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Investigations on sugar-induced GLP-1 secretion and DPPIV activity in NCI-H716 cells

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Abstract

Glucagon-like peptide-1 (GLP-1) plays a central role in maintaining glucose homeostasis and is a key therapeutic target in type 2 diabetes. Although the link between dietary sugars and GLP-1 secretion is well established, the specific and concentration-dependent effects of common sugars such as sucrose, glucose, and fructose remain poorly understood.

This study aimed to investigate how these carbohydrates influence GLP-1 secretion and dipeptidyl peptidase-4 (DPPIV) activity using the human enteroendocrine NCI-H716 cell line. It was hypothesized that sucrose would stimulate GLP-1 secretion in a dose-dependent manner and that different sugars would differentially modulate DPPIV activity. Cells were treated with defined concentrations (50–250 mM) of each sugar. GLP-1 secretion was quantified via ELISA, DPPIV activity measured enzymatically, and the expression of key genes (*SLC2A1*, *SLC2A5*, *DPP4*, *SI*) assessed by RT-PCR.

The results demonstrated that sucrose at 100 mM significantly enhanced GLP-1 secretion ($p < 0.0001$), followed by a decline at higher concentrations. Glucose and fructose did not significantly stimulate GLP-1 secretion within the tested concentration range. The absence of *SLC2A5* expression in NCI-H716 cells suggests fructose uptake occurs via alternative, non-specific pathways. Furthermore, DPPIV activity increased in response to sucrose and fructose, but not glucose or mannitol, indicating a carbohydrate-specific regulation.

In conclusion, sucrose at 100 mM showed a peak stimulation of GLP-1 secretion followed by a reduction at higher concentrations. Distinct regulatory effects of different sugars on DPPIV activity were observed. These findings contribute to a better understanding of sugar-induced enteroendocrine responses and highlight the need for further mechanistic studies to validate these *in vitro* findings under physiologically relevant conditions.

Zusammenfassung

Glucagon-like Peptide-1 (GLP-1) spielt eine zentrale Rolle bei der Aufrechterhaltung der Glukosehomöostase und ist ein wichtiges therapeutisches Ziel bei Typ-2-Diabetes. Obwohl der Zusammenhang zwischen ernährungsrelevanten Zuckern und der GLP-1-Sekretion gut erforscht ist, sind die spezifischen und konzentrationsabhängigen Wirkungen gängiger Zucker wie Saccharose, Glukose und Fruktose nach wie vor kaum bekannt.

In dieser Studie wurde untersucht, wie diese Kohlenhydrate die GLP-1-Sekretion und die Aktivität von Dipeptidylpeptidase-4 (DPPIV) beeinflussen, wobei die menschliche enteroendokrine Zelllinie NCI-H716 verwendet wurde. Die Hypothese war, dass Saccharose die GLP-1-Sekretion dosisabhängig stimuliert und dass verschiedene Zucker die DPPIV-Aktivität unterschiedlich modulieren. Die Zellen wurden mit bestimmten Konzentrationen (50-250 mM) der einzelnen Zucker behandelt. Die GLP-1-Sekretion wurde mittels ELISA quantifiziert, die DPPIV-Aktivität enzymatisch gemessen und die Expression von Schlüsselgenen (*SLC2A1*, *SLC2A5*, *DPP4*, *SI*) mittels RT-PCR untersucht.

Die Ergebnisse zeigten, dass Saccharose in einer Konzentration von 100 mM die GLP-1-Sekretion signifikant erhöhte ($p < 0,0001$), gefolgt von einem Rückgang bei höheren Konzentrationen. Glukose und Fruktose stimulierten die GLP-1-Sekretion innerhalb des getesteten Konzentrationsbereichs nicht signifikant. Das Fehlen der *SLC2A5*-Expression in NCI-H716-Zellen deutet darauf hin, dass die Fruktoseaufnahme über alternative, unspezifische Wege erfolgt. Darüber hinaus stieg die DPPIV-Aktivität als Reaktion auf Saccharose und Fruktose, nicht aber Glucose oder Mannitol, was auf eine kohlenhydratspezifische Regulierung hindeutet.

Zusammenfassend induzierte Saccharose bei 100 mM die höchste GLP-1-Sekretion, gefolgt von einer Abnahme bei höheren Konzentrationen. Darüber hinaus wurden unterschiedliche regulatorische Effekte verschiedener Zucker auf die DPPIV-Aktivität beobachtet. Diese Ergebnisse tragen zu einem besseren Verständnis der zuckerinduzierten enteroendokrinen Reaktionen bei und unterstreichen die Notwendigkeit weiterer mechanistischer Studien, um diese *In-vitro*-Ergebnisse unter physiologisch relevanten Bedingungen zu validieren.

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Abbreviations

Symbol/Abbreviation	Meaning
AV	Average value
α -gustducin	Alpha-gustducin
bp	Base pairs
BSA	Bovine serum albumin
$\text{CaCl}_2 \cdot \text{H}_2\text{O}$	Calcium chloride dihydrate
Caco-2	Colon cancer cell line, clone 2
cAMP	Cyclic adenosine mono phosphate
CBBG 250	Coomassie Brilliant Blue Dye-G250
CCK	Cholecystokinin
CCK-8	Cell counting Kit - 8
cDNA	Complementary Deoxyribonucleic acid
C_{final}	Final concentration
CH_3COOH	Acetic acid
CS	Cell suspension
ddH ₂ O	Double distilled water
DF	Dilution factor
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DPPIV	Enzyme: dipeptidyl peptidase-4
<i>DPP4</i>	Gene: <i>dipeptidyl peptidase-4</i>
EECs	Enteroendocrine cells

ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FW Primer	Forward Primer
g	g-Forces
<i>GAPDH</i>	<i>Glycerinaldehyd-3-phosphat-Dehydrogenase</i>
GIP	Glucose-dependent insulintropic polypeptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GLUT	Glucose transporter
GLUT1	Glucose transporter 1
GLUT2	Glucose transporter 2
GLUT5	Glucose transporter 5
GPR40	G-protein coupled receptor 40
GPR120	G-protein coupled receptor 120
G β 3	G protein beta 3 subunit
G γ 13	G protein gamma 13 subunit
HBSS	Hank's Balanced Salt Solution
HCl	Hydrochloric acid
HEPES	2-(4-(2-Hydroxyethyl)-1-piperazinyl)- ethane sulfonic acid
HRP	Horseradish peroxidase
H ₃ PO ₄	Phosphoric acid
IC50	Half-maximal inhibitory concentration
K _{ATP} channel	ATP-sensitive potassium channel

KCl	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
KRPH	Krebs–Ringer phosphate -HEPES buffer
LS	N-lauroylsarcosine
MgSO ₄	Magnesium sulfate
mM	millimolar
mRNA	Messenger ribonucleic acid
Na ⁺ /K ⁺ -ATPase	Sodium/Potassium-ATPase
NaCl	Sodium chloride
NAD ⁺ /NADH	Nicotinamide adenine dinucleotide (oxidized/reduced form)
NADP ⁺ /NADPH	Nicotinamide adenine dinucleotide phosphate (oxidized/reduced form)
NaHCO ₃	Sodium hydrogen carbonate
Na ₂ HPO ₄	Disodium hydrogen phosphate
NCI-H716	Primarily used cell line: National Cancer Institute-human-716 (identifier number)
NFW	Nuclease-free water
NTC	Non template control
NR	Neutral red
NRU	Neutral red uptake assay
PBS	Phosphate-Buffered Saline
Pen-Strep	Penicillin-Streptomycin
PLCβ2	Phospholipase C beta 2

PMS	Phenazinium methylsulfate
PYY	Peptide YY
qPCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
RNase	Ribonuclease
RPMI	Roswell Park Memorial Institute Medium 1640
RSD	Relative standard deviation [%]
RT	Room temperature
RT+	Reverse transcriptase positive (+)
RT-	Reverse transcriptase negative (-)
RV Primer	Reverse Primer
SD	Standard deviation
SGLT1	Sodium Dependent Glucose Co-transporter 1
SGLT2	Sodium-Glucose Co-Transporter 2
<i>SI</i>	Gene: <i>sucrose isomaltase</i>
<i>SLC2A1</i>	Gene: <i>solute carrier family 2 member 1</i>
<i>SLC2A2</i>	Gene: <i>solute carrier family 2 member 2</i>
<i>SLC2A5</i>	Gene: <i>solute carrier family 2 member 5</i>
<i>SLC5A1</i>	Gene: <i>solute carrier family 5 member 1</i>
ssDNA	Single-stranded deoxyribonucleic acid
TAS1R2	Taste receptor type 1 member 2
TAS1R3	Taste receptor type 1 member 3
TMB	3,3',5,5'-Tetramethylbenzidine
TMD	C-terminal 7-transmembrane domain

TRIS	Tris(hydroxymethyl)aminomethane
TRPM5	Transient Receptor Potential channel M5
VDCCs	Voltage-dependent Ca ²⁺ channels
VFT	Venus flytrap domain

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1 Introduction

Carbohydrates are essential for energy production and supporting vital processes in humans. Their recognition and influence have evolved over time but are affected by genetics, environment, and lifestyle factors like diet and inactivity. The constant availability and overconsumption of energy-rich foods are increasingly linked to metabolic disorders such as obesity, which can subsequently lead to type 2 diabetes. (summarized by Zheng *et al.*, 2018)

In maintaining human health, it is critical that blood glucose levels remain constant. Glucose homeostasis is tightly regulated by hormones like insulin and glucagon, produced by the pancreas. Insulin lowers blood sugar levels by promoting glucose absorption into cells, while glucagon acts as a counter-regulatory hormone, increasing blood glucose levels. Prior to the release of these hormones, incretins such as glucagon-like peptide-1 (GLP-1) and gastric inhibitory polypeptide (GIP) are secreted. Among these, GLP-1 plays a particularly important role in amplifying glucose-stimulated insulin secretion from pancreatic cells and inducing satiety responses. (Müller *et al.*, 2019)

Understanding GLP-1 secretion requires recognition of both established knowledge and current limitations regarding the complex mechanisms underlying this physiological process. It is well known that sugars, particularly glucose, stimulate GLP-1 release from enteroendocrine L-cells (Müller *et al.*, 2019). However, the detailed intracellular signaling pathways and their precise role in regulating glucose homeostasis are not yet fully understood. Additionally, whether the incretin response is substrate-specific and how it depends on stimulus intensity remain open questions. Addressing these gaps could facilitate the development of targeted therapies against obesity and type 2 diabetes. This need is underscored by the widespread consumption of sugar-sweetened products, which significantly contribute to the increasing global prevalence of metabolic disorders (Tappy, 2021).

Therapeutic agents targeting the incretin system, such as GLP-1 receptor agonists and dipeptidyl peptidase-4 (DPP-4) antagonists, which prolong the half-life of endogenous GLP-1 by preventing its enzymatic degradation, are already employed in the treatment of type 2 diabetes mellitus (Deng *et al.*, 2020). Investigating the mechanisms that regulate GLP-1 secretion and degradation therefore holds translational potential.

Against this background, the present study aimed to investigate the concentration-dependent effects of dietary sugars, specifically the disaccharide sucrose and the monosaccharides glucose and fructose, on GLP-1 secretion in a subclone of NCI-H716 cells. These human enteroendocrine cells represent a well-established *in vitro* model for sweet taste receptor-mediated GLP-1 release. A particular focus was placed on the relationship between sugar exposure and DPPIV activity to better understand the potential interaction between stimulus and degradation. All experiments were designed to assess concentration dependence, including cell viability, GLP-1 secretion, and DPPIV activity, although the latter was investigated using a limited high to low concentration approach.

2 Scientific background

2.1 Intestinal nutrient sensing

Intestinal nutrient sensing is an important physiological process whereby specific cells within the gastrointestinal tract (GIT) detect the presence of nutrients, primarily via specialized cells known as enteroendocrine cells (EECs). These cells are located in the intestinal epithelium and play an essential role in regulating digestion, appetite, and metabolic health by secreting specific hormones in response to various nutrients. (summarized by Sanchez *et al.*, 2022) Although EECs respond to a variety of nutrients, a particular emphasis is placed on the sensing of carbohydrates and their impact on metabolic regulation.

During nutrient absorption in the small intestine, EECs recognize the presence of various nutrients and subsequently synthesize peptides that interact with the brain and liver via vagal afferent neurons and the circulatory system (Duca *et al.*, 2021). This endocrine signaling helps suppress the liver's natural nutrient production while regulating dietary intake (Breen *et al.*, 2013).

Upon detecting nutrients, EECs initiate a series of intracellular responses. L-cells and K-cells, respond to various nutrients such as carbohydrates, fats, and proteins through multiple signaling pathways. For instance, lipids are sensed in the small intestine by G-protein coupled receptors GPR40 and GPR120, with GPR120 stimulating GLP-1 secretion and initiating a signaling cascade throughout the body. (Efeyan *et al.*, 2015)

Dietary proteins are broken down in the ileum into peptides and amino acids by protease. These amino acids are sensed by the Taste 1 Receptor Member 1/Taste 1 Receptor Member 3 (TAS1R1/TAS1R3) heterodimeric umami receptor, which activate G-protein signaling pathways, leading to neurotransmitter release. (Efeyan *et al.*, 2015)

Carbohydrates, such as starches and disaccharides, are hydrolyzed into monosaccharides, primarily glucose and fructose, which are absorbed by enterocytes and enteroendocrine cells. The primary receptor for sweet taste detection is the Taste 1 Receptor Member 2/Taste 1 Receptor Member 3 (TAS1R2/TAS1R3) heterodimer. Additionally, sodium-glucose co-transporter 1 (SGLT1) and glucose transporters may also function as sensors, although their signaling roles require further investigation. (Sclafani & Ackroff, 2012)

2.2 L-cells: key enteroendocrine regulators of appetite

Among the various types of EECs, L-cells are particularly important as they regulate appetite and energy homeostasis through hormone secretion, making them essential contributors to metabolic health. The GIT is the body's largest endocrine organ. EECs are distributed throughout the GIT and constitute less than 1 % of the cell population in the intestinal epithelium. L-cells are predominantly distributed within the distal ileum and proximal colon, embedded throughout the intestinal epithelium, with notable aggregation in the crypts and along the villi. These cells are crucial for homeostasis as they produce approximately 30 hormones that play essential roles in digestion, appetite, and metabolism, mainly through their interactions within the gut-brain axis. (Reimann *et al.*, 2012)

EECs modulate food intake by detecting luminal contents and releasing hormones, with a primary focus on two types: open-type and closed-type EECs. Open-type EECs have a cone-shaped structure with one extremity close to the basal lamina and microvilli at the other end in contact with luminal contents. This configuration allows them to sense nutrients and promote hormone release from secretory granules into adjacent blood vessels. (Cummings & Overduin, 2007) Conversely, closed-type EECs respond to neuronal or circulatory signals instead of directly interacting with luminal nutrients. The hormones secreted by EECs can also exert paracrine effects on adjacent cells, including neurons. Recent research indicates that EECs connect directly with neurons via synapse-like structures known as neuropods. (Bohórquez *et al.*, 2011; Bohórquez *et al.*, 2015)

L-cells, designated as "L" for "large," are distinguished by their mature form and size in comparison to other EEC types. They express the preproglucagon gene and are predominantly located in the intestinal epithelium. L-cells express the preproglucagon gene and are predominantly located in the intestines. Their cone-shaped structure facilitates the release of hormones such as GLP-1, glicentin, oxyntomodulin, and GLP-like peptide 2 (GLP-2) via posttranslational processing. (Eissele *et al.*, 1992; Petersen *et al.*, 2015)

L-cells vary in their release of peptides depending on their anatomical location. For instance, L-cells in the lower small intestine often co-localize with peptide YY (PYY) and cholecystikinin (CCK), while those in the upper small intestine exhibit co-localization with gastric inhibitory peptide (Grandt *et al.*, 1994; Guarita *et al.*, 2000). The co-expression of PYY and GLP-1 in L-cells is fundamental for regulating energy balance and maintaining energy homeostasis. PYY exists primarily in two forms: the full-length variant (PYY₁₋₃₆), which engages various Y receptors (Y1, Y2, and Y5), and the truncated form (PYY₃₋₃₆), which selectively targets the Y2 receptor. GLP-1 primarily operates through the GLP-1 receptor. (Grandt *et al.*, 1994; Guarita *et al.*, 2000)

Furthermore, L-cells co-expressing GLP-1 and PYY are distributed throughout the gastrointestinal epithelium, beginning in the proximal jejunum and increasing in density towards the distal intestines, reflecting their interaction with ingested nutrients (Eissele *et al.*, 1992; Larsson *et al.*, 1975). After food intake, circulating levels of GLP-1 and PYY rise within 15 minutes, typically correlating with caloric intake. Peak plasma concentrations occur at approximately 40 minutes for GLP-1 and 90 minutes for PYY following a mixed meal, which levels gradually stabilize. (Batterham & Bloom, 2003; Todd *et al.*, 1998) In contrast, during fasting, these hormones are present in modest levels in the blood, but postprandial increases in humans are significantly higher than in rodents (Elliott *et al.*, 1993; Ronveaux *et al.*, 2014).

The release of GLP-1 and PYY exhibit a two-phase release profile. The initial rapid rise is driven by L-cells in the upper small intestine and is thought to be mediated through neural or hormonal signals in response to nutrient sensing (Elliott *et al.*, 1993; Nauck *et al.*, 1996). A second, delayed phase is triggered by direct contact of nutrients with L-cells in the distal gut, where specific nutrient receptors on the apical membrane initiate hormone secretion (Pilichiewicz, Chaikomin, *et al.*, 2007; Pilichiewicz, Papadopoulos, *et al.*, 2007).

Once released into circulation, GLP-1 and PYY undergo enzymatic cleavage predominantly by DPPIV within the intestinal endothelium and liver. This enzyme converts GLP-1 into its inactive form and processes PYY₁₋₃₆ into its active variant PYY₃₋₃₆. (Spreckley & Murphy, 2015)

Understanding the two-phase release mechanism and hormonal dynamics of EECs is crucial for elucidating their roles in regulating hunger, food intake, and energy homeostasis. This fundamental knowledge underpins the relevance of the NCI-H716 cell line, which serves as a valuable model for studying the functions of L-cells and the secretion of hormones related to nutritional intake and metabolic health.

2.3 NCI-H716: a suitable model for studying GLP-1 secretion *in vitro*

The NCI-H716 cell line is derived from the small intestinal tissue of a 33-year-old Caucasian male patient with colorectal adenocarcinoma and was established in 1984. This cell line exhibits endocrine characteristics, enabling it to secrete GLP-1. (Park *et al.*, 1987; Reimer *et al.*, 2001) It is important to note that this cell line is tumor-derived, and its characteristics may differ from those of healthy cells. Since NCI-H716 are suspension cells, they need a membrane coating to promote adhesion and horizontal growth. While initial studies suggested that differentiation occurs post-attachment, later findings indicated that GLP-1 secretion remains unaffected by differentiation. (Anini & Brubaker, 2003; de Bruïne *et al.*, 1992)

Building on the foundational understanding of intestinal nutrient sensing, it is essential to investigate the mechanisms by which EECs, particularly L-cells, secrete GLP-1 in response to nutrient stimuli. Mechanisms of GLP-1 secretion have been extensively characterized using various cell and animal models, including three notable *in vitro* GLP-1 secreting cell lines: GLUTag and STC-1, which are murine-derived, and NCI-H716, a human-derived cell line. (de Bruïne *et al.*, 1992; Drucker *et al.*, 1994; Rindi *et al.*, 1990)

These models share a common secretion mechanism that is dependent on increased cytoplasmic calcium levels, which can arise from intracellular stores or influx through the plasma membrane (Tolhurst *et al.*, 2009).

Moreover, NCI-H716 cells express key components of the taste transduction pathway, including the sweet taste receptor subunits TAS1R2 and TAS1R3, as well as signaling molecules such as alpha-gustducin (α -gustducin), G protein beta 3 subunit (G β 3), G protein

gamma 13 subunit (Gγ13), phospholipase C beta 2 (PLCβ2), and transient receptor potential cation channel subfamily M member 5 (TRPM5) (Jang *et al.*, 2007). These characteristics make them a valuable model for studying sweet taste receptor-mediated regulation of GLP-1 (Reimer *et al.*, 2001).

2.4 Glucagon-like Peptide 1 (GLP-1)

GLP-1 is an incretin hormone that plays a crucial role in metabolic regulation. Synthesized from the proglucagon gene located on chromosome 17, GLP-1 consists of 30 amino acids and exerts multiple metabolic effects, including the enhancement of insulin secretion from the pancreas and the inhibition of glucagon release, ultimately regulating to promote satiety. (Holst *et al.* 1987)

After synthesis, GLP-1 undergoes post-translational modifications by specific proprotein convertases, particularly PC1/3, which cleave proglucagon into bioactive peptides including GLP-1 and GLP-2. Notably, GLP-1 is amidated at the C-terminus, a modification critical for its insulinotropic and glucagon-inhibiting activities (Gupta, 2013).

Research indicates that GLP-1 not only helps regulate blood sugar levels but also modulates appetite by stimulating specific receptors in the hypothalamus and modifying neural pathways involved in appetite and energy balance (Flint *et al.*, 1998). The importance of incretins like GLP-1 in glucose homeostasis is highlighted by studies showing that intravenous glucose administration results in significantly lower insulin release compared to equivalent oral glucose doses, attributing up to 70 % of insulin secretion after oral glucose intake to the incretin effect (Kreymann *et al.*, 1987; Nauck *et al.*, 1986).

GLP-1 is predominantly secreted by enteroendocrine L-cells in the intestinal ileum, with additional production occurring in the hypothalamus, taste bud cells, and pancreatic tissue (Gupta, 2013; Jensterle *et al.*, 2021). It exists in several forms, with amidated GLP-1 (7-36) and GLP-1 (7-37) being the main bioactive variants produced from the proglucagon precursor, an 18 kDa protein, via post-translational processing. Notably, GLP-1 (1-36 amide) is mainly secreted in the pancreas, while GLP-1 (1–37) is produced in the ileum and hypothalamus. Approximately 80 % of GLP-1 in circulation is in its amidated form, GLP-1 (7-36). (Orskov *et al.*, 1994)

GLP-1 has a very short half-life of less than 2 minutes due to rapid metabolism by dipeptidyl peptidase 4 (DPPIV), which converts it into inactive forms with low GLP-1 receptor affinity (Deacon, 2004). It is estimated that only 10-15 % of biologically active GLP-1 is absorbed into the systemic circulation (Hansen *et al.*, 1999). Additionally, GLP-1 serves as a substrate for neutral endopeptidase, and the inhibition of this enzyme has been shown to enhance the stability and bioavailability of both endogenous and exogenous GLP-1 *in vivo* (Plamboeck *et al.*, 2005).

Circulating GLP-1 interacts with class 2 G-protein-coupled receptors known as GLP-1 receptors (GLP1R), which are distributed in the GIT, pancreatic islets, hypothalamus, stomach, lungs, brain, heart, and kidneys. This distribution reflects a remarkable conservation of homology among these organs. The GLP1R consists of 463 amino acids and contains eight hydrophobic domains, with the N-terminal extracellular domain likely functioning as the signal sequence. (Holz, 2004) The binding domain for GLP-1 (7–36) amide spans amino acids 7-21, with the positively charged imidazole side chain of histidine at position 7 mediating receptor interaction. Other key amino acid side chains important for receptor binding include those at positions 10 (glycine), 12 (phenylalanine), 13 (threonine), 15 (aspartate), as well as side chains at positions 28 and 29 in the C-terminus. (Gupta, 2013)

When blood glucose levels rise, activation of GLP1R in pancreatic β -cells triggers a cascade of signaling pathways that promote insulin secretion. These include pathways involving protein kinase A (PKA), Ca^{2+} /calmodulin-dependent protein kinase (CaMK), and mitogen-activated protein kinases (MAPK, ERK1/2), along with phosphatidylinositol 3-kinase (PI-3K) and protein kinase B (PKB/Akt). The extensive presence of GLP1R suggests that GLP-1 influences various physiological processes beyond glucose metabolism, underscoring its role as a multifaceted regulatory hormone and its potential trophic effects on β -cells, promoting growth, differentiation, and survival. (Holz, 2004)

GLP-1 facilitates insulin secretion in response to glucose by stimulating metabolic pathways within the L-cells, leading to increased ATP production inside these cells. The elevated ATP levels inhibit ATP-sensitive potassium (K_{ATP}) channels, causing membrane depolarization and the activation of voltage-dependent calcium channels (VDCCs). This depolarization triggers calcium influx, which promotes the exocytosis of insulin-containing granules from pancreatic β -cells. Additionally, GLP-1 enhances the efficiency of these pathways by acting on K_{ATP} channels, VDCCs, intracellular calcium stores, and the

transport or priming of insulin granules, thereby amplifying insulin secretion (Eliasson *et al.*, 2003; Holz *et al.*, 1999; Holz *et al.*, 1993)

Altering specific amino acid residues in GLP-1 may improve its resistance to DPPIV-mediated degradation, potentially extending its half-life and enhancing its therapeutic effectiveness (Gupta, 2013). Interestingly, the release of GLP-1 is also implicated in the response to sweet taste. Von Molitor *et al.* (2021) describe an 'alternative pathway' in type II taste bud cells where glucose influx via GLUTs and/or SGLT1 increases ATP, inhibits K_{ATP} -mediated K^+ outflow, and consequently induces GLP-1 release. This is illustrated in Figure 1.

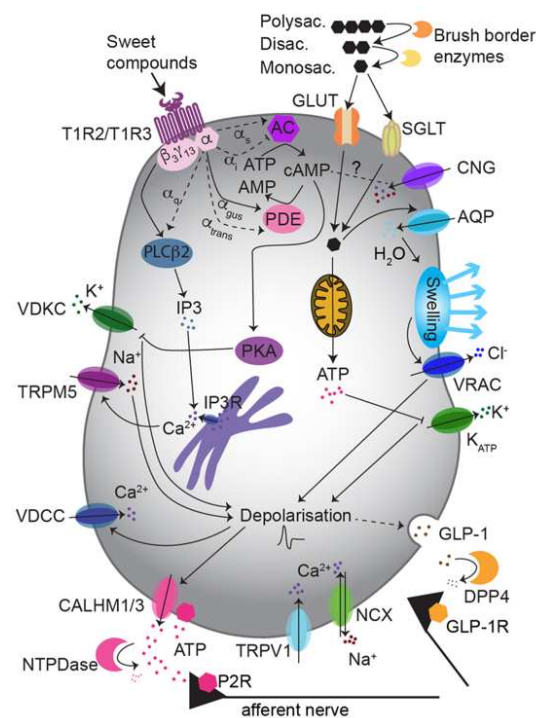


Figure 1: Sweet taste transduction uses multiple pathways in type II taste bud cells.

The “canonical pathway” involves activation of gustducin by the T1R3/T1R2 receptor, leading to an increase in intracellular Ca^{2+} via $PLC\beta_2/IP_3$ signaling, cell depolarization via TRPM5, and ATP release. Non-caloric sweeteners may primarily activate the $PLC\beta_2/IP_3$ pathway, whereas sugars might mainly stimulate a cAMP/PKA pathway, depolarizing the cell through inhibition of K^+ channels. The “alternative pathway” may involve glucose influx via GLUTs and/or SGLT1, resulting in increased ATP production and inhibition of K_{ATP} -mediated K^+ outflow, potentially inducing GLP-1 secretion. Abbreviations: AQP, aquaporins; DPP4, dipeptidyl peptidase 4; CALHM1/3, Ca^{2+} homeostasis modulator 1/3; VDKC, voltage-dependent K^+ channel; P2R, purinergic receptor class 2; GLP-1R, GLP-1 receptor. Reproduced from Molitor *et al.* (2021) under the Creative Commons Attribution License (CC BY). Copyright © 2021 by Molitor, Riedel, Krohn, Hafner, Rudolf, and Cesetti. Used with permission.

The characterization of GLP-1's metabolic role and its rapid degradation has led to the development of new classes of antidiabetic medications designed to enhance GLP-1 receptor activation. This can be accomplished either through the inhibition of the DPPIV

enzyme, which is responsible for GLP-1 degradation, or by formulating synthetic GLP-1 receptor agonists that possess a longer half-life than naturally occurring GLP-1. (Andersen *et al.*, 2018)

By enhancing GLP-1 receptor activation, these medications offer promising therapeutic approaches for diabetes management and metabolic health. Further investigations examining the effects of sucrose, glucose, and fructose on GLP-1 secretion and DPPIV activity in the NCI-H716 cell model, with an emphasis on concentration-dependent responses, could provide important insights into the cellular mechanisms underlying enteroendocrine signaling. Moreover, understanding the role of DPPIV in this context is crucial, as its activity directly impacts GLP-1 levels and, consequently, the overall metabolic effects associated with these sugars.

2.5 Dipeptidyl Peptidase-4 (DPPIV)

Dipeptidyl peptidase 4 (DPPIV), also referred to as CD26, is a transmembrane serine protease that plays an essential role in glucose metabolism by rapidly inactivating glucagon-like peptide-1 (GLP-1) and glucose-dependent insulintropic peptide (GIP). This inactivation occurs within few minutes by the cleavage of N-terminal dipeptides. (Rasmussen *et al.*, 2003)

DPPIV specifically cleaves GLP-1 and GIP, which start with Tyr-Ala, His-Ala, or His-Ser, at their N-terminal end, targeting the penultimate alanine residue. Additionally, DPPIV recognizes peptides with penultimate proline (Mentlein *et al.*, 1993). Importantly, DPPIV exists as both a membrane-bound protein and a soluble circulating enzyme in the body (Drucker, 2007). In this study, the primary focus is on the intricate mechanisms of GLP-1 inactivation by DPPIV, as these processes are directly linked to its therapeutic effects on glucose metabolism.

DPPIV catalyzes the conversion of active GLP-1 (7-36) and (7-37) into inactive forms, specifically GLP-1 (9-36) and (9-37), which exhibit low affinity for the GLP-1 receptor. The kidneys play a major role in the clearance of these inactive peptides and metabolites from the bloodstream, thus regulating GLP-1 availability and its physiological effects. (Deacon, 2004)

Structurally, DPPIV exists in two primary forms: a membrane-anchored protein that can stimulate intracellular signaling independent of its enzymatic activity, and a circulating soluble enzyme that retains its enzymatic functions (Lambeir *et al.*, 2003). Genetic studies highlight DPPIV's essential role in glucose homeostasis. For instance, Fischer 344/CRJ rats show increased GLP-1 levels and reduced glycemic excursions following glucose challenges (Nagakura *et al.*, 2001). Similarly, mice with targeted DPPIV gene inactivation demonstrate improved glucose tolerance and enhanced levels of GLP-1, GIP, and insulin, alongside increased insulin sensitivity and resistance to diet-induced obesity (Conarello *et al.*, 2003; Marguet *et al.*, 2000).

Despite its well-documented enzymatic functions, DPPIV cleaves numerous peptides and chemokines containing alanine or proline at the second position (Kang & Park, 2021), thereby complicating the identification of specific substrate peptides that mediate the glucose-lowering effects of DPPIV inhibitors. Notably, DPPIV inhibitors show reduced efficacy in lowering blood glucose levels in mice that are genetically deficient in both GLP-1 and GIP receptors (Hansotia *et al.*, 2004).

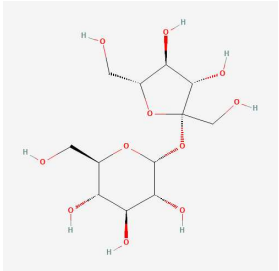
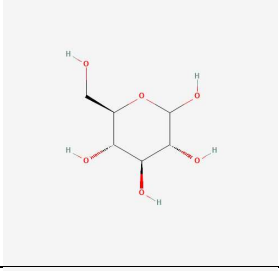
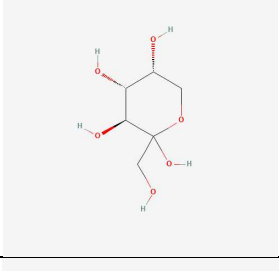
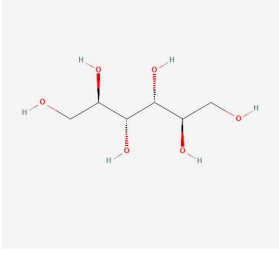
Further investigations reveal that while non-selective inhibitors can lead to significant toxicity, selective DPPIV inhibitors can be administered safely at high doses in animal models without affecting human T cell activation *in vitro*. These findings highlight the importance of selectivity in optimizing the safety profile of antihyperglycemic agents (Lankas *et al.*, 2005). However, the long-term impact of selective DPPIV inhibition on the biological activities of other peptides, such as pituitary adenylate cyclase-activating polypeptide, stromal cell-derived factor-1, or substance P, remains uncertain (Ahrén & Hughes, 2005; Guieu *et al.*, 2006; Gupta & Sen, 2019).

Chemical inhibitors of DPPIV effectively prevent the inactivation of GLP-1 and GIP, leading to reduced blood glucose levels in both preclinical (Deacon & Holst, 2006) and human studies (Ahrén *et al.*, 2005; Ahrén *et al.*, 2011). Long-term DPPIV inhibition in clinical settings has been associated with reduced HbA1c levels, prevention of weight gain, enhanced β -cell function, and suppression of plasma glucagon in individuals with type 2 diabetes (Ahrén *et al.*, 2004; Ahrén *et al.*, 2005).

2.6 Stimulating agents used in the study

Commonly occurring sweet tasting substances were used to analyze their effects on the biological processes under investigation. In addition to sugars such as sucrose, glucose, and fructose, mannitol served as an osmotic control agent. This approach aims to determine whether GLP-1 secretion is directly induced by sugars or if it results from osmotic changes in the cellular environment (see Table 1 for IUPAC names and structures of the compounds used).

Table 1: IUPAC names and structures of compounds used in this study
computed by Lexichem TK 2.7.0 (PubChem release 2021.10.14)

Sucrose	(2 <i>R</i> ,3 <i>R</i> ,4 <i>S</i> ,5 <i>S</i> ,6 <i>R</i>)-2-[(2 <i>S</i> ,3 <i>S</i> ,4 <i>S</i> ,5 <i>R</i>)-3,4-dihydroxy-2,5-bis(hydroxymethyl)oxolan-2-yl]oxy-6-(hydroxymethyl)oxane-3,4,5-triol	
Glucose	(3 <i>R</i> ,4 <i>S</i> ,5 <i>S</i> ,6 <i>R</i>)-6-(hydroxymethyl)oxane-2,3,4,5-tetrol	
Fructose	(3 <i>S</i> ,4 <i>R</i> ,5 <i>R</i>)-2-(hydroxymethyl)oxane-2,3,4,5-tetrol	
Mannitol	(2 <i>R</i> ,3 <i>R</i> ,4 <i>R</i> ,5 <i>R</i>)-hexane-1,2,3,4,5,6-hexol	

2.6.1 Sucrose

Sucrose (C₁₂H₂₂O₁₁) is a non-reducing disaccharide composed of glucose and fructose linked by a glycosidic bond, commonly found in plants, cyanobacteria, and algae, as a

product of photosynthesis. Industrially, sucrose is primarily derived from the crops of sugarcane (*Saccharum* spp.) and sugar beets (*Beta vulgaris*). (Ni *et al.*, 2022)

As a commonly occurring sugar, sucrose serves as a readily available source of energy in human nutrition (Göbel & Fichtner, 2023). Sucrose binds to both subunits of the heterodimeric sweet taste receptor TAS1R2/TAS1R3, with a greater affinity for the N-terminal domain of TAS1R3 (Nie *et al.*, 2005). The hydroxyl groups in sucrose facilitate hydrogen bonding with hydrophilic amino acids, positioning sucrose the agonist-hinge structure found in venus fly trap (VFT) domains (Zhang *et al.*, 2010).

After ingestion, sucrose is hydrolyzed by the enzyme sucrase-isomaltase at its non-reducing ends, producing glucose and fructose in the brush border membrane of the intestinal lumen (Hauri *et al.*, 1979). This breakdown is crucial because glucose serves as a primary energy source for animal tissues and can be stored in the body as glycogen or triglycerides. Notably, glucose binds to the same site on the sweet taste receptor as sucrose (Nie *et al.*, 2005).

In the intestine, glucose is transported via the SGLT1 transporter on the apical side and GLUT2 on the basolateral side, while fructose is absorbed through the GLUT5 transporter and then transported to the liver via GLUT2 (Cheeseman, 1993; Muriel *et al.*, 2021).

The physiological effects of sucrose include a rapid increase in blood sugar levels, prompting insulin release for cellular glucose uptake. Additionally, sucrose stimulates GLP-1 secretion at varying concentrations (Jang *et al.*, 2007).

The osmotic properties of sucrose (Junqueira *et al.*, 2016) can impact cell hydration and physiological responses, raising questions about whether changes in GLP-1 secretion result from the sugar itself or osmotic effects.

2.6.2 Glucose

Glucose ($C_6H_{12}O_6$) is a monosaccharide that serves as an essential source of energy for the human body, supporting the metabolic processes that fuel the cells composing tissues and organs. In the digestive system, glucose is generated by the breakdown of carbohydrates, such as starches and sugars. Once absorbed into the bloodstream, glucose is transported to the cells, where it is used to produce energy in the form of adenosine triphosphate (ATP). (Gribble & Reimann, 2016)

Glucose plays a key role in several metabolic pathways, including glycolysis, gluconeogenesis, and the citric acid cycle, all essential for energy production within the body. Excess glucose can be stored as glycogen in the liver and muscle tissues through glycogenesis, providing energy reserves during fasting or increased energy demands. Conversely, when blood glucose levels drop, glycogen is broken down back into glucose via glycogenolysis. (Kanungo *et al.*, 2018)

The transepithelial transport of glucose in the small intestine is facilitated by *SGLT1* on the apical side of intestinal enterocytes, which enables the active transport of glucose into the cell coupled with the inward transport gradient of two Na^+ ions. Following a carbohydrate-rich meal or free sugars, the increased glucose influx through *SGLT1* in L-cells generates sufficient sodium ion flow to induce membrane depolarization and hormone secretion. (Gribble & Reimann, 2016) Glucose then exits the enterocyte through the GLUT2 transporter located on the basolateral membrane, playing a critical role in regulating blood glucose levels (Santer *et al.*, 2003).

In *SGLT1*-deficient mice, peak levels of GLP-1 and GIP triggered by glucose are reduced, highlighting *SGLT1*'s essential role of hormone release (Gorboulev *et al.*, 2012). Interestingly, elevated GLP-1 levels have been observed in *SGLT1*-knockout mice at later stages following a glucose challenge, possibly due to unabsorbed glucose fermenting in the distal intestine (Powell *et al.*, 2013).

Insulin is primarily responsible for regulating blood glucose levels. When blood sugar levels rise, insulin is secreted to stimulate glucose uptake by cells, effectively enabling the utilization of glucose for energy production. Additionally, insulin also interacts with signaling pathways that enhance glucose transport mechanisms in target tissues (Rahman *et al.*, 2021). Glucose also modulates the secretion of gut hormones such as GLP-1, influencing appetite and prompting insulin sensitivity (Jang *et al.*, 2007).

In scientific research, glucose is frequently used as a positive control in metabolic assays due to its well-characterized properties. This provides a benchmark for validating results in enzymatic assays or cell-based experiments (Johnson & Besselsen, 2002), particularly in studies comparing the effects of disaccharides like sucrose to monosaccharides like glucose and fructose on GLP-1 secretion.

2.6.3 Fructose

Fructose ($C_6H_{12}O_6$) is a monosaccharide commonly found in fruits, honey, and root vegetables. It is known for its high sweetness level, which has led to its widespread use in the food industry as a sweetening agent (Muriel *et al.*, 2021). However, rising fructose consumption poses significant health risks. Numerous epidemiological studies have associated high fructose intake with the development of metabolic syndrome. This syndrome includes conditions such as obesity, insulin resistance, hyperglycemia, dyslipidemia, hypertension, nonalcoholic fatty liver disease, gout, and hyperuricemia. (Caliceti *et al.*, 2017; Jegatheesan & De Bandt, 2017; Stanhope, 2012)

The metabolism of fructose differs significantly from that of glucose, particularly in the initial phosphorylation stages (Hannou *et al.*, 2018). While glucose is phosphorylated at the C-6 position to yield fructose-6-phosphate, fructose undergoes phosphorylation at the C-1 position by the enzyme ketohexokinase to form fructose-1-phosphate. This metabolic pathway bypasses the regulatory step involving phosphofructokinase, potentially leading to excessive ATP consumption and the accumulation of intermediates, which can disrupt metabolic homeostasis. (Jang *et al.*, 2018)

Fructose is absorbed in the small intestine via the GLUT5 transporter located on the apical membrane of enterocytes. GLUT5 is a member of the facilitative glucose transporter (GLUT) family and is the only transporter with a specificity for fructose, unable to transport glucose or galactose. (Manolescu *et al.*, 2007). Fructose is transported passively via GLUT5, assisted by concentration gradients (Douard & Ferraris, 2008). Once absorbed, fructose is transferred to the liver via the basolateral GLUT2 transporter. GLUT2 is a low-affinity transporter that can recognize glucose and galactose and is involved in fructose uptake across the hepatic plasma membrane into the liver, it functions bidirectionally at the basolateral membrane of intestinal and renal epithelial cells (Leturque *et al.*, 2005; Wood & Trayhurn, 2003).

In the liver, fructose is rapidly metabolized into various metabolites that are crucial for central metabolic pathways. This rapid conversion contributes to *de novo* lipogenesis, particularly with high fructose consumption, resulting in elevated triglyceride levels (Softic *et al.*, 2020). Excessive fructose consumption can overwhelm intestinal absorption and clearance capacity, allowing more fructose to access the liver and potentially causing metabolic disturbances. Notably, although fructose and glucose have the same chemical

formula, they have quite different physiological effects *in vivo*, which may contribute to the development of metabolic diseases. (Samuel, 2011)

While fructose affects the secretion of gut hormones, particularly GLP-1, it does not directly stimulate insulin release and primarily acts independently of the glucose-mediated responses. Although fructose can enhance GLP-1 secretion, especially in individuals with type 2 diabetes, the increase in plasma insulin concentrations after fructose ingestion was not significantly correlated with glucose levels. This difference in hormonal response plays an important role in appetite regulation and energy homeostasis within the body. (Vozzo *et al.*, 2002)

2.6.4 Mannitol

Mannitol ($C_6H_{14}O_6$) is a sugar alcohol that occurs naturally in various plants and can be synthesized by several microorganisms. It is one of the key compounds in the sugar alcohol category, alongside sorbitol, xylitol, and erythritol. Among them, it is unique as it is the only non-hygroscopic crystal. (Livesey, 2003; Song & Vieille, 2009)

Mannitol's molecular structure differs from that of sorbitol primarily in the orientation of hydroxyl groups at the C-2 position. With a molecular weight of 182.17 g/mol, mannitol has a sweetness level of 50-70 % compared to sucrose, exhibits good stability in dilute acid/alkaline solutions, being resistant to oxidation in air. Additionally, it is soluble in glycerin and slightly soluble in ethanol, making it useful for various applications (Chen *et al.*, 2020).

