plasma membrane.[72,77,99]. (Figure 2)

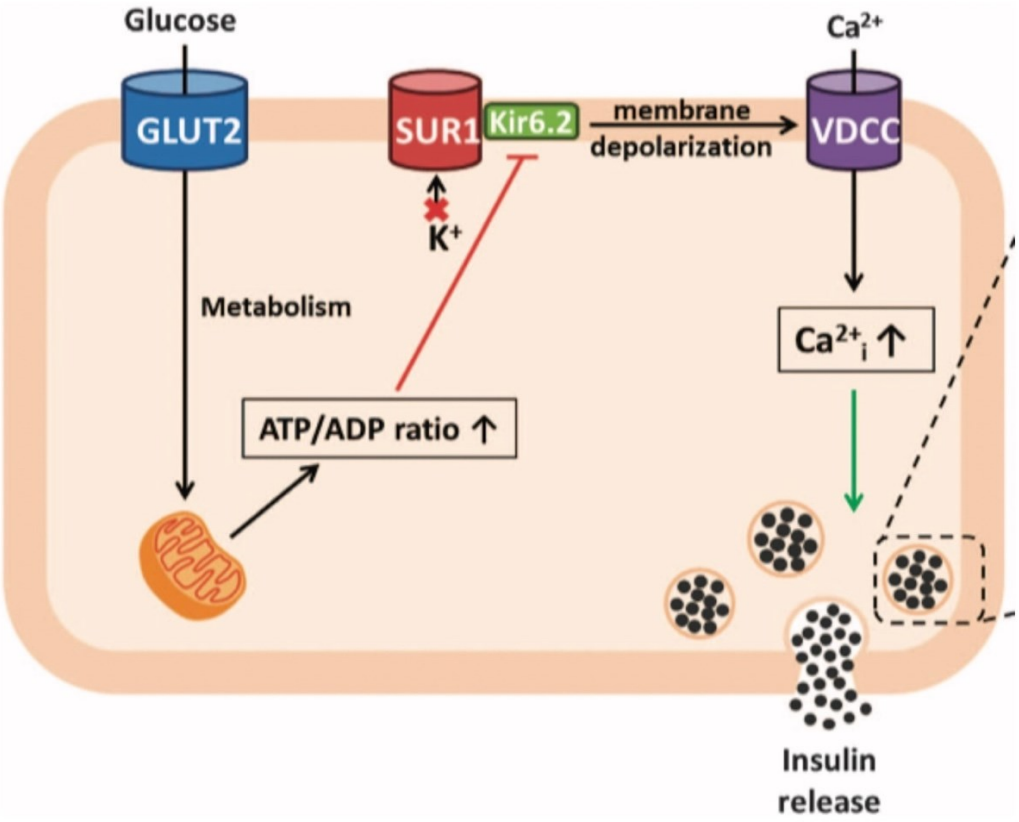


*Figure 2. Glucose-stimulated insulin release from a pancreatic β-cell (adapted from Röder et al., 2016)* [72]

### 1.3.3. Insulin signaling pathways.

Insulin, orchestrates metabolic processes by engaging with its specific receptor, commonly termed the insulin receptor (IR). This receptor, prominently localized in adipose tissues, hepar, and skeletal muscle, serves as a cornerstone for metabolic modulation[32,101].

Structurally, the IR is a heterotetrameric assembly constituted of dual alpha and beta subunits. The former, the alpha subunits, are strategically positioned external to the cell and are primed for insulin binding. Conversely, the beta subunits traverse the cell's lipid bilayer and are endowed with intrinsic tyrosine kinase functionality [32,102,103].

Upon insulin's engagement with the IR, a sequence of intracellular events is initiated. This involves the autophosphorylation of the beta subunits, subsequently summoning key substrates such as IRS-1 and IRS-2. Through this, the cellular machinery is primed to initiate both metabolic and growth-associated pathways [32,101,104,105].

***In the metabolic pathway***, IRS-1 and IRS-2 facilitate the activation of the enzyme phosphatidylinositol-3-kinase (PI3K). This, in turn, augments the activity of AKT, a serine/threonine kinase. AKT's primary role involves prompting the movement of the glucose transporter 4 (GLUT4) to the cell membrane, which is pivotal for glucose uptake. Moreover, AKT plays an instrumental role in bolstering protein production, amplifying glycogen, and lipid biogenesis, and triggering the mTOR pathway [104,106,107]

***The mitogenic pathway*** *(growth and proliferation-associated)* is set into motion through the activation of the Ras protein. This engenders a series of phosphorylation events along the MAP kinase trajectory, culminating in cellular growth and replication. Of note, ERK1/2 (p44/p42MAPK) delineates the growth-response to insulin and IGFs [104].

***Transcriptional regulation.*** Intriguingly, insulin's influence isn't confined to immediate cellular responses. It has the prowess to modulate transcriptional landscapes. For instance, it can temper the activity of Foxo1, a transcription factor that is inherently attuned to genes responsive to fasting [108]. An intricate play is observed with peroxisome proliferator-activated receptors (PPARs) - nuclear receptors that govern metabolic gene sets. The interactions of PPARs with co-regulators like PGC1, which is under the purview of pathways including insulin signaling, further elucidate the intricate dynamics of insulin's influence [109].

***Insulin signaling deactivation*** However, the insulin signaling trajectory is not perpetual. Post insulin engagement, the IR, and the ensuing signaling constituents undergo rapid inactivation.

This could manifest through insulin's dissociation from its receptor or potential degradation of the receptor. Certain entities, such as the protein tyrosine phosphatase 1B (PTP1B), can attenuate the receptor's activity. Furthermore, the suppressor of cytokine signaling (SOCS) proteins can hinder the interplay between the phosphorylated receptor and IRS proteins, stymieing the downstream signaling cascade [103].

Finally, various serine/threonine kinases, including AKT, phosphorylate the insulin receptor and its active substrates, blocking insulin signalling [110]. These pathways may contribute to insulin resistance (described before).

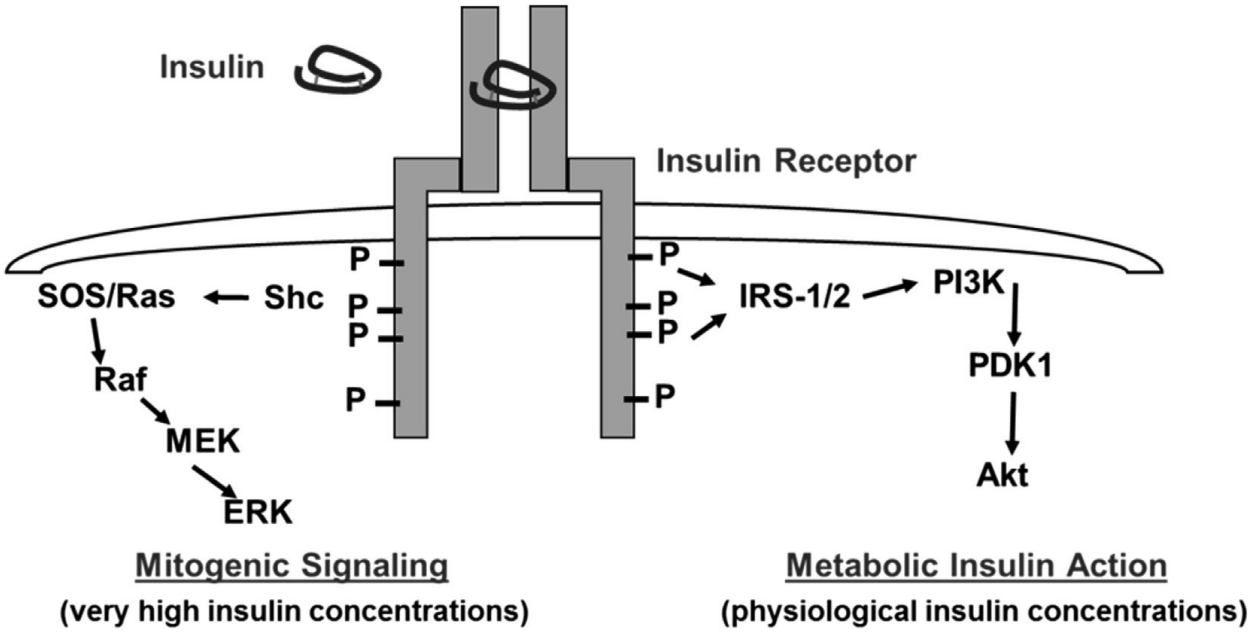


*Figure 3. Basic schematic of the bifurcated insulin signaling pathway. Adapted from Bedinger &Adams. 2015* [104]

*Insulin Receptor Substrate (IRS), phosphatidylinositol-4,5-bisphosphate-3-kinase (PI3K), 3-phosphoinositide dependent protein kinase-1 (PDK1), Son of Sevenless (SOS). Mitogen/Extracellular signal-regulated Kinase (MEK), Extracellular Signal-Regulated Kinase (ERK)*

Regulation of insulin secretion involves other factors categorized as direct stimulants, amplifiers, and inhibitors which are summarized in Table 2.

*Table 2 Insulin release regulation*

| Category | Factor | Effect on Insulin Release | Citation |
|---|---|---|---|
| Stimulants of insulin release | Glucose | Enhance | [111,112] |
| | Amino acids: Leucine | Enhance | [113] |
| | Neural: Vagal stimulation, Acetylcholine | Enhance | [114] |
| | Drugs: Sulfonylureas, Meglitinides | Enhance | [115,116] |
| Amplifiers of glucose-induced insulin release | Enteric hormones: Glucagon-like peptide 1 (7-37) (GLP1), Gastric inhibitory peptide (GIP), Cholecystokinin, Gastrin, Secretin | Enhance | [117,118] |
| | Neural: β-adrenergic effect of catecholamines | Enhance | [114] |
| | Amino acids: Arginine | Enhance | [113] |
| | Drugs: GLP1 agonists | Enhance | [117,118] |
| Inhibitors of insulin release | Neural: α-adrenergic effect of catecholamines | Inhibit | [114] |
| | Humoral: somatostatin | Inhibit | [119,120] |
| | Drugs: Diazoxide, Thiazides, β-blockers, Clonidine, Phenytoin, Vinblastine | Inhibit | [121-125] |

### 1.4. Pancreatic regeneration: Mechanisms and implications for diabetes treatment

Pancreatic regeneration is crucial for the restoration of functional β-cell mass in individuals with diabetes. In type 1 diabetes, the immune-mediated destruction of β-cells necessitates the regeneration of new insulin-secreting cells to replace the lost ones. Similarly, in type 2 diabetes, the decline in β-cell mass and impaired β-cell function contribute to the development and progression of the disease. Promoting pancreatic regeneration offers a potential therapeutic approach to replenish β-cell mass, enhance insulin secretion, and improve glucose homeostasis in individuals with diabetes.

Pancreatic regeneration can occur through various mechanisms, including differentiation of pluripotent stem cells, β-cell replication, transdifferentiation and reprogramming of non-pancreatic cells. These mechanisms involve complex molecular and cellular processes orchestrated by a network of signaling pathways, transcription factors, and growth factors. Understanding these mechanisms is essential for developing targeted strategies to promote efficient and functional pancreatic regeneration.

#### 1.4.1 Differentiation of pluripotent stem cells

Pancreatic regeneration is imperative for restoring functional β-cell mass in diabetes patients, both in type 1, where immune-mediated β-cell destruction occurs, and type 2, characterized by declining β-cell mass and function [126-128]. The culture of human embryonic stem cells (hES cells) and induced pluripotent stem cells (transdifferentiation cells offer a prominent solution by differentiating into functional pancreatic islets [129,130].

The differentiation of human embryonic stem cells (hES cells) into pancreatic endocrine cells has been guided by four-stage protocols, culminating in β-like cells [131,132].

Despite early differentiations resulting in mixed hormone expressions, subsequent protocols improved in vitro differentiation [132].

Recent advancements have created more intricate protocols, generating cellular clusters that closely resemble pancreatic islets [133,134]. These clusters have been transplanted successfully in mouse models, reversing experimental diabetes. Additionally, pancreatic progenitor cells have emerged as a promising therapeutic product [132,135]. Challenges persist, however, including large-scale differentiation, elimination of unwanted cells, and immune rejection Solutions may lie in encapsulation in durable biomaterials or genetic modification. Despite hurdles, the field remains promising for future diabetes treatment.

### 1.4.2. β-Cell replication: regulation, factors, and implications in diabetes and therapeutic strategies

Postnatal β-cell growth primarily occurs through replication, contrary to earlier theories of stem cell differentiation [136,137]. Various factors influence this, such as the transcription factor Nkx6.1, crucial for β-cell development [138,139], and physiological adaptations during pregnancy [140,141]. In adulthood, β-cell replication is influenced by aging, reducing β-cell replication rates [142,143] , and growth factors like hepatocyte growth factor, insulin-like growth factor, and connective tissue growth factor [144,145]. Obesity also affects β-cell mass, mainly through hyperplasia [146].

In diabetes, strategies targeting β-cell replication, such as ER stress reduction and encapsulation of β-cells, could enhance β-cell survival [147-150]

Complex interplays involving the mTOR pathway and cyclin D2 contribute to glucose-induced β-cell proliferation [151], while inhibition of DYRK1A and GSK3B has stimulated β-cell replication [152].

In summary, the complexity of β-cell replication and the various factors influencing it hold substantial potential for therapeutic interventions. Further research is essential to fully harness β-cell replication in treating diabetes.

### 1.4.3. Transdifferentiation of pancreatic cells: mechanisms, factors, and prospects

Transdifferentiation refers to the transformation of one differentiated cell type into another and emerges as a groundbreaking approach for creating functional β-cells as a part of diabetes treatment. Through the investigation of pancreatic cell transdifferentiation, we might uncover innovative avenues for regenerative interventions.

Recent research has yielded evidence for the transdifferentiation of distinct cell types within the pancreas into β-cells. For instance, acinar cells, comprising the pancreas' exocrine portion, have been effectively reprogrammed into β-like cells both in vivo and in vitro [153,154]. Additional evidence shows potential for ductal cells' transdifferentiation into β-cells [155,156], and other cell types, including gastrointestinal cells and stomach tissue [156-159], also gallbladder cells [160], have been transformed into insulin-secreting β-like cells**.**

The orchestration of transdifferentiation processes relies on defined transcription factors: Pdx1[155,161], Ngn3 [155], and MafA [155,162,163] coupled with signaling pathways like Notch[156,164], Wnt [156], and Hedgehog [156,165] facilitate the transdifferentiation of pancreatic cells into β-cells. Transdifferentiation's efficiency and scale are modulated by various elements, including cellular plasticity, epigenetic alterations, and the pancreatic microenvironment [166-168].

The activation of certain signaling pathways and presence of transcription factors further influence these events [156].

Despite the potential of transdifferentiation, challenges remain, such as identifying optimal transcription factor combinations and modulating signaling pathways to enhance efficiency.

Comprehending the underlying mechanisms of cellular plasticity and epigenetic modifications, along with pioneering delivery methods and gene expression control techniques, might contribute to the triumph of transdifferentiation strategies.

Continued exploration and development of inventive strategies will likely drive the progress of transdifferentiation in the treatment of diabetes.

### 1.4.4. Intrinsic and extrinsic factors influencing pancreatic regeneration: insights from age, genetics, environment, and the microenvironment

Pancreatic regeneration represents a vital, dynamic process in upholding pancreatic functionality. Discerning the factors influencing pancreatic regeneration is indispensable for formulating therapeutic strategies for pancreatic ailments.

*Aging* has been identified to negatively impact pancreatic regeneration, with a marked decrease in the proliferative and differentiative abilities of pancreatic progenitor cells [169-171]

*Genetic components*, including variations in genes like Pdx1 and Ngn3 [172,173], modulate the regenerative prowess of pancreatic cells.

*Environmental factors* such as diet, toxins, and metabolic conditions affect pancreatic regeneration. HFD and toxin exposure have been linked with diminished regenerative response [174,175]

*Hormones*, such as insulin, glucagon, and growth hormone, play crucial roles in pancreatic regeneration. Insulin has been shown to promote β-cell proliferation and neogenesis [175], while glucagon receptor antagonism ameliorates hyperglycaemia and promotes β cell regeneration in mouse models of type 2 diabetes [176]

*Various growth factors,* including epidermal growth factor (EGF), fibroblast growth factor (FGF), and transforming growth factor-β (TGF-β), have been implicated in pancreatic

regeneration. These growth factors regulate cell proliferation, differentiation, and survival during pancreatic regeneration [177]

*Cytokines*, such as interleukin-6 (IL-6) interleukin-10 (IL-10) and tumor necrosis factor-alpha (TNF-α), contribute to the inflammatory responses that accompany pancreatic regeneration. They play roles in immune modulation, cell signaling, and tissue repair [177,178]

*Inflammation* triggers the recruitment of immune cells, release of cytokines, and activation of regenerative signaling pathways. However, chronic inflammation can hinder regeneration and lead to fibrosis [177,179]

*The extracellular matrix (ECM) and microenvironment* are crucial for cell interactions and the overall regenerative ability of the pancreas [177,180].

The regulation of pancreatic regeneration is multifaceted, encompassing intrinsic and extrinsic factors such as age, genetics, environment, hormones, growth factors, cytokines, inflammation, and the microenvironment. Understanding these complex interactions will necessitate further research to unravel the underlying mechanisms and cultivate therapeutic interventions to augment pancreatic regeneration for the treatment of pancreatic disorders.

### 1.5. Cellular Communication Network (CCN) factor family

CCN proteins were first discovered in the late 1980s and early 1990s as small proteins (30–40 kDa) enriched in cysteine content (10% by mass, 34–38 conserved residues) and characterized by a tetramodular structure. CCN family was born as the acronym of three classical members namely, cysteine-rich angiogenic inducer 61 (**C**YR 61, also known as CCN1), connective tissue growth factor (**C**TGF, also known as CCN2) and nephroblastoma overexpressed (**N**OV, also known as CCN3)[181-183], based on their compelling structural similarities. After the establishment of this family, three additional members were added completing the present form of this family

of six members. Each of the CCN family members had multiple names as multiple groups discovered the members independently while working with different biological systems [184-186]. The various names are listed in the table below (table 3)

A consensus was reached on October 2018, the Human Genome Organization (HUGO) Gene Nomenclature Committee approved the new CCN nomenclature proposed by the ICCNS Scientific Committee when the genes referred to as CYR61, CTGF, NOV and WISP1–3 were respectively designated by the gene symbols CCN1–6 with corresponding gene names « **c**ellular **c**ommunication Q2 **n**etwork factor 1–6 » [187,188]

*Table 3 Cellular C0mmunication Network Nomenclature*

| Family Member | Previous Names and Abbreviations | References |
|---|---|---|
| CCN1 | CYR61, CEF10, βIGM1, IGFBP9, IGFBP-rP4 | [187] |
| CCN2 | CTGF, FISP12, Hcs24, βIG-M2, HBGF-0.8, ecogenin, IGFBP8, IGFBP-rP2 | [187] |
| CCN3 | NOV, IGFBP9, IGFBP-rP3 | [187] |
| CCN4 | ELM-1, WISP-1 | [187] |
| CCN5 | rCOP-1, WISP-2, CTGF-L, HICP | [187,189] |
| CCN6 | WISP- 3 | [187] |

Abbrevations:

**CYR61** (Cysteine-rich angiogenic inducer 61),

**CEF10** (Chicken embryo fibroblast-derived protein 10),

**βIG-M1** (B-inducible gene-h3 module),
**IGFBP9** (Insulin-like growth factor-binding protein 9),
**IGFBP-rP4** (Related protein to IGFBP)
**CTGF** (Connective tissue growth factor)
**FISP12** (Fibroblast-inducible secreted protein-12)
**Hcs24** (Hypertrophic chondrocyte-specific protein 24)
**HBGF-0.8** (Heparin-binding growth factor 0.8)
**NOV** (Nephroblastoma overexpressed)
**ELM-1** (Elm1 protein)
**WISP-1** (WNT1 inducible signaling pathway protein 1)
**WISP-2** (WNT1 inducible signaling pathway protein 2)
**CTGF-L** (CTGF-like),

### 1.5.1. Structure of CCN proteins

The CCN family of proteins consists of six members, each exhibiting a modular structure with four conserved domains, except for CCN5, which uniquely lacks the fourth CT domain, resulting in a lower molecular weight. These proteins, rich in cysteine residues, demonstrate an interesting evolutionary strategy where individual exons encode each domain, allowing for reshuffling and leading to diverse products [183,190,191]. Adding complexity, a flexible hinge region separates each molecule into two halves, maintaining a conserved structure across the family [192,193]. Despite these commonalities, significant sequence variations between members lead to functional divergence [183].Overall, sharing approximately 50-60% identical sequences, including 38 cysteines, CCN proteins either act as matricellular proteins or growth factors, interacting with receptors and playing a key role in regulating growth factors, cytokines, and matrix metalloproteinases (MMPs) [194-196]. This elegant combination of consistency and variability within the CCN family reflects the intricate nature of biological design.

#### *1.5.1.1. IGFBP domain*

The IGFBP domain of the CCN proteins displays strong sequence homology with the N-terminal domain of conventional IGFBPs [181]. However, despite this sequence similarity, the CCN proteins bind IGF with a very low affinity that is 100-fold lower than the classic IGFBPs [197]. The literature lacks any valuable information regarding the specific role of the IGFBP domain in CCN proteins.

#### *1.5.1.2. The von Willebrand factor C repeat*

The von Willebrand factor type C domain (VWC) is a common motif found in numerous ECM proteins [198]. It is known to regulate many of CCN 's biological functions. Specifically, the VWC regulates TGF-ß signaling and bone morphogenic proteins (BMPs), which are key growth factors in several organs and tissues [199,200]. CCN2 is among the CCN proteins that has been extensively studied for its interactions with BMPs and TGF-ß [199]

#### *1.5.1.3. The thrombospondin type-1 repeat (TSP-1) module.*

The thrombospondin type-1 repeat module was named after the ECM glycoprotein TSP-1, which is an adhesive glycoprotein with a molecular weight of ~450 kDa. It a member of the thrombospondin family of proteins that bind to fibrinogen, fibronectin, laminin, collagen, and integrin. TSP-1 is endocytosed after interacting with cell surface receptors such as low-density lipoprotein-related protein (LRP- [201-203]. Thrombospondin-1 is a trimeric protein, composed of multiple domains including N- and C-terminal globular domains, a procollagen-likedomain, and three types of repeated sequence motifs (type 1, 2, and 3 repeats) which are essential for its function [204,205]. This module has been implicated in cell adhesion, angiogenesis, binding, and regulating growth factors and other ECM proteins activities [206].

### *1.5.1.4. The cysteine knot C-terminal domain*

The CT (or heparin-binding domain ) is conserved between many ECM proteins and growth factors such asVEGF, TGFβ and BMPs [207]. The CT domain is thought to serve as a dimerization module due to its unique structure that consists of a cysteine knot motif of six conserved cysteine residues [181]. This domain interacts with HSPGs, Notch1, and integrins, which are involved in cell proliferation, apoptosis [208] and differentiation [209]. The CT domain exerts a role in the initial dimerization and receptor binding [210-212].

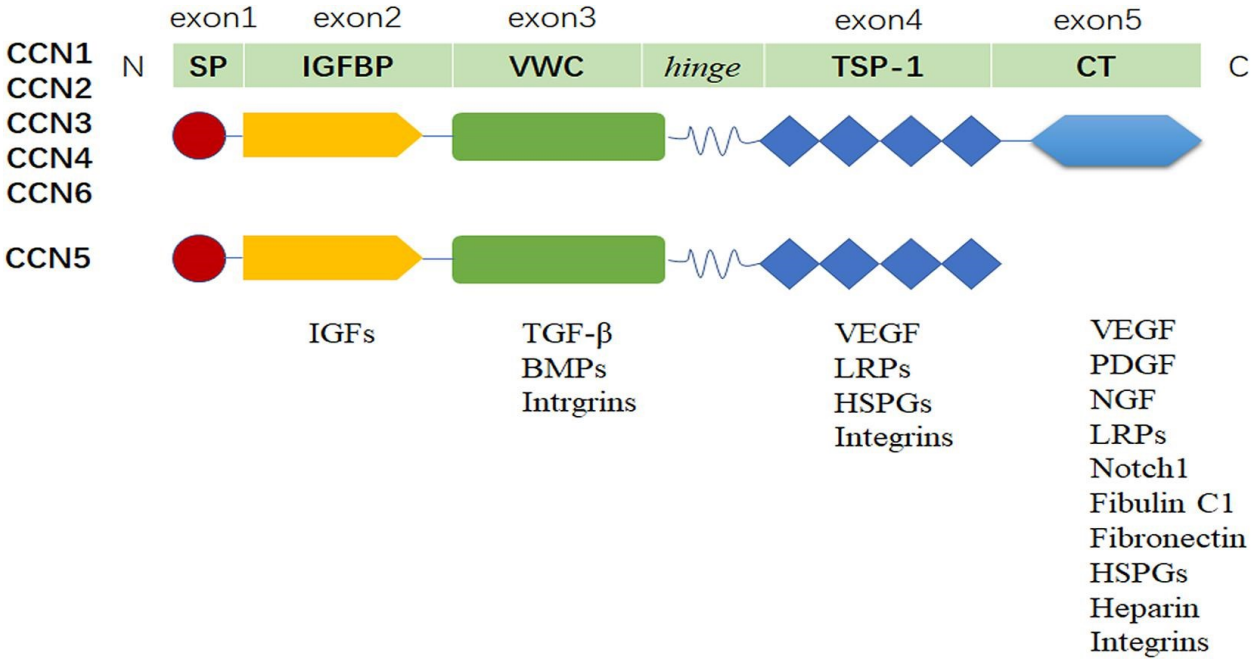


*Figure 4. Structure of CCN proteins.*

Schematic of four conserved functional domains coded by associated exons. CCN proteins could serve different functions via interactions with a variety of cell surface receptors and extracellular ligands (e.g., integrins, HSPGs, LRPs, TGFβ, VEGF, and PDGF). Adapted from Jia et al. 2021 [213].

### 1.5.2. Function of CCN proteins

Collectively, CCN proteins have multiple biological functions such as cell growth, differentiation, proliferation, apoptosis, adhesion, and migration of numerous cell types [214,215]. Consequently, they regulate diverse physiological processes, including skeletal development, stem cell differentiation, angiogenesis, chondrogenesis, and wound repair [215].

There have been reports of abnormal CCN protein secretion in fibrogenesis, carcinogenesis, and atherosclerosis[215,216].

### 1.5.3. CCN5

#### *1.5.3.1. Discovery of CCN5 (Wisp2) gene*

CCN5 was originally cloned and published by several groups in the late 1990s. Delmolino and Castellot first reported the discovery of CCN5 in 1997 as part of a screen to identify heparin-regulated genes in VSMC [217]. Heparin down-regulates proliferation in VSMC, thus this screen was able to identify downstream effectors of heparin that may also be able to inhibit VSMC mitogenesis [218]. CCN5 was found to be upregulated in a subtractive hybridization array comparing heparin treated VSMC with untreated cells. This gene, first identified in this screen as hicp and later renamed Cop-1, had features of a growth arrest-specific gene.

Independently, another group identified the rat orthologue of CCN5 (WISP-2), rCop-1 in Rat Embryonic Fibroblasts (REFs). The study found a significant correlation between loss of expression of the CCN5 with cell transformation by inactivation of P53 and concomitant expression of a constitutively active H-Ras. Thus, this study suggested that CCN5 may be a negative regulator of tumorigenesis. In the same year, CCN5 (WISP-2) and CCN4 (WISP-1), were identified in a genetically engineered mouse model. These studies indicated that CCN5/WISP-2 is a Wnt inducible gene [185].

The human CCN5 gene is mapped to chromosome 20 at position 20q12-q13.1. The other five known CCN genes are located on different chromosomes (chromosomes 1, 6, and 8). CCN5 has four exons of 96, 216, 254, and 842 bp separated by three introns. Several different CCN5 cDNA clones have been sequenced and taken together strongly suggest that the processed mRNA sequence includes 1708 nucleotides, including a 261 nucleotide 5' UTR, the 750 nucleotide ORF encoding the 250 amino acid residues, a 730 nucleotide 3' UTR beginning with a TAA stop codon at nucleotide 1011, and an mRNA poly-A addition consensus site at nucleotide 1710 [186,219]. The rat and human CCN5 amino acid sequences share approximately 74% similarity whereas mouse and human CCN5 have 73% similarity [220]

***1.5.3.2. Structure of CCN5***

CCN5 is a secreted protein with 251 amino acids and a molecular weight of approximately. 28kDa. The reported molecular weights vary from 26 kDa [220], 28 kDa [221,222], 31 kDa [186,219,223]. The nucleotide and protein sequence of CCN5 exhibits a 30–40% sequence homology with other family members, largely CCN4 (WISP-1) and CCN6 (WISP-3). The modular architectures of CCN5 are identical with other family members except in their C-terminal Cysteine-knot (CT) domain, which is absent in the CCN5 gene [185]. This may render an important biological function to CCN5, as the CT domain is involved in binding matrix proteins, integrins and important signaling molecules [192]. Indeed, domain analysis has indicated that each domain may serve a distinct function for the protein, and in certain cell types there are splice variants that create proteins comprised of the individual CCN domains [224].

Although other domains have not been investigated for CCN5, other CCN family member proteins have been shown to have domain-specific functions. CCN2 has been shown to induce

angiogenesis during breast cancer. Removing the CT domain abrogates this activity, suggesting that CCN5 may negatively regulate angiogenesis since it is missing the CT domain [225].

#### *1.5.3.3. CCN5 receptor and signaling pathways regulating CCN5 expression*

#### 1.5.3.3. 1. Receptor

A high-affinity specific CCN5 receptor has not yet been identified. However, there is evidence of specific binding with a few membrane receptors.

*One potential candidate receptor for CCN5 is LRP5/6,* which is a co-receptor for canonical Wnt signaling. Canonical Wnt proteins usually require acetylation for secretion and binding to the heterodimeric receptor complex composed of Frizzled (FZD) and LRP5/6 proteins [226]. However, CCN5 does not require acetylation for its secretion and binding to FZD receptors, suggesting that CCN5 may bind directly to the LRP5/6 receptor or activate it through alternative signaling pathways. The interaction between CCN5 and LRP5/6 has been studied in adipocytes [227,228]. CCN5 has been identified as an autocrine Wnt ligand that inhibits the differentiation of mesenchymal precursor cells into adipocytes. This process involves the activation of canonical Wnt signaling and potentially involves binding to LRP5/6 [229].

*Integrins* are another class of potential receptors for CCN5. CCN5 has been shown to interact with different integrins depending on the specific cellular context. In vascular smooth muscle cells (VSMCs), CCN5 binds to integrin αvβ3, which regulates the matrix degradation ability of podosomes, cellular structures involved in matrix remodeling [230]. In triple-negative breast cancer cells (MDA-MB-231), CCN5 interacts with integrin α6β1, leading to the inhibition of cell proliferation through the regulation of the cyclin-dependent kinase inhibitor (CKI) p27Kip1 [231]

#### 1.5.3.3.2. Wnt signalling

Previous studies, which first identified the CCN5 gene in a mouse mammary tumor model, indicated that CCN5 is the second family member of Wnt-1-induced signaling proteins, and thus the name of this protein was WISP-2 [185]. Wnt signaling, an essential pathway in cell development, differentiation, and oncogenesis, has been associated with the regulation of CCN5 expression. Pennica et al. (1998) demonstrated that overexpression of Wnt-1 in mouse mammary epithelial cell lines led to the up-regulation of CCN5 at both mRNA and protein levels [185]. Wnt proteins bind to FZD and LRP5/6, triggering a signaling cascade that involves the phosphorylation of LRP5/6, recruitment of signal transducers DVL and AXIN, inhibition of GSK-3B and CK1, and stabilization of β-catenin. The stabilized β-catenin translocates to the nucleus, where it interacts with transcription factors TCF/LEF to induce gene expression. Wnt signaling thus plays a significant role in development and oncogenesis.[232,233].

Moreover, physiological activators of Wnt signaling have also been shown to induce CCN5 expression in different cell lines:

Upregulated expression of the Wnt-1 protein in the hepatocellular carcinoma cell line Huh-7 and treatment of the osteoblast cell line with a small molecule inhibitor of the GSK-3 enzyme caused an increase in CCN5 mRNA transcription. Therefore, we can anticipate that CCN5 regulation by Wnt1 could be mediated through GSK-3β/ β-catenin pathway [234].

Mouse Pluripotent Progenitor Cells: Overexpression of Wnt3A led to a 2.5-fold increase in CCN5 expression [235].

Synovial Fibroblasts: Expression of β-catenin led to an up-regulation of CCN5 by 2.9-fold, while inactivated cells showed down-regulation which express constitutively downstream [236].

Childhood Adrenocorticoid Tumors (ACT): ACT samples showed increased CCN5 expression, implicating Wnt overactivation controls [234].

Salivary Gland Tumor Development: A 4-fold increase in CCN5 expression in tumor-derived cell lines, although down-regulated compared to normal cells, suggesting a role in tumor progression (168,172,173)

Effects of Small Signaling Molecules like PKA and PKC: Modulation of the Wnt pathway through these molecules could induce CCN5 expression, with PKC acting as a negative regulator of β-catenin stability [237,238].

The evidence collectively highlights the multifaceted nature of CCN5 regulation by Wnt signaling. This complex interaction spans various cellular contexts and physiological conditions, including mechanical strain, estrogen enhancement, different cancer types, and the influence of small signaling molecules.

**1.5.3.3.3. IGF-1 signaling**

Our lab was the first to identify CCN5 as a signaling molecule integral to the mitogenic action of the IGF-1 axis in pancreatic islets [239]. CCN5 expression was elevated in the islets of mice that overexpressed IGF-I compared to wild-type mice. Moreover, freshly isolated islets from wild-type mice cultured with IGF-I induced CCN5 mNA and protein levels. Furthermore, siRNA-mediated suppression of CCN5 in MIN6 insulinoma cells blocked IGF-I's proliferative effect [239]. Interestingly, a 2-kb region of the CCN5 promoter region has a similar structure to the IGF-I promoter [240]. It is known that the CCN5 promoter contains binding sites for specificity proteins 1(Sp1) and activator protein (Ap1), which are believed to mediate the cross-talk between CCN5 and IGF-I [241].

Signaling crosstalk could also occur between the IGF-I, insulin, and Wnt pathways. Together, these signals regulate cat/T cell factor (TCF)-mediated gene transcription via Akt-mediated phosphorylation of GSK-3, as well as phosphorylation and stabilization of β- catenin [242,243], suggesting that WNT, insulin, and IGF-1 regulate islet function in collaboration (Figure 5).

***1.5.3.3.4. Estrogen signaling.***

Role of estrogen-mediated regulation of CCN5 expression was evident from early studies carried out independently in different labs [223,244-247]. CCN5 is overexpressed in estrogen receptor alfa (ER-α) positive breast cancer cell lines compared to other breast cell lines [223,247]. Also, it was shown that treatment of ER-α-positive cells with estrogen leads to an increase in mRNA transcript and protein levels of CCN5 [221,244,246]. 17β-estradiol raises CCN5 mRNA and protein in MCF-7 cells in culture in a dose- and time-dependent manner, reaching maximal levels by 72 h [221,244,246]. The hormones that interact specifically with ER-α can induce the expression of CCN5. Dexamethasone and tri-iodothyronine do not influence CCN5 expression, although certain xenoestrogens may [221]. Also, upregulation of CCN5 expression by estrogen was entirely blocked by the pure anti-estrogen inhibitor, ICI 182,780 [244,246]. One early study found a high positive connection between CCN5 protein expression and estrogen receptor alpha (ER-α) positivity in human breast cancer samples [244]. Furthermore, studies have shown that CCN5 gene promoter has estrogen response elements (EREs). ER-α which is the direct mediator of estrogen action binds to the CCN5 promoter in an estrogen-dependent fashion [240]. Progesterone also induces CCN5 protein in ER-positive breast cancer cells [244]. Progesterone effects on CCN5 appear to be cell-specific [248]. Combining estrogen and progesterone increased CCN5 expression in uterine smooth muscle cells [248]

### 1.5.4. CCN5 role in metabolism

#### *1.5.4.1. CCN5 and adipogenesis*

Adipogenesis, the process by which preadipocytes differentiate into mature adipocytes, plays a pivotal role in metabolic regulation and the pathophysiology of conditions like obesity and metabolic syndrome and is crucial for maintaining metabolic homeostasis. [249,250]. Dysregulation of adipogenesis has been linked to various metabolic disorders, highlighting the importance of understanding the underlying mechanisms [251].

CCN5 has attracted attention owing to its possible significance in this process**.**

The regulatory role of CCN5 in adipogenesis has been gleaned primarily from in-vitro studies using 3T3-L1 preadipocyte cell lines [252] and in-vivo studies in CCN5 knockout (KO)[253] and transgenic mice models [228],

It has distinct functions attributed to its secreted and cytosolic forms. The complex interplay between these forms and their interactions with various signaling pathways may contribute to the nuanced effects observed in CCN5 KO mice:

Early Phase of Adipogenesis (Commitment Phase)

- *Cytosolic CCN5*: The cytosolic form of CCN5 may regulate adipogenic commitment by interacting with BMP4, playing a role in the early stages of adipogenesis [227,229,252] where it acts as a gatekeeper, deciding the fate of preadipocytes.

- *Secreted CCN5*: The secreted form of CCN5 may inhibit the commitment of mesenchymal stem cells to the adipocyte lineage by activating the WNT pathway, maintaining mesenchymal precursor cells in an undifferentiated state [227,229,252].

Late Phase of Adipogenesis (Differentiation Phase):

-Potential dual role: The *secreted* form of CCN5 may inhibit adipogenic differentiation by activating the WNT pathway, while the *cytosolic* form's role in this stage is not explicitly provided in the existing literature at least in my knowledge [227,229,252]

Most of the studies are focused on the inhibitory role of adipogenesis:

***In vitro studies****: Inhibition of adipogenic differentiation:* In 3T3-L1 preadipocyte cell lines, CCN5 has been found to be downregulated during adipocyte differentiation, suggesting a potential inhibitory role in adipogenesis. The overexpression of CCN5 inhibits adipocyte differentiation, marked by the reduction of key adipogenic transcription factors like peroxisome proliferator-activated receptor gamma (PPARγ) and CCAAT/enhancer-binding protein alpha (C/EBPα) [227,228]. Furthermore, the lipid accumulation, often taken as a marker of adipogenesis, is also suppressed upon CCN5 overexpression, thus suggesting its anti-adipogenic role.

***In vivo, studies***. In parallel, studies involving CCN5 KO mice reveal an interesting phenotype. When fed with a HFD, these mice showed higher adiposity compared to their wild-type counterparts. Moreover, adipocytes from KO mice were found to be larger in size, indicating enhanced adipocyte hypertrophy. Taken together, these observations provide compelling in-vivo evidence of CCN5's inhibitory role in adipogenesis.[253] Further supporting this hypothesis, transgenic mice overexpressing CCN5 exhibited resistance to diet-induced obesity. These mice maintained a lean phenotype even under a HFD, suggesting that CCN5 might prevent excessive adipose tissue expansion, possibly by inhibiting adipogenesis.[228]

Impact on hyperplastic BAT and WAT: WISP2 transgenic mice were characterized by increased proliferation and growth of mesenchymal precursor cells, leading to markedly increased and hyperplastic brown adipose tissue (BAT) and "healthy" hyperplastic WAT. This phenotype indicates a role for CCN5 in promoting hyperplasia in adipose tissues [227]

In summary, CCN5/WISP2 appears to play a multifaceted role in adipogenesis, influencing preadipocyte commitment, and differentiation, and impacting hyperplasia in adipose tissues. The complex interplay between cytosolic and secreted forms and their interactions with various signaling pathways may contribute to the nuanced effects of CCN5.

#### *1.5.4.2. CCN5 role in β cells proliferation*

Our lab has shown that CCN5/WISP2 is normally expressed in mouse islet β-cells and that IGF-I directly stimulates its expression. CCN5 overexpression increases the proliferation of insulinoma cells, activates Akt and Erk2 kinases, and inhibits streptozotocin-induced caspase-3 activation and apoptosis. Recombinant CCN5 protein seems to reproduce the proliferative effect and the stimulation on Akt phosphorylation. These findings suggest that CCN5 is capable of regulating islet cell proliferation and survival and that increased CCN5 expression may contribute to IGF-I–stimulated islet cell growth and/or survival [239].

Kaddour et al. 2019 show that recombinant protein CCN5 promotes islet cell proliferation and survival in vitro. The results were consistent with in vivo transgenic mice: Rh - CCN5 stimulates the proliferation of INS 832/13 cells and primary mouse islets. Pretreatment with CCN5 protects INS 832/13 cells and primary mouse islets against lipotoxicity, glucolipotoxicity and streptozotocin-induced cell death. CCN5 treatment enhances ß cell identity and function in primary islets cells [254].

#### *1.5.4.3. Glucose metabolism in CCN5 knockout and transgenic mice*

Kim et al. 2018 studied CCN5 knockout mice and observed that CCN5-knockout mice fed a normal-chow diet (NCD) displayed mild hyperglycemia and insulin resistance. On the other hand, HFD-fed CCN5 knockout mice exhibited a mild diabetic phenotype and higher insulin levels. Remarkably, when fed with NCD, the knockout (KO) mice exhibited significantly

elevated fasting blood glucose levels in comparison to the wild-type (WT) mice. However, this discrepancy was not observed when both KO and WT mice were subjected to an HFD.
Insulin Tolerance Tests (ITT) and Glucose Tolerance Tests (GTT) were conducted at week 12 and 24 of NCD and HFD. During week 12, CCN5 KO mice revealed decreased insulin tolerance and glucose tolerance compared to the WT mice when fed a HFD. Furthermore, the KO mice showed elevation in plasma insulin levels compared to their WT counterparts on the same diet. At the 24-week examination, no notable disparities in ITT and GTT were detected between the KO and WT mice, although an acute impairment in ITT was observed in KO mice fed with a NCD within 30 minutes post insulin injection. Elevated plasma insulin was also detected in KO mice on NCD. Collectively, these findings hint at the possibility that the absence of CCN5 in KO mice could contribute to the development of a mild form of type 2 diabetes [253].
On the other hand, aP2-CCN5 transgenic mice displayed increased lean body mass, expanded brown adipose tissues (BAT), enhanced glucose uptake by adipose cells and skeletal muscle, decrease blood glucose levels and improved insulin sensitivity (Grünberg et al., 2017)
In summary, the exploration of CCN5's function in metabolism, specifically in glucose regulation and insulin sensitivity, has uncovered nuanced relationships within knockout and transgenic mouse models. Together, these findings contribute to a complex understanding of CCN5's role in metabolic processes and hold potential therapeutic implications for metabolic disorders like type 2 diabetes.

### 1.6. The roles of sex hormones in metabolism

Sexual hormones (primarily androgens, estrogens, and progesterone), not only regulate sexual functions but also have a significant impact on metabolic activities, thereby linking them to MetS

[255-258] They function as endogenous signaling molecules that orchestrate a range of physiological processes, including but not limited to, reproductive functions, somatic growth, and the development of secondary sexual traits. Sexual hormones are predominantly produced by the gonads, and to a lesser extent, by the adrenal glands.

In the context of MetS, both testosterone and estrogen are of particular interest. Testosterone modulates muscle composition, fat distribution, and insulin sensitivity. Contrarily, estrogen is crucial for controlling body weight and energy balance. Its influence becomes especially significant during menopause when changes in estrogen levels are associated with an increased risk of MetS [259-261].

### 1.6.1. Sex hormone-binding globulin (SHBG)

Primarily synthesized in the liver, SHBG is responsible for the transport and bioavailability of sexual hormones in the circulatory system [262]. Existing research indicates that diminished levels of SHBG could elevate the risk of MetS and type 2 diabetes, [256,263], even more than low testosterone levels [264-266]. A study by Ding et al. (2009) further corroborated the protective effects of elevated SHBG levels. However, the intricate interplay between SHBG, sexual hormones, and metabolic disorders necessitates ongoing research for a comprehensive understanding. (11-13).

### 1.6.2. Sex hormones and body weight

The influence of sex hormones on body weight is notably sex specific. Males tend to accumulate visceral fat, whereas females display subcutaneous fat accumulation [267]. The android pattern in men is associated with higher risk of developing MetS, while the gynoid pattern in women may offer protection [268-270]. Furthermore, sex differences in metabolism are reflected in variations in

energy expenditure and feeding patterns between male and female mice [271,272]. The distribution of body fat seems to influence cardiometabolic risk more than total adiposity [273-276].

*In murine models*, sex differences in metabolism are partly attributed to differences in physical activity, energy expenditure, and feeding patterns between male and female mice. Importantly, rodents have a higher ratio of brown adipose tissue (BAT) to white adipose tissue (WAT), which significantly impacts their metabolic responses. BAT, which is more metabolically active than WAT, plays a more prominent role in rodents than in adult humans, where it is relatively scarce [277,278]. Therefore, this might cause differences in disease progression and response to metabolic challenges.

### 1.6.3. Sex hormone in glucose homeostasis

There are sex differences in the genetic programs of pancreatic endocrine cells. Yong et al. (2022) combined pancreas perfusion data and single-cell genomic data to explore the sex differences in the human pancreas at the single-cell type and single-cell level. The study found that female endocrine cells have a higher secretion capacity than male endocrine cells. Single-cell RNA-sequencing analysis suggested that endocrine cells in male controls have molecular signatures that resemble type 2 diabetes (T2D). The authors also identified genomic elements associated with genome-wide association study T2D loci that have differential accessibility between female and male delta cells. These genomic elements may play a sex-specific causal role in the pathogenesis of T2D [279].

#### *1.6.3.1. Estrogens*

Estrogens' relationship with insulin resistance and type T2D has been well-investigated in molecular and clinical contexts.

*On a molecular* level, both ERα and ERβ receptors are associated with metabolic protection. ERα knockout mice exhibit peripheral insulin resistance, further highlighting the role of this pathway [280]. ERβ, like ERα, plays a key role in metabolic regulation. Its activation is associated with improved lipid profiles and adipocyte differentiation. ERβ knockout mice have been observed to develop more severe metabolic syndrome under a HFD compared to their wild-type counterparts, suggesting the protective role of ERβ in the metabolic health [281]. Estradiol may reduce hepatic glucose production, which helps to maintain glucose homeostasis. This effect appears to be mediated, at least in part, by suppression of the expression of gluconeogenic enzymes.[282]

*Clinically*, estrogen increases the glucose uptake capacity in premenopausal women [283]. The loss of this increased sensitivity as women age is postulated to be a function of the loss of estrogen's protective action following the onset of menopause [284,285]. In contrast, mice, specifically laboratory strains, may not completely recapitulate this pattern due to differences in fat distribution and hormonal regulation.

The interplay between estrogens and metabolic diseases continues to be an active area of study.

***1.6.3.2. Androgens***

The influence of androgens, specifically testosterone, on glucose regulation is intricate, multi-dimensional, and subject to subtle variations.

*From a molecular perspective*, testosterone amplifies insulin sensitivity in skeletal muscle tissues, primarily by *enhancing the PI3K-AKT signaling cascade*, a pivotal pathway for insulin-mediated actions. Research by Pal et al. (2019) demonstrated that testosterone supplementation ameliorated insulin sensitivity in male mice subjected to HFD, a model mimicking T2D, and potentiated insulin signaling in myocytes. Additionally, testosterone *modulates the p85 gene*, an

integral part of the PI3K complex, thereby improving overall insulin sensitivity—a crucial factor in both the prevention and management of T2D [286]. Intriguingly, testosterone appears to *reduce hepatic glucose production* while simultaneously elevating hepatic insulin resistance, a paradox that may serve as a compensatory mechanism to sustain glucose equilibrium [287].

*Clinically*, hypogonadism, marked by diminished testosterone levels, is frequently correlated with compromised glucose tolerance and increased insulin resistance in men, thereby escalating the risk of T2D [259,288,289] suggesting a protective role of testosterone against MetS.

Testosterone replacement therapy has been shown to mitigate insulin resistance, underscoring its role in glucose metabolism [290,291]. However, the relationship between testosterone and metabolic syndrome is far from straightforward. Some studies indicate that elevated testosterone levels could also exacerbate metabolic syndrome, particularly in women with conditions like polycystic ovary syndrome (PCOS) [292]. Thus, testosterone's effects on insulin sensitivity manifest differently in men and women: while elevated testosterone levels in men may confer protection against diabetes, in women, they are associated with an increased risk.

The interplay between testosterone and glucose regulation is modulated by a numerous factor, including general health, body composition, and hormonal equilibrium. Further investigations are imperative to decode the intricate relationships and their ramifications for metabolic disorders.

#### *1.6.3.3. Progesterone*

Progesterone, well-known for its critical roles in female reproduction, has recently been spotlighted for its broader influence on metabolism, particularly glucose homeostasis.

*At the molecular level*, progesterone acts through the progesterone receptor (PGR), found in metabolic tissues including the liver, adipose tissue, and skeletal muscle. Its binding leads to transcriptional modulation of genes associated with insulin signaling and glucose regulation [293]. This hormone's influence extends to direct stimulation of insulin production within pancreatic islets and hepatic glycogen storage, yet intriguingly, it may inhibit insulin's function in adipose tissues and skeletal muscle [294,295]. The presence of progesterone receptors in rat adipocytes provides further insight into its potential suppression of lipolysis [296]. Contemporary research suggests progesterone's role in elevating blood glucose levels by upregulating genes linked to gluconeogenesis, a mechanism driven by hepatic progesterone receptor membrane component 1 PGRMC1, especially in insulin-resistant states [297,298]. Additionally, progesterone's activation of glycogen phosphorylase contributes to increased blood sugar levels [299].

*From a clinical perspective*, studies involving postmenopausal women have indicated that progesterone may not significantly alter lipid profiles [300,301], suggesting its potential utility in hormone replacement therapy (HRT) because, unlike other progestogens, progesterone does not seem to modify estrogen's impact on blood lipids.

During pregnancy, heightened progesterone levels correlate with gestational diabetes onset [298,302], a relationship evidenced by increased insulin resistance linked to diminished Glut4 expression in skeletal muscles [299].

Variations in progesterone levels across the menstrual cycle, with a peak in the luteal phase, have been correlated with impaired glucose tolerance and decreased insulin sensitivity [303]; however, the clinical relevance of these observations requires further examination

*In murine models*, the absence of PR has been linked to improved glucose tolerance and reduced fasting blood sugar levels [304], attributed to β-cell mass expansion and accelerated β-cell proliferation [299].

In conclusion, testosterone, estradiol, and progesterone play specific and significant roles in the regulation of β cell function, glucose levels, and insulin sensitivity. However, the relationships between these hormones and metabolic processes are complex and influenced by various factors.

## 7. Rationale and objectives

*Rationale*

The increasing prevalence of metabolic syndrome (MetS) and type 2 diabetes (T2D) has underscored the need for a deeper understanding of the underlying mechanisms that contribute to these conditions. A critical aspect of this understanding lies in the exploration of factors that influence β-cell proliferation and regeneration. Adipose tissue, with its multifaceted roles in energy homeostasis, insulin resistance, and secretion of adipokines, has emerged as a central player in metabolic regulation.

Within this context, CCN5, an adipokine, has been identified as a potential growth factor for β-cells. Previous findings from our lab and others have indicated the potential of CCN5 in promoting β-cell proliferation and survival, thereby attenuating the pathogenesis of type 2 diabetes mellitus (T2D). However, the biological endogenouseffects of CCN5 remain to be fully elucidated, warranting further investigation.

*Hypothesis*

CCN5 promotes β-cell proliferation and survival in vivo, thereby attenuating the pathogenesis of T2D. Lack of systemic CCN5 could result in a deficit of β cell mass, leading to increased risk for developing diabetes mellitus and other metabolic changes.

*Objectives*

Characterization of CCN5-KO Mice: To establish and characterize CCN5 knockout (KO) mice, understanding the metabolic phenotype at different ages, sexes, and diets.

The metabolic phenotype at different ages in both sexes in the chow diet

Metabolic responses to HFD-induced obesity (ITT and GTT)

Changes in the percentage of β cell and insulin and glucagon levels in WT and CCN5 KO mice in both sexes in a normal-chow diet (NCD)

Changes in the percentage of β cell, insulin and glucagon levels in WT and CCN5 KO mice in both sexes on a high-fat diet (HFD)

The proposed research aims to shed light on the role of CCN5 in β-cell proliferation and survival, offering a novel perspective on the complex interplay between adipokines, β-cells, and metabolic disorders. By exploring the systemic effects of CCN5 gene deficiency and its impact on metabolic responses, this study could pave the way for innovative therapeutic strategies targeting metabolic syndrome and type 2 diabetes.

# Chapter 2. Materials and Methods

## 2.1. Generation of CCN5/WISP2 knockout mice

CCN5 KO was generated using embryonic stem cells (ES) in which exons 2-5 of the CCN5 gene were replaced by a lacZ cassette (Knockout Mouse Project (KOMP)(Jiang et al., 2017).

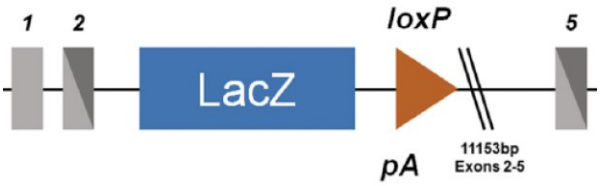


*Figure 5. Schematic of reporter-tagged deletion allele for CCN5 using embrionic stem cells obtained from IMPC.*

LacZ cassette is inserted directly behind ATG starting codon located in Exon2. Adapted from Jiang et al. (2018). [305]

## 2.2. Animals

We used CCN5/WISP 2 knockout, heterozygous and homozygous, donated from the University of California, Los Angeles. The knockout strain was rederived by in vitro fertilization by Mitra Cowan, Jade Desjardins and Nobuko Yamanaka at McGill Integrated Core for Animal Modeling (MICAM). We maintained the colony in animal facilities of the Research Institute of McGill University Health Centre (RIMUHC) and mapped and genotyped them by PCR to confirm their status (wild-type – WT or knockout – KO). All animal procedures were carried out in accordance with the recommendations of the Canadian Council on Animal Care (CCAC), and they have been approved by the McGill University Animal Care Committee.

We feed them either a normal-chow diet (18,6% crude protein and 6.2% fat) or a high-fat diet (60% kcal as fat, Research Diets, Inc.; D12492)

### 2.3. Genotyping

#### 2.3.1. Tail Lysis

Tail samples, approximately 2mm in length, were lysed in a solution containing 100uL of 50nM NaOH. The lysis was performed at 98ºC for a duration ranging from 30 minutes to 1 hour. Following lysis, the samples were neutralized with 75uL of 1M Tris-HCl (pH 7.5).

#### 2.3.2. PCR Mix Preparation

For each sample, the PCR mix was prepared as follows:

- 5uL DreamTaq Green PCR MasterMix (2X)
- 3uL Sterile water
- 0.5uL Primer Master Mix
- 2uL Tail Lysate

#### 2.3.3. Primers

The primers used in the study were as follows:

- Forward: TGGGATGGAGGTCTTTCTTG
- Reverse: CATTCAAGACCAGGATGCCT
- Lac Z R: GTCTGTCCTAGCTTCCTCACTG

The primers were initially designed as two separate sets but were later optimized into a single set. The original wild-type (WT) set included:

- Forward: TTGAGCCAATGCAGTGTTGAG
- Reverse: CCTCTCCAGCACCATGTTC

The original mutant set included:

- Forward: TGGGATGGAGGTCTTTCTTG

- Reverse: GTCTGTCCTAGCTTCCTCACTG

#### *2.3.4. PCR Protocol*

The PCR protocol was carried out as follows:

- Initial denaturation: 95°C for 3 minutes

- Denaturation: 95°C for 30 seconds

- Annealing: 59°C for 30 seconds

- Extension: 72°C for 2 minutes

- Final extension: 72°C for 5 minutes

The denaturation, annealing, and extension steps were repeated for a total of 34 cycles.

This genotyping method allowed for the precise identification and characterization of wild- type, (WT), knockout (KO) and heterogenous (HE) genetic variations within the samples,

The WT band is 1088 base pairs, the KO band is 500 base pairs and the HE mice genotyping contains both the WT band and KO band.

### 2.4. Body composition

The body composition of adult male and female mice was determined by EchoMRI (Echo Medical Systems). This was performed when normal-chow diet mice were 5, 9 and 16 months and at week 12 of high-fat-diet-fed mice.

### 2.5. Blood samples

All blood samples were collected between 9:00 am and 12:00 pm. Blood glucose was measured from the tail tip by the glucose oxidase method using a OneTouch Verio Flex glucometer from Life Scan, Inc. Blood plasma samples were collected by cardiac puncture using 25–27-gauge needles and plasma was separated by centrifugation 5k/10min and stored in aliquots at −20◦C. Insulin levels were measured using Mouse Ultrasensitive Insulin ELISA from ALPCO. Glucagon levels were quantified using a mouse Glucagon ELISA kit from Elabscience.

### 2.6. Insulin tolerance test (ITT)

We performed ITT when normal-chow diet (NCD) mice were 6 months old (24 weeks) and at week 12 of high-fat diet (HFD) group, injecting 0.75 UI/kg Insulin Human Recombinant from SIGMA, intraperitoneally. After that, we measured blood glucose levels at 0, 15, 30, and 60 min after the injection. The degree to which blood glucose concentrations fell following insulin administration, represented the efficiency of whole-body insulin action.

### 2.7. Glucose tolerance test (GTT)

We performed GTT when NCD mice were 6 months old (24 weeks) and at week 14 of HFD group administrating 10 % glucose (1g /kg) intraperitoneally. After that, we measured blood glucose levels at 0, 15, 30, 60, 90, and 120 min after the injection. Subsequent glucose uptake, primarily conducted by muscle, fat tissue, and liver tissue led to a gradual decrease of the blood glucose concentration.

### 2.8. Collection of tissues

After sacrificing mice, we collected the pancreas and saved it in formaldehyde 10% at 4°C for immunohistochemistry.

### 2.8.1. Immunohistochemistry

We fixed pancreatic tissues in 10% formalin and sent them to the core facility where they sectioned them at a thickness of 5 μm. Later we stained either with hematoxylin-eosin (H&E) for routine histopathology evaluation or with 4590S anti-rabbit antibody from Cell Signaling Technology for determining the percentage of islet β cells in the pancreas. The immunohistology samples were prepared on the histological platform of RIMUHC.

### 2.8.2. Imnunofluorescence

We used Insulin 4590S antirabbit antibody from cell signalling technology and insulin combined with Ki 67 antibody for determination of β cell replicative rate. The combination of these antibodies provided insights into both insulin expression and proliferative activity within the β cells. This intricate analysis was performed on the Invivoex platform.
(http://www.invivoex.com/)

### 2.9. Data analyses

Multi-analyses of variance (MANOVA) by using PROC GLM (general linear model) were used to determine between-group differences and within-group changes over time. Additionally, least-squares means post hoc for multiple comparisons of means (LSMEANS statement with Bonferroni correction) was applied. All statistical analyses were performed using Statistical Analysis System software, version 9.4 (SAS Institute, Cary, NC), with P values $<0.05$ considered significant.

***Nikon Elements D*** software was used to determine β-cell percentage in the pancreas.

***GraphPad Prism*** version 5.0 was used to visually represent the experimental results.

# Chapter 3. Results

## 3.1. Genotyping Results.

The genotyping of the mice was performed to confirm the presence of the WT, KO, and heterozygous (HE) genotypes within the study population. The PCR amplification of specific genomic regions allowed us to distinguish between these genotypes.

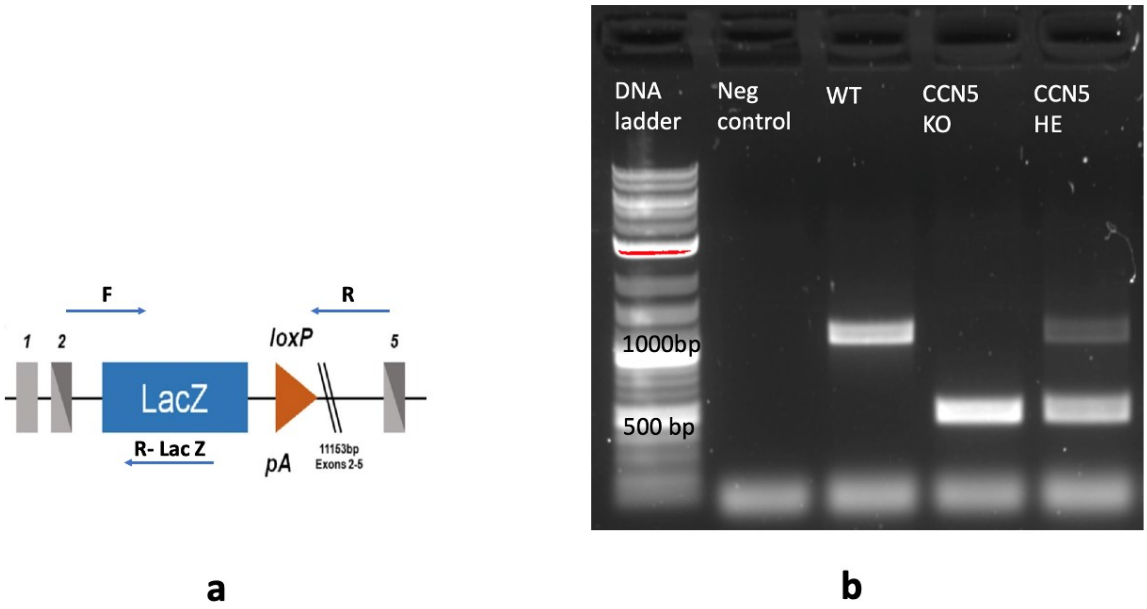


*Figure 6 .Location of three primers (a) and PCR Genotyping results of CCN5 gene(b)*

*The gel image shows the PCR products for wild-type (WT), knockout (KO), and heterozygous (HE) genotypes. WT shows a single band at 1088 bp, KO shows a single band at 500 bp, and heterozygous shows two bands at 500 bp and 1088 bp. The DNA ladder is included for size reference, and the negative control confirms the specificity of the amplification.*

The lanes are as follows:
-DNA Ladder: A molecular weight marker used to estimate the size of the PCR products.
-Neg Control: No bands are observed, confirming the absence of contamination and the specificity of the PCR reaction
- WT (1088 bp): A single band is observed at 1088 base pairs, representing the wild-type allele.
- KO (500 bp): A single band is observed at 500 base pairs, representing the knockout allele.

- HE (500 bp and 1088 bp): Two bands are observed in the heterozygous sample, one at 500 base pairs (representing the knockout allele) and the other at 1000 base pairs (representing the wild-type allele).

These banding patterns confirm the presence of the respective genotypes and provide insights into the genetic variations within the samples. The DNA ladder and negative control serve as essential references for the analysis.

### 3.2. CCN5 knockout mice both in normal-chow diet and high-fat diet are not significantly obese.

CCN5 KO mice of both NCD and HFD were viable, fertile, and showed no observable abnormalities.

**3.2.1. Normal-chow diet knockout mice show similar weight with their wild-type counterparts** demonstrated by the results of EcoMRI. At the age of 5 months, both male and female KO mice demonstrated a slight increase in average body weight compared to the WT mice, although the difference was not statistically significant. As the mice aged, et the age of 9 months, KO females on average weighed 3.57g more than WT females. However, by 16 months, KO and WT female body weights were nearly identical. For males, KO mice maintained slightly higher body weight than WT mice at 16 months, but this was not statistically significant.

In terms of fat mass percentage, 5-month-old KO female mice showed a slightly higher percentage than WT mice.2 As the mice aged, the difference in fat mass percentage between WT and KO groups increased. By 9 months, KO females had a fat mass percentage approximately 7% higher than WT females. By 16 months, the difference in fat mass percentage between KO and WT females had reduced to less than 1%. For males, the difference in fat mass percentage between KO and WT at 16 months was higher than the females. Across all different ages, fat mass percentage of CCN5 KO was not significantly different from WT mice.

We didn't see any difference between WT and CCN5 KO mice in lean mass percentages of all ages, both in males and females. These results are represented in Figure 7 and Table 4.

### 3.2.2. High-fat diet-fed CCN5 knockout mice show similar weight to their wild-type counterparts.

Upon 27 weeks of a high-fat diet, all mice exhibited weight gain: female KO mice showed a 2.7-fold increase in weight while their WT counterparts showed a 2.4-fold increase. Male KO mice showed a 2-fold increase, and their WT counterparts showed a 1.9-fold increase but there was no significant change between WT and the mice lacking CCN5 gene (Figure 9).

EcoMRI analysis conducted at week 12 of a HFD confirmed the lack of significant differences in total weight between WT and KO mice. It also showed that there is no difference between KO and WT mice regarding the percentage of fat mass and lean mass. The HFD did not appear to significantly exaggerate the differences between KO and WT mice compared to the normal diet (Figure 8 and Table 5).

These results indicate that the absence of the CCN5 gene does not have a significant impact on obesity in mice fed either NCD or a HFD, or that the KO genotype might influence these parameters, but it is not the only determinant.

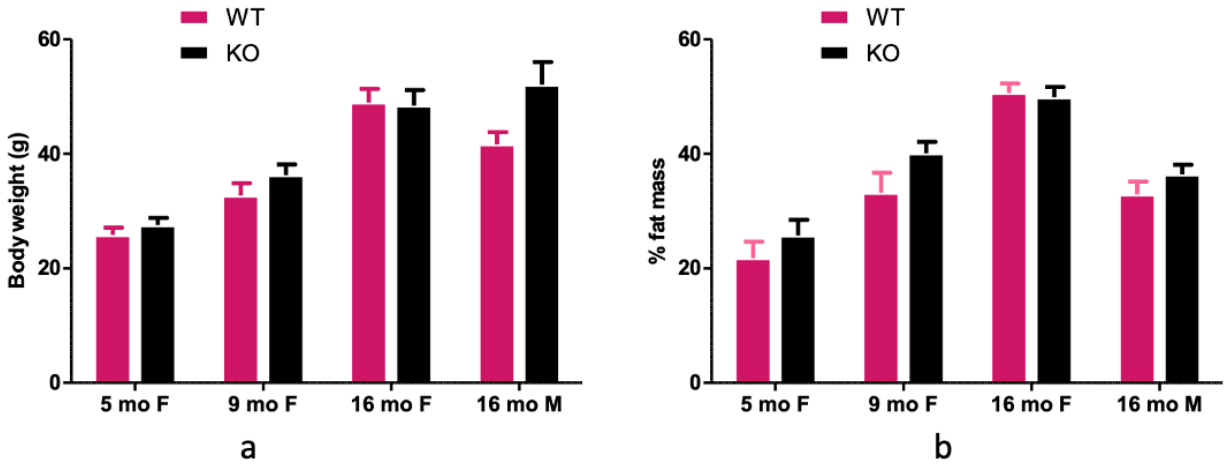


*Figure 7. Age-related changes in body composition parameters in wild-type and knockout mice on a normal-chow diet (M-male, F- female)*

*The graphs illustrate the trends in (a) body weight (g), and (b) fat mass percentage (%) for WT and KO mice, for females at 5 months, 9 months and 16 months and males at 16 months.*

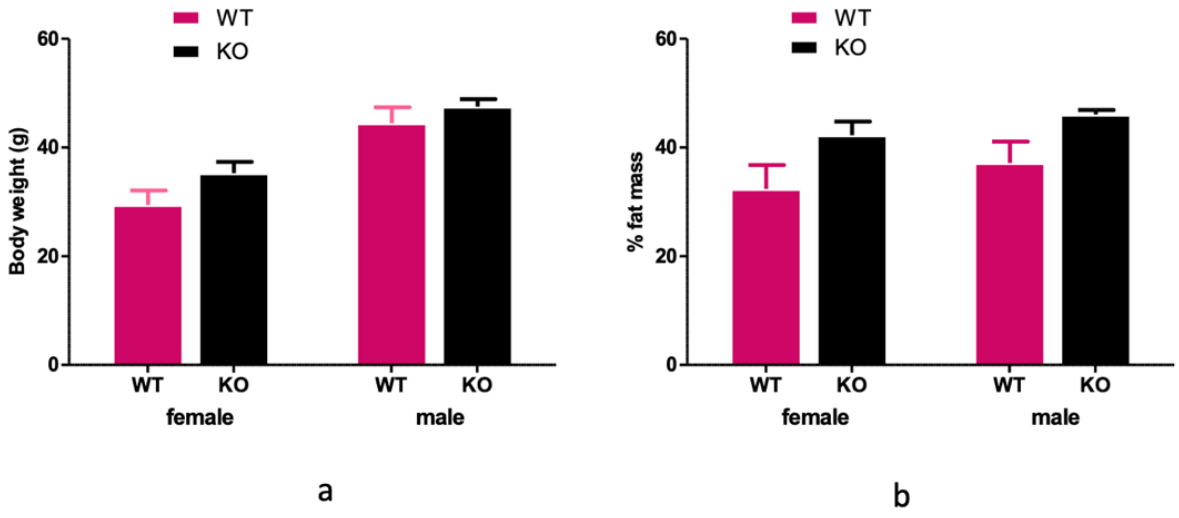


*Figure 8. Body composition parameters measured by EchoMRI in 6 month wild-type and knockout mice on a high-fat diet.*

*The graphs display the differences in **(a)** body weight, and **(b)** fat mass percentage (%), between WT and KO, both male and female*

*Table 4. Age-related changes in body composition parameters in wild-type and knockout mice on a normal-chow diet*

| | Age & Sex | WT (Mean±SE(n)) | KO (Mean±SE(n)) | P-value |
|---|---|---|---|---|
| Body Weight (g) | | | | |
| | 5 mo F | 25.82±1.26(8) | 27.59±1.24(9) | NS |
| | 9 mo F | 32.71±2.15(8) | 36.28±1.88(8) | NS |
| | 16 mo F | 49.00±2.34(5) | 48.46±2.68(5) | NS |
| | 16 mo M | 41.67±2.10(4) | 52.1±2.10(3) | NS |
| Fat Mass % | | | | |
| | 5 mo F | 21.79±2.86(8) | 25.73±2.73(9) | NS |
| | 9 mo F | 33.20±3.48(8) | 40.10±1.98(8) | NS |
| | 16 mo F | 50.63±1.67(5) | 49.91±1.75(5) | NS |
| | 16 mo M | 32.92±2.26(4) | 36.39±1.73(3) | NS |
| Lean Mass % | | | | |
| | 5 mo F | 74.22±2.65(8) | 70.52±2.38(9) | NS |
| | 9 mo F | 63.69±3.05(8) | 57.32±1.80(9) | NS |
| | 16 mo F | 47.63±1.59(5) | 49.56±1.73(5) | NS |
| | 16 mo M | 66.44±2.43(4) | 59.65±1.62(3) | NS |

*The data presents body weight (g), fat mass percentage (%), and lean mass percentage (%) for WT and KO mice, both male and female, at 5, 9, and 16 months of age. Values are presented as mean ± SEM..P values represent the statistical significance between the WT and KO groups for each sex and were calculated using SD.*

*Table 5. Body composition parameters in wild-type and knockout mice on a high-fat diet in 6-month age mice*

| | WT Female (n=7) | KO Female (n=9) | WT Male (n=6) | KO Male (n=7) | P-value |
|---|---|---|---|---|---|
| Body Weight (Mean±SE) | 29.44±1.26 | 35.29±2.08 | 44.44±2.96 | 47.54±1.33 | NS |
| Fat Mass % (Mean±SE) | 32.40±4.36 | 42.25±2.55 | 37.14±3.95 | 46.09±0.82 | NS |
| Lean Mass% (Mean±SE) | 63.58±3.86 | 55.24±2.31 | 61.21±3.64 | 52.65±0.70 | NS |

*The data presents body weight (g), fat mass percentage (%), and lean mass percentage (%) for WT and KO mice, both male and female. Values are presented as mean ± SEM. P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD.*

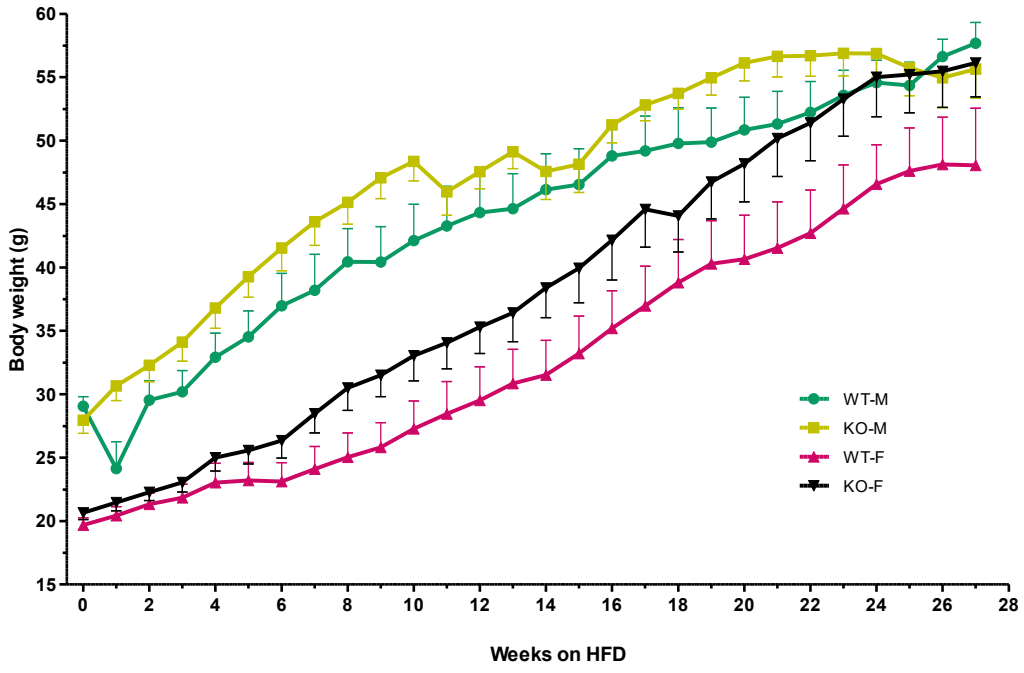


*Figure 9. Changes in body weight for both male and female* wild-type *and CCN5* knockout *mice, monitored over 27 weeks on high-fat-diet. The measurements are presented in grams and include the standard error of the mean (SEM).*

*Table 6. The overall increase in body weight after 27 weeks of high-fat diet*

| | WT | N | KO | N |
|---|---|---|---|---|
| Male 0 wk | 29.1 ± 1.8 | 6 | 27.9 ± 2.7 | 7 |
| Male 27 wk | 54.4 ± 4.5 | | 55.8 ± 5.8 | |
| Fold change | 1.87 | | 2.00 | |
| Female 0 wk | 19.7 ± 1.5 | 7 | 20.6 ± 1.6 | 9 |
| Female 27 wk | 48.1 ± 11.9 | | 56.1 ± 8.1 | |
| Fold change | 2.44 | | 2.72 | |

### 3.3. Lack of diabetes mellitus onset in wild-type and CCN5 knockout mice during 27 weeks on a high-fat diet.

During 27 weeks of HFD, we measured blood glucose levels every week. The glucose stayed steady at around 10-12 mmol/l in the male mice and in the first half, which was followed by a slight elevation to ~13 mmol/l during the 2$^{nd}$ half. Male CCN5 KO mice showed no more elevation vs. WT controls, if anything, a slight *decrease* in blood glucose level towards the last 5 weeks. Female mice had larger variations in the first half which was stabilized at ~9 mmol/l during the 2$^{nd}$ half. In female KO mice, no significant differences were observed when compared to WT counterparts, except the last week (Figure 10). While the Figure 9 indicated an increase in

female body weight, it did not correlate with the onset of diabetes in these females.

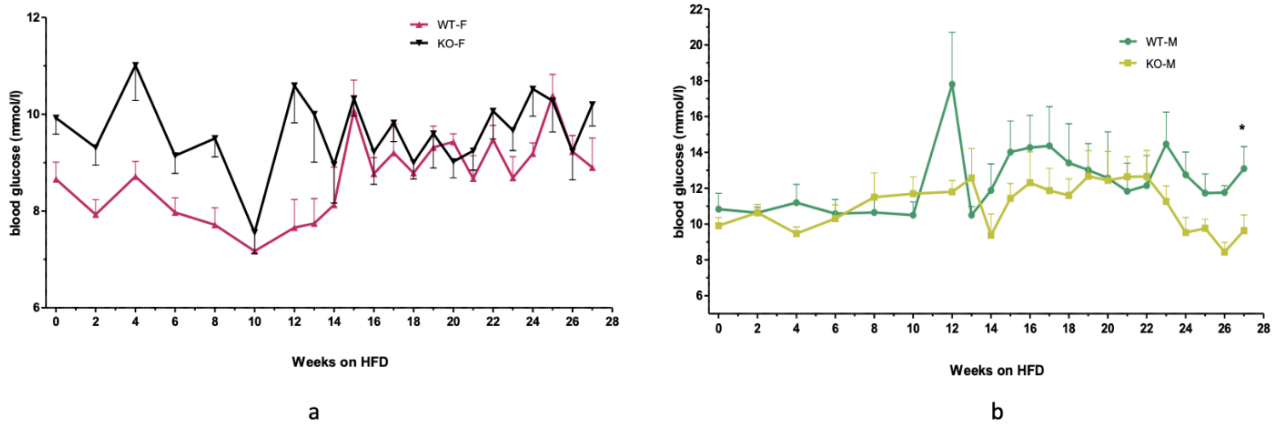


*Figure 10. Weekly blood glucose levels during 27 weeks of high-fat diet in female (a) and male (b) mice*

*The graph illustrates the initial variations in female mice stabilizing at ~9 mmol/l and the consistent glucose levels in male mice (10-12 mmol/l) with a slight elevation in the second half. Male CCN5 KO mice showed a slight decrease in blood glucose levels in the last 5 weeks, while no significant difference was observed in female KO mice compared to WT controls. P values represent the statistical significance between the WT and KO groups for each sex and were calculated using SD.*

### 3.4. CCN5 gene deficiency improved insulin sensitivity and glucose tolerance in diet-induced obesity, especially in male mice

#### 3.4.1. Normal insulin sensitivity in normal-chow diet.

Insulin tolerance test (ITT) was performed on 6-month-old mice in a NCD and in the 12th week of the HFD group and a glucose tolerance test (GTT) was conducted 1-2 weeks after ITT in all groups. The ITT measures how quickly glucose is cleared from the bloodstream after the administration of insulin, which is a key indicator of insulin sensitivity. The GTT is a measure of how effectively the body's cells can utilize glucose after it enters the bloodstream. No difference was observed in ITT and GTT in NCD-fed mice.

In the ITT conducted on both male and female mice, when they were at the age of 6 months, the baseline glucose levels were similar between the WT and KO groups. Following insulin administration, female KO mice exhibited slightly higher glucose levels at each measured time point (15, 30, and 60 minutes) compared to their WT counterparts. In male group the levels of glucose were higher in KO mice only in min 60. However, the results in both males and females were not statistically significant. (Figure 11 and Table 7).

The GTT shows sexual dimorphic changes in a normal-chow diet, but only in min 60 and 120, these changes were statistically significant. The peak of the blood glucose levels of 18-19 mmol/l was reached approximately 15 min after glucose administration, for both males and females, without significant differentiation between WT and KO. However, distinct patterns emerged at later time points. At the 60-minute mark, KO females exhibited a significant decrease in glucose levels compared to their WT counterparts (P=0.034). Conversely, KO males showed a significant elevation in glucose levels at the 120-minute time point compared to WT males (P=0.0083). Following these divergent responses, glucose levels in all groups trended back toward baseline levels by the 120-minute mark, suggesting effective glucose clearance (Figure 12 and Table 8). We also tested areas under the curve (AUC) and did not see any statistical significance between WT and KO mice in both ITT and GGT in NCD.

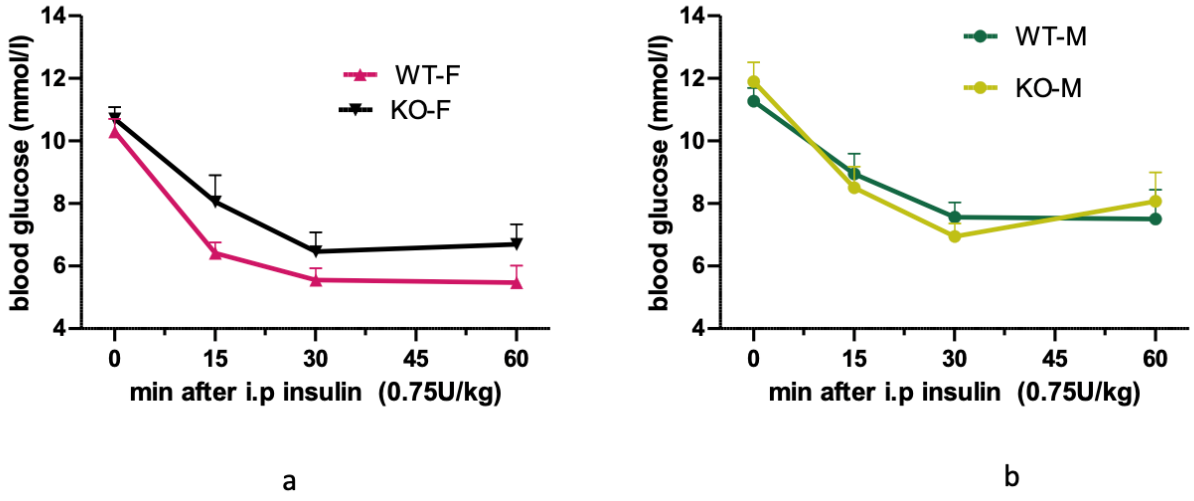


*Figure 11. Insulin tolerance test performed in 6-month-old mice female (a) and male (b) fed with normal-chow diet.*

*This graph illustrates the response of blood glucose levels to an intraperitoneal injection of insulin (0.75 UI/kg) in both wild-type and CCN5-KO mice. Blood glucose levels were measured at 0, 15, 30, and 60 minutes after the injection. The decline in blood glucose concentrations over time represents the efficiency of whole-body insulin action, providing insights into the insulin sensitivity of the mice. There is no difference between WT and KO mice.*

*Table 7. Insulin Tolerance Test results in 6-month-old mice fed with normal-chow diet.*

| ITT in 6 mo. mice | WT- F (7) Mean ±SE (mmol/l) | KO-F (10) Mean ±SE (mmol/l) | P | WT-M (8) Mean ±SE (mmol/l) | KO-M (4) Mean ±SE (mmol/l) | P |
|---|---|---|---|---|---|---|
| 0 min | 10.30±0.40 | 10.70±0.37 | NS | 11.27±0.41 | 11.90±0.61 | NS |
| 15 min | 6.41±0.33 | 8.05±0.85 | NS | 8.95±0.64 | 8.50±0.67 | NS |
| 30 min | 5.55±0.36 | 6.46±0.62 | NS | 7.56±0.47 | 6.95±0.41 | NS |

*Note for figure 12 and table 9:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SEM.*

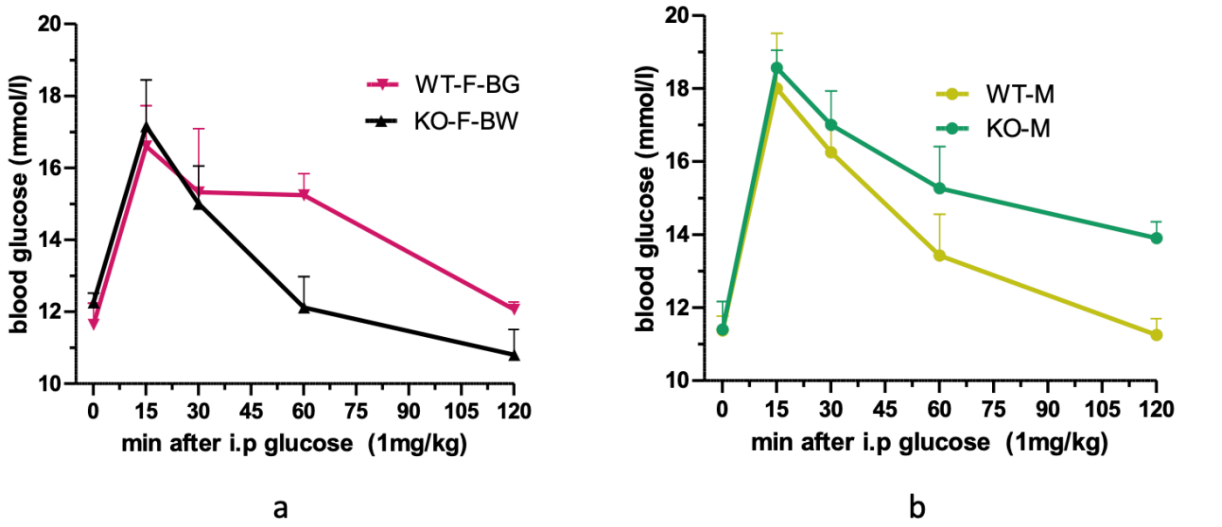


*Figure 12. Glucose tolerance test in 6-month-old mice females (a) and males (b) fed with normal-chow diet.*

*This graph illustrates the response of blood glucose levels to an intraperitoneal injection of glucose in the study subjects. Blood glucose levels were measured at specific time intervals (0, 15, 30, 60, and 120 minutes) after the injection. The curve represents the body's ability to clear glucose from the blood over time.*

*Table 8. Glucose Tolerance Test results in 6- month- old normal-chow diet control mice*

| GTT in 25 wk. mice NCD | WT- F (5) Mean ±SE (mmol/l) | KO-F (5) Mean ±SE (mmol/l) | P | WT-M (4) Mean ±SE (mmol/l) | KO-M (3) Mean ±SE (mmol/l) | P |
|---|---|---|---|---|---|---|
| 0 min | 11.64±0.59 | 12.28±0.25 | NS | 11.37±0.39 | 11.40±0.76 | NS |
| 15 min | 16.60±1.12 | 17.16±1.28 | NS | 18.00±1.50 | 18.56±0.48 | NS |
| 30 min | 15.32±1.76 | 15±1.05 | NS | 16.25±0.78 | 17.00±0.92 | NS |
| 60 min | 15.24±0.59 | 12.12±0.85 | 0.034 | 13.42±1.13 | 15.26±1.14 | NS |
| 120 min | 12.06±0.21 | 10.8±0.71 | NS | 11.25±0.44 | 13.90±0.45 | 0.0083 |

*Note for figure 12 and table 8:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SE.*

### 3.4.2. Increased insulin sensitivity and improved glucose tolerance in HFD-fed mice

Between the 11$^{th}$ and 12th week of the HFD experiment, we conducted ITT on our mice. Before we administered insulin (0 min), the female CCN5 KO mice had an average blood glucose level of 11.33 ± 0.78, which was slightly lower than the female WT, who had an average level of 12.25 ± 1.38, but the differences didn't reach significance. Among the males, again, the CCN5 KO mice had a lower average blood glucose level (10.90 ± 0.44) compared to the WT (14.51 ± 1.16), but these differences weren't statistically significant. Following insulin administration, both female and male KO mice demonstrated reduced average blood glucose levels compared to the WT mice. However, the observed difference was statistically significant only in male mice at 15, 30, and 60 minutes, with p-values of p=0.0353, p=0.0185, and p=0.0184, respectively. Consistent with these findings, the area under the curve (AUC) analysis further corroborated these results.

This suggests that, under HFD, the KO mice may have better insulin sensitivity than the WT. The details are presented in Figure 13 and Table 9.

The glucose tolerance test performed at week 14 of high-fat diet in male and female mice shows again sexual dimorphic changes but the male CCN5 KO group was significantly more glucose tolerant than its WT counterpart only in min 120 (p=0.03). (Figure 14 and Table 10)

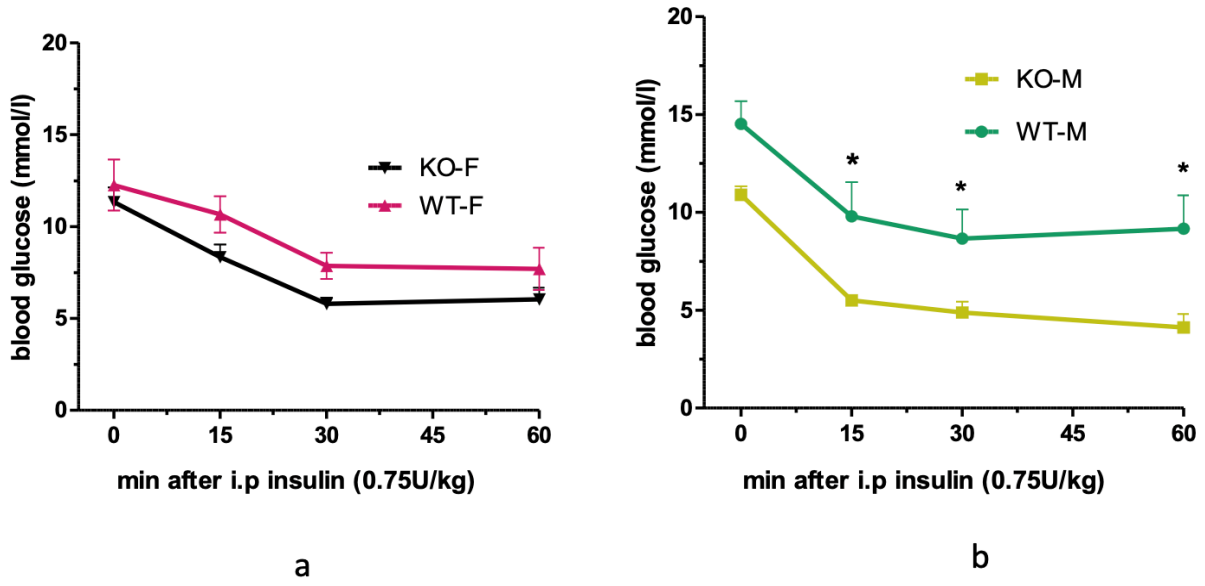


*Figure 13. Insulin tolerance test performed in weeks 11-12 of high-fat diet in 6-month-old mice female (a) and male (b).*

*This graph illustrates the response of blood glucose levels to an intraperitoneal injection of insulin (0.75 UI/kg) in both wild-type and CCN5-KO mice. Blood glucose levels were measured at 0, 15, 30, and 60 minutes after the injection. The decline in blood glucose concentrations over time represents the efficiency of whole-body insulin action, providing insights into the insulin sensitivity of the mice. Male KO mice are more insulin sensitive in all time stamps.*

*Table 9. Insulin Tolerance Test results in weeks 11-12 of high-fat diet fed mice (6 months old)*

| ITT in week 11-12 of HFD | WT- F (7) Mean ±SE (mmol/l) | KO-F (9) Mean ±SE (mmol/l) | P | WT-M (6) Mean ±SE (mmol/l) | KO-M (7) Mean ±SE (mmol/l) | P |
|---|---|---|---|---|---|---|
| 0 min | 12.25±1.38 | 11.33±0.78 | NS | 14.51±1.16 | 10.90±0.44 | NS |
| 15 min | 10.65±0.98 | 8.33±0.68 | NS | 9.80±1.74 | 5.50±0.23 | 0.035 |
| 30 min | 7.85±0.71 | 5.80±0.33 | NS | 8.66±1.48 | 4.88±0.54 | 0.019 |
| 60 min | 7.70±1.14 | 6.03±0.63 | NS | 9.16±1.71 | 4.11±0.68 | 0.018 |

*Note for figure 14 and table 10:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SEM.*

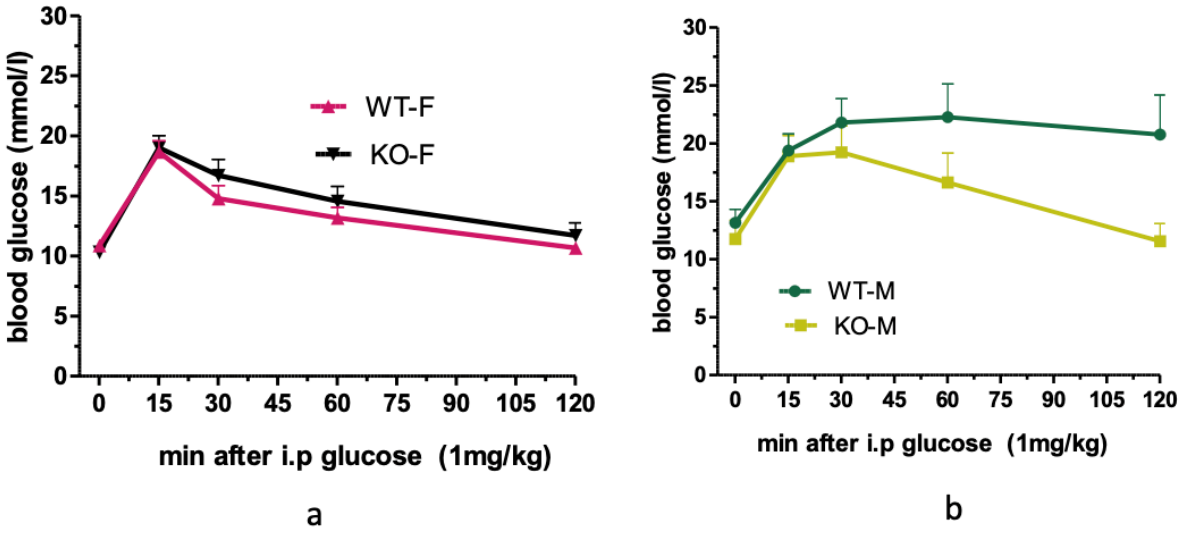


*Figure 14. Glucose tolerance test performed in week 14 in 6.5-month-old mice females (a) and males (b) fed with a high-fat diet.*

*This graph illustrates the response of blood glucose levels to an intraperitoneal injection of glucose in the study subjects. Blood glucose levels were measured at specific time intervals (0, 15, 30, 60, and 120 minutes) after the injection. The curve represents the body's ability to clear glucose from the blood over time. The KO male mice are more glucose tolerant than WT in min 120.*

*Table 10. Glucose Tolerance Test results in week 14 of high-fat diet fed mice (6.5 months old).*

| GTT in week 14 HFD | WT- F (7) Mean ±SE (mmol/l) | KO-F (9) Mean ±SE (mmol/l) | P | WT-M (4) Mean ±SE (mmol/l) | KO-M (7) Mean ±SE (mmol/l) | P |
|---|---|---|---|---|---|---|
| 0 min | 10.87±0.45 | 10.32±0.35 | NS | 13.15±1.16 | 11.74±1.62 | NS |
| 15 min | 18.65±0.93 | 19.00±1.01 | NS | 19.38±1.43 | 18.88±1.76 | NS |
| 30 min | 14.78±1.08 | 16.72±1.32 | NS | 21.8±2.06 | 19.22±2.28 | NS |
| 60 min | 13.17±0.87 | 14.54±1.24 | NS | 22.26±2.86 | 16.62±2.54 | NS |
| 120 min | 10.67±0.42 | 11.7±1.05 | NS | 20.76±3.41 | 11.55±1.52 | 0.03 |

*Note for figure 15 and table 7:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SEM.*

### 3.4.3. Differential insulin and glucagon responses in knockout and wild-type mice under normal-chow and high-fat diets

We explored how diet and the lack of the CCN5 gene influenced key metabolic hormones insulin and glucagon, in our test mice. Our comparison groups were KO and WT mice, and we studied them under both a NCD and an HFD.

**Insulin Responses**

Under the NCD, insulin levels in the KO and WT mice were similar, with no significant variations observed between the groups. The transition to the HFD, however, revealed distinct differences. First HFD caused an increase of insulin levels in both WT and CCN5 KO mice. Secondly, we noted that both female and male KO mice had lower insulin levels compared to their WT counterparts (Figure 15 and Table 11). This was particularly interesting considering the previous ITT results, where male KO mice exhibited increased insulin sensitivity. Furthermore, as is shown later in Figure 17, it is consistent with the decreased β-cell mass.

**Glucagon Responses**

When assessing glucagon levels, we didn't observe any significant variations between the KO and WT groups, irrespective of diet or sex (Figure 16 and Table 12). This implies that glucagon levels were not significantly affected by genetic modifications or dietary changes.

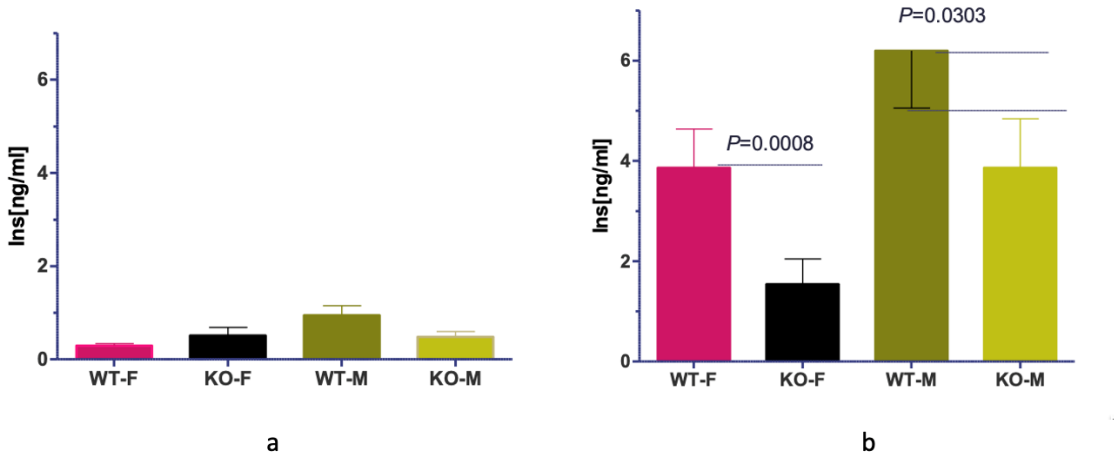


*Figure 15. Comparative insulin levels in 9 months old WT and KO mice under normal-chow diet (NCD) panel (a) and high-fat diet (HFD) panel (b).*

*Notably, a significant difference in insulin levels is observed between WT and KO mice under HFD, highlighting the potential impact of CCN5 gene deficiency on insulin regulation in response to a high-fat dietary condition.*

*Table 11. Insulin levels in normal-chow diet and high-fat diet of 9-month age mice*

| Insulin | WT-F | KO-F | P | WT-M | KO- M | P |
|---|---|---|---|---|---|---|
| NCD | Mean ±SE(n)<br>0.29±0.047(7) | Mean ±SE(n)<br>0.5±0.17 (8) | NS | Mean ±SE(n)<br>0.94 ±0.2 (5) | Mean ±SE(n)<br>0.48 ±0.11 (7) | NS |
| HFD | Mean ±SE(n)<br>3.86 ± 0.23 (7) | Mean ±SE(n)<br>1.72 ± 0.20 (9) | 0.0008 | Mean ±SE(n)<br>6.45 ± 0.39 (5) | Mean ±SE(n)<br>3.71 ± 0.21 (7) | 0.03 |

*Table 12. Glucagon levels in normal-chow diet and high-fat diet of 9-month age mice*

| Glucagon | WT-F | KO-F | P | WT-M | KO-M | P |
|---|---|---|---|---|---|---|
| NCD | Mean ±SE(n)<br>16.35± 0.597(7) | Mean ±SE(n)<br>15.8 ± 0.39 (6) | NS | Mean ±SE(n)<br>15.97± 0.42(7) | Mean ±SE(n)<br>15.0 ± 0.44(7) | NS |
| HFD | Mean ±SE(n)<br>14.96± 0.5(7) | Mean ±SE(n)<br>13.37± 0.58 (8) | NS | Mean ±SE(n)<br>14.2 ± 0.64 (5) | Mean ±SE(n)<br>12.36 ± 1.94 (7) | NS |

*Note for figure 16 and tables 8 & 9:*

*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*

*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*

*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SEM.*

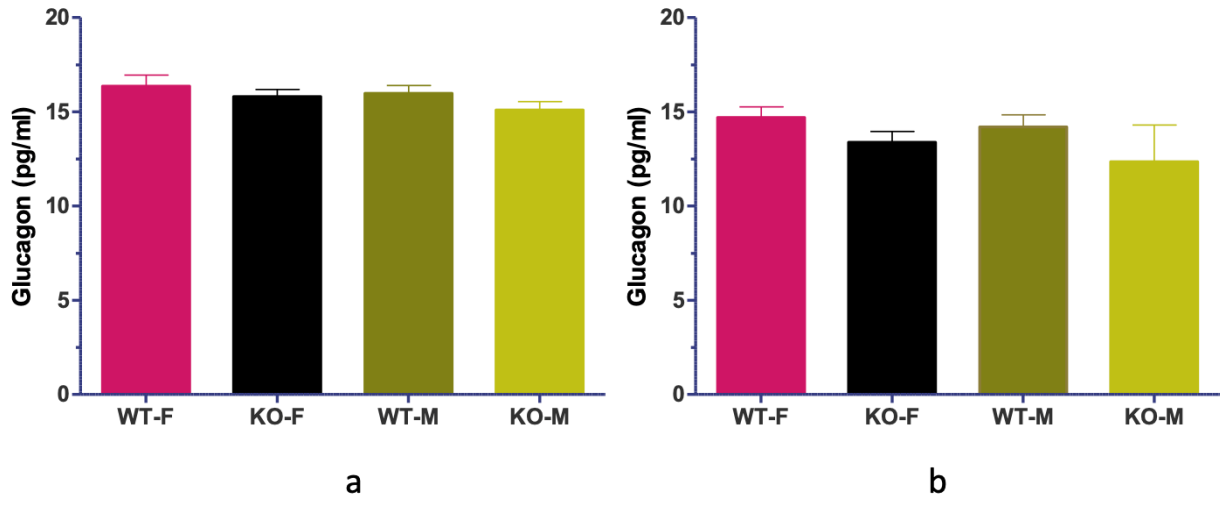


*Figure 16. Comparative glucagon levels in 9 months old WT and KO mice under normal-chow diet (a) and high-fat diet (b)*

*No significant variations between the KO and WT groups, irrespective of diet or sex*
*Note:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the wild-type and knockout groups for each sex and were calculated using SD. The table and corresponding graph are represented with SE.*

### 3.5. The Impact of CCN5 deficiency on β cell percentage and proliferation.

#### 3.5.1. Significant reduction in both male and female knockout mice

We have discovered CCN5 as a target of IGF-1 overexpression in pancreatic β-cells; both in vitro overexpression of CCN5 cDNA and treatment using recombinant CCN5 protein stimulated β-cell proliferation [239,254,306]. In the pursuit of understanding the potential deficiencies in normal β-cell growth associated with a general knockout, a comprehensive examination was conducted on a cohort of 9-month-old mice. The selected approach involved the deployment of immunohistochemistry (IHC) targeting insulin, a critical player in β-cell functionality. The findings corroborated the initial hypothesis, revealing a noticeable reduction in the percentage of

β-cell area in CCN5 KO mice across both sexes. Figure 17 showcases the insulin staining process, distinctly differentiating between WT and CCN5 KO male mice. Further, Figure 18 and Table 13 provide a visual and tabulated representation of the data, respectively, calculated using Nikon Elements software. This concerted analysis underscores the significant influence of CCN5 on β-cell growth and offers valuable insights into the intricacies of β-cell development and function.

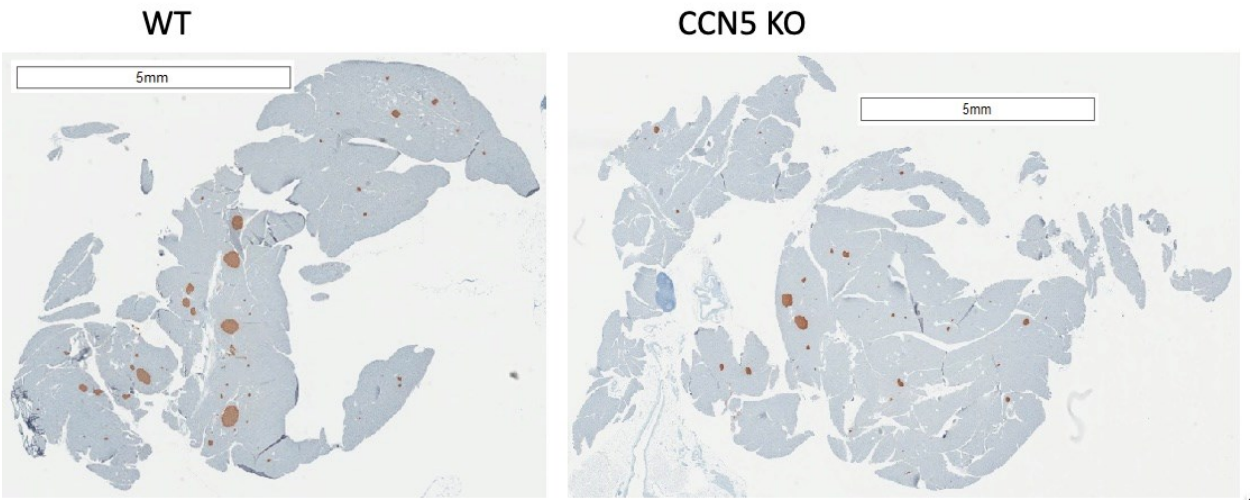


*Figure 17. Insulin staining of β-cells in 9-month-old male wild-type and CCN5 knockout mice in normal-chow diet. Brown stain indicate insulin.*

The image illustrates the staining using 4590S anti-rabbit antibody from Cell Signaling Technology. A visual comparison reveals a significant decrease in β cell percentage in CCN5 KO male mice as compared to WT. The staining technique provides a detailed view of the cellular structure, emphasizing the impact of CCN5 deletion.

In an insightful exploration of β cell percentages among **female mice**, WT group (n=7) exhibited an average percentage of 1.067 ± 0.19. In contrast, the CCN5 KO female group (n=8) revealed a lesser average of 0.6125 ± 0.19. A p-value of 0.037 confirmed the statistical significance of this

difference, inviting further investigation into the role of CCN5 in female mice (Figure 18 and Table 13).

**The male cohort** presented even more compelling findings. WT males (n=7), with a β cell percentage of 3.4 ± 0.57, stood in stark contrast to the CCN5 KO males (n=6), who exhibited a markedly reduced percentage of 0.56 ± 0.09. This difference, highly significant with a p-value of 0.0002, emphasizes the profound impact of CCN5's absence in male mice, offering a striking demonstration of sex-specific variations in β-cell function (Figure 18 and Table 13).

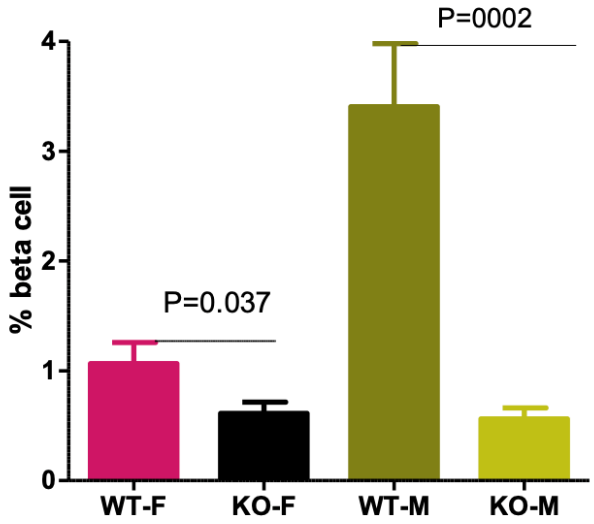


*Figure 18. Comparison of β-cell percentage in male and female wild-type and CCN5 knockout mice at 9-months of age on a normal-chow diet.*

*The graph represents the quantitative analysis of insulin staining using 4590S anti-rabbit antibody. Both male and female CCN5 KO mice exhibited a significant decrease in β cell percentage compared to WT, with p-values of 0.0002 for males and 0.038 for females. The data underscores the marked effect of CCN5 deletion on β cell proliferation, with clear gender-specific variations.*

*Notes:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*- P values represent the statistical significance between the WT and KO groups for each sex and were calculated using SD. The table and corresponding graph are represented with SEM.*

*Table 13. Comparison of β cell percentages in wild-type and CCN5 knockout mice, analyzed separately for males and females.*

| Genotype | B cell % (mean ±SE) | P value | Sample size |
|---|---|---|---|
| WT-F | 1.067 ± 0.19 | 0.037 | 7 |
| KO-F | 0.6125± 0.19 | | 8 |
| WT-M | 3.4± 0.57 | 0.0002 | 7 |
| KO-M | 0.56± 0.09 | | 8 |

*Notes:*
*- WT-F and KO-F represent the female wild-type and knockout groups, respectively.*
*- WT-M and KO-M represent the male wild-type and knockout groups, respectively.*
*The mean percentage of β cells is presented along with the standard error of the mean (SE). P-values were calculated according to standard deviation (SD), while the table and graph representations were made with the standard error of the mean (SEM).*

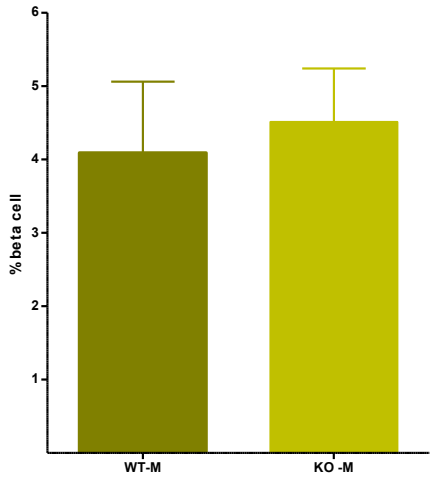


*Figure 19. Comparison of β-cell percentage in male wild-type and CCN5 knockout mice on high-fat diet.*

*The graph represents the quantitative analysis of insulin staining using 4590S anti-rabbit antibody. When examining male CCN5 KO mice on an HFD, no significant changes in β cell percentage were observed compared to their WT counterparts.*

*Table 14. Comparison of β cell percentages in wild-type and CCN5 knockout male mice, fed with HFD.*

| Genotype | B cell % (mean ±SE) | P value | Sample size |
|---|---|---|---|
| WT-M | 4.09 ± 0.97 (5) | 0.745 | 5 |
| KO- M | 4.51 ± 0.78 (5) | | 5 |

*The mean percentage of β cells is presented along with the standard error of the mean (SE). P-values were calculated according to standard deviation (SD), while the table and graph representations were made with the standard error of the mean (SEM).*

### 3.5.2. Examination of β-cell islet structure in response to diet-induced obesity: A comparative analysis of male wild-type and CCN5 knockout mice after 27 weeks on high-fat diet

As CCN5 is required for normal β-cell growth in the chow diet (Figure 17), we assessed and compared its potential role in compensatory β-cell compensation in response to diet-induced obesity. We performed pancreas immunohistochemistry on male WT and CCN5 KO mice after 27 weeks on HFD.

In a meticulous examination of β cell islets, the use of higher magnification (scale bars ranging from 2 or 3 mm to 200 nm) unveiled remarkable details within the cellular architecture. The observations included a unique phenomenon where four substantial islets appeared to merge (Figure 20). Within this merged complex, three of the islets displayed large areas where the β-cells had been conspicuously destroyed and depleted, as indicated by asterisks (*). These areas, once occupied by functioning β-cells, were now filled with structures that were either empty or deceased. These remnants exhibited distinctive red staining when subjected to hematoxylin-eosin staining (data not shown), reflecting the cellular devastation within these regions.

Furthermore, other parts of the islets revealed clear indications of fibrosis, as demarcated by red arrows. The presence of fibrosis within the islets is a sign of tissue scarring and may represent a

chronic response to the underlying destruction and depletion of β-cells. This scarring process often results in the formation of non-functional, fibrous tissue that replaces the normal insulin-producing β-cells (Figure 20).

Remarkably, the examination did not reveal any distinct differences between the WT and CCN5 KO, even after 27 weeks on HFD. Both groups displayed similar patterns of β-cell destruction, depletion, and fibrosis.

These findings present a vivid illustration of the profound alterations within the islet structure. The combined evidence of β-cell destruction, depletion, and subsequent fibrosis paints a compelling picture of the dynamic and sometimes deleterious changes that can occur within the pancreatic islets. These phenomena raise questions regarding the underlying mechanisms responsible for these changes and open avenues for further exploration. Understanding the factors contributing to β-cell destruction and fibrosis could have significant implications for diabetes research and treatment, particularly in the context of dietary interventions such as HFD.

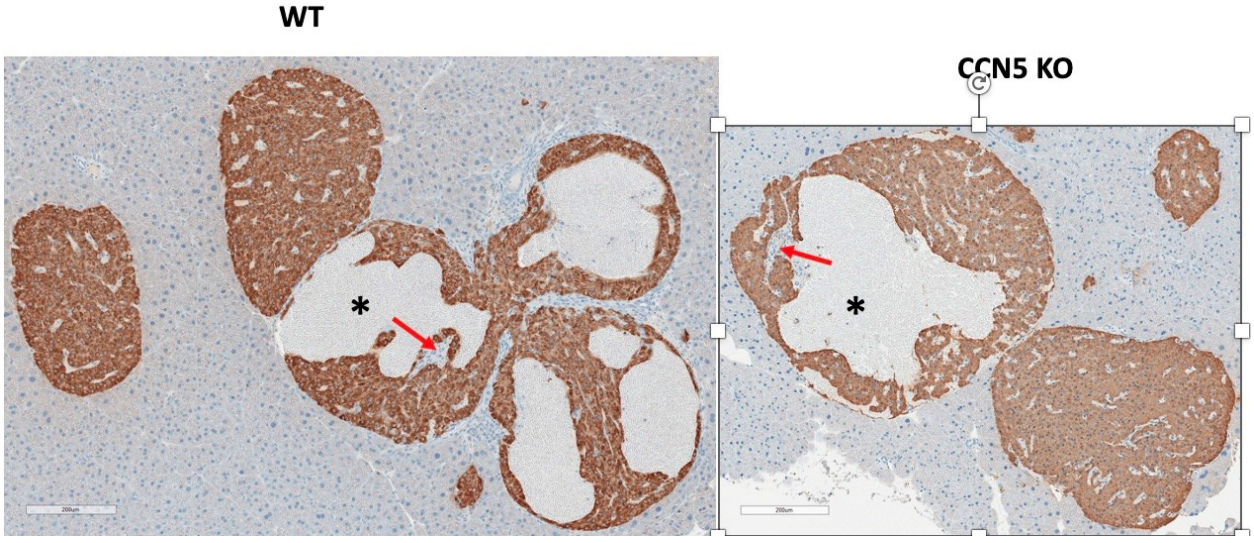


*Figure 20. Insulin staining of pancreatic β-cell islets in male wild-type and CCN5 knockout mice after 27 weeks on HFD, using 4590S anti-rabbit antibody from Cell Signaling Technology.*

*Higher magnification images (scale bars ranging from 2 or 3 mm to 200 nm) unveil remarkable details within the cellular architecture. Four substantial islets appear to merge, with areas of β-cell destruction and depletion marked by asterisks (*). Regions of fibrosis are indicated by red arrows, reflecting tissue scarring and chronic response to the destruction and depletion of insulin-producing β-cells.*

### 3.5.3. Knockout caused no significant decrease in β-cell proliferation in 3-week-old mice.

The impact of CCN5 KO on β-cell proliferation was investigated in 3-week-old mice, a developmental stage known for active β-cell proliferation. The study aimed to elucidate whether the apparent decrease in β-cell mass in adult CCN5 KO could be traced back to early postnatal development. Mixed sexes were utilized for this study, as sexual differentiation had not yet occurred.

As depicted in Fig 21, WT islets exhibited a minimal presence of Ki67-labeled β-cells (red), accounting for approximately 4.5% of all β-cells. In contrast, this ratio was observed to be reduced by half in CCN5 KO mice (bottom panels, only 2.0%). However, the decrease in % β-cell area from 1.28% to 0.95% at this stage was not found to be statistically significant (Table 15).

Although the rate of β-cell proliferation was somewhat decreased in CCN5 KO mice, with values for %Ki67 and Ki67+ per β-cell area lower than those in WT mice, these differences were not statistically significant ($P > 0.05$). Thus, the KO induced no significant decrease in β-cell proliferation in 3-week-old mice. It's important to note that the sample size in this study was relatively small, which may have influenced the statistical outcomes.

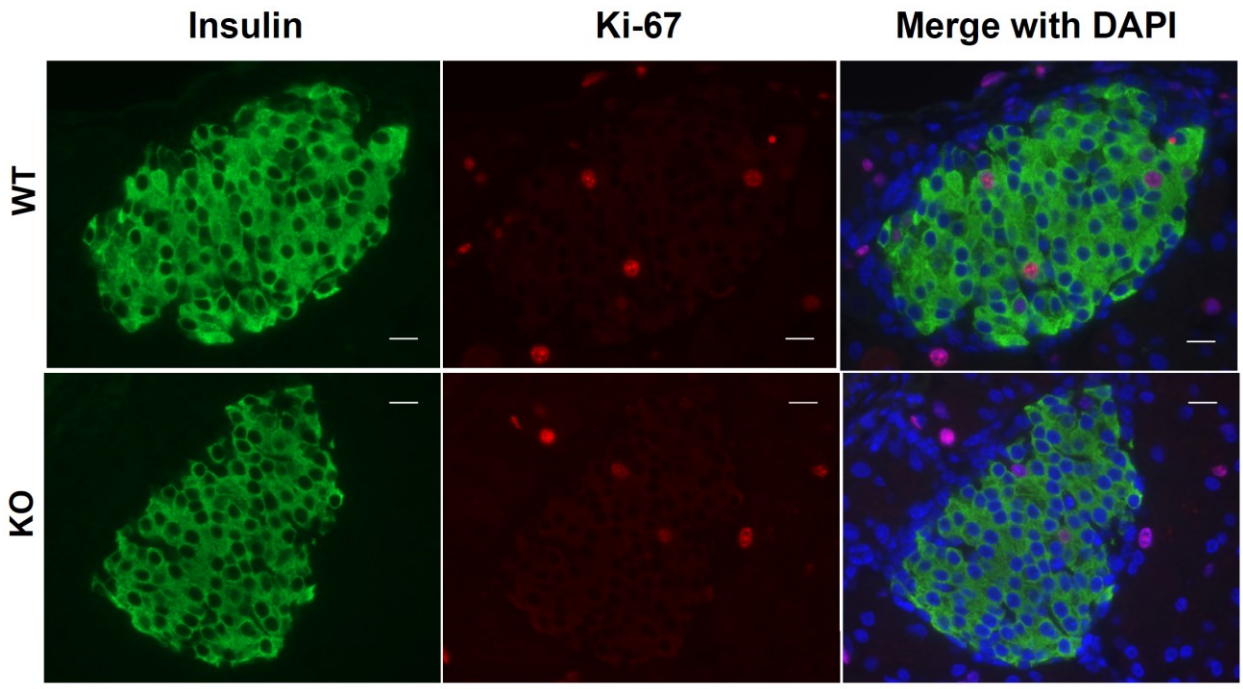


*Figure 21. Immunofluorescence analysis of pancreatic islet β-cell proliferation in 3-week-old pups*

*Top panel: wild-type, Bottom panel knockout*
*Left (green) Insulin staining which identifies β-cells within the pancreatic islet.*
*Middle (red): Ki67 staining which marks cell proliferation within the islet.*
*Right: Merged with DAPI which visualizes cell nuclei, providing structural context.*
*Comparison: Although differences in β-cell proliferation between WT and KO mice were observed, they were not found to be statistically significant*.

*Table 15. The comparison of β cell proliferation between* wild-type *mice and CCN5 knockout mice, measured by Ki67 staining. The percentage of Ki67 positive cells and the Ki67 positive cells per β-area (in 1,000 microns) were assessed. The P values provided indicate the statistical significance of the differences between the two groups and were calculated using SD. The table is represented with SEM.*

| | Tag | %Ki67 | Ki67+ per β-cell area |
|---|---|---|---|
| WT mice | 1 | 5.51 | 0.80 |
| | 2 | 2.85 | 0.33 |
| | 3 | 6.03 | 0.94 |
| | 4 | 3.44 | 0.47 |

| | | | |
|---|---|---|---|
| Mean ± S.E. | | 4.46 ± 0.78 | 0.64 ± 0.14 |
| CCN5 KO | 5 | 0.35 | 0.04 |
| | 7 | 2.84 | 0.41 |
| | 8 | 4.37 | 0.59 |
| Mean ± S.E. | | 2.03 ± 1.17 | 0.35 ± 0.16 |
| P value | | 0.21 | 0.24 |

**%Ki67:** Ki67 positive cells per total DAPI counts**.**

**Ki67+ per β-cell area:** Ki67 positive cells per β-cell area (1,000 microns**)**

These findings support the notion that the reduced β-cell mass in adult CCN5 KO mice may not be solely attributed to diminished proliferation during early development. The decreased rate of β-cell proliferation early in life adds complexity to our understanding of the role of CCN5 in β-cell mass regulation and warrants further investigation into other potential factors, such as cell size or increased cell death that may contribute to this phenotype in adulthood. Those factors coupled with the small sample size, underscores the need for further research to untangle the underlying mechanisms contributing to the observed differences in β-cell mass in adulthood.

# Chapter 3. Discussion

The primary focus of our study was to understand the role of Cellular Communication Network factor 5 (CCN5) in β-cell growth and insulin resistance, particularly in the context of diet-induced obesity. The specific research problem addressed was the lack of comprehensive understanding of the mechanisms through which CCN5 influences metabolic processes and contributes to obesity. We aimed to investigate the effects of CCN5 knockout on lean/fat mass, insulin sensitivity, and glucose tolerance under a NCD and an HFD

The methodology employed in this study involved the use of CCN5 KO mice and WT counterparts, which were subjected to both a NCD and an HFD. The mice were then analyzed for changes in body weight, insulin and glucose tolerance, expression, and proliferation of β cells.

This chapter will begin with a summary of our findings, followed by an analysis of the effects of CCN5 KO on total and lean/fat mass, insulin sensitivity, glucose tolerance and β-cell maintenance. The chapter will then delve into the potential mechanisms underlying these effects, compare them with previous studies and conclude with a discussion on the implications of the findings for future research and the conclusion.

### 4.1. Summary of key findings:

Our findings suggest that CCN5 is essential for normal β-cell growth, but its expression appears to have detrimental effects on insulin and glucose tolerance when subjected to HFD.

Interestingly, our CCN5 KO mice did not exhibit significant changes in lean/fat mass, insulin sensitivity, or glucose tolerance when fed an NCD, despite a ~50% reduction in β-cell area and serum insulin level at 9 months. Upon HFD feeding, CCN5 KO mice demonstrated sexual dimorphic changes in insulin sensitivity, with male KO mice displaying significant improvements in insulin sensitivity and glucose tolerance.

### 4.2. Interpretation and explanation of findings

#### 4.2.1. The lack of weight gain in CCN5 knockout mice.

##### 4.2.1.1. CCN5 plays a multifaceted role in adipogenesis.

It has distinct functions attributed to its secreted and cytosolic forms. The complex interplay between these forms and their interactions with various signaling pathways may contribute to the nuanced effects observed in CCN5 KO mice:

Early Phase of Adipogenesis (Commitment Phase)

- *Cytosolic CCN5*: The cytosolic form of CCN5 may regulate adipogenic commitment by interacting with BMP4, playing a role in the early stages of adipogenesis [227,229,252]

- *Secreted CCN5*: The secreted form of CCN5 may inhibit the commitment of mesenchymal stem cells to the adipocyte lineage by activating the WNT pathway, maintaining mesenchymal precursor cells in an undifferentiated state [227,229,252].

Late Phase of Adipogenesis (Differentiation Phase):

- Potential dual role: The *secreted* form of CCN5 may inhibit adipogenic differentiation by activating the WNT pathway, while the *cytosolic* form's role in this stage is not explicitly provided in the existing literature at least in my knowledge [227,229,252]

CCN5 KO effect on adipogenesis:

- *Potential weight gain in early phase*: The absence of secreted CCN5 in the commitment phase may promote adipogenic differentiation by removing the inhibitory effect on the WNT pathway, potentially leading to weight gain

- *Complex effects in late phase*: The effects of CCN5 KO in the differentiation phase are complex and may depend on the balance between the secreted and cytosolic forms, as well as other signaling interactions. Our study's findings suggest that CCN5 KO might specifically

interfere with the later stages of adipocyte differentiation. This interference could be either direct, through an unknown function of CCN5, or indirect, through alteration of other signaling molecules or pathways.

So, the role of CCN5 in adipogenesis is multifaceted and stage-specific, and the effects of CCN5 KO on weight are likely to be complex and context-dependent. The absence of CCN5 may disrupt the regulatory balance in both the commitment and differentiation phases of adipogenesis, leading to varied effects on weight. Further in-depth study using a combination of genetic, biochemical, and cellular approaches would be required to fully elucidate the mechanisms underlying these observations.

**4.2.1.2. Compensatory inhibition of adipogenesis**:

*Wnt Signaling Pathway:* The Wnt/β-catenin signaling pathway's inhibition of adipogenic differentiation during the early stages of preadipocyte commitment is well-established. It keeps preadipocytes undifferentiated and suppresses key transcription factors like PPARγ and C/EBPα [232]. CCN5's interaction with the Wnt pathway is nuanced and context-dependent, potentially affecting its activation or inhibition [193].

Other proteins or factors might be compensating for the absence of CCN5 and inhibiting adipogenesis. For example, proteins in the Wnt signaling pathway, such *as Wnt10b*, have been shown to inhibit adipogenesis [232,307]. If these proteins are upregulated in the absence of CCN5, they could potentially compensate for its absence and prevent an increase in adipogenesis. Wnt10b acts on specific receptors like Frizzled and the co-receptors LRP5/6 on the cell surface, leading to processes that inhibit adipogenesis and promote osteogenesis [308].

In the absence of Wnt signaling, β-catenin is phosphorylated by a destruction complex that includes the proteins Axin, APC, GSK3β, and CK1. Phosphorylated β-catenin is recognized by

the E3 ubiquitin ligase β-TrCP, which targets it for proteasomal degradation. This process is inhibited when Wnt ligands bind to their receptors, leading to the accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus [226]

The potential effect of CCN5's absence: If Wnt10b is upregulated in the absence of CCN5, it could potentially inhibit adipogenesis, thereby preventing weight gain in CCN5 knockout mice. This is supported by the fact that CCN5/WISP2 is associated with WNT-regulated genes such as *cyclin D1* [252]. Cyclin D1, a cell cycle regulator, may have a complex effect on adipogenesis It's been shown to both promote (by enhancing the expression of PPARγ, a master regulator of adipogenesis and inhibit this process possibly through its effects on cell cycle progression [309,310]. If Wnt10b's upregulation in the absence of CCN5 leads to increased Cyclin D1, it could have various effects on fat cell development.

Moreover, Wnt10b's overexpression might also affect *mesenchymal stem cells' (MSCs')* differentiation into osteoblasts rather than adipocytes [308], possibly contributing to the lack of weight gain in CCN5 KO mice.

However, it's essential to recognize that the impact of Wnt10b upregulation might vary with other factors like the presence of other Wnt proteins and the organism's metabolic state.

#### 4.2.1.3. Increased energy expenditure

##### *4.2.1.3.1. Physical activity:*

Based on the observed expression patterns of CCN5, it is hypothesized that CCN5 KO mice may exhibit increased physical activity. This hypothesis arises from the fact that CCN5 is expressed more substantially in muscle tissues and minimally in the brain, as documented in the Genotype-Tissue Expression (GTEx) portal (www.gtexportal.org/home/gene/WISP2). Consequently, the absence of CCN5 in KO mice might influence factors such as neurological alterations,

behavioral modification, and physiological changes in muscle function. These speculations offer an intriguing avenue for exploration but would require empirical substantiation through further experimental research.

***4.2.1.3.2. Basal Metabolic Rate (BMR):*** A higher BMR in CCN5 KO mice might result in more energy burned at rest, possibly contributing to the lack of weight gain on HFD. This could be due to altered energy utilization or thermogenic tissue activity [311]. Direct measures and exploration of underlying mechanisms are needed.

**4.2.1.3.3. Thermogenesis*:*** Enhanced thermogenesis in CCN5 KO mice might prevent weight gain through increased energy expenditure via brown adipose tissue (BAT) activity or "browning" of white adipose tissue (WAT) [312]. Specific studies, such as measuring physical activity levels and BAT activity, are warranted.

**4.2.1.4. Altered food intake.**

CCN5's role in appetite regulation might lead to reduced food intake in CCN5 KO mice, preventing weight gain. Investigating feeding behavior, neurotransmitters, or hormones involved in hunger and satiety signals might reveal underlying mechanisms [313,314].

**4.2.1.5 Changes in gut microbiota.**

Alterations in gut microbiota in the absence of CCN5 might affect energy balance and weight gain, possibly through mechanisms like energy extraction from diet and modulation of host metabolism [315-319]. Empirical verification, such as comparative studies and microbiota transplantation, is required [320].

**4.2.1.6. Altered lipid metabolism.**

Lipid metabolism, critical for various physiological functions, may be influenced by the absence of CCN5, possibly leading to increased lipid oxidation and subsequent weight loss prevention [321-

[323]. While there are no explicit studies on CCN5 in lipid metabolism, its potential impact through associated cellular processes, such as cell proliferation and differentiation, forms a plausible hypothesis requiring further investigation.

### 4.2.2. Improved insulin sensitivity in CCN5 knockout male mice on high-fat diet

All the hypotheses on why CCN5 KO doesn't have higher body weight than WT stands also for the increased insulin sensitivity in CCN5 KO mice. Maintaining body weight and not increasing it can lead to increased insulin sensitivity through several mechanisms.

*Reduction of adipose tissue inflammation*: Excess adiposity is associated with increased inflammation in adipose tissue. This inflammation can impair insulin signaling, leading to insulin resistance [45]. By maintaining body weight, inflammation in adipose tissue might be reduced, promoting insulin sensitivity.

*Improved lipid metabolism:* Weight maintenance can lead to a healthier lipid profile, with lower levels of circulating fatty acids. Elevated fatty acids are associated with impaired insulin signaling in peripheral tissues like muscle and liver [324]. Maintaining body weight may reduce fatty acid levels and thus improve insulin sensitivity.

*Enhanced glucose uptake*: Adipocytes play a vital role in glucose homeostasis. In a state of weight maintenance, adipocytes may function more effectively in taking up glucose in response to insulin, leading to increased insulin sensitivity [55].

*Decreased ectopic fat accumulation*: Excessive weight gain often leads to ectopic fat deposition in non-adipose tissues like liver and muscle. This ectopic fat can impair insulin action in these tissues [32]. By preventing weight gain, ectopic fat accumulation might be avoided, promoting insulin sensitivity.

*Positive effects on other hormones and cytokines*: Weight maintenance may positively influence the secretion of adipokines and hormones that modulate insulin sensitivity, such as adiponectin, which enhances insulin action [325].

*Potential effects on CCN5*: In the context of CCN5, maintaining body weight in CCN5 KO mice might indicate a modulation in adipogenic pathways that not only prevents weight gain but also facilitates proper insulin signaling, though this aspect would require further investigation specific to CCN5 KO model.

Additionally, the loss of CCN5 in HFD could potentially affect the interaction between *integrins and the insulin receptor*, thereby influencing insulin sensitivity. CCN5 is known to interact with multiple integrins, and these interactions can modulate various cellular functions, including insulin signaling. The loss of CCN5 could potentially affect the interaction between integrins and the insulin receptor, thereby influencing insulin sensitivity. Active β1 and β3 integrins interact with the insulin receptor to modulate insulin action. This interaction was demonstrated through a proximity ligation assay between the insulin receptor with both β1 and β3 integrins in subcutaneous white adipocytes [326]. Moreover, the overall adipose tissue phenotype of Kind2adipo-cre mice, which have a loss of Kindlin-2 that regulates the activation of β1 and β3 integrins, showed many similarities to the adipose tissue knockout of the insulin receptor. This suggests an interaction between integrin and insulin signaling [326]. Furthermore, it has been shown that α5β1 integrin activation enhances basal insulin receptor phosphorylation as well as insulin-stimulated insulin receptor substrate-1 (IRS-1) phosphorylation and recruitment of PI3-kinase to IRS-1 [327]. This suggests that integrins can enhance insulin signaling, which could potentially explain the increased insulin sensitivity observed in CCN5 KO mice in HFD. However, these are hypotheses and would need to be tested experimentally to confirm. It's also

important to note that the effects of CCN5 loss on insulin sensitivity could be tissue-specific, as suggested by Ruiz-Ojeda al., (2021), which found that loss of integrin activity affected mTORC1 activity and insulin resistance restricted in white adipose tissue but not in brown adipose tissue [326]. CCN5 is known to interact with integrins, including β1 integrins. In the absence of CCN5, the interaction between CCN5 and β1 integrins would be disrupted. According to Draicchio et al. (2022), the absence of β1 integrins in mice fed a HFD led to improved insulin sensitivity [328]. Therefore, it's possible that the disruption of the interaction between CCN5 and β1 integrins in CCN5 KO mice could have a similar effect, leading to improved insulin sensitivity despite the HFD. However, this is a hypothetical explanation and would need to be confirmed with further research. It's also important to note that the role of integrins in insulin action and glucose uptake is complex and may be influenced by many factors, including other proteins, and signaling pathways, the specific type of diet, and the duration of the diet, among others.

The diminished β-cell area and serum insulin level observed in CCN5 KO mice align with our prior findings, confirming CCN5 as a β-cell derived growth factor. Regulated by IGF-I and Wnt signaling, CCN5 functions through Akt, FAK, ERK pathways, increasing cyclin D1 and CDK4 levels. It promotes β-cell proliferation, DNA synthesis and protects β-cells from apoptosis [239] This reduction in the β-cell area likely translates to decreased insulin production, which accounts for the lower insulin levels in CCN5 KO mice. Intriguingly, these reduced insulin levels might enhance insulin sensitivity, possibly stemming from decreased downregulation of insulin receptors due to the reduced insulin presence. Additionally, CCN5's absence might induce alterations in adipogenesis, potentially through shifts in protein expression governing this

process. This may further contribute to the lack of marked obesity in CCN5 KO mice when on a HFD.

*Hypothetical upregulation of CCN2 and CCN4 in CCN5 KO mice*

In CCN5 KO mice, the absence of CCN5 might trigger compensatory mechanisms involving the upregulation of other CCN family members, such as CCN2 and CCN4. CCN2, known for its role in the proliferation of embryonic pancreatic cells, might influence β cell function and insulin sensitivity [329]. Similarly, CCN4's involvement in β cell proliferation could further contribute to enhanced insulin responsiveness [330]. These hypothetical interactions could lead to a complex phenotype in CCN5 KO mice, where the upregulation of other CCN proteins compensates for the loss of CCN5. This would require further experimental investigation, considering the multifaceted roles and interactions of CCN proteins in various biological processes.

**4.2.3. No difference in glucagon levels in both the normal-chow diet and high-fat diet between CCN5 knockout mice and wild-type mice in both sexes.**

The role of glucagon in metabolism is complex and multifaceted. Glucagon is a hormone that is produced by the alpha cells of the pancreas and plays a crucial role in maintaining glucose homeostasis. It primarily acts to increase blood glucose levels, counteracting the effects of insulin, which lowers blood glucose levels [32]. Glucagon achieves this by stimulating glycogenolysis and gluconeogenesis in the liver. It also promotes lipolysis in adipose tissue [76]. The unchanged glucagon levels in CCN5 KO mice in NCD and HFD compared to WT mice in HFD in our study could suggest that the regulation of glucagon secretion is not significantly affected by the absence of CCN5. This could be due to a variety of factors, including the potential compensatory mechanisms that might be in place to maintain glucose homeostasis in the absence of CCN5 [32]. However, the exact mechanisms through which CCN5 might not

influence glucagon secretion or action are not clear and would require further investigation. It's also important to note that the regulation of glucagon secretion is influenced by a multitude of factors, including blood glucose levels, insulin levels, and the nutritional status of the organism, among others. Therefore, the unchanged glucagon levels observed in our study could also be reflective of these other regulatory influences [89,118]. It's also worth noting that while glucagon primarily acts to increase blood glucose levels, it also has other metabolic effects that could potentially influence the overall metabolic phenotype of the CCN5 KO mice. For instance, glucagon has been shown to promote ketogenesis in the liver [331], which could potentially contribute to the energy metabolism of the mice [76].

In conclusion, while the unchanged glucagon levels in our study suggest that the regulation of glucagon secretion might not be significantly affected by the absence of CCN5, the potential influence of glucagon on the metabolic phenotype of the CCN5 KO mice is complex and likely involves multiple factors and pathways. Further studies would be needed to fully elucidate these mechanisms.

**4.2.4. Effects of sexual hormone on insulin sensitivity**

We found that only CCN5 KO male mice were more insulin sensitive than WT and for the female, the results were not statistically significant. This shows that sex hormones also have an impact on the results. According to Yong, 2022 women and men have intrinsic differences in their pancreas biology. Specifically, women appear to have higher capacities for insulin secretion than men. This phenomenon has been reported both in vivo and in perfused islets in vitro [279]. In the context of CCN5 KO male mice in HFD being more insulin sensitive than WT and KO females in HFD, it could be hypothesized that the increased insulin sensitivity in male CCN5 KO mice could be due to the intrinsic differences in the pancreas biology and the genetic

predisposition of males to diabetes. The male CCN5 KO mice might have developed adaptive mechanisms to cope with the high-fat diet, leading to increased insulin sensitivity. However, this is a complex issue and further research would be needed to fully understand the mechanisms involved. Also, testosterone can improve skeletal muscle insulin responsiveness in HFD by potentiating the PI3K-AKT pathway [286] and enhance insulin signaling in the skeletal muscle by positively regulating p85 gene expression. It reduces hepatic glucose output despite increasing hepatic insulin resistance [287]. But those are cases of testosterone supplementation in normal mice fed with HFD. Further investigation should be made into the role of testosterone in CCN5 KO mice.

In conclusion, the variances in insulin sensitivity between male and female CCN5 KO mice on a HFD may be traced back to an interplay of factors, such as innate differences in pancreas biology, genetic inclination, and variations in body fat distribution. However, the complete understanding of the underlying mechanisms calls for further in-depth research.

**4.3. Comparison with other studies**

To the best of our knowledge, the seminal work by Kim et al. (2018) represents the only investigation specifically focused on the role of CCN5 KO mice in β-cell function. [253]. Other studies are done in vitro or in transgenic mice. In the study by Kim et al. (2018), ITT and GTT revealed significant impairments in KO mice compared to WT mice when fed HFD at 12 weeks. However, by week 24, these differences were no longer statistically significant. In contrast, our study demonstrates an improvement in insulin sensitivity in male KO mice at the 12-week mark on HFD. Notably, the KO mice in Kim et al. (2018)'s study exhibited higher body weight compared to their WT counterparts. However, we did not observe a statistically significant difference in body weight between the KO and WT mice in our study.

The divergence in metabolic and weight outcomes between our study and that of Kim et al. (2018) could be attributed to a variety of variables:

**1.** ***Age of mice*****:** Age affects metabolic processes, including insulin sensitivity. In Kim et al., (2018), the ITT and GTT were performed after 11 weeks and 23 weeks of NCD or HFD feeding. However, the document does not specify the initial age of the mice before the feeding period started. Given that mice are typically weaned at 3 weeks of age and if the feeding period started immediately after weaning, the mice would have been approximately 14 weeks old at the time of the first ITT and GTT (after 11 weeks of feeding) and 26 weeks old at the time of the second ITT and GTT (after 23 weeks of feeding). However, this is just an assumption, and the actual ages could be different if the feeding period did not start immediately after weaning. In our study, the ITT and GTT were performed when the mice were 6 months old (24 weeks old), similar to the second set of tests in Kim et al. (2018). but differing from their initial 14-week set. This age difference may explain some variance.

***2. Diet*****:** Both our study and Kim et al., 2018 used the same high-fat diet (HFD; 60% kcal as fat, Research Diets, Inc.; D12492), which significantly influences metabolic processes, including insulin sensitivity. However, NCD varied between the two studies. In our research, the NCD consisted of 18.6% crude protein and 6.2% fat, whereas Kim et al. (2018) did not mention the exact composition of their NCD. The discrepancies in the NCD composition might have contributed to differences in the metabolic responses observed, particularly considering the variety of standard NCD formulations available for research.

***3. Environmental factors:*** Factors such as housing conditions (light, noise, cage cleaning), stress levels and handling can also influence metabolic processes in mice [332]

***4. Experimental procedures***: Differences in fasting, insulin or glucose dosage, and administration methods may also account for variations in results [333]. Notably, glucose administration differed between our study (10% glucose) and Kim et al. (2018) (20% glucose).

**5. *Biological variability***: Inherent variations in biological systems may yield different results even under identical conditions [333]**.**

**6.*Sample size*:** Our study included 13-16 mice per group, more than Kim et al.(2018)'s 6-12, and considered both sexes. The larger sample size might enhance reliability and precision, reducing the impact of random variations [333].

### 4.4. Limitations

The study was conducted using a mouse model, which may not fully replicate the complex metabolic processes in humans. Furthermore, the study did not directly measure the effects of CCN5 KO on adipogenesis or energy expenditure, which could be important factors contributing to the observed effects.

### 4.5. Recommendations for future research

4.5.1. Elucidating mechanisms of CCN5 in insulin sensitivity and glucose tolerance:

Future research should aim to understand the mechanisms through which CCN5 regulates insulin sensitivity and glucose tolerance. This might involve in-depth research on the effects of CCN5 deletion on adipogenesis, especially in later stages, and its impact on energy expenditure. The use of associated substrates to identify metabolic pathways might give new insights.

4.5.2. Indirect calorimetry analyses:

To better understand the metabolic implications of CCN5 KO, future studies could employ indirect calorimetry to measure energy expenditure, respiratory quotient, and substrate utilization in both KO and WT mice.

4.5.3. Monitoring of locomotor activity and food intake:

Assessing physical activity and food intake could provide additional insights into the metabolic phenotype of CCN5 KO mice. Automated systems could be used to monitor these parameters over an extended period.

4.5.4. Inclusion of ovariectomized female models:

Considering observed sex-specific metabolic responses, the incorporation of ovariectomized female models could offer valuable insights into hormonal influences, particularly estrogen, on metabolic outcomes.

4.5.5. Longitudinal studies: A longitudinal study could provide insights into how the metabolic effects of CCN5 KO evolve over time, especially in relation to aging.

4.5.6. Studies involving humans

Studies using human samples could provide further insights into the role of CCN5 in obesity and insulin resistance.

By addressing these areas, future research could provide a more comprehensive understanding of the role of CCN5 in metabolism and its potential as a therapeutic target for metabolic disorders.

**4.6. Conclusion**

In conclusion, this study provides valuable insights into the role of CCN5 in regulating body weight, β cell maintenance, insulin sensitivity and glucose tolerance. Our research reveals a correlation between the absence of CCN5 in male mice and enhanced insulin sensitivity, especially when they are subjected to a high-fat diet and are at least 6 months old. Furthermore, our data also indicate a crucial role of CCN5 in β-cell growth, as evidenced by observations made at 9 months. This finding is particularly significant given the critical role β cells play in

insulin production and regulation, which is directly linked to diabetes. These results collectively indicate that CCN5 may serve as an important regulator in the metabolic pathways associated with obesity and insulin resistance. Its impact is dual-faceted, influencing both the specific functions of β-cells and the overall metabolic activities. They underscore the importance of further investigation into CCN5's role and its potential as a therapeutic target in managing metabolic disorders like obesity and diabetes.

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