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Ing. Lucie Holá (roz. Černá)

Vzájemná interakce ghrelinu a jeho nového endogenního antagonisty LEAP2: možná úloha v patologii obezity

Interplay between ghrelin and its novel endogenous antagonist LEAP2: possible role in the pathology of obesity

Dizertační práce

Vedoucí dizertační práce: RNDr. Lenka Maletínská, DSc.

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ABSTRAKT

Zvyšující se počet osob s nadváhou a obezitou se v naší společnosti stal závažným zdravotním problémem. Obezita je často způsobena nadměrnou hyperfagií, a proto je důležité komplexně rozumět regulaci příjmu potravy, abychom mohli toto chronické onemocnění úspěšně léčit. Ghrelin, periferní peptidový hormon zodpovědný za zvýšení příjmu potravy, přímo ovlivňuje hypotalamus prostřednictvím GHSR (zkratka anglického názvu growth hormone secretagogue receptor). Nedávno bylo zjištěno, že LEAP2 (zkratka anglického názvu liver expressed antimicrobial peptide 2) přirozeně inhibuje konstitutivní aktivitu GHSR jako inverzní agonista. Proto je LEAP2 potenciálně využitelný kandidát pro vývoj antiobezitního léčiva.

Tato disertační práce zkoumá interakci mezi ghrelinem a LEAP2 v kontextu regulace příjmu potravy a obezity. Nejprve se zaměřuje na modifikovaný zkrácený N-terminální peptid LEAP2(1-14) a jeho lipidizované analogy a zkoumá jejich afinitu ke GHSR a jeho aktivaci *in vitro* a *in vivo*. Výsledky ukazují, že palmitovaný LEAP2(1-14) (palm-LEAP2(1-14)) v porovnání s ostatními analogy vykazuje nejvýraznější afinitu ke GHSR, působí jako inverzní agonista GHSR, snižuje příjem potravy, inhibuje uvolňování růstového hormonu navozeného ghrelinem a vykazuje zvýšenou stabilitu v potkaní plasmě. Tato zjištění naznačují, že palm-LEAP2(1-14) by mohl být slibným lékem proti obezitě.

Studie dále zkoumá vliv potravy s vysokým obsahem tuků na obezitu a rozvoj rezistence vůči ghrelinu a LEAP2 u myší. Výsledky ukazují, že podávání vysokotukové diety snižuje aktivní a celkový ghrelin v plasmě, zvyšuje *LEAP2* mRNA v játrech a vede ke glukózové intoleranci. Přechod na standardní dietu normalizuje expresi mRNA *LEAP2* v játrech a hladinu aktivního ghrelinu, nikoli však celkového ghrelinu v plasmě. Studie dále prokazuje rezistenci vůči palm-LEAP2(1-14) vyvolanou vysokotukovou dietou a také rezistenci vůči stabilnímu GHSR agonistovi [Dpr^3]Ghrelinu která je reverzibilní po přechodu na standardní dietu.

Nakonec byl hodnocen potenciál palm-LEAP2(1-14) potlačit vliv vysokotukové diety na nárůst tělesné hmotnosti a normalizovat morfometrické a metabolické parametry spojené s obezitou. Palm-LEAP2(1-14) mírně snížil přírůstek tělesné hmotnosti vyvolaný podáváním vysokotukové diety a snížil hladinu leptinu v plasmě. Celkově však palm-LEAP2(1-14) nebyl schopen potlačit účinek vysokotukové diety pravděpodobně v důsledku rezistence k palm-LEAP2(1-14).

Tato zjištění přispívají k lepšímu pochopení patofyziologie obezity a naznačují nutnost dalšího zkoumání alternativních strategií ke zlepšení účinnosti léčby obezity zaměřené na dráhy ghrelinu a LEAP2.

Klíčová slova:

ghrelin, LEAP2, palm-LEAP2(1-14), obezita, ghrelinová rezistence, inverzní agonismus

ABSTRACT

The increasing number of overweight and obese individuals has become a major health issue in our society. The etiology of obesity often involves excessive hyperphagia, highlighting the importance of comprehensive understanding the regulation of food intake regulation in order to effectively treat this chronic condition. Ghrelin, a peripheral peptide hormone responsible for increasing food intake, directly affects the hypothalamus through the growth hormone secretagogue receptor (GHSR). Recently, it was found that liver expressed antimicrobial peptide 2 (LEAP2) naturally counteracts the effects of the GHSR as an inverse agonist. This makes LEAP2 a potential candidate for the development of anti-obesity treatment.