Mannitol has numerous physiological functions that make it valuable in the food and pharmaceutical industries. With a caloric value of 1.99 kcal/g and a glycemic index of zero, it is suitable for diabetics and dieters (Meng *et al.*, 2017). The Food and Drug Administration (FDA) has classified mannitol as Generally Recognized As Safe (GRAS), allowing its use in food products. For example, it serves as a sweetener or anti-sticking agent in sugar-free chewing gum and enhances the firmness in flavored ice cream and chocolate structure (Saha & Racine, 2011). Its non-reducing properties help to maintain the color of baked goods by preventing Maillard reactions. Moreover, mannitol stimulates superoxide dismutase and inhibits the accumulation of lipid peroxides, showing promise in reducing free radicals. (Shen *et al.*, 1997)

In cell culture studies, elevated sugar concentrations in the medium can boost metabolite production due to increased energy availability and osmotic pressure. Mannitol can be employed as an osmotic control to differentiate these effects. (Tholakalabavi *et al.*, 1994) As a cell-impermeant and nonmetabolized compound, mannitol creates a hypertonic environment that induces water movement away from the cell, leading to plasmolysis. When used alongside sugars like sucrose, it allows researchers to determine whether the observed effects on exocytosis are attributable to the sugars' osmotic impacts or their metabolic activity. (Malek *et al.*, 1998)

2.7 Previous results of the project

Notably, prior work from the research group, confirmed that this subclone of NCI-H716 expresses both proglucagon and GLP-1 receptor encoding genes, underpinning its relevance for research on GLP-1 regulation (Dür, 2023).

Investigations into GLP-1 secretion evaluated sucrose, sucralose, sodium cyclamate, neohesperidin dihydrochalcone, and rebaudioside M. Only 150 mM sucrose significantly increased GLP-1 secretion by 1.9-fold compared to the control. To isolate osmotic effects, 150 mM mannitol was used as a control and showed no significant increase in secretion, supporting that sucrose's effect is not solely due to osmotic pressure. Further investigations found that sweeteners equivalent to 5 % sucrose did not trigger GLP-1 secretion, while sucrose at 150 mM did, suggesting a preference for carbohydrates. Pathway analysis with inhibitors prior to sucrose treatment did not significantly influence GLP-1 secretion (Uusitalo, 2025). These inhibitors included 1 mM lactisole, which blocks the sweet taste receptor TAS1R3 (Jiang *et al.*, 2005), 250 μ M phloridzin, an inhibitor of SGLT1 (Bisignano *et al.*, 2018), and 10 μ M voglibose, an α -glucosidase inhibitor that targets carbohydrate digestion and uptake pathways (Dabhi *et al.*, 2013).

Gene expression analysis via droplet digital PCR with cDNA from NCI-H716 cells revealed a substantial increase in *TAS1R3* (Taste receptor type 1 member 3) subunit droplets, exceeding RT- droplets by over four-fold. However, no mRNA expression was detected for *TAS1R2* (Taste receptor type 1 member 2), *SLC5A1*, or *SLC2A2*, with *HPRT1* serving as a control gene. This suggests that alternative mechanisms for glucose transport may be active in this cell line (Uusitalo, 2025).

The canonical sweet taste receptor is formed by a heterodimeric complex of TAS1R2 and TAS1R3 subunits (Morini & Temussi, 2005), but only TAS1R3 is expressed by the NCI-H716 subclone used in the present studies (Uusitalo, 2025).

2.8 Aims and hypotheses

This study aims to enhance the understanding of GLP-1 secretion mechanisms and their regulatory pathways.

In the research field of enteroendocrine cells and intestinal sweet sensing-induced GLP-1 secretion, gaps remain in understanding the mechanisms influencing blood glucose homeostasis. The study uses human enteroendocrine cells, NCI-H716, that have been optimized previously in the research group for studying the sweet taste receptor mediated GLP-1 secretion.

Important investigations include clarifying the specific signaling pathways mediating GLP-1 secretion, examining how varying sweetness levels influence this process, and elucidating the role of DPPIV in regulating GLP-1 activity.

Hypotheses:

- *The genes SI (sucrose isomaltase), SLC2A5, SLC2A1 and DPP4 are expected to be expressed in the cell line under baseline conditions, regardless of sucrose treatment, indicating constitutive expression.*
- *Exposure to various sugars will differentially influence GLP-1 secretion rates, with sucrose expected to enhance secretion in a dose-dependent manner.*
- *DPPIV activity will vary with different carbohydrate exposures, suggesting a potential role for DPPIV in the regulation of GLP-1 metabolism.*

3 Materials and methods

3.1 Devices

Table 2: List of devices used in this study

Devices	Manufacturer
Analytical balance	Pioneer™ OHAUS, Parsippany, US
Biological Safety Cabinet MSC Advantage	Thermo Fisher Scientific, Waltham, US
Box Storage System Tank, BSS-750	Thermo Fisher Scientific, Waltham, US
C1000 Touch™ Thermal Cycler	Bio-Rad Laboratories, Inc. in Hercules, USA
Centrifuge 5418R	Eppendorf AG, Hamburg, DE
Centrifuge 5804R	Eppendorf AG, Hamburg, DE
Centrifuge 5810R	Eppendorf AG, Hamburg, DE
CO ₂ Incubator, C150	BINDER GmbH, Tuttlingen, DE
Electronic Multichannel Pipette, Xplorer, 8-Channel (100 µL, 1200 µL)	Eppendorf AG, Hamburg, DE
FlexStation 3 Multi-Mode Microplate Reader	Molecular Devices, San Jose, US
Hemocytometer, Counting Chamber, Neubauer® improved bright-line, 0.1 mm Depth, 0.0025 mm ²	BRAND GmbH + Co. KG, Wertheim, DE
Mechanical pipette, single-channel, Research plus®, (0.1 - 2.5 µL; 0.5 - 10 µL; 2 - 20 µL; 10 - 100 µL; 20 - 200 µL; 100 - 1000 µL; 0.5 - 5 mL)	Eppendorf AG, Hamburg, DE
Microcentrifuge PerfectSpin Mini	Peqlab, VWR International GmbH, Darmstadt, DE
Microplate Shaker, PMS-1000i	Grant Instruments™, Cambridge, UK
Microscope, IT400 Trino	VWR International GmbH, Darmstadt, DE
Multichannel Pipette (300 µL)	STARLAB INTERNATIONAL GmbH, Hamburg, DE
Multimode-Microplate-Reader Tecan Spark®	Tecan Group Ltd., Männedorf, CH
pH-Meter, pHenomenal™	VWR International GmbH, Darmstadt, DE
Pipette Controller, PipetBoy, acu 2	INTEGRA Biosciences AG, Zizers, CH

qPCR, Linegene_17201	Bioer, Hangzhou, CHN
StepOnePlus™, Real-Time PCR System	Applied Biosystems™, Foster City, US
Tecan NanoQuant® Plate	Platten Tecan Group Ltd., Männedorf, CH
Ultrapure Water Sytem, Arium® Pro	Satorius AG, Göttingen, DE
Ultrasonic Cleaning Bath	VWR International GmbH, Darmstadt, DE
Vacuum Pump, Vacuum Aspiration Systems 815	DITABIS AG, Pforzheim, DE
Vacuum filtration system, sterile	Sartorius Stedim, Goettingen, DE
Vortexer, RS-VA10	Phoenix Instruments, Garbsen, DE
Waterbath, ecoline staledition 003	LAUDA Dr. R. Wobser GmbH & Co. KG, Lauda-Königshofen, DE
Waterbath, open bath steel	VWR International GmbH, Darmstadt, DE

3.2 Materials

Table 3: List of materials used in this study

Material	Manufacturer
Aspiration Pipette, glas, unplugged, sterile, 2mL	Pioneer™ OHAUS, Parsippany, US
Cell culture plate, flat base, sterile (96-well, 24-well)	Sarstedt AG & Co. KG, Nümbrecht, DE
Cell culture flask, surface: Suspension, Filter cap (T25, T75, T175)	Sarstedt AG & Co. KG, Nümbrecht, DE
Cover Glasses, round, „Menzel Gläser“	Thermo Fisher Scientific, Waltham, US
Cryogenic Vials, round bottom, inside thread, 2 mL	Carl Roth GmbH + Co. KG, Karlsruhe, DE
CytoOne® Dish (100 x 20 mm), non-treated, sterile	STARLAB INTERNATIONAL GmbH, Hamburg, DE
Disposable cannula, Sterican® standard cannulas, size 2, sterile	B. Braun Melsungen AG, Melsungen, DE
Disposable Syringe, Injekt® Solo, sterile, 2 mL	B. Braun Melsungen AG, Melsungen, DE
Disposable Gloves, Microflex® XCEED® nitrile gloves, powder-free	Thermo Fisher Scientific, Waltham, US
Disposable tissues, Rotizell®-Tissues	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Whatman™ Filter paper, Qualitative Diameter 125 mm, 100 circles	Cytiva, Vienna, AT

Membrane filter paper (MicronSep Nitrocellulose 47 mm 0.22 µm Membrane Disks)	GVS Life Sciences, Bologna, IT
PARAFILM®, sealing film, 120 µm	VWR International GmbH, Darmstadt, DE
PCR plate, 96-well, frosted, "Fast" Type, Semi Sub Skirt, Low Profile, Eckschnitt A1	Biozym Scientific GmbH, Oldendorf, DE
Pipette Tips, TipOne® (20 µL; 200 µL; 300 µL; 1000 µL; 5 mL)	STARLAB INTERNATIONAL GmbH, Hamburg, DE
Precision Wipes	Kimtech Science, Kimberly-Clark, Irving, US
Screw cap tube (15 mL, 50 mL)	Sarstedt AG & Co. KG, Nümbrecht, DE
Serological Pipettes, plugged, sterile (5 mL; 10 mL; 25 mL; 50 mL)	Sarstedt AG & Co. KG, Nümbrecht, DE
SuperLock® Microcentrifuge Tubes, 1.5 mL	STARLAB INTERNATIONAL GmbH, Hamburg, DE

3.3 Chemicals

Table 4: List of chemicals and molecular biological materials used in this study

Chemicals	Manufacturer
Acetic acid, anhydrous	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Bovine Serum Albumin (BSA), ≥ 96 %,	Sigma-Aldrich, Darmstadt, DE
Caco-2 cell line	CLS Cell Lines Service GmbH, Eppelheim, DE
Calcium chloride dihydrate (CaCl ₂ H ₂ O) ≥ 99 %, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Coomassie Brilliant Blue G-250	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Corning® Matrigel® (GFR) Basement Membrane Matrix, phenol red-free	Thermo Fisher Scientific, Waltham, US
D (+) - Glucose, waterfree	Carl Roth GmbH + Co. KG, Karlsruhe, DE
D (-) - Mannitol, ≥ 98 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Dimethyl sulfoxide, 99.5 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
<i>DPP4</i> Forward Primer	Sigma-Aldrich, Darmstadt, DE
<i>DPP4</i> Reverse Primer	Sigma-Aldrich, Darmstadt, DE
Erythrosine Red B	Sigma-Aldrich, Darmstadt, DE
Ethanol ≥ 96 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE

Fetal Bovine Serum (FBS)	Thermo Fisher Scientific, Waltham, US
D (-) - Fructose, pure	AppliChem GmbH, Darmstadt, DE
<i>GAPDH</i> Forward Primer	Merck KGaA, Darmstadt, DE
<i>GAPDH</i> Reverse Primer	Merck KGaA, Darmstadt, DE
Hanks' Balanced Salt Solution (HBSS)	Sigma-Aldrich, Darmstadt, DE
HEPES, $\geq 99.5\%$	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Hydrochloric acid	VWR International GmbH, Darmstadt, DE
Luna® Universal qPCR Master Mix	New England Biolabs® GmbH, Massachusetts, US
Magnesium sulfate hydrate, $\geq 99\%$, dried, pure	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Medium: Gibco™ Roswell Park Memorial Institute (RPMI) 1640 (1X) + GlutaMAX™-I + 25 mM HEPES	Thermo Fisher Scientific, Waltham, US
NCI-H716 cell line	ATCC®, Manassas, US
Neutral red	Sigma-Aldrich, Darmstadt, DE
N-lauroylsarcosine sodium salt	Sigma-Aldrich, Darmstadt, DE
Nuclease-free, autoclaved, diethyl dicarbonate-treated water	Carl Roth GmbH + Co. KG, Karlsruhe, DE
OneTaq® Hot Start 2x Master Mix with Standard Buffer	New England Biolabs® GmbH, Massachusetts, US
Penicillin-streptomycin, sterile filtered, 10000 units penicillin, 10 mg/mL streptomycin, 0.9 % NaCl	Sigma-Aldrich, Darmstadt, DE
Phosphate-Buffered Saline (PBS), sterile	Sigma-Aldrich, Darmstadt, DE
Potassium chloride (KCl), $\geq 99.5\%$, p.a.	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Potassium dihydrogen phosphate (KH ₂ PO ₄)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Recombinant Human DPPIV/CD26 (High Purity Dimer) Protein	R&D Systems, Inc., Minneapolis, US
RNase AWAY surface decontamination agent	Thermo Fisher Scientific, Waltham, US
ROTI®Nanoquant	Carl Roth GmbH + Co. KG, Karlsruhe, DE
<i>SI</i> Forward Primer	Merck KGaA, Darmstadt, DE

<i>SI</i> Reverse Primer	Merck KGaA, Darmstadt, DE
<i>SLC2A1</i> Forward Primer	Sigma-Aldrich, Darmstadt, DE
<i>SLC2A1</i> Reverse Primer	Sigma-Aldrich, Darmstadt, DE
<i>SLC2A5</i> Primer Mix	Bio-Rad Laboratories, California, USA
Sodium chloride (NaCl)	Carl Roth GmbH + Co. KG, Karlsruhe, DE
Sodium hydrogencarbonate (NaHCO ₃)	Sigma-Aldrich, Darmstadt, DE
Sucrose, ≥ 99.5 %	Sigma-Aldrich, Darmstadt, DE
TRIS (C ₄ H ₁₁ NO ₃), ≥ 99.3 %	Carl Roth GmbH + Co. KG, Karlsruhe, DE
WST-8 (Cell counting kit-8)	Sigma-Aldrich, Darmstadt, DE

3.4 Kits

Table 5: List of kits used in this study

Kit	Manufacturer
DPPIV-Glo™ Protease Assay G8350	Promega GmbH, Walldorf, DE
GLP-1 Total ELISA Kit Cat. #EZGLP1T-36K	Merck KGaA, Darmstadt, DE
LunaScript™ RT SuperMix Kit	New England Biolabs® GmbH, Massachusetts, US
Monarch® PCR & DNA Cleanup Kit (5 µg)	New England Biolabs® GmbH, Massachusetts, US
Monarch® Total RNA Miniprep Kit	New England Biolabs® GmbH, Massachusetts, US

3.5 Composition of solutions

Krebs Ringer Phosphate HEPES buffer

Table 6: Composition of KRPB buffer

Chemical	Amount
NaCl	7.480 g
KCl	0.140 g
KH ₂ PO ₄	0.160 g
CaCl ₂ • 2H ₂ O	0.470 g
MgSO ₄	0.156 g
NaHCO ₃	2.180 g
HEPES	2.383 g
ddH ₂ O	1000 mL

The pH of the solution was adjusted to 7.4.

Phosphate-buffered saline (unsterile)

Table 7: Composition of unsterile PBS (10 X, 500 mL)

Chemical	Amount
NaCl	40.03 g
Na ₂ HPO ₄	7.10 g
KCl	1.006 g
KH ₂ PO ₄	1.361 g
ddH ₂ O	dissolved in 500 mL

The pH of the solution was adjusted to 7.4.

Sucrose

Table 8: Composition of sucrose stock solution 1.5 M

Chemical	1.5 M in 3 mL (CCK-8 & NRU)	1.5 M in 15 mL (GLP-1 & DPPIV)
Sucrose, $\geq 99.5\%$	1.545 g	7.701 g
KRPH	dissolved in 3 mL	dissolved in 15 mL

Fructose

Table 9: Composition of fructose stock solution 1.5 M

Chemical	1.5 M in 3 mL (CCK-8 & NRU)	1.5 M in 15 mL (GLP-1 & DPPIV)
D (-) - Fructose, pure	0.811 g	4.068 g
KRPH	dissolved in 3 mL	dissolved in 15 mL

Glucose

Table 10: Composition of glucose stock solution 1.5 M

Chemical	1.5 M in 3 mL (CCK-8 & NRU)	1.5 M in 15 mL (GLP-1 & DPPIV)
D (+) - Glucose, waterfree	0.812 g	4.052
KRPH	dissolved in 3 mL	dissolved in 15 mL

Mannitol

Table 11: Composition of mannitol stock solution 1 M

Chemical	1.0 M in 3 mL (CCK-8 & NRU)	1.5 M in 15 mL (GLP-1 & DPPIV)
D (-) - Mannitol, $\geq 98\%$	0.820 g	4.085
KRPH	3 mL	dissolved in 15 mL

Neutral red stock solution (4 mg/mL)

Table 12: Composition of neutral red stock solution

Chemical	Amount
Neutral red	40 mg
PBS	10 mL

Neutral red media (40 µg/mL)

Table 13: Composition of neutral red media (dilution 1:100)

Chemical	Amount
RPMI w/o FBS	12 mL
Neutral red stock solution	0.12 mL

Neutral red destain solution

Table 14: Composition of neutral red destain solution

Chemical	Amount
C ₂ H ₅ OH	10 mL
ddH ₂ O	10 mL
CH ₃ COOH, waterfree	0.2 mL

1 % N-lauroylsarcosine

Table 15: Composition of cell lysis solution 1 % N-lauroylsarcosine

Chemical	Amount
N-lauroylsarcosine sodium salt	0.551 g
PBS	55 mL

10 mM TRIS-HCl + 0.1 % BSA

Table 16: Composition of 10 mM TRIS-HCl + 0.1 % BSA

Chemical	Amount
TRIS	0.048 g
ddH ₂ O	40 mL
BSA	0.041 g
HCl [5 M & 0.1 M]	to adjust pH to 7.4-7.5

The pH of the solution was adjusted to 7.4-7.5.

ROTI®Nanoquant 5 X (acc. to protocol)

Table 17: Composition of Bradford protein reagent

Chemical	Amount
ROTI®Nanoquant	20 mL
ddH ₂ O	80 mL

The Bradford reagent was prepared by mixing 20 mL ROTI®Nanoquant with 80 mL double-distilled water.

3.6 Programs

Table 18: List of software used in this study

Programme	Manufacturer
BLAST®	National Library of Medicine, Bethesda, US
CLC Sequence Viewer 8.0	Informer Technologies Inc., Los Angeles, US
EndNote 21.5	Clarivate, London, UK
Gene9660	Bioer, Hangzhou, CHN
Goodnotes 6	GoodNotes Limited, London, UK
LinRegPCR Version 2021.2	Heart Failure Research Center, Academic Medical Center Amsterdam, NL
Lucidchart	Lucid Software Inc., Utah, US
Microsoft 365 Excel Version 16.91	Microsoft Corporation, Redmond, US
Prism 10.2.3	GraphPad Software Inc, San Diego, US
Sigmaplot 13.0	Systat Software Inc., San Jose, US
SparkControl™ Softwarei-control™ 1.6 software V 3.1 SP1	Tecan Trading AGTecan Group Ltd., Männedorf, CH
StepOne™ Software Version 2.1	Applied Biosystems™, Foster City, US

3.7 Methods used in this study

The experimental methods are broken down into individual steps in Figure 2, which depicts the schematic workflow used in the laboratory procedures of this master's thesis.

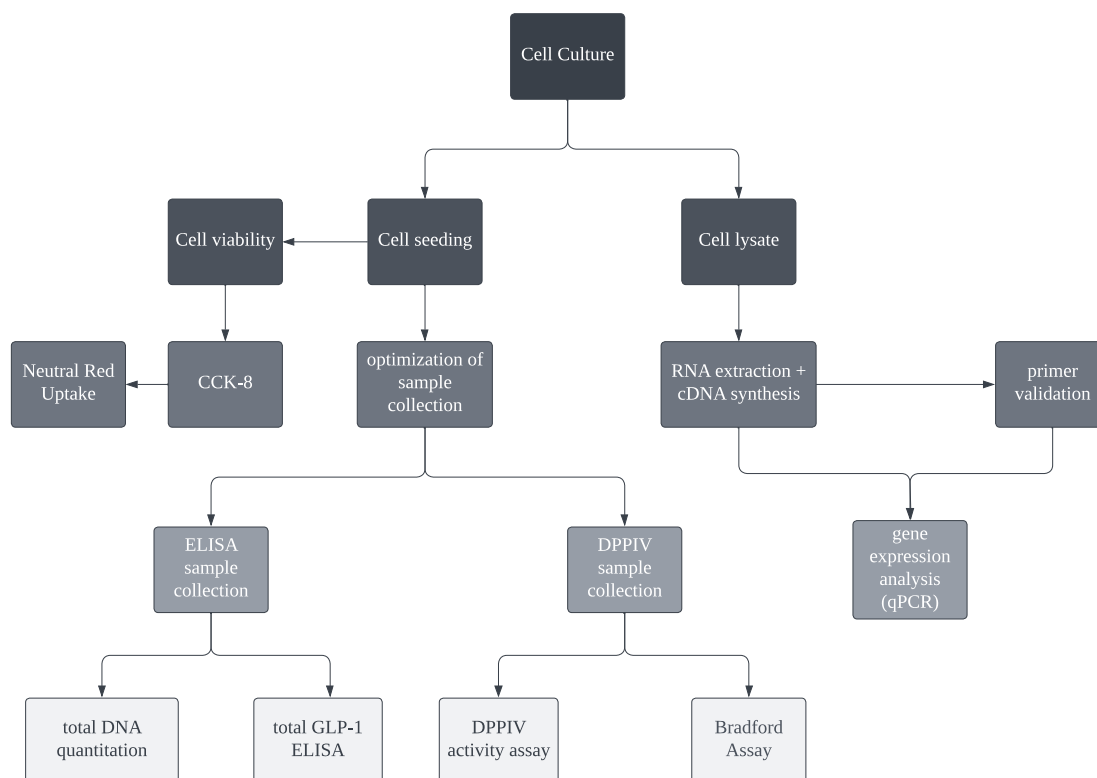


Figure 2: Schematic illustration of the workflow used.

(created with Lucidchart)

Initially, cell viability was assessed at selected concentrations of the treatments including sucrose, fructose, glucose, and mannitol, using concentrations of 50, 100, 150, 200, and 250 mM. Subsequently, gene expression levels of target genes were evaluated in cultured cells using a presence-absence design without any treatment. These untreated NCI-H716 cells served as baseline controls, while Caco-2 cells were used as a positive control for gene expression analysis. Primer validation was conducted to ensure accurate quantification of gene expression. Next, a total GLP-1 ELISA was conducted, and the results were normalized to the total DNA content. Based on the GLP-1 measurements, concentrations of 100 mM and 250 mM were selected for the DPPIV activity assay, with normalization to total protein content assessed by Bradford assay. These experiments aimed to further elucidate the complex mechanisms and interactions between GLP-1 and DPPIV.

It should be noted that the sample collection for GLP-1 and DPPIV experiments was conducted separately in accordance with the sample collection protocol, detailed in sections 3.12.2 (Sample collection of total GLP-1 secretion) and 3.13.2 (Sample collection of DPPIV activity).

3.8 Cell Culture of NCI-H716 cells

3.8.1 Thawing cells

Prior to thawing the cells, separate batches of 9 and 10 mL of + 37 °C culture medium (Gibco™ Roswell Park Memorial Institute (RPMI) 1640 (1X) + GlutaMAX™-I + 25 mM HEPES) containing 1 % penicillin/streptomycin were made in screw cap tubes. NCI-H716 (ATCC®, No. CCL-251; Manassas, US) cells were kept in liquid nitrogen at - 196 °C and were aliquoted in 10 % dimethyl sulfoxide (DMSO) as a cryoprotectant. The cryotube was thawed while swirling in a water bath at + 37 °C. Since the concentration of DMSO at + 37 °C has a harmful effect on the cells, this process needed to be done as soon as possible (Hartwig, 2009).

The cells (1 mL) were added to the 9 mL medium as soon as they had thawed, reducing the DMSO concentration to 1 % and eliminating the toxic effect. The cells were then centrifuged at + 25 °C and 120 x g for 7 minutes. The supernatant was removed and discarded. The cell pellet was resuspended with the 10 mL medium and transferred to a T25 cell culture flask for suspension cells. The bottle was stored at + 37 °C and 5 % CO₂ in the incubator.

The medium was changed after 3-4 days. To prepare for this, the medium was warmed in a water bath. An equal amount of medium, containing 1 % Pen-Strep, was prepared. The medium containing the cells was centrifuged at + 25 °C and 120 x g for 7 minutes to pellet the cells. After centrifugation, the supernatant was carefully removed, and the cell pellet was resuspended in fresh medium and transferred to a cell culture (surface: suspension, filter cap). The flask was then incubated at + 37 °C in a humidified atmosphere with 5 % CO₂ until the next splitting day.

3.8.2 Passaging cells

First, the cells were observed under a microscope before commencing any procedures. Preparation involved setting up the hood and preparing the medium. For passaging

suspension cells, medium and antibiotics were needed. The cell suspensions were collected in 50 mL screw cap tubes and were centrifuged. Setting conditions to + 25 °C, 120 x g (rcf), and spinning for 7 minutes. Meanwhile, a suitable amount of fresh media (RPMI + 10 % FBS) with antibiotics (1 % Pen-Strep) was prepared in a screw cap tube. After centrifugation the old medium was carefully aspirated by using a Pasteur pipette, without disrupting the cell pellet. The cells were then resuspended several times using 10 mL of the prepared fresh medium and a 10 mL serological pipette. Further separation of the cells was done by using a 1000 µL pipette, conducting approximately 30-35 up-and-down cycles. The cells were counted in accordance with the protocol outlined in the manufacturer's instructions. Suspension cell passaging is not based on confluency, but on the doubling time of the cells, hence the cells were split to the desired or recommended density after counting, e.g., 0.5×10^6 cells/ml for NCI-H716 to ensure optimal growth rate (ATCC®, No. CCL-251; Manassas, US).

The cell concentration of the cell suspension per milliliter was calculated using the following formula.

$$\text{cell number per mL} = \frac{\text{cell number per square}}{\text{number of squares}} * 10 * 0.4 \text{ or } 0.5$$

The multiplication by 10 accounts for the dilution process, in which 200 µL of media was combined with 50 µL of the cell suspension, resulting in a 1:5 dilution with Erythrosine Red B (0.1 % in PBS, sterile filtered). The cell number per square was obtained from hemocytometer counts and was then multiplied by the dilution factor (10) to determine cells per milliliter. Additionally, a correction factor was applied to account for cell loss during passaging: a factor of 0.4 or 0.5 was used depending on the interval between splits. Specifically, a factor of 0.4 was applied for a 2-day interval between splitting days (e.g., Monday to Thursday), and a factor of 0.5 was used for a 3-day interval (e.g., Thursday to Monday).

The desired amount of cell suspension was transferred into the new flask. Each flask was used up to two times for passaging cells and were properly labelled. The cells were put to a humidified incubator at + 37 °C with 5 % CO₂. The hood was cleaned thoroughly with 70 % EtOH, then the metal parts with 2 % Hexaquart, followed by a 1 h UV-treatment.

3.8.3 Matrigel coating of well plates

Matrigel aliquots were thawed on ice to prevent premature polymerization and maintain handling stability. Then Matrigel stock was diluted in ice-cold, sterile Hanks' Balanced Salt Solution (HBSS) to achieve a final concentration of 1 mg/ml. Throughout the procedure, well plates were kept on ice. The wells were coated with the prepared Matrigel solution using the volumes specified in Table 19.

Table 19: Specified coating volumes for cell culture plates

Culture plates		
Format	Surface area (cm ²)	Coating solution (mL)
24-well	1.96	0.25
96-well	0.32	0.05

After coating, the Matrigel was incubated at room temperature to facilitate gelation for a period of 1 hour. After gelation, double the amount of the coating solution was used to rinse the culture surface with HBSS. The last wash was then removed, and the coated wells were allowed to air-dry for 30 minutes in the laminar hood while they were left uncovered. If the plate was not immediately used, the coated surface was covered with sterile Phosphate-Buffered Saline (PBS) the same amount as HBSS, sealed with parafilm and stored in a clean plastic bag in the fridge at + 4 °C. Coated plates were used within 14 days of preparation.

3.8.4 Seeding cells for experiments

For subsequent experiments, the cell suspension was spread in 12-well or 96-well plates, depending on the assay requirements. The number of cells per well was calculated according to the specifications of the respective assay. For example, Cell Counting Kit-8 (CCK-8) and neutral red uptake (NRU) viability assays were performed in a 96-well plate and required 1×10^6 cells/well. Whereas the sample collection for GLP-1 and DPPIV were performed in a 24-well plate required 1.26×10^6 cells/well. The values required for the calculations are summarized in Table 20, which has been previously optimized in the research group.

Table 20: Volumes of calculated cell suspension per well plate

Plate type	Total volume per well
24-well plate	1 mL
96-well plate	0.2 mL

The following formula is an example for seeding 22 wells with 1 mL excess for stimulating NCI-H716 cells for GLP-1 ELISA.

$$cell\ number\ per\ mL = \frac{1.26 * 23\ mL}{counted\ cell\ number}$$

The amount of cell suspension was calculated to achieve a final concentration of 1.26×10^6 cells/mL, with 1 mL allocated per well. The seeding medium was prepared by mixing RPMI with 10 % FBS and 1 % Pen-Strep, and the cell suspension was adjusted accordingly based on this calculation. The Matrigel coated plates were either filled with 200 μ L cell suspension/well for 96-well plates or 1 mL cell suspension/well for 24-well plates. The plates were gently agitated to facilitate uniform cell distribution across the wells. After 15 minutes the plates were stored in the incubator at + 37 °C and 5 % CO₂ for a cultivation period of 24 h prior to the treatment of the cells.

3.9 Cell Counting Kit-8 viability assay

A viability assay assesses the survival or activity of cells or organisms and is used to evaluate toxicity, treatment effectiveness, or cell culture health.

3.9.1 Principle

The Cell Counting Kit-8 (CCK-8) uses Water-Soluble Tetrazolium-8 (WST-8), which generates a formazan dye when reduced by cellular dehydrogenases in the presence of an electron carrier. The amount of dye directly correlates with the number of viable cells. CCK-8 is stable and mildly cytotoxic, allowing for 48-hour incubations. This kit allows for high-sensitivity colorimetric assays, outperforming other tetrazolium salts such as MTT, XTT, MTS, and WST-1. (Sigma-Aldrich, 2018). The principle of the mechanism is shown in Figure 3.

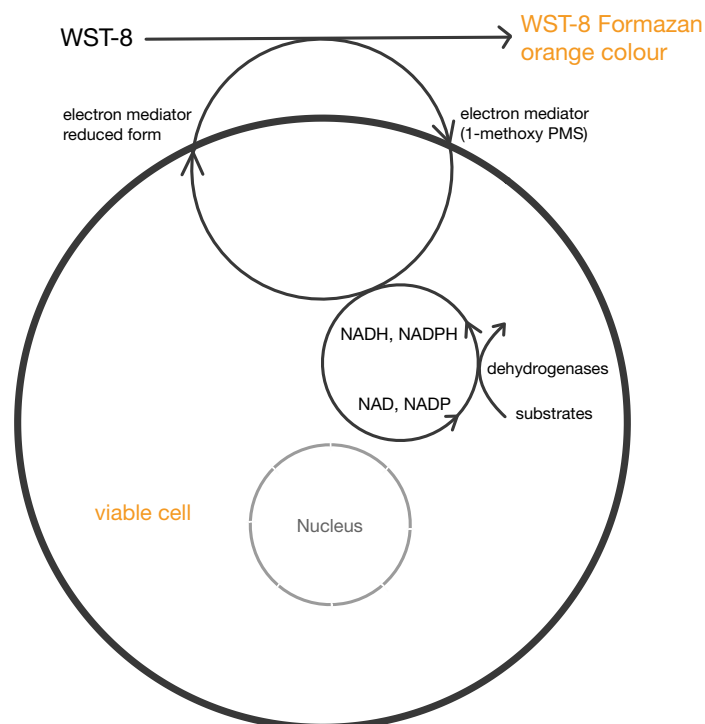


Figure 3: CCK-8- principle of detection mechanism.

(created with Goodnotes 6, based on concepts from ApexBio Technology, 2013)

As shown in the Figure 3, cellular activities produce NADH and NADPH, which converts WST-8 to an orange-colored formazan through 1-methoxy PMS. Cellular dehydrogenase activity determines how much WST-8 formazan is produced. The absorbance can be measured at 450 nm, corresponding to the peak maximum, with a reference measurement at 650 nm for each well to account for background noise.

3.9.2 Procedure

The experiment was conducted 24 h after seeding the cells in 96-well plate. The buffers Krebs-Ringer phosphate -HEPES buffer and Phosphate buffered saline (KRPH, PBS) were placed in a water bath at + 37 °C to warm up. Cell confluency was checked under the microscope and any wells with lower cell numbers were noted. The centrifuge and balance were prepared, with a 96-well plate filled with water serving as the balancing medium. The plate was then centrifuged at room temperature at 133 x g for 1 minute. After discarding the medium, each well was washed once with 200 µl of pre-warmed PBS using a multi-channel pipette. Next, 90 µl of KRPH was added to the control and starvation wells. The volumes were adjusted as necessary based on calculations to achieve treatment concentrations of 50, 100, 150, 200, and 250 mM for each of the treatment agents: mannitol, glucose, and sucrose.

After an incubation period of 1 h, the effects on cellular confluency were observed again under the microscope to assess any morphological changes or cell density variations, as this observation was necessary since the viability results were not normalized. After that, the plate was incubated with 5 % CO₂ for 1 h at + 37 °C. After incubation, 10 µl of KRPH and 10 X concentrated solutions of mannitol, glucose and sucrose at concentrations of 50, 100, 150, 200, 250 mM were added to the control and stimulation wells, respectively.

In addition, each well received 7.5 µl of CCK-8 solution, followed by a 2 h incubation period. Making bubbles was avoided since they can interfere with optical density measurements. The absorbance was measured at 450 nm using a microplate reader, referencing 650 nm for each well.

3.9.3 Data analysis

Cell viability was quantified by subtracting the mean absorbance of the blank wells from each sample, followed by normalization to the mean of the control wells containing only starvation buffer (KRPH). This value was then multiplied by 100 to calculate cell viability as a percentage of control. Outliers from three biological replicates and three technical replicates were identified using the ROUT outlier test (Q = 5%). The data were processed using Microsoft 365 Excel. Subsequent statistical analyses were conducted using GraphPad Prism.

3.10 Neutral red uptake viability assay

After observing negative results in the CCK-8 assay due to fructose reacting with the reagent (as evidenced by the fructose blanks), the subsequent step involved conducting the neutral red uptake assay.

3.10.1 Principle

The principle of the neutral red uptake assay (NRU assay) relies on the uptake and retention of the vital dye neutral red by viable cells, serving as an indicator of cellular health and viability. Cultured cells are treated with a cell-permeable dye neutral red (NR), which is taken up by healthy, active cells via endocytosis. The dye accumulates within the lysosomes of these viable cells due to their intact membrane and metabolic activity, as shown in Figure 4. Cells with compromised membrane integrity, such as damaged or dead cells, exhibit reduced uptake and retention of neutral red. The absorbance of the extracted dye

solution directly correlates with the number of viable cells present in the culture. (Repetto *et al.*, 2008)

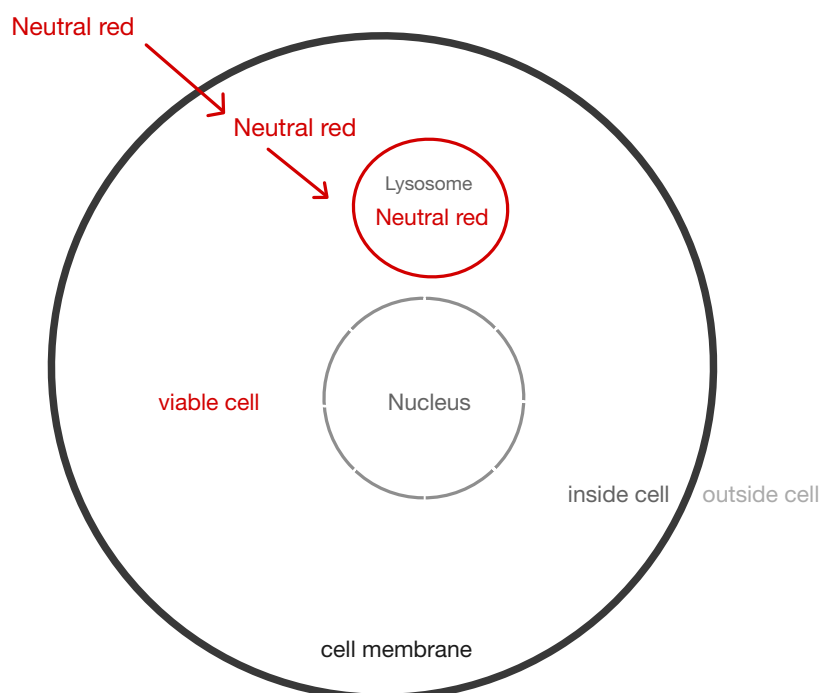


Figure 4: Neutral red uptake assay principle.

Living cells incorporate neutral red into lysosomes, and the dye's accumulation reflects cell viability. (created with Goodnotes 6)

Living cells absorb neutral red, which is fixed in lysosomes at an acidic pH. The absorbance can be measured at 540 nm (Repetto *et al.*, 2008), corresponding to the peak maximum.

3.10.2 Procedure

The protocol closely followed the procedure described in section 3.9.2, particularly the initial incubation steps. However, the subsequent steps after the 2 h fructose treatment differed. Unlike the CCK-8 assay, where the reagent is directly added to the treatment followed by a 2-hour incubation, this approach was not applied here. Instead, the procedure was performed according to Repetto *et al.* (2008), as this method is specifically optimized for the detection of cytotoxicity using the neutral red uptake assay. The variation reflects methodological differences between the two assay systems and their respective detection principles.

In the neutral red protocol, warm neutral red medium was prepared (see composition in chapter 3.5, Table 13) and then centrifuged at 600 x g for 10 minutes to remove any precipitated crystals. The centrifuged neutral red medium was then filtered through a filter

paper (Whatman™ Filter paper, Qualitative Diameter 125 mm, 100 circles). The medium was subsequently removed from the wells, and 100 µL of neutral red medium was pipetted into each well. The plate was then incubated for another 2 hours in the incubator at + 37 °C with 5 % CO₂. Afterwards, the neutral red medium was removed from the wells. Next, 150 µL of unsterile PBS was pipetted into each well, and the plate was gently shaken before removing the PBS. Then, 150 µL of neutral red destain solution was pipetted into each well, followed by confluence check. The plate was shaken on a plate shaker for 10 minutes. Finally, absorption at 540 nm was measured using a Tecan plate reader.

3.10.3 Data Analysis

Cell viability was quantified by subtracting the mean absorbance of the blank wells from each sample, followed by normalization to the mean of the control wells containing only starvation buffer (KRPH). This value was then multiplied by 100 to calculate cell viability as a percentage of control. Outliers from three biological replicates and three technical replicates were detected using ROUT outlier test (Q = 5 %). The data were processed using Microsoft 365 Excel. Subsequent statistical analyses were conducted using GraphPad Prism.

3.11 Gene expression using presence-absence design

Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR) is a method for amplifying and detecting gene expression. First, RNA is extracted from the sample and transformed to cDNA with reverse transcriptase. During amplification, an intercalating dye labels the DNA for quantification of the product (Adams, 2020). A presence/absence strategy for qPCR in NCI-H716 and Caco-2 cells, in conjunction with NTC (No Template Control), RT+ (Reverse Transcription positive), and RT- (Reverse Transcription negative) controls, was performed.

3.11.1 Sample collection

A sample from the cultured NCI-H716 cells was taken for RNA isolation. Three biological replicates from different passages were collected. After counting the cells with fresh cell medium, 1.5 mL of the cell suspension was transferred to a microreaction tube. The sample was then centrifuged at room temperature at 500 x g for 1 minute. The supernatant was aspirated, and the cell pellet was snap-frozen in liquid nitrogen before being stored at -80 °C until RNA isolation was performed.

3.11.2 RNA isolation

The Monarch® Total RNA Miniprep Kit from New England Biolabs® GmbH was used to isolate the RNA. RNase-free micro reaction tubes were used for all subsequent steps and work surfaces, equipment and materials were cleaned with RNase-away. The samples were thawed at room temperature. The cell lysates were purified according to the manufacturer's instructions. All centrifugation steps were performed at RT and 16000 x g.

3.11.3 Determination of RNA concentration

To determine the RNA concentration, the absorbance was measured at 230 nm, 260 nm and 280 nm using the Tecan Spark plate reader. To determine the blank value, 2 µL of nuclease-free water (NFW) was first applied in duplicate to a NanoQuant® plate and the absorbance was measured. Following purification, 2 µL of each sample was applied in duplicate. The RNA concentration was determined in ng/µL after subtracting the blank value using absorbance measurements at specific wavelengths (230 nm for organic compounds, 260 nm for nucleic acids, and 280 nm for proteins). The A_{260}/A_{280} and A_{260}/A_{230} ratios were assessed for sample purity. Optimal purity was indicated by A_{260}/A_{280} ratios between 1.8 and 2.0, and A_{260}/A_{230} ratios > 1.8. Values outside these ranges indicate impurities from proteins, phenols, or residual solvents (Paola Cruz-Tapias, 2013). If the values were within the desired range, cDNA synthesis was carried out.

3.11.4 cDNA synthesis

For RT-qPCR, cellular mRNA was converted to cDNA for stability using reverse transcriptase, an RNA-dependent 5' → 3' DNA polymerase. The LunaScript® Reverse Transcription SuperMix Kit from New England Biolabs® was used for cDNA synthesis, following to the manufacturer's protocol. Sample volumes were calculated based on RNA concentrations to achieve 1 µg of RNA. In a 96-well plate (PCR plate, Biozym Scientific GmbH), the sample volume was adjusted to 16 µL with nuclease-free water. LunaScript® RT SuperMix or No-RT Control Mix (for assessing genomic DNA contamination) was added to each well. Details of the synthesis preparations can be found in Table 21.

Table 21: Synthesis approaches cDNA C1000 Touch™ thermal cycler

synthesis approach	sample [μL]	NFW [μL]	LunaScript RT Supermix [μL]	No-RT Control Mix [μL]
with reverse transcriptase	variable, volume, $C_{\text{final}} = 1 \mu\text{g}$ needed	variable, 16 - sample volume	4	-
w/o reverse transcriptase	variable, volume, $C_{\text{final}} = 1 \mu\text{g}$ needed	variable, 16 - sample volume	4	-

The 96-well plate was sealed with parafilm and centrifuged at 1000 x g for 1 minute. The cellular RNA was then transcribed into cDNA in the C1000 Touch™ Thermal Cycler. The cycle steps of the program used are presented in Table 22. At the end of the program, the plate was centrifuged again for 1 minute at 1000 x g and stored at - 20 °C until further processing of the samples. Before performing RT-qPCR, 10 μL of the resulting cDNA was diluted with 90 μL NFW (1:10) in a fresh micro reaction tube.

Table 22: Temperature program cDNA synthesis C1000 Touch™ thermal cycler

Cycle steps	Duration [min]	Temperature [°C]
Primer annealing	2	25
cDNA synthesis	10	55
Heat denaturation	1	95

3.11.5 Primer validation

Primers for gene expression analysis were ordered from Sigma-Aldrich for subsequent RT-qPCR. The primer sequences were taken from publications (e.g., Gu *et al.*, 2007) and are listed in Table 23 along with other properties. In this subchapter, sucrose isomaltase (*SI*) was selected as a representative target gene to exemplify the primer validation process described in this section.

Table 23: Properties of the used *SI* primers

Gene	Sequence (5' → 3')	Length [bp]	T _m [°C]	nmol
<i>h_SI</i>	FW: CATCCTACCATGTCAAGAGCCAG	23	66.7	52.1
	RV: GCTTGTTAAGGTGGTCTGGTTT	22	63.1	45.4

The primers were diluted with NFW according to the manufacturer's instructions so that solutions with a concentration of 100 µM were available. From this starting solution, 5 µL were taken and diluted with 45 µL NFW to a 10 µM working solution. The working solutions were stored at - 20 °C until use.

To validate the primers, RT-qPCR was performed to test whether expression of the corresponding gene can be observed at a primer concentration of 200 nM. For this purpose, a master mix was prepared for a sample and non-template control. This consisted of the Luna® Universal RT-qPCR Master Mix from New England Biolabs®, the primer pairs and NFW (see Table 24). A cDNA sample of Caco-2 cells (CLS Cell Lines Service GmbH, Eppelheim, DE; passage 22 in DMSO) was used as a positive control to validate the *SI* primer. Isolation of RNA and synthesis of cDNA were performed as described for NCI-H716 cells.

Table 24: Master mix qPCR C1000 Touch™ thermal cycler

Components	Volume [µL]	<i>SI</i> _C _{final}
Luna® Universal qRT-PCR 2x Master Mix	35.4	1x
Primer FW (10 µM)	0.7	200 nM
Primer RV (10 µM)	0.7	200 nM
NFW	26.9	

In micro-reaction tubes the reaction mix was prepared, 27.9 µL of the master mix were mixed with 3.3 µL of the sample or NFW for the control. Subsequently, 10 µL of the reaction mixtures were applied in triplicates to a 96-well plate. The plate was sealed with a foil and centrifuged at + 4 °C, 1000 x g and 1 minute. Gene expression was then measured in the StepOnePlus™ according to the temperature program in Table 25.

Table 25: Temperature program RT-qPCR in StepOnePlus™

Cycle steps	Temperature [°C]	Time [s]	Cycles
Initial Denaturation	95	60	1
Denaturation	95	15	45
Elongation	60	30	
Melting curve	60-95		1

LinRegPCR was used to evaluate the measured data. As the primers showed a PCR efficiency > 1.8, a further PCR was carried out in the C1000 Touch™ Thermal Cycler. For this purpose, a reaction mixture was prepared from OneTaq® Hot Start 2x Master Mix with Standard Buffer from New England Biolabs®, the primer pairs, the sample and NFW. The exact composition can be found in Table 26.

Table 26: Reaction mix qPCR C1000 Touch™ thermal cycler primer validation

Components	Volume [μL]
OneTaq® Hot Start 2x Master Mix with standard buffer	13.75
Primer FW	0.56
Primer RV	0.56
Sample	2.75
NFW	9.89

From the reaction mixture, 25 μL were transferred to a 96-well plate well and sealed with foil. The C1000 Touch™ Thermal Cycler was used to run the temperature program indicated in Table 27. The primer annealing temperature for the PCR cycle was determined using the primer sequences and the T_m Calculator (Version 1.16.5) from New England Biolabs®. The duration of the elongation time during PCR is determined by the length of the amplicon (196 bp), which was calculated using CLC Sequence Viewer 8.0 software.

Table 27: Temperature program for *SI* qPCR C1000 Touch TM thermal cycler

Cycle steps	Temperature [°C]	Time [s]	Cycles
Initial Denaturation	94	30	1
Denaturation	94	15	45
Primer Annealing	52	40	
Elongation	68	20	
Final Extension	68	300	1

The generated amplicons were purified using the Monarch® PCR & DNA Cleanup Kit (5 µg) from New England Biolabs ®. All centrifugation steps were carried out at room temperature and 16000 x g for one minute. 25 µL of sample were mixed with 125 µL of Cleaning Buffer in a micro reaction tube by pipetting up and down several times. Then the mixture was pipetted onto a column with a suitable collection tube. The sample was centrifuged and the flow-through discarded. This was followed by two wash steps in which 200 µL of DNA Wash Buffer was added and the sample was centrifuged. The column was placed on a micro reaction tube and incubated with 14 µL DNA Elution Buffer for one minute at RT. After centrifugation, the RNA concentration and purity were determined analog to the determination of the RNA concentration during the sample preparation for cDNA synthesis (see chapter 3.11.3). The sample was diluted with NFW, resulting in duplicates of a 15 µL solution containing 1 ng/µL DNA. To one solution 2 µL of the forward primer and to the other 2 µL of reverse primer were added. The micro reaction tubes were barcoded and delivered to Eurofins Genomics using the TubeSeq service. Sanger sequencing of the primers was done there. The results were confirmed with Blast (NCBI) and CLC Sequence Viewer 8. Because the sequences acquired and the target genes were identical, the primers could be used for RT-qPCR.

3.11.6 RT-qPCR

A presence/absence qPCR approach was conducted on NCI-H716 cells, with Caco-2 cells serving as a positive control, alongside NTC (No Template Control), RT+ (Reverse Transcription positive), and RT- (Reverse Transcription negative) controls. This approach was used to determine whether specific gene transcripts are constitutively present or absent in NCI-H716 cells under basal culture conditions.

Controls are essential for validating qPCR results: the NTC control, which contains neither cDNA nor gDNA, ensures no contamination or nonspecific amplification; the RT+ control, containing cDNA synthesized from RNA, confirms actual gene expression; and the RT- control, which includes RNA but no reverse transcriptase, verifies that the signals aren't due to contaminating genomic DNA (Paola Cruz-Tapias, 2013).

Other primers like *GAPDH* (Walker *et al.*, 2017), *DPP4* (Hernández *et al.*, 2017) and *SLC2A1* (Zopun *et al.*, 2018) which are listed in the Table 28 for RT- qPCR have already been validated in previous studies. The *SI* (Gu *et al.*, 2007) primer pairs were diluted with nuclease free water to a concentration of 100 μ M according to the manufacturer's instructions. The working solutions were stored at -20 °C until use. Since the initially used primer for *SLC2A5* did not show any detectable expression, a commercially available, validated primer mix from Bio-Rad was ordered to verify the result. The *SLC2A5* primer mix was delivered at room temperature by postal service and subsequently stored at + 4 °C until use.

Table 28: Primer sequences and concentrations of the primers used

Protein	Primer	Sequence	C _{final} [nM]	Reference
Glyceraldehyde 3-phosphate dehydrogenase	<i>GAPDH</i>	FW: AGGTCGGAGTCAACGGATTTG	200	(Walker <i>et al.</i> , 2017)
		RV: GGGGTCATTGATGGCAACAATA		
Dipeptidyl Peptidase-4	<i>DPP4</i>	FW: AGTGGCGTGTTCAAGTGTGG	100	(Hernández <i>et al.</i> , 2017)
		RV: CAAGGTTGTCTTGTGGAGTTGG		
Sucrose Isomaltase	<i>SI</i>	FW: CATCCTACCATGTCAAGAGCCAG	200	(Gu <i>et al.</i> , 2007)
		RV: GCTTGTTAAGGTGGTCTGGTTT		
Glucose Transporter 1	<i>SLC2A1</i>	FW: ATTGGCTCCGGTATCGTCAAC	200	(Zopun <i>et al.</i> , 2018)
		RV: GCTCAGATAGGACATCCAGGGTA		
Glucose Transporter 5	<i>SLC2A5</i>	FW: n/a	n/a	Bio-Rad
		RV: n/a		

For RT-qPCR, samples and primers were thawed on ice. A master mix was prepared using Luna® Universal qRT-PCR Master Mix from New England Biolabs®, nuclease-free water and the respective primer.

Table 29 shows the diverse master mix preparations calculated by the StepOnePlus™ software. For example, for gene *DPP4* (100 nM) = 21 reactions + 15 % excess, for the reference gene *GAPDH* (200 nM) = 39 reactions + 15 % excess, and for the genes *SI* and *SLC2A1* (200 nM) = 39 reactions + 10 % excess. The genes (*GAPDH*, *DPP4*, *SI*, *SLC2A1*) were prepared analogously, considering the final concentrations. Due to limitations in fitting all reactions and samples per gene within a 96-well plate, the remaining samples/reactions were distributed across additional plates according to the correct calculated volume of reactions and subsequently replicated.

Table 29: Master mix calculations according to StepOnePlus™ software

Components	<i>DPP4</i> V [μL] C _{final} = 100 nM	<i>GAPDH</i> V [μL] C _{final} = 200 nM	<i>SI & SLC2A1</i> V [μL] C _{final} = 200 nM
Luna® Universal qRT-PCR Master Mix	120.8	224.2	224.2
NFW	91.8	161.5	161.5
Primer FW	2.4	9.0	9.0
Primer RV	2.4	9.0	9.0
Total Reaction Mix Volume	217.4	403.6	403.6

39 reactions per gene, including one NTC sample, 3 samples of NCI-H716 cells each (±) and 3 samples of Caco-2 cells each (±). These were applied as technical triplicates, i.e. $6 + 6 = 12 \rightarrow 12 * 3 = 36$ and the NTC sample also as a technical triplicate, resulting in 39 reactions.

Table 30 shows the Reaction Mix for the validated primer mixture (200 x 20 μL reaction) of *SLC2A5* + 10 % excess.

Table 30: Master mix for *SLC2A5*

Components	V [μ L] C _{final} = 200 nM
2 x Luna® Universal qRT-PCR Master Mix	224.2
NFW	134.6 + 22.4 = 157
Primer FW + RV	22.4
Total Reaction Mix Volume	403.6

29.7 μ L of the diverse master mixes were transferred to micro reaction tubes with a save lock and 3.3 μ L of the respective sample or nuclease free water (NTC_sample) were added. After mixing and brief centrifugation, 10 μ L of the reaction mixtures were applied in triplicates to a 96-well plate kept on ice. The plate was sealed and centrifuged at + 4 °C for 1 minute at 1000 x g. Subsequently, the plate was measured in the BIOER Linegene_17201 qPCR instrument using the Gen9660 software, following the temperature program listed in Table 31. Based on trends from prior experimental results, the number of amplification cycles for the third biological replicates (*SI* primer pairs of NCI-H716 p 8 $\pm$ and Caco-2 passage 14 $\pm$) was decreased from 45 to 40.

Table 31: Temperature program Gen9660 software

Cycle steps	Temperature [°C]	Time [s]	Cycles
Initial Denaturation	95	60	1
Denaturation	95	15	40 - 45
Elongation	60	30	
Melting curve	60 - 95		1

The measurement was performed in the presence/absence design, where all samples were uniformly treated with the forward and reverse primer. The absence of detectable gene expression in the RT- samples indicates that any genomic DNA was present only at levels below the detection limit. Consequently, the DNA digestion process, conducted following the established RNA isolation protocol, was considered as successful.

The Caco-2 cells were used as a positive control, whereby the cell lysate of the Caco-2 cells was produced by a colleague of the working group as part of another project and was kindly

made available for this study, to verify the functionality of the primers and the PCR reaction.

3.11.7 Data analysis

The data was transported from the Gen9660 software to an Excel file. Afterwards the ratio (Rn) of SBYR/ROX was calculated and the remaining data was put into an appropriate form for LinReg, namely WELL/CYCLE/TARGET/Rn. The baseline was determined, and the analyzed data was saved to the Excel file. Then the data, which was created by LinReg was checked according to the listed parameters (Nolan *et al.*, 2006) shown in Table 32.

Table 32: Data check qPCR data

Analytical parameters	Target data
PCR eff.	> 1.8
R ²	≥ 0.99
NTC	opt. = 0 or very high cycle number, similar as RT (-)
RT+ to RT-	≥ 5 cycle difference RT+ to RT-

Further statistical analyses were performed using Excel to determine the average (AV), standard deviation (SD), and relative standard deviation (RSD) of the N0 (mean efficiency) values. Outliers were identified and excluded when their RSD exceeded 15 %. The relative gene expression was then determined by dividing the mean N0-value of the target gene by the mean N0-value of the reference gene, as described in the following formula:

$$relative\ gene\ expression = \frac{target\ gene}{ref.\ gene}$$

GraphPad Prism was used to analyze the outliers (ROUT, Q = 5 %) of the biological replicates and to present the data in a graphic.

3.12 Determination of total GLP-1 secretion by sandwich ELISA

3.12.1 Principle

The assay is based on a Sandwich ELISA technique to quantify total GLP-1 levels. In this method, a microtiter plate is pre-coated with a capture antibody, an anti-GLP-1 polyclonal antibody, that binds specifically to GLP-1 molecules present in the samples. After adding

the samples, unbound components are washed away. Next, a biotinylated anti-GLP-1 monoclonal antibody, acting as the detection antibody, binds to the GLP-1 molecules already captured on the plate. Unbound detection antibodies are removed through a second wash step. Horseradish peroxidase (HRP) is then conjugated to the immobilized biotinylated antibodies. After washing away any free enzyme conjugates, the HRP activity is measured in the presence of the substrate 3,3',5,5'-tetramethylbenzidine (TMB). HRP catalyzes the conversion of TMB, producing a colorimetric change. The enzyme activity is determined spectrophotometrically by measuring the rise in absorbance at 450 nm, corrected for absorbance at 590 nm, following acidification of the generated products. Since the amount of GLP-1 in the sample is proportional to the increase in absorbance, its concentration is determined by comparison to a standard curve generated from known GLP-1 standards (Merck, 2013). The assay measures total GLP-1, including both cleaved and uncleaved forms, which should be carefully considered during data analysis and interpretation.

3.12.2 Sample collection

NCI-H716 cells were seeded at 1.26×10^6 cells/well onto Matrigel-coated 24-well plates and incubated at + 37 °C with 5 % CO₂ for 24 hours. Cell confluence was observed under a microscope, noting any wells with lower cell density. The plate was then centrifuged at room temperature for 1 minute at 133 x g after 24 h of cultivation. After removing the medium, cells were washed once with 1 mL of pre-warmed PBS. Next, 100 µl of KRPH buffer (for control wells) or 10 X concentrated treatment solutions (sucrose, fructose, glucose, mannitol) were added to the appropriate wells, adjusting volumes when treatments were not precisely 10 X concentration. The plate was incubated for 2 h at + 37 °C with 5 % CO₂ for stimulation.

The supernatant was collected and centrifuged at 1000 x g, + 4 °C for 5 min to remove cell debris. The clarified supernatant was transferred to the fresh new micro reaction tubes (500 µL each) for long term storage at - 80 °C. The remaining cell pellets were lysed by adding 840 µl of PBS and 60 µL of 1 % N-lauroylsarcosine solution per well, ensuring thorough resuspension. The plate was sealed with parafilm, placed in a clean plastic bag, and stored at - 20 °C until DNA measurement using Tecan equipment.

3.12.3 GLP-1 ELISA assay

The total GLP-1 content of the supernatants was determined using Merck's total GLP-1 ELISA (MERCK, Cat #EZGLP1T-36K Rev. 8-May-17). The assay was carried out in accordance with the manufacturer's instructions to maintain standardized procedures and ensure experimental consistency. Key components emphasized in the instructions were the preparation of blank samples, the creation of a standard series, the specific concentration of the reconstituted standard (1.149 pm), and the method of sample application. The reconstituted standard was used to create the standard curve, which was applied to both the initial plate and the second ELISA plate. After use, the reconstituted standard was sealed with parafilm, placed in a clean plastic bag, and stored in the freezer at -20 °C for preservation until needed for the next run.

3.12.4 Determination of total DNA content

A crucial normalization step and a method for estimating the number of cells in a sample is to determine the total DNA content. The frozen 24-well plates were thawed at room temperature. Blanking was done with 2 µL PBS. Shortly before application, the samples were properly resuspended and applied as technical duplicates with 2 µL of the suspension. If the RSD of the technical duplicates was greater than 10 %, samples were remeasured.

3.12.5 Data analysis

SigmaPlot 13.0 software was used to analyze data from the GLP-1 determination. A sigmoidal 4-parameter logistic function was created from the data of the standards. The GLP-1 concentrations of the samples were calculated using the formula below.

$$y = \min - \frac{(\max - \min)}{1 + \left(\frac{x}{EC_{50}}\right)^{-Hillslope}}$$

The measured absorbances of the standard series were used to establish a standard curve. The corresponding linear equation was set up to determine the GLP-1 content of the samples. The calculated GLP-1 concentrations of the samples were set in relation to the corresponding DNA content (ng/µL $\triangleq$ µg/mL), allowing for representation of values as GLP-1 content in pM per µg DNA.

Data analysis was performed in GraphPad Prism, where outliers within the biological replicates were identified using the ROUT method (Q = 5 %). Statistical significance

among the treatment groups, including control and mannitol as an osmotic control, was assessed using one-way ANOVA followed by Tukey's test for multiple comparisons. A line graph was then generated to provide a more compact representation of the GLP-1 secretion, with statistical significance determined at $p < 0.05$. Data processing and graphing were conducted using Microsoft 365 Excel and GraphPad Prism.

3.13 Determination of DPPIV activity

3.13.1 Principle

The DPPIV-Glo™ Protease Assay uses luminescence to measure dipeptidyl peptidase IV (DPPIV) activity. The assay uses a luminogenic substrate, Gly-Pro-aminoluciferin, in a buffer optimized for DPPIV and luciferase activity. By adding the DPPIV-Glo™ Reagent, the substrate is cleaved, generating a luminescent signal that directly correlates with DPPIV activity (Promega_Corporation, 2015), as seen in Figure 5.

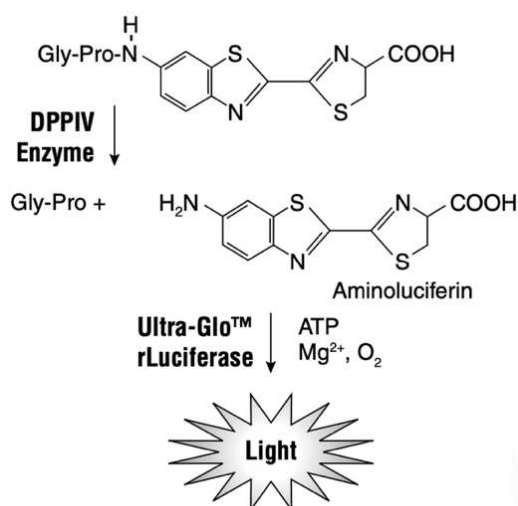


Figure 5: Principle of the reaction mechanism for the determination of DPPIV activity.

The luminogenic substrate, which contains the Gly-Pro sequence recognized by DPPIV, is cleaved by the enzyme, releasing aminoluciferin. This substrate then serves as a target for luciferase, leading to a reaction that produces luminescence (Promega_Corporation, 2015).

3.13.2 Sample collection

The treatment concentrations for DPPIV analysis were chosen after comparing each concentration to the mannitol control using ELISA results. The protocol followed was identical to that outlined in Section 3.12 (Determination of GLP-1 secretion by sandwich ELISA), specifically in Section 3.12.2 (Sample collection), first paragraph. However the procedure used after the two-hour incubation with the treatments differed.

The supernatant was carefully removed and discarded. Subsequently, 1 mL of cold PBS was added to each well to rinse and remove any residual treatment, followed by aspiration. Next, 200 μ L of a 10 mM Tris-HCl solution containing 0.1 % BSA (pH 7.4–7.5) was pipetted into each well, subsequently referred to as lysis buffer. The 24-well plate was then placed on ice for 1.5 hours at + 4 °C to facilitate the liquefaction of the Matrigel. After this incubation, a 2 mL disposable syringe fitted with a cannula containing the liquefied Matrigel, cells, and lysis buffer (10 mM Tris-HCl) was used to lyse the cells. The sample was subjected to 10 up-and-down mixing cycles to ensure proper homogenization. The cell lysate was collected into microreaction tubes and centrifuged at + 4 °C for 10 minutes at 16000 x g, yielding a pellet of cell debris and a supernatant containing solubilized DPPIV proteins. The supernatant was then transferred to two new micro reaction tubes, with the volume divided in half, while the cell debris pellet remained. Finally, the microreaction tubes were stored at - 20 °C until the analysis was carried out.

3.13.3 DPPIV activity assay

Assay was performed according to the manufacturer's protocol (Promega_Corporation, 2015). Before performing the assay, the kit components were warmed to room temperature. Then the DPPIV Glo substrate was diluted with ddH₂O to produce a 10 mM starting solution. Similarly, the luciferin detection reagent was reconstituted with 10 mL of DPPIV Glo buffer. To prepare the DPPIV Glo reagent, DPPIV Glo substrate and luciferin detection reagent were mixed in a 1:500 ratio and incubated for 60 min at room temperature. During the incubation period, the other substances required for the assay were prepared. 1 μ L of DPPIV enzyme was withdrawn under sterile conditions and transferred to a microreaction tube. The enzyme had an initial concentration of 0.2 μ mol. To achieve a final concentration of 1 ng/mL in the well, the enzyme was diluted accordingly with lysis buffer. However, since the enzyme was added to the well in a 1:3 dilution, a stock solution with a concentration of 3 ng/mL was prepared. After the incubation period, a white microtiter plate was pipetted according to the scheme in Table 33 and the samples were applied in duplicate.

Table 33: Composition of reaction mixtures for DPPIV activity the determination

	DPPIV Glo reagent [μL]	Tris buffer [μL]	diluted DPPIV enzyme [μL]	sample [μL]
Blank	50	50	-	-
Positive Control	50	20	30	-
Sample	50	30	-	20

The microtiter plate was then placed on a plate shaker (Microplate Shaker, PMS-1000i) for 30 seconds at 400 rpm to ensure that the solutions in the wells were mixed. The plate was then incubated for 30 min at RT. The luminescence signal was measured using the Flex Station III plate reader, following the manufacturer's instructions.

3.13.4 Determination of total protein content

Prior investigations assessed the effects of Matrigel and ensured that signal overlap was ruled out. Additionally, these effects were measured in relative terms rather than absolute values.

DPPIV activity was measured and normalized to the protein concentration of the cell lysates, as determined by the Bradford assay. Protein quantification is an essential step for data normalization. The same microreaction tube was used for both the DPPIV activity assay and the protein measurement.

The principle of the Bradford assay is based on the binding of the dye Coomassie Brilliant Blue G250 to the basic amino acid residues of the cell proteins. The binding takes place in an acidic environment. The color changes from red brown at 470 nm to blue at 595 nm. This is determined by photometric measurement, whereby the absorbance and protein content of the sample correlate proportionally to each other (Bradford 1976).

ROTI® Nanoquant solution is a modification to Bradford's protein test. There are three different states of Coomassie Brilliant Blue Dye-G250, each with a distinct absorption wavelength as seen in Figure 6.

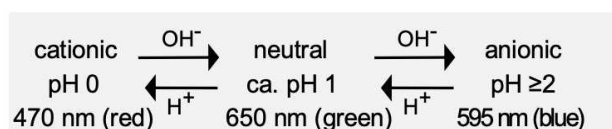


Figure 6: Absorbing states of CBBG 250

(Carl_Roth, 2021).

The dye changes from a cationic to an anionic state upon binding to a protein, with an absorption peak occurring at 595 nm, which is directly proportional to the concentration of the protein. This method, initially described by Bradford, is most effective when measuring against a calibration curve using BSA, as the dye primarily binds to basic amino acids.

Reproducible protein concentrations up to 200 ng (200 μ l with $c = 1$ μ g/ml in cuvette assays or 50 μ l with $c = 4$ μ g/ml in 96-well culture plates) can be analyzed in aqueous solutions with ROTI®Nanoquant. Assay was performed according to (Carl_Roth, 2021). The samples were diluted in accordance with the guidelines, as a larger protein concentration in the samples was expected, a 1:40 dilution with sterile PBS was selected. Then the samples were measured at 590 nm and 450 nm, with linearity determined by the quotient OD590/450.

3.13.5 Data analysis

To analyze the DPPIV assay results, the blank was removed from the raw data, and the positive control displayed the highest luminescence signal. The average values of the replicates were determined. A standard curve was established using the measured absorbances of the standard series to determine the protein content of the samples. The corresponding standard curve was established to determine the protein content of the samples and the dilution factor of 40 was considered. The luminescence values obtained were related to the corresponding protein content so that the results were presented as DPPIV activity per mg protein. This data was again related to the respective control (KRPH only). Statistical significance was determined using a one-way ANOVA. Outliers were identified using ROUT ($Q = 5\%$). The data was processed using Microsoft 365 Excel and GraphPad Prism.

4 Results

4.1 Cell viability assays

To investigate the effects of various substances on cell viability, both the CCK-8 and a neutral red uptake assay were performed. Sucrose, glucose, fructose and mannitol were tested at concentrations of 50, 100, 150, 200, and 250 mM. Mannitol served as an osmotic control. Initial results from the CCK-8 assay indicated that fructose reacted with the CCK-8 reagent. Consequently, the NRU assay was selected as a more reliable method for evaluating cell viability in the presence of fructose.

Figure 7 shows the effects of sucrose, glucose, mannitol, and fructose (50-250 mM) on NCI-H716 cell viability after a 2-hour incubation, determined by using CCK-8 (A-C) and NRU (D) assays. Cell viability is expressed as a percentage of the control. The dashed line represents the cutoff value of 70 %, and values above this threshold were considered acceptable for further analysis, as defined by ISO 10993-5:2009 (International Organization for Standardization, 2009).

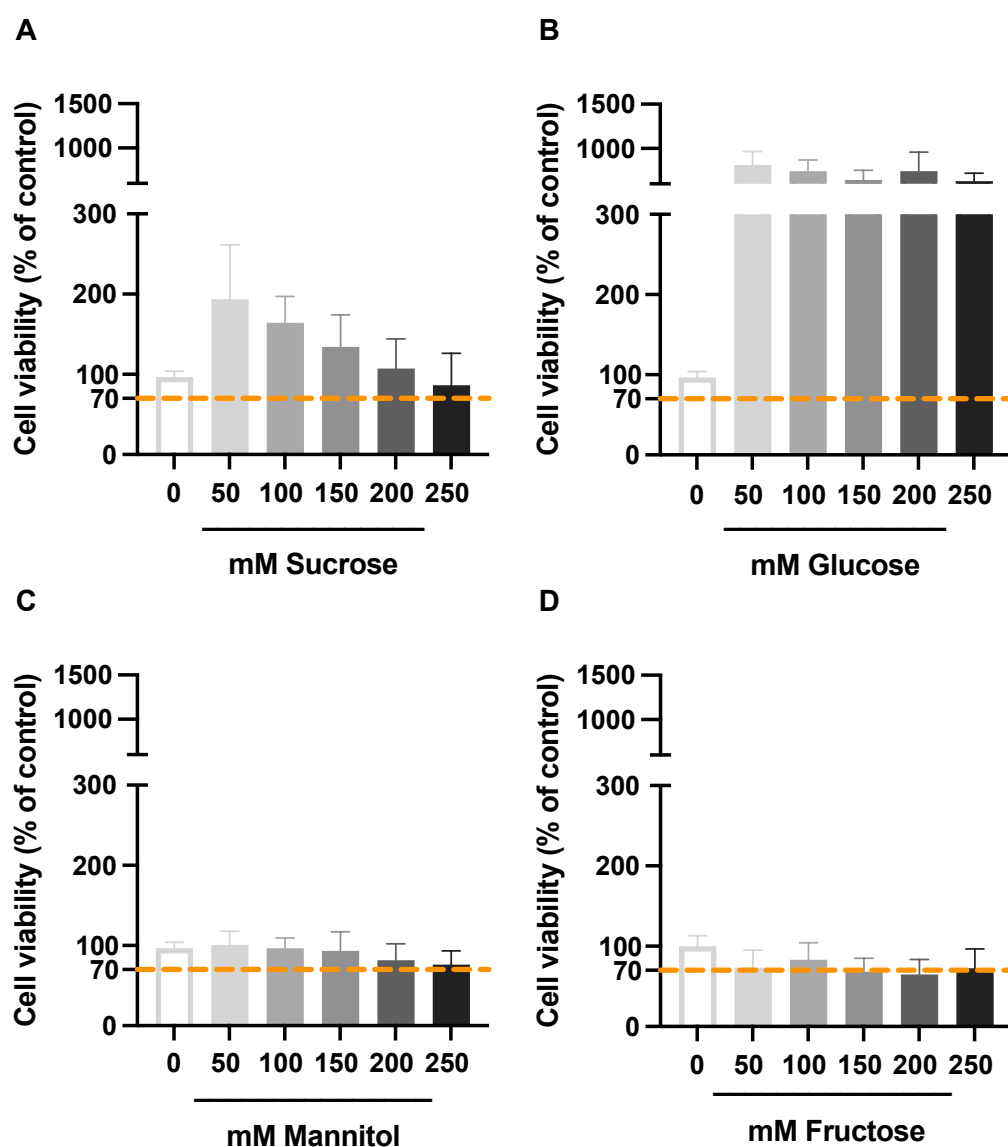


Figure 7: Cell viability of NCI-H716 cells.

Percentage of control cell viability of NCI-H716 cells compared to the control (= 0 mM) after 2 hours of incubation with 50 mM, 100 mM, 150 mM, 200 mM, and 250 mM of sucrose, glucose, mannitol, and fructose. Graphs A-C present results from the CCK-8 assay, while Graph D shows results from the NRU assay. Values are expressed as percentage of the control and presented as mean + SD (biological replicates = 3, technical replicates = 3). The dashed line represents the cutoff value of 70 %. Outliers were identified using ROUT (Q = 5 %).

In summary, the 2-hour incubations with sucrose, glucose, fructose, and mannitol at various concentrations showed no negative effects on cell viability compared to the KRPH control. A cutoff threshold of 70 % cell viability, as indicated by the dashed line in the figure, was used to evaluate cytotoxicity. Except for the sucrose treatment, none of the other treatments exhibited a dose-dependent effect on cell viability. Although cell viability in the 150 mM and 200 mM fructose groups was just below the 70 % threshold, the mean value for the 250 mM group remained above it. Therefore, the entire concentration range of each substance was included in subsequent GLP-1 secretion experiments.

4.2 Gene expression (presence/absence design)

RT-qPCR was conducted to determine the genes present in the NCI-H716 cell line that are involved in the metabolism of glucose, sucrose and fructose. For this purpose, the genes *SI*, *SLC2A5*, *SLC2A1* and *DPP4* were analyzed. *GAPDH* was used as a reference gene because of its consistent and stable expression across tissues and experimental conditions, making it a reliable internal control for normalization in gene expression experiments (Kozera & Rapacz, 2013). The results of the RT-qPCR for *SI*, *SLC2A1* and *DPP4* show that the investigated genes were detectable in NCI-H716 cells, and the Caco-2 cell line served as a positive control.

Figure 8 shows the relative gene expression of *SI*, *SLC2A5*, *SLC2A1* and *DPP4*, presented as the average N0_(mean efficiency) of each target gene normalized to the average N0_(mean efficiency) of the reference gene *GAPDH*.

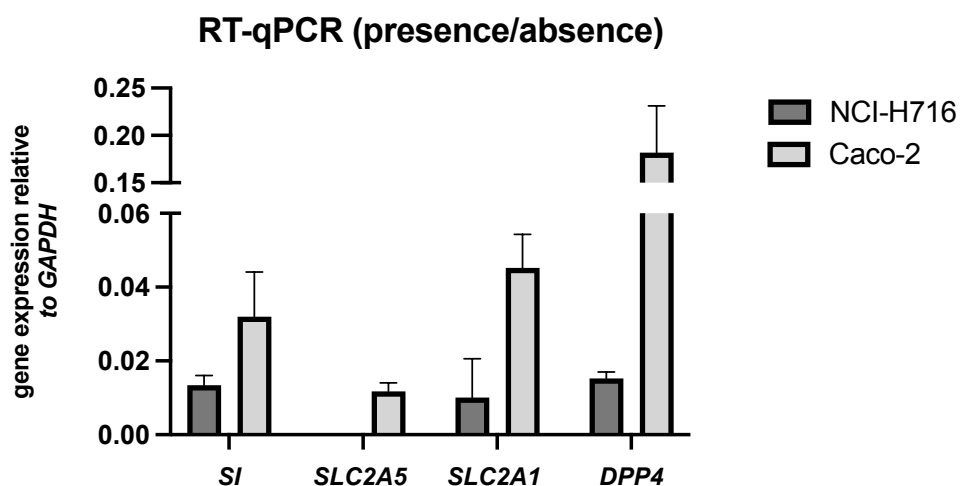


Figure 8: Relative gene expression of targeted genes.

SI, *SLC2A5*, *SLC2A1*, *DPP4*. N0_mean eff. of each target gene normalized to the reference gene *GAPDH*. Values are shown as mean + SD (biological replicates= 3, technical replicates= 2-3). Caco-2 cells served as a positive control.

In addition to the N0-value, qualitative assessment of gene expression was supported by evaluating the difference in cycle thresholds (Ct) between RT+ and RT- controls, as well as the non-template control (NTC). In Caco-2 cells, all target genes showed clear amplification with Ct differences greater than five cycles between RT+ and RT- controls, confirming reliable expression and serving as a positive control for assay performance.

In the NCI-H716 cell line, *SI*, *SLC2A1*, and *DPP4* were confirmed to be expressed based on clear RT+ amplification and Ct differences exceeding five cycles compared to RT- and NTC. For *SLC2A5*, however, amplification was not observed in RT+ samples or closely matched that of the RT- and NTC, with Ct differences below five. This suggests that *SLC2A5* is not expressed in NCI-H716 cells under basal conditions.

In summary, *SI*, *SLC2A1*, and *DPP4* were detected in NCI-H716 cells, while *SLC2A5* was not expressed under basal conditions. Caco-2 cells served as a positive control and showed expression of all investigated genes. These results served as the molecular basis for subsequent experiments investigating the functional relevance of the identified genes in GLP-1- and DPPIV-related pathways.

4.3 Total GLP-1 secretion

The total GLP-1 secretion in NCI-H716 cells was quantified using ELISA, with concentrations determined from a four-parameter logistic standard curve. All sample concentrations fell within the assay's dynamic range, although in the lower range (0.4-3.6 pM). To account for variations in cell number, GLP-1 secretion was normalized to cellular DNA content, measured photometrically at 260 nm after lysis with 1 % N-lauroylsarcosine.

Figure 9 illustrates the normalized GLP-1 secretion (pM/mg DNA) following a 2-hour incubation with 50–250 mM sucrose, fructose, glucose, and mannitol. Data are presented as mean + SD (n = 3 biological replicates with 2 technical replicates each). Outliers were identified and removed using the ROUT method (Q = 5 %). One-way ANOVA with Tukey's post-hoc test ($\alpha = 0.05$) was performed to determine statistically significant differences between treatment groups (Table 34).

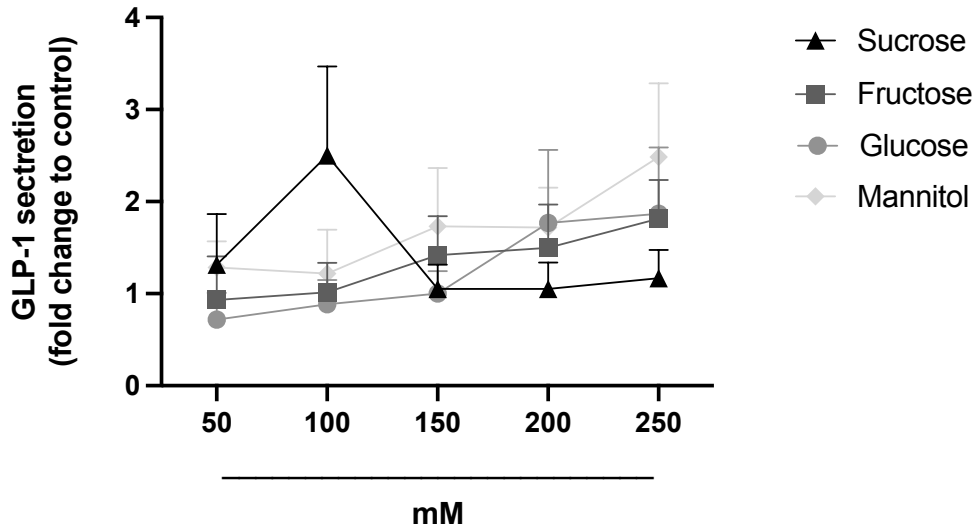


Figure 9: GLP-1 secretion of NCI-H716 cells.

Concentration of GLP-1 secretion normalized to DNA content (expressed as pM per mg DNA) after treatment with 50, 100, 150, 200, 250 mM sucrose, fructose, glucose and mannitol. The data is presented as a line graph, displaying mean values + SD (biological replicates = 3, technical replicates = 2). Outliers were identified using the ROUT (Q = 5 %) outlier test based on targeted data.

A trend of increasing GLP-1 secretion was observed for glucose, fructose and mannitol with increasing concentrations. Sucrose exhibited a peak secretion level at 100 mM, followed by a decline at concentrations ranging from 150 mM to 250 mM. Minimal variation in GLP-1 secretion was noted for fructose and glucose. In contrast, mannitol induced a concentration-dependent increase in GLP-1 secretion, with the most substantial increase observed at 250 mM. Table 34 presents the statistical results and significant differences between groups, indicated by distinct letters.

Table 34: ANOVA results and post-hoc groupings for GLP-1 secretion

Treatment	F-statistic	p-value	R ²	0	50	100	150	200	250
				mM					
Sucrose	10.140	< 0.0001	0.5848	B	B	A	B	B	B
Fructose	8.205	< 0.0001	0.5326	BC	C	C	BC	AB	A
Glucose	5.597	0.0006	0.4374	BC	C	BC	AC	A	AB
Mannitol	9.378	< 0.0001	0.5726	C	BC	BC	AB	AB	A

In summary, all treatments stimulated GLP-1 secretion within the lower range of the ELISA's dynamic range. The choice of 100 mM and 250 mM for subsequent DPPIV activity analysis was based on these notable trends: the peak in sucrose-induced GLP-1 secretion at 100 mM and the marked increase in mannitol-induced GLP-1 secretion at 250 mM, likely attributable to osmotic effects.

4.4 DPPIV activity measurement

DPPIV activity in NCI-H716 cell lysates was determined using a luminescence assay. These concentrations were selected based on the GLP-1 secretion results (Figure 9, Table 34). DPPIV activity values were normalized to total protein content (Bradford assay).

Figure 10 shows the normalized DPPIV activity (% of control) for each treatment. One-way ANOVA with Dunnett's multiple comparisons test ($\alpha = 0.05$) was performed. Outliers were identified using ROUT (Q = 5 %).

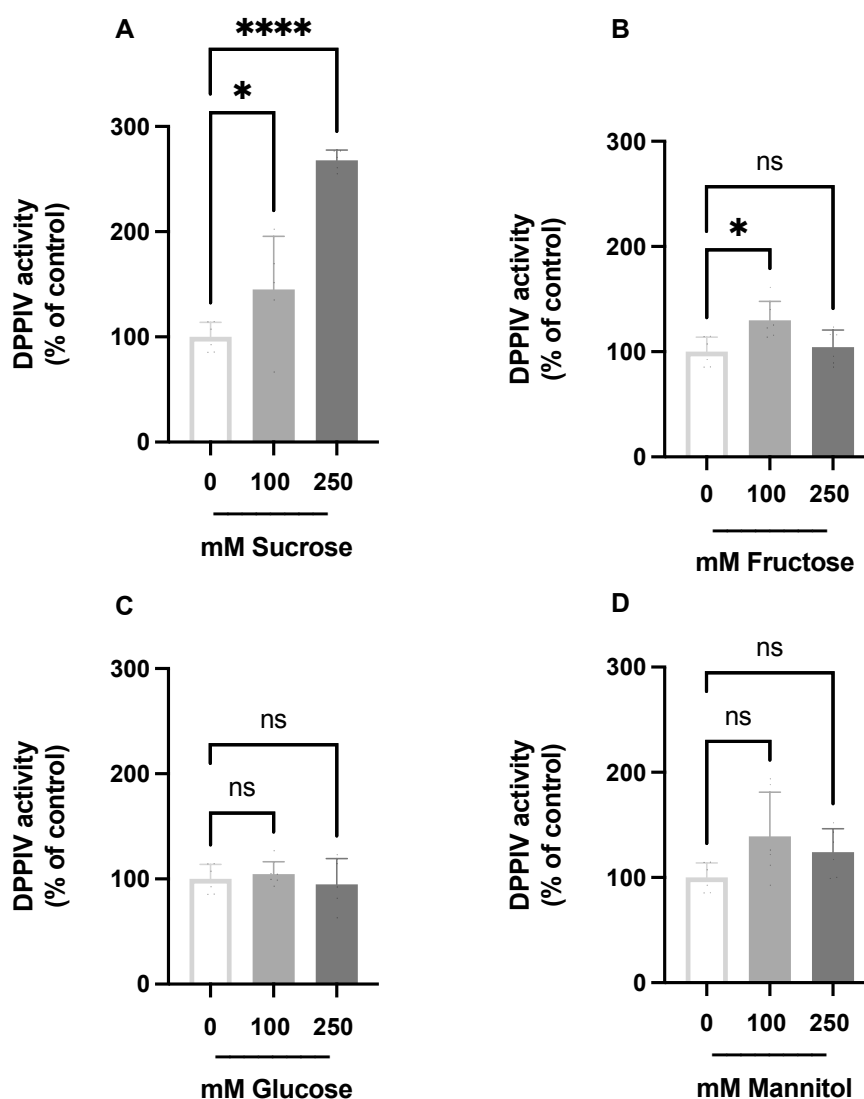


Figure 10: Normalized DPPIV activity in NCI-H716 cell lysates.

Normalized DPPIV activity (% of control, KRPH only) in NCI-H716 cell lysates following a 2-hour incubation with 100 mM and 250 mM sucrose, fructose, glucose, and mannitol. Graphs A-D present the data as mean + SD (n=3 biological replicates with 2 technical replicates each). One-way ANOVA with Dunnett's multiple comparisons test ($\alpha = 0.05$) was performed. * $p < 0.05$; **** $p < 0.0001$; ns = not significant. Outliers were identified using ROUT (Q = 5 %).

Figure 10 shows that sucrose significantly increased DPPIV activity at both 100 mM (* $p < 0.05$) and 250 mM (**** $p < 0.0001$) compared to the control. Fructose showed a significant increase at 100 mM (* $p < 0.05$) but not at 250 mM. In contrast, neither glucose nor mannitol significantly affect DPPIV activity at either of the tested concentrations.

In summary, these findings indicate that sucrose and fructose treatments, at specific concentrations, significantly modulated DPPIV activity, suggesting sugar-mediated regulation of DPPIV. This effect was not observed with glucose or mannitol.

4.5 GLP-1 and DPPIV correlation

To further investigate the relationship between GLP-1 secretion and DPPIV activity, Figure 11 displays the association between GLP-1 secretion (fold change to control) and DPPIV activity (% of control) across treatments involving 100 mM and 250 mM sucrose, fructose, glucose, and mannitol.

It is crucial to emphasize that, due to the separate sampling times for GLP-1 secretion and DPPIV activity measurements, no direct correlation between these two parameters can be calculated from these data. Therefore, the averages of 6 replicates (3 biological replicates and 2 technical replicates) for both GLP-1 and DPPIV were calculated and are illustrated in Figure 11.

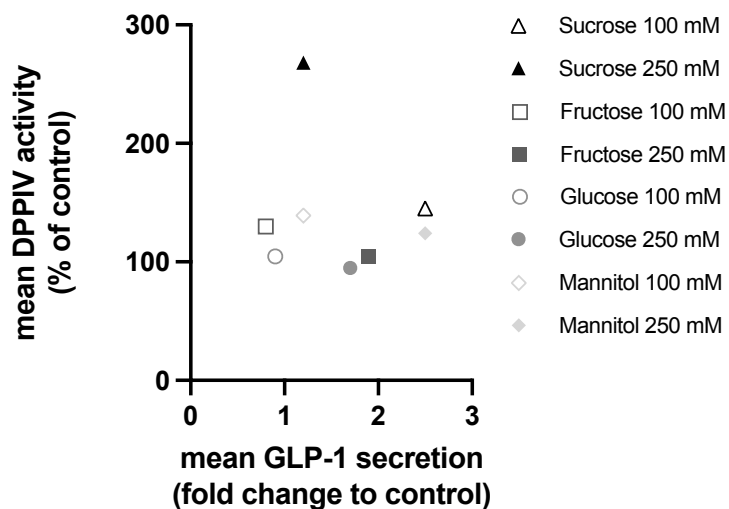


Figure 11: Correlation of GLP-1 secretion and DPPIV activity.

GLP-1 secretion is shown on the x-axis (fold change to control) and DPPIV activity on the y-axis (% of control). Each data point represents the mean of GLP-1 secretion (biological replicates = 3, technical replicates = 2) and DPPIV activity (biological replicates = 3, technical replicates = 2) for treatments with sucrose, fructose, glucose, and mannitol at 100 mM and 250 mM. Sucrose is indicated by triangles, fructose by squares, glucose by circles, and mannitol by diamonds. Outlined symbols represent the lower concentration (100 mM), while filled symbols indicate the higher concentration (250 mM).

Figure 11 presents the mean GLP-1 secretion and DPPIV activity for each treatment condition. Pearson correlation analysis did not indicate a statistically significant relationship between these two parameters.

Qualitative assessment reveals notable differences in both GLP-1 secretion and DPPIV activity across sugar types and concentrations. Specifically, at 250 mM, fructose (filled squares) and glucose (filled circles) tends to show higher GLP-1 secretion and relatively lower DPPIV activity compared to a treatment with 100 mM. A similar, though less defined trend is observed for mannitol. In contrast, sucrose at 250 mM (filled triangles) exhibits lower GLP-1 secretion but higher DPPIV activity compared to 100 mM, where it demonstrates higher GLP-1 secretion and lower DPPIV activity. This inconsistent pattern across sugar types highlights the complexity of the relationship between GLP-1 secretion and DPPIV activity, indicating a definitive relationship cannot be established.

5 Discussion

These investigations provide valuable insights into the characteristics of the NCI-H716 cell line, which is frequently utilized as one of the human-derived *in vitro* models for studying GLP-1 regulation (Gagnon & Brubaker, 2015). Elucidating the mechanisms involved after food intake, particularly nutrient sensing and the physiology of EECs, is essential. A better understanding of these mechanisms, especially those related to commonly occurring sugars such as sucrose, glucose, and fructose, could facilitate the development of more targeted diabetes treatments, including personalized dietary strategies as well as modified or new pharmaceuticals.

The sugars sucrose, glucose, and fructose were tested at concentrations of 50, 100, 150, 200, and 250 mM, with mannitol serving as an osmotic control at the same concentrations. All tested sugars exhibited non-toxic effects. However, sugar exposure led to an overall increase in cell viability. Notably, sucrose and glucose, had a more pronounced impact on cellular activity compared to the control, supporting the hypothesis that glucose is required for energy production. This is further supported by the highest viability observed in response to the monosaccharide glucose. Nevertheless, due to the relatively high variability in the data, no definitive conclusions regarding a dose-dependent effect can be drawn for glucose.

This variability and the influence of glucose on cell viability are particularly relevant when considering the physiological roles of glucose in metabolic regulation. For instance, it has been established that endogenous GLP-1 enhances insulin secretion and inhibits postprandial glucagon secretion in a glucose-dependent manner, thereby playing a crucial role in maintaining glucose homeostasis. GLP-1 also slows gastric emptying, reduces food intake, and promotes weight loss, effects that are mirrored by GLP-1 receptor agonists (Drucker, 2003; Holst *et al.*, 2008). Notably, the suppression of glucagon by GLP-1 does not occur at hypoglycemic glucose levels. Hence, exogenous GLP-1 does not impair the physiological mechanisms that counteract hypoglycemia (Nauck *et al.*, 2002). This insight underscores the importance of glucose not only as an energy source for cellular function but also as a critical component in broader endocrine regulation within metabolic pathways.

Notably, the disaccharide sucrose shows a slight, concentration-dependent decrease in cell viability suggesting that there is an inverse trend with sucrose, thus higher sugar concentrations leading to a decreased cell viability. For instance, a study examining different types of maple syrup on two colorectal cancer cell lines found that high concentrations of sucrose in maple syrup significantly inhibited the growth of the colorectal cancer cell lines. This result suggests that the high concentration (10 %) of sucrose in maple syrup has cytotoxic effects due to high osmotic pressure (Yamamoto *et al.*, 2015). This finding aligns with the observation of sucrose's impact, supporting the notion that elevated levels of this disaccharide can impair cell survival.

Furthermore, the results of the mannitol treatments consistently showed average cell viability above the 70 % threshold across the tested concentrations (50, 100, 150, 200, and 250 mM) within the NCI-H716 subclone samples. This indicates that the osmotic conditions of the experiments did not induce significant cell death. The absence of a dose-response relationship suggests that osmotic stress did not significantly affect cell viability within the tested concentration range.

While negative effects were observed with fructose in conjunction with the CCK-8 reagent, this prompted a transition to the NRU assay for further assessment. In this assay, cell viability results indicated $68.3 \% \pm 15.5 \%$ at 150 mM and $65.0 \% \pm 15.5 \%$ at 200 mM, both approaching the threshold of decreased cell viability. Notably, cell viability at 250 mM remained above this threshold. This pattern may reflect limitations in the methodology, particularly the multiple washing steps required in the NRU assay, could have led to the

loss of viable cells. As a result, the observed reduction may not necessarily indicate cell death.

The results of the CCK-8 viability as well as NRU assay showed that the different concentrations of the tested substances such as sucrose, fructose, glucose and mannitol had a distinct and significant effect on the cell viability of NCI-H716 cells. Certain concentrations were found to be non-cytotoxic, emphasizing their suitability for further studies on GLP-1 secretion. These findings suggest that sugars, particularly sucrose and glucose at defined concentrations may serve as effective stimulators or positive controls for the NCI-H716 cell line in future studies. While most published research has focused on lower glucose concentrations, for example proliferative effects were observed at 2.5 mM glucose in NCI-H716 cells (Chandramoorthy *et al.*, 2022), systematic analyses using higher concentrations, such as those tested here (50–250 mM), are still lacking and require further investigation.

Gene expression analyses confirmed the suitability of the Caco-2 cell line as a positive control, as these cells exhibited clear amplification of *SI* (Zhou *et al.*, 1998), *DPP4* (Preinfalk *et al.*, 2024), and *SLC2A1* (Zopun *et al.*, 2018), consistent with previously reported expression patterns in the literature. Based on this validated benchmark, the NCI-H716 cells also showed expression of *SI*, *DPP4*, and *SLC2A1* under basal conditions. These findings support the initial hypothesis that these genes are constitutively expressed in NCI-H716 cells, regardless of sucrose treatment. Moreover, the results align with previous reports describing the presence of key glucose and disaccharide metabolism-related genes in human enteroendocrine cell models (Blanchet *et al.*, 2025).

Interestingly, *SLC2A5*, was not detected in any of the analyzed NCI-H716 samples, despite initial expectations based on its well-characterized role in intestinal fructose uptake in other human cell models, such as Caco-2 cells (Ji *et al.*, 2019). This absence of *SLC2A5* suggests that fructose may not utilize this dedicated transport mechanism in NCI-H716 cells. Furthermore, as outlined in the “Previous results” (Chapter 2.7), *SLC2A2* had already been excluded in earlier analyses, confirming that it was not present in the NCI-H716 cells. This reinforces the notion that fructose uptake may not occur via these specific transporters. Instead, fructose uptake appears to mimic that of mannitol, indicating that it might be taken up non-specifically, potentially through osmotic mechanisms during the cell viability and

GLP-1 assays. The observed increase in GLP-1 secretion compared to controls does not strongly support an alternative pathway for fructose transport.

The absence of these key fructose transporters highlights the need to consider broader implications for fructose metabolism in the NCI-H716 cell line. Studies indicate that *SLC2A5* expression is substantial in Caco-2 cells and rapidly proliferating cancer cells, serving as an indicator of malignancy or elevated proliferation rates (Douard & Ferraris, 2008). The absence of *SLC2A5* in certain cancer cell lines, such as NCI-H716, suggests that these cells may lose the regulatory factors that suppress high *SLC2A5* expression seen in normal cells. This highlights the necessity for further investigation not only into the regulatory mechanisms behind *SLC2A5* expression, but also into which other transporters may facilitate fructose uptake in the NCI-H716 cell line, and the underlying mechanisms involved.

SLC2A1 primarily facilitates glucose uptake in tissues with high energy demands, such as the brain and enterocytes (Vulturar *et al.*, 2022). While there is some evidence suggesting *SLC2A1* can also mediate the fructose uptake, this occurs to a limited extent, with a significantly lower affinity for fructose than *SLC2A5* (Ferraris *et al.*, 2018). Previous studies indicate that human *SLC2A7* can transport both glucose and fructose with high affinity (K_m of ~ 0.2 mM). Sequence alignments indicate that *SLC2A2*, *SLC2A5*, and *SLC2A7* possess an isoleucine (Ile) at the position homologous to Val-290 in *SLC2A1*, suggesting a potential role for this Ile in substrate specificity. Structural comparisons show that both Val-290 in *SLC2A1* and Ile-314 in *SLC2A7* face the aqueous pore (Manolescu *et al.*, 2005), highlighting a structural adaptation in *SLC2A7* that supports its capacity to effectively transport fructose alongside glucose.

Considering the distinct roles and substrate affinities of glucose and fructose transporters, it would be interesting to explore specific inhibitors targeting *SLC2A1*, which has been confirmed to be expressed. As *SLC2A5* could not be detected in the NCI-H716 cell line, *SLC2A7* may be of interest for further investigations.

Recent studies have identified two natural product-derived inhibitors, Ast6G (Astragalin 6 glucoside, 20 mM) from *Astragalus* species and Rub (Rubusoside, 20 mM) from *Rubus* plants, which exhibit different specificity profiles. Ast6G selectively inhibits *SLC2A5* without affecting *SLC2A1*, while Rub demonstrates inhibitory activity against

both transporters. Although the IC₅₀ (half-maximal inhibitory concentration) values for these inhibitors are moderate (~ 6 mM for Ast6G and ~ 5 mM for Rub), they are closely aligned with the K_m (Michaelis-Menten constant) values for fructose and glucose transport, respectively, indicating their physiological relevance. Further insights can be gained from mutational analyses of key residues, such as W₃₈₈ in *SLC2A1* and A₃₉₆ in *SLC2A5*, as these modifications can influence inhibitor specificity and substrate accessibility (George Thompson *et al.*, 2015). This underscores the potential benefits of leveraging these inhibitors in future research to fully understand the substrate specificity of sugar transporters and their roles in metabolic processes.

In addition to the glucose and fructose transporters previously discussed, SGLT2 (Sodium-Glucose Co-Transporter 2) also warrants consideration in the context of glucose metabolism and GLP-1 secretion. Given that the expression and activity of SGLT2 can impact overall glucose homeostasis, examining its potential role in the NCI-H716 cell model would be valuable, especially since the gene expression of *SGLT2* has not yet been investigated within this research group. SGLT2 inhibitors, commonly referred to as gliflozins, such as dapagliflozin or empagliflozin, have gained prominence in diabetes treatment by reducing glucose reabsorption in the kidneys, leading to increased glucose excretion and improvements in glycemic control (Iqbal *et al.*, 2023). Future studies could focus on the effects of SGLT2 inhibitors on glucose uptake and their potential interactions with GLP-1 secretion. Additionally, understanding how SGLT2 inhibitors may modify metabolic processes such as lipolysis and ketogenesis in the liver, as suggested by recent findings, could provide deeper insights into metabolic regulation and therapeutic strategies for type 2 diabetes (Tsushima *et al.*, 2022).

The quantitative PCR analysis revealed that *SI* and *DPP4* are also present in the NCI-H716 cell line. Additionally, the results demonstrated that sucrose can be hydrolyzed in NCI-H716 cells, indicating active expression of specific enzymes or transporters. The ability to hydrolyze sucrose is significant for understanding the metabolic processes within this cell line, particularly when compared to Caco-2 cells, which have shown differing capacities in sucrose utilization (de Bruïne *et al.*, 1992; Lundquist & Artursson, 2016). Future investigations should aim to elucidate the underlying enzymatic mechanisms and the regulation of these processes to further clarify the role of sucrose hydrolysis in nutrient uptake and cellular metabolism. The presence of *DPP4* indicates an additional layer of

functionality, suggesting the cell line's capability to process and utilize various substrates, though specific contributions will be explored further in subsequent analyses.

Overall, these findings suggest that *SI*, *DPP4*, and *SLC2A1* may be involved in glucose and sucrose metabolism and could influence GLP-1 secretion. However, as these results are based on gene expression data, further investigations are required to elucidate the underlying mechanisms of sugar uptake and their potential impact on GLP-1 secretion in NCI-H716 cells, which represent a valuable *in vitro* model for studying enteroendocrine cell functions.

The hypothesis that different sugars (sucrose, glucose, fructose) would induce distinct GLP-1 secretion rates was partially confirmed. Notably, sucrose treatment resulted in a peak secretion at 100 mM, followed by a decrease at higher concentrations, a pattern that was unanticipated. As shown in Figure 9 and summarized in Table 34, sugar type and concentration had significant effects on GLP-1 secretion (all $p < 0.001$ except glucose, $p = 0.0006$). However, the response patterns were complex and did not fully align with the original hypothesis, indicating a nuanced relationship between sugar type, concentration and GLP-1 release (Kjems *et al.*, 2003).

While a general trend of increased GLP-1 secretion with increasing concentration was observed for mannitol, this is likely reflective of osmotic effects rather than a specific sugar-dependent response. This concentration-dependent increase in GLP-1 secretion with mannitol supports previous findings that demonstrated osmotic effects can influence hormone secretion.

For instance, research has shown that GLP-1 receptor (GLP-1R) signaling can inhibit the vasopressin system, with increased expression of GLP-1R observed in stimulated vasopressin neurons. Notably, treatment with the GLP-1R agonist liraglutide was found to decrease vasopressin synthesis and release, suggesting a complex interplay between osmotic regulation and GLP-1 signaling pathways. Additionally, liraglutide was shown to initiate hyperphosphorylation of presynaptic active zone proteins that control vasopressin exocytosis, indicating that hydration status may further influence the pharmacodynamics of GLP-1R agonists, which should be considered in current therapeutic strategies. (Greenwood *et al.*, 2023)

Analysis revealed only a marginal increase in GLP-1 secretion with fructose compared to mannitol, primarily at the highest concentration (250 mM). This modest increase, largely osmotically driven, suggests that fructose does not significantly enhance GLP-1 secretion and does not support the notion of an active fructose transport mechanism within the tested conditions. Moreover, minimal variation in GLP-1 secretion was observed for fructose and glucose across the tested concentration range (50, 100, 150, 200, 250 mM), indicating that these sugars do not substantially influence GLP-1 secretion.

The observation that fructose has no significant effect on GLP-1 secretion in NCI-H716 cells supports the well-established hypothesis that fructose is predominantly metabolized in an insulin-independent manner. Fructose is primarily metabolized in the liver, where it is processed through specific enzymatic pathways that do not require insulin (Vozzo *et al.*, 2002). In contrast, sucrose is a disaccharide composed of glucose and fructose, which can lead to a more significant insulin response due to the glucose component when metabolized (Jang *et al.*, 2007). Despite the theoretical foundation that glucose metabolism is closely linked to insulin secretion and the associated GLP-1 release, the current results suggest that glucose, within the tested concentration range (50-250 mM), does not significantly stimulate GLP-1 secretion in NCI-H716 cells. This contrasts with previous findings using lower glucose concentrations, which reported a more pronounced effect (Müller *et al.*, 2019). Fructose prompts only modest insulin secretion and can enter cells without insulin, allowing it to be rapidly absorbed by liver cells and converted to fructose-1-phosphate, thereby bypassing the initial steps of glucose metabolism (Bantle, 2009). This suggests that fructose's metabolic mechanisms operate independently of the insulin-dependent signaling pathway.

Notably, comparisons between glucose and mannitol revealed significant differences only at 50 mM and 150 mM, with mannitol inducing higher GLP-1 secretion levels. In contrast to the initial hypothesis and previous findings from the research group, which reported a significant increase in GLP-1 secretion at 150 mM sucrose, the current study revealed peak secretion at 100 mM sucrose, followed by a decrease at higher levels (Table 34, Figure 9).

These observations demand further investigation, as they may relate to potential alterations in cell metabolism or signaling pathways resulting from the different metabolic pathways of sucrose compared to fructose and glucose. Given that NCI-H716 cells are derived from colon cancer, it raises the possibility that their response to osmotic changes may play a

more significant role in regulating GLP-1. This aligns with findings by Sun *et al.* (2017), which indicated that intestinal glucose stimulates GLP-1 secretion through mechanisms involving SGLT1 and GLUT2. Notably, this study reported that while glucose effectively stimulated GLP-1 release in the duodenum and ileum, there was no significant stimulation in colon tissue (Sun *et al.*, 2017). This suggests that the hormonal regulation of GLP-1 in colorectal cells may be influenced more by osmotic effects than by the direct action of sugars, as indicated by the results.

Importantly, the data indicate that the tested monosaccharides, including glucose and fructose, led to increased GLP-1 concentrations in a concentration-dependent manner. Similarly, mannitol, as a sugar alcohol, exhibited an increase in GLP-1 concentrations alongside rising concentrations, suggesting a potential osmotic influence. In contrast, the disaccharide saccharose did not follow this trend, highlighting a distinct behavior among the tested sugars. These findings emphasize the complexity of GLP-1 release potentially influenced by osmotic changes, indicating that the response may not solely depend on the specific type of sugar present.

Therefore, future research should further investigate the mechanisms by which osmoregulation influences GLP-1 release in NCI-H716 cells. Potential avenues could include exploring specific signaling pathways that mediate GLP-1 secretion in response to osmotic changes. Understanding these mechanisms could provide insights into metabolic regulation in relation to diabetes and may inform therapeutic strategies targeting GLP-1 and DPPIV pathways, ultimately contributing to improved management of diabetes and related metabolic disorders.

Building on these observations, the subsequent analysis evaluated the effects of the various sugars on DPPIV activity. Given the peak in sucrose-induced GLP-1 secretion observed at 100 mM and the significant increase in mannitol-induced GLP-1 secretion at 250 mM, these concentrations were selected for DPPIV activity analysis to explore the potential interplay between GLP-1 secretion and DPPIV activity under varying conditions.

The findings indicate that DPPIV activity is significantly influenced by the type of sugar present. DPPIV activity in NCI-H716 cell lysates was determined after a 2-hour incubation with 100 mM and 250 mM concentrations of sucrose, fructose, glucose, and mannitol, in alignment with the timing of GLP-1 sample collection. Analysis involved normalizing

DPPIV activity to total protein content and performing one-way ANOVA with Dunnett's multiple comparisons test ($\alpha = 0.05$). The results (Figure 10) support the initial hypothesis, demonstrating that DPPIV activity is indeed modulated by different carbohydrates.

Notably, sucrose markedly increased DPPIV activity at both tested concentrations (100 mM: $p < 0.05$; 250 mM: $p < 0.0001$), suggesting either a direct effect of sucrose on DPPIV or an indirect effect due to altered cellular processes. The concentration-dependent effect observed at 100 mM should correspond to the peak in sucrose-induced GLP-1 secretion. However, it is critical to acknowledge that the GLP-1 and DPPIV assays were performed on separately collected samples at different time points and with distinct lysis buffers, which may limit the direct comparability of the results and should be considered in the interpretation.

Additionally, the observed increase in DPPIV activity could arise from a direct action of sucrose on the enzyme or from indirect effects related to metabolic changes within the cells, such as variations in DPPIV expression, post-translational modifications, or alterations in subcellular localization. While fructose also significantly increased DPPIV activity at 100 mM ($p < 0.05$), glucose and mannitol did not show significant effects.

Importantly, the modulation of DPPIV activity may play a crucial role in regulating GLP-1's biological effects. While GLP-1 secretion can be influenced by osmotic effects, DPPIV acts to inactivate GLP-1 by cleaving it, thereby reducing its availability and activity. However, GLP-1 conveys its functions significantly through the stimulation of the nervous system and thus the rapid degradation of GLP-1 by DPPIV may not be as critical, as it can still communicate a satiety signal via the activation of intestinofugal enteric neurons. (Zhang *et al.*, 2022) This perspective suggests that the differential regulation of DPPIV activity may impact not only GLP-1 levels but also the overall metabolic response to different carbohydrates, highlighting the complexity of these interrelated pathways.

These findings support the hypothesis that DPPIV activity is influenced by carbohydrate type, emphasizing its potential role in regulating GLP-1 secretion. Future study should focus on clarifying the underlying processes by which different carbohydrates alter DPPIV activity, as well as investigating how these influences may differ in different physiological or pathological conditions.

Despite these findings, the separation of GLP-1 and DPPIV analyses allowed for only a vague assessment of their correlation. This methodological choice, while necessary, limited the ability to establish a direct causal link between GLP-1 secretion and DPPIV activity, underscoring the challenges inherent in examining their interactions under the experimental conditions. Preliminary tests aimed at developing a protocol for simultaneous sample collection to measure GLP-1 and DPPIV from the same sample did not yield useful results.

The evaluation of different lysis buffers – specifically PBS for GLP-1 and 10 mM Tris-HCl with 0.1 % BSA for DPPIV – revealed considerable fluctuations in the results. Although separate samples were ultimately collected for DPPIV analysis, complications persisted. Normalization of the samples prior to analysis was essential to ensure accurate and reliable comparisons between the wells. Normalization corrects for variations in sample concentration and provides a consistent basis for measuring changes in secretion levels (O'Rourke *et al.*, 2019). For GLP-1, secretion levels were normalized to DNA content, while DPPIV results were normalized to protein content using the Bradford assay.

This process posed several challenges, particularly because NCI-H716 cells grow in suspension and require an extracellular matrix coating, such as Matrigel, for optimal cultivation (Gagnon & Brubaker, 2015). To dissolve the matrix, samples were placed on ice, which was necessary to enable cell processing steps such as mechanical lysis. Consequently, Matrigel was present in all samples. Sample loss also posed a challenge, as variable retention within the syringe needle complicated handling. Although the initial volume calculations based on the buffer employed were accurate, ensuring sufficient volume remained for both the DPPIV activity assay and the Bradford protein quantification proved difficult.

Moreover, the Tris buffer contains small amounts of BSA, which is intended to stabilize DPPIV, but may also lead to artificially elevated protein content. However, since these issues were consistent across all wells and unavoidable during sample collection, it effectively cancels out when comparing all samples to the control. These methodological precautions were essential to ensure data reliability under the given experimental conditions, allowing for a more accurate interpretation of the biological significance of GLP-1 and DPPIV secretion levels.

The results indicate significant variations in GLP-1 secretion and DPPIV activity across different sugar types and concentrations (Figure 11). Some trends suggest a potential inverse relationship between GLP-1 secretion and DPPIV activity, particularly for fructose and glucose at 250 mM. However, this was inconsistent and not statistically significant, highlighting the need for further investigation.

Furthermore, the limited sample size and differences in sampling time points restricted the ability to perform a robust correlation analysis. As a result, the data are presented as average values for each sugar type and concentration (sucrose, fructose, glucose, mannitol at 100 mM and 250 mM; biological replicates = 3, technical replicates = 2). Variability among biological replicates may also have contributed to the observed expression patterns. Additionally, differences in sampling time points such as varying passage numbers and the use of non-identical sample sets for GLP-1 and DPPIV measurements may have introduced confounding factors, suggesting that observed effects could reflect natural variation rather than a clear causal relationship.

To address these limitations, future research should aim to increase sample sizes and control additional variables to draw more definitive conclusions. Implementing simultaneous sample collection for GLP-1 and DPPIV measurements from the same samples, would allow for comprehensive assessments of dynamic interactions over time.

Additionally, the effects of GLP-1 receptor agonists, particularly Semaglutide, warrant further investigation, especially regarding their roles in weight loss and potential implications in aiding alcohol dependence (Chuong *et al.*, 2023). Given the limited number of publications in this area, this represents a promising direction for future research. Other GLP-1 agonists like Liraglutide, along with DPPIV antagonists such as Linagliptin and Sitagliptin, could provide further insights into the regulatory mechanisms involved. The incorporation of these pharmacological agents in the examination of the NCI-H716 cell model could enhance understanding of the interactions between GLP-1 secretion and DPPIV activity.

In summary, while these insights contribute to understanding the variables affecting GLP-1 secretion and DPPIV activity, more robust experimental designs are essential to fully elucidate these complex relationships and their practical applications in metabolic disorders like type 2 diabetes.

6 Conclusion

This study provides insights into the roles of commonly occurring sugars, particularly sucrose, glucose, and fructose, in regulating GLP-1 secretion and DPPIV activity within the NCI-H716 cell line.

The gene expression analyses confirmed constitutive expression of *SI*, *SLC2A1*, and *DPP4*, supporting the hypothesis that these genes are expressed regardless of sugar treatment. Notably, *SLC2A5* was not detected, suggesting alternative pathways for fructose uptake or transport in these cells. Indicating a significant deviation from expected fructose transportation mechanisms, leading to the conclusion that fructose may be absorbed non-specifically, similar to mannitol or via other, yet unidentified, transport pathways.

The investigation into sugar-induced GLP-1 secretion was based on the hypothesis that exposure to various sugars would differently influence secretion rates, with sucrose expected to enhance secretion in a dose-dependent manner. This hypothesis was only partially confirmed. Sucrose at 100 mM triggered the highest secretion, with a peak followed by a decline at higher concentrations, indicating a dose-dependent and complex relationship between sugar type, concentration, and hormone release. This pattern highlights the nuanced effects of different sugars on GLP-1 secretion. While the research confirms the role of sucrose in stimulating GLP-1 release, it also raises significant questions regarding the effects of fructose and glucose. Notably, the observed lack of significant stimulation of GLP-1 secretion by glucose challenges the established understanding that glucose directly influences GLP-1 release, a finding that varies from past reports (Müller *et al.*, 2019). This inconsistency indicates that factors such as cellular context, sugar concentration, and experimental conditions must be considered when interpreting the effects of these carbohydrates on hormone secretion.

Furthermore, the modulation of DPPIV activity by different sugars underscores its potential role in GLP-1 metabolism. Sucrose notably influenced DPPIV activity, supporting the hypothesis that carbohydrate exposure can affect this enzyme's activity and, consequently, GLP-1 bioavailability. These findings suggest an intricate interplay between sugar type and metabolic regulation, with implications for therapeutic strategies targeting GLP-1 pathways.

However, several unexpected results warrant further investigation. The absence of *SLC2A5* expression prompts questions about fructose transport mechanisms, and the limited impact of glucose on GLP-1 secretion challenges existing understanding of glucose's role in hormone regulation. These discrepancies may be influenced by cellular context, experimental conditions, or concentration effects.

Despite the valuable contributions of this study, several limitations need to be addressed in future research. The separation of GLP-1 and DPPIV analyses hindered the ability to establish direct associations between these factors. Developing protocols that allow simultaneous measurement of both from the same samples would reduce variability caused by different sample preparation techniques and improve data accuracy. Additionally, methodological challenges such as sample handling issues related to the presence of Matrigel must be resolved to ensure the reliability and reproducibility of the results.

In summary, this research underscores the differential impacts of specific sugars on GLP-1 secretion and DPPIV activity, emphasizing the complex regulatory pathways involved. Further elucidation of these mechanisms is essential, especially for developing targeted interventions to improve metabolic regulation and treat conditions like type 2 diabetes. Continued investigation will deepen the understanding of sugar metabolism and hormone regulation, paving the way for more effective therapeutic strategies.

AI tools

DeepL Translate, DeepL SE: <https://www.deepl.com/translator>

Translation of text passages to enhance clarity and accuracy.

ChatGPT, OpenAI: <https://www.openai.com/chatgpt>

Assistance with text formulation, particularly in maintaining a scientific tone.

Lucidchart: <https://www.lucidchart.com/pages>

Diagram-based visualization of methodology and study structure.

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Appendix A: raw data

Table 1: Raw data of relative cell viability (%) of NCI-H716 cells after 2 h incubation with 50, 100, 150, 200, and 250 mM sucrose, assessed by CCK-8 assay compared to KRPB control (outliers removed by ROUT, Q = 5 %)

0	50	100	150	200	250
mM					
	289.8	168.4	182.3	140.3	113.4
92.2	296.1	199.3	167.6	128.8	127.4
81.1	260.8	184.2	155.5	127.8	103.5
101.3	155.2	199.6	157.6	135.3	101.4
95.3	148	172.7	151.7	128.5	103.4
103.4	147.7	185.8	144.2	127.7	124.6
98.8	147	124.9	82	51.3	30.5
97.2	168.2	119.9	79.9	56.1	34.6
103.9	131.9	122.9	88.6	68.7	39.4

Table 2: Raw data of relative cell viability (%) of NCI-H716 cells after 2 h incubation with 50, 100, 150, 200, and 250 mM glucose, assessed by CCK-8 assay compared to KRPB control (outliers removed by ROUT, Q = 5 %)

0	50	100	150	200	250
mM					
	928.2	865.9	616.1	738.3	709.1
92.2	947.3	790	644.9	770.6	698
81.1		750.2			631.5
101.3	908	822.6	740.5	949	707.6
95.3	894.1	807.7	790.5	958.5	693.9
103.4	938.6	903.6	766.8	1003	692.4
98.8	604.4	589.7	532.5	511.8	510.7
97.2	624.9	576.2	541.1	494.4	502.3
103.9	652.3	584.4	537.5	514	535.3

Table 3: Raw data of relative cell viability (%) of NCI-H716 cells after 2 h incubation with 50, 100, 150, 200, and 250 mM mannitol, assessed by CCK-8 assay compared to KRPB control (outliers removed by ROUT, Q= 5 %)

0	50	100	150	200	250
mM					
	125.4	101.4	129.8	117.9	106.4
92.2	105.2	104.5	108.6	105.2	85.2
81.1	103.2	100.5	106.3	75.9	84.3
101.3	106.3	107.9	110.6	81.6	83.1
95.3	117.5	105.1	96.5	86.5	80.3
103.4	108.5	109.7	95	87.1	78.4
98.8	83.7	81.8	66.4	59.9	53.3
97.2	76.1	76.7	66.4	59	56.8
103.9	81.6	83.7	61.3	60.3	56.7

Table 4: Raw data of relative cell viability (%) of NCI-H716 cells after 2 h incubation with 50, 100, 150, 200, and 250 mM fructose, assessed by NRU assay compared to KRPB control (outliers removed by ROUT, Q = 5 %)

0	50	100	150	200	250
mM					
98.88	105.97	122.8	92.94	77.44	98.56
96.93	76.07	100.45	84.99	92.05	96.93
104.19		93.83	79.7	85.89	107.84
90.91	47.69	61.65	44.42		55.37
99.46	57.45	73.41	58.86	54.37	54.6
109.63	97.42	69.25	65.64	43.94	82.19
125.32	66.72	62.95	59.87	47.9	47.24
77.72	86.13	81.26	78.24	67.91	62.48
96.96	47.4		50.01	50.21	46.27

Table 5: Raw data of relative gene expression in NCI-H716 and Caco-2 cells: N0 mean values calculated as expression of target genes (*SI*, *SLC2A5*, *SLC2A1*, *DPP4*) normalized to the housekeeping gene (*GAPDH*). Outliers are removed using the ROUT (Q = 5 %).

<i>SI</i>		<i>SLC2A5</i>		<i>SLC2A1</i>		<i>DPP4</i>	
NCI-H716	Caco-2	NCI-H716	Caco-2	NCI-H716	Caco-2	NCI-H716	Caco-2
0.0158	0.0203	0.0000	0.0115	0.0235	0.059	0.0157	0.1568
0.0169	0.026	0.0000	0.0116	0.0242	0.0531	0.0168	0.1401
0.0166			0.0108	0.0244			0.1552
	0.0174		0.0092	0.0023	0.0329	0.013	
0.0106	0.0268	0.0000	0.0102	0.0023	0.0419	0.0128	0.1286
0.0118	0.0274	0.0000	0.0095	0.0026	0.0426	0.0151	0.1566
0.0112	0.0457	0.0001	0.0163	0.0035		0.0149	0.2296
0.0112	0.049	0.0001	0.0122	0.0035	0.0378	0.0159	0.2589
0.0132	0.0432		0.0143	0.0043	0.0492	0.018	0.2297

Table 6: Raw data of GLP-1 secretion in NCI-H716 cells normalized to DNA content (expressed as pM per mg DNA) after treatment with 50, 100, 150, 200, and 250 mM sucrose, fructose, glucose, and mannitol. Values are expressed as percentage change relative to the control (KRPB only). Outliers were identified using ROUT (Q = 5 %).

mM	Sucrose						Fructose						Glucose						Mannitol					
50	1	1.2	2.1	0.9	0.8	1.9	0.4	0.7	0.9	1	0.8	1.8	0.6	0.6	1.3	0.5	0.6	0.7	0.9	1.2	1.3	1.1	1.5	1.7
100	1.9	4.1	2.1	1.3	2.8	2.8	0.5	1	1.2	0.8	1.3	1.3	0.7	0.9	1.2	0.6	0.7	1.2	0.7	1.5	1.9	1	1	
150	1	1.4	1.1	0.6	1.2	1	0.7	1.6	1.3	1.5	1.4	2	0.6	1	1	0.9	1.3	1.2	1	1.4	2.8	1.6	1.5	2.1
200	0.8	1.3	1.4	0.7	0.9	1.2	0.6	1.6	1.4	1.8	1.8	1.8	1	1.6	1.4	1.5	3.3	1.8	1.2	1.5	2	1.3	2.1	2.2
250	1	1.3	0.8	1.5	1.5	0.9	1.7	2.2	1.3	1.4	2.3	2	1.5	2.2	1.5	1.4	3.2	1.4	2.3	2.1	3.6	1.3	2.5	3.1

Table 7: Raw data of DPPIV activity in NCI-H716 cells normalized to protein content after treatment with 100 250 mM sucrose, fructose, glucose, and mannitol. Values are expressed as percentage change relative to the control (KRPB only). Outliers were identified using ROUT (Q = 5 %).

KRPB	Sucrose		Fructose		Glucose		Mannitol	
0	100	250	100	250	100	250	100	250
mM								
114.62	66.7	255.2	113.9	85.3	104.6	63	193.9	99.2
114.47	169.5		161.1	95.6	93.2	92.3	121.9	100.2
107.27	202.4	260.9	140.2	89.4	126.8		188.4	133.6
85.38	151.7	270.7	115.6	116.1	99.7	123.1	92.5	143.1
85.53		276.5	122.7	116.4	105.2	114.6	126.1	117.1
92.73	135.2	276.6	125.4	123.3	98.8	81.6	111.5	152

Table 8: Summary of GLP-1 secretion and DPPIV activity in NCI-H716 cells after treatment with 100 mM and 250 mM sucrose, fructose, glucose, or mannitol. Values are calculated from previously obtained raw data and represent means of biological replicates, each based on technical triplicates. Outliers were excluded using the ROUT method (Q = 5 %).

Treatment	mean GLP-1 secretion	mean DPPIV activity
100 mM Sucrose	2.5	145.1
250 mM Sucrose	1.2	268
100 mM Fructose	0.8	129.8
250 mM Fructose	1.9	104.4
100 mM Glucose	0.9	104.7
250 mM Glucose	1.7	94.9
100 mM Mannitol	1.2	139.1
250 mM Mannitol	2.5	124.2

Appendix B: „Erklärung zur Verwendung generativer KI-Systeme“

Bei der Erstellung der Arbeit habe ich die folgenden auf künstlicher Intelligenz (KI) basierten Systeme¹ benutzt:

1. **DeepL Translate, DeepL SE:** <https://www.deepl.com/translator>
2. **ChatGPT, OpenAI:** <https://www.openai.com/chatgpt>
3. **Lucidchart:** <https://www.lucidchart.com/pages>

Ich erkläre weiterhin, dass ich

- ☒ mich aktiv über die Leistungsfähigkeit und Beschränkungen der oben genannten KI-Systeme informiert habe.
- ☒ die mithilfe der oben genannten KI-Systeme generierten Inhalte nicht als Zitate kenntlich gemacht habe, da die Systeme ausschließlich zur stilistischen Überarbeitung und zur Hebung des wissenschaftlichen Ausdrucks einzelner, von mir verfasster Passagen verwendet wurden. Eine exakte Abgrenzung der durch KI beeinflussten Textstellen ist nicht mehr möglich, da die Überarbeitungen kontinuierlich und fließend in meinen eigenen Schreibprozess integriert wurden.
- ☒ überprüft habe, dass die mithilfe der oben genannten KI-Systeme generierten und von mir übernommenen Inhalte faktisch richtig sind.
- ☒ mir bewusst bin, dass ich als Autor:in dieser Arbeit die Verantwortung für die in ihr gemachten Angaben und Aussagen trage.

Die oben genannten KI-Systeme habe ich wie im Folgenden dargestellt eingesetzt.

Arbeitsschritt	Eingesetzte(s) KI- System(e)	Beschreibung der Verwendungsweise
Generierung von Ideen und Konzeption der Arbeit		
Literatursuche und -analyse		
Auswahl von Methoden und Modellen		
Datensammlung und -analyse		
Erstellung von Visualisierungen	Lucidchart	Lucidchart wurde verwendet, um das methodische Vorgehen in Form eines Diagramms übersichtlich darzustellen und die Struktur der Arbeit für Leser:innen nachvollziehbarer zu machen.
Interpretation und Validierung		
Redigieren des Texts (Strukturierung und Formulierung)	ChatGPT, OpenAI	Textpassagen wurden in ChatGPT eingefügt mit der Aufgabe, den wissenschaftlichen Ton zu verbessern („enhance the scientific tone“). Die inhaltliche Grundlage stammt vollständig von mir; ChatGPT diente ausschließlich der sprachlichen Optimierung.
Übersetzung des Texts der Arbeit	DeepL Translate, DeepL SE	Die KI wurde durchgängig zur sprachlichen Überarbeitung und zur Übersetzung ins Englische verwendet, um Klarheit, Stil und wissenschaftliche Ausdrucksweise zu verbessern.

¹ Wenn Sie unsicher sind, ob Sie eine verwendetes KI-System angeben müssen, wenden Sie sich an Ihre:n Betreuer:in.