This thesis explores the interaction between ghrelin and LEAP2 in the context of food intake regulation and obesity. Firstly, it focuses on modified N-terminal peptide LEAP2(1-14) and its lipidized analogs, examining their affinity to and activation of GHSR *in vitro* and *in vivo*. The results demonstrate that palmitoylated LEAP2(1-14) (palm-LEAP2(1-14)) exhibits the most pronounced affinity for GHSR, acts as GHSR inverse agonist, reduces food intake, inhibits growth hormone release, and shows increased stability in rat plasma. These findings suggest that palm-LEAP2(1-14) holds promise as an anti-obesity treatment.

Furthermore, the study investigates the impact of a high-fat (HF) diet on obesity and the development of ghrelin and LEAP2 resistance in mice. The results reveal that HF diet feeding decreases active and total plasma ghrelin, increases liver *LEAP2* mRNA expression, and leads to glucose intolerance. The switch to a standard diet normalizes liver *LEAP2* mRNA expression and active ghrelin levels but not total ghrelin. Furthermore, the study demonstrates resistance to palm-LEAP2(1-14) induced by the HF diet and also resistance to GHSR stable agonist [Dpr^3]Ghrelin, which is reversible upon switching to a standard diet.

Lastly, the potential of palm-LEAP2(1-14) to counteract the effects of a HF diet on body weight gain and normalize morphometric and metabolic parameters associated with obesity was evaluated. Palm-LEAP2(1-14) slightly reduced the body weight gain induced by HF diet feeding and decreased plasma leptin level. But overall, palm-LEAP2(1-14) was not able to suppress the effect of HF diet due to palm-LEAP2(1-14) resistance.

These findings advance our comprehension of obesity pathophysiology and indicate the necessity for additional investigation into alternative approaches to improve the efficiency of anti-obesity treatments that target the ghrelin and LEAP2 pathways.

Key words:

LEAP2, ghrelin, palm-LEAP2, obesity, ghrelin resistance, inverse agonist

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CONTENTS

ABBREVIATIONS

ACN	Acetonitrile
AgRP	Agouti-related protein
AKT	Serine/threonine-specific protein kinase
AMPK	5' AMP-activated protein kinase
ANOVA	Analysis of variance
ARC	Arcuate nucleus
AUC	Area under the curve
B2m	Beta-2-microglobulin
CaM	Calmodulin
CaMKII	Calcium calmodulin-dependent protein kinase II
CART	Cocaine- and amphetamine-regulated transcript
CNS	Central nervous system
CSF	Cerebrospinal fluid
DCM	Dichloromethane
des-acyl ghrelin	Ghrelin without the acyl group
DIEA	N,N-Diisopropylethylamine
DIO	Diet-induced obesity
DMF	Dimethylformamide
Dpr	Diaminopropionic acid
ECL	Extracellular loop
ELISA	Enzyme-linked immunosorbent assay
EMA	European Medicines Agency
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal-regulated kinase 1/2
ESI	Electrospray ionization
eWAT	Epididymal white adipose tissue
FDA	U.S. Food and Drug Administration
FRET	Fluorescence resonance energy transfer
GAPDH	Glyceraldehyde3-phosphate dehydrogenase
GH	Growth hormone
GHRP-6	Growth hormone-releasing peptide 6
GHS	Growth hormone secretagogues
GHSR	Growth hormone secretagogue receptor 1a
GLP-1	Glucagon-like peptide 1
GOAT	Ghrelin O-acyl-transferase
HATU	Hexafluorophosphate azabenzotriazole tetramethyl uronium
HF	High-fat
HPLC	High-performance liquid chromatography
IBMM	Institut des Boimolécules Max Mousseron
ICL	Intracellular loop
ICV	Intracerebroventricularly
IKEM	Institute for Clinical and Experimental Medicine
IOCB	Institute of Organic Chemistry and Biochemistry
IP3	Inositol-1,4,5-triphosphate
KD	Dissociation constant
LEAP2	Liver-expressed antimicrobial peptide 2
MBOAT	Membrane-bound O-acyl transferase
MCFA	Medium-chain fatty acid

MS	Mass spectrometry
myr	Myristoyl
Nle	Norleucine
NPY	Neuropeptide Y
OGTT	Oral glucose tolerance test
palm	Palmitoyl
pAMPK	Phosphorylated 5' AMP-activated protein kinase
PC1/3	Prohormone convertase 1/3
PI3K	Phosphoinositide 3-kinase
PKA	Protein kinase A
PKCβ	Protein kinase C β
PKCε	Protein kinase C ε
PLC	Phospholipase C
POMC	Proopiomelanocortin
SC	Subcutaneous
SPa	[D-Arg^{1}, D-Phe^{5}, D-Trp^{7,9}, Leu^{11}]-substance P
St	Standard
stear	Stearoyl
TAG	Triglycerides
TFA	Trifluoroacetic acid
TIS	Triisopropylsilane
TM	Transmembrane domains
α-MSH	α-melanocyte-stimulating hormone

1 Introduction

1.1 Obesity and its treatment

The increase in overweight and obese individuals is one of the most serious health problems in our society. The prevalence of obesity has tripled worldwide since 1975 (Muller et al., 2022). Obesity is strongly associated with an increased risk of many health problems such as type 2 diabetes, and hypertension (Fruh, 2017). Currently, the mostly recommended approach to treat and manage obesity is to modify lifestyle by diet and exercise (Wadden et al., 2012). However, not everyone is able to achieve significant weight loss through lifestyle changes alone and therefore pharmaceutical treatment is recommended in individuals, who are at risk of obesity comorbidities (Krentz et al., 2016).

In recent years, certain medications have been utilized to manage severe obesity. Unfortunately, a majority of these drugs have been withdrawn from the market due to their significant side effects, especially with regard to cardiovascular complications (Coulter et al., 2018). European Medicines Agency (EMA) has approved only four anti-obesity drugs for long-term use in Europe. Orlistat (brand name Alli) inhibits gastric and pancreatic lipases, which leads to reduced absorption of triglycerides, but regrettably causes digestive problems such as increased defecation, fecal urgency, and fatty stools (Tak and Lee, 2021). Naltrexone/bupropion (brand name Mysimba) is combination of opioid and alcohol addiction drug naltrexone and antidepressant bupropion that have together synergistic anorexigenic effect. Its typical side effects include dizziness, headache, dry mouth and digestive problems (Apovian et al., 2015). Liraglutide (brand name Saxenda) is a glucagon-like peptide 1 (GLP-1) derivative, which after daily subcutaneous (SC) injections induces postprandial satiety, decreases appetite and slows gastric emptying. Liraglutide was first used for type 2 diabetes treatment and later in higher doses for treatment of obesity. Similarly as previous drugs, liraglutide has also side effects such as nausea, vomiting, diarrhea and constipation (Khera et al., 2016). Recently, another GLP-1 derivative semaglutide (brand name Wegovy) was approved as type 2 diabetes and chronic obesity treatment. Semaglutide is SC injected only once a week, however, its gastrointestinal side effects (typically nausea, diarrhea, vomiting, and constipation) are very similar to liraglutide (Wilding et al., 2021).

Therefore, it is still important to work on the development of new anti-obesity treatments with improved safety profiles and high efficacy. Since obesity is frequently caused by

hyperphagia, there is a need to fully understand food intake regulation to treat this chronic disease.

The only known peripherally released orexigenic peptide hormone is ghrelin. Ghrelin acts directly in the hypothalamus through the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999, Andrews, 2011). GHSR is known to be a constitutively active G-protein-coupled receptor with intrinsic activity in a non-active state (Holst et al., 2004). Therefore, attention has turned to the reducing the high constitutive activity of GHSR by use of inverse agonists. Although several GHSR inverse agonists and antagonists have been shown to function *in vitro* and *in vivo*, no drug that reduces body weight by targeting GHSR has been developed yet (Schalla and Stengel, 2019). Recently, liver-expressed antimicrobial peptide 2 (LEAP2) was identified as an endogenous inverse agonist of GHSR (Ge et al., 2018), which makes it potential candidate for anti-obesity drug development.

1.2 Growth hormone secretagogue receptor (GHSR)

1.2.1 GHSR history

GHSR is the only recognized ghrelin receptor (Mani and Zigman, 2017). The discovery of GHSR is a typical example of reverse pharmacology because GHSR was identified earlier than its ligand ghrelin (Nikolopoulos et al., 2010). Bowers et al. (Bowers et al., 1984b) discovered that certain opioid peptide derivatives, which lacked opioid properties, had weak growth hormone (GH)-releasing activity and were therefore named GH secretagogues (GHS). The first GHS was derived from met-enkephalin (Tyr-Gly-Gly-Phe-Met-COOH), with D-Trp instead Gly^2 and amide at the C-terminus. After the discovery of ghrelin (Kojima et al., 1999), it was found that hydrophobic N-terminus is important for GHSR activation, thus D-Trp was probably fundamental structure in GHS responsible for its interaction with the GHSR (Kojima and Kangawa, 2005).

Although the GH-releasing activity of early GHSs was minimal and observed only in *in vitro* studies, their discovery prompted synthesis of more potent peptide GHS analogs, such as GH-releasing peptide 6 (GHRP-6) (Bowers et al., 1984a) or first non-peptide GHS analog MK-0677 (Smith et al., 1993), which were able to induce GH release in rats, monkeys, lambs, and calves, (Bowers et al., 1984a) and in dogs, respectively (Patchett et al., 1995). Due to the potential for therapeutic targeting, there was an intense effort to identify GHS receptor. That was eventually discovered in the pituitary and *arcuate nucleus*

(ARC) of swine and humans in 1996 and named GHS receptor (GHSR) (Howard et al., 1996).

Endogenous ligand of GHSR was discovered by Kojima et al. in 1999 (Kojima et al., 1999). Surprisingly, it was discovered in the stomach, specifically in gastric entero-endocrine cells, and not in the brain, where GHSR expression was detected. Ghrelin is a 28-amino acid peptide that has a fatty acid (n-octanoyl) side chain at its third serine residue, which is unique among peptide hormones and essential for binding to the receptor. It was named after the Proto-Indo-European word "ghre," which means "grow."

Almost twenty years later, another natural GHSR ligand LEAP2 was discovered (Ge et al., 2018). It was initially known as an antimicrobial peptide (Krause et al., 2003), but it was later found to have the ability to act as both an antagonist and an inverse agonist of GHSR (M'Kadmi et al., 2019).

1.2.2 GHSR structure

GHSR belongs to G protein-coupled receptors. GHSR has seven α-helical domains that span across the membrane, with its N- and C-terminal ends located on the extracellular and intracellular sides, respectively.

Nucleotide sequence analysis revealed two types of GHSR cDNA derived from the same gene. The human full-length type GHSR1a cDNA encodes the 366 amino-acids protein with all seven transmembrane domains (TM). Type 1b encodes a truncated protein of 289 amino acids with only five TM (Davenport et al., 2005). GHSR1b binds neither ghrelin nor synthetic GHS. GHSR1a is considered to be the active form of GHSR (Li et al., 2016), and therefore GHSR1a is called GHSR nowadays.

The structure of GHSR shows the presence of two cavities that bind ghrelin (Figure 1). Additionally, the receptor has a wide gap (crevasse) between TM6 and TM7 that is rich in hydrophobic amino acid residues, which probably recognize the octanoyl group of ghrelin (Shiimura et al., 2020). Binding pocket of GHSR interacts with hydrophobic N-terminal region of ghrelin which includes the octanoyl moiety and the residues Phe^4 and Leu^5. Binding of ghrelin to the receptor leads to the conformational change in ghrelin that structures its central region and forms well-defined hydrophobic core. Octanoylation of ghrelin is essential for forming its hydrophobic core and promotes access of ghrelin to the receptor ligand-binding pocket (Ferre et al., 2019). GHSR also interacts with N-terminal part of LEAP2 which includes hydrophobic residues Met^1, Pro^3, Phe^4, and Trp^5 (Li et al.,

2021, M'Kadmi et al., 2019). This interaction reduces GHSR intrinsic activation. Both ghrelin and LEAP2 bind to the GHSR with similar affinity with K_D in a nM range (M'Kadmi et al., 2019).

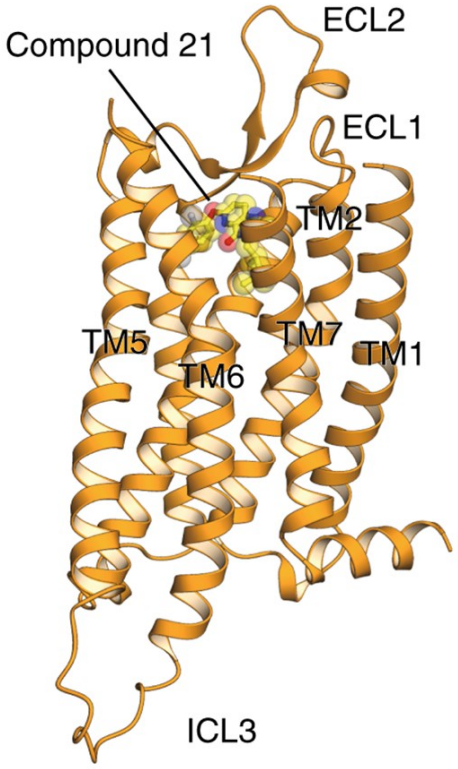

Figure 1: Structure of GHSR. Structure of human GHSR in complex with an antagonist molecule compound 21. GHSR is shown in cartoon representation in orange and compound 21 is shown as spheres and sticks with carbon atoms in yellow, oxygen in red, and nitrogen in blue. ECL – extracellular loop, ICL – intracellular loop, TM – transmembrane domains (Shiimura et al., 2020).

1.2.3 GHSR expression

GHSR is expressed in both the central nervous system (CNS) and the periphery. It is primarily found in neurons in the hypothalamus, hippocampus, dorsal and medial raphe nuclei, substantia nigra pars compacta, lower brain stem regions, and autonomic preganglionic neurons. Additionally, it is also present in vagal afferent (nodose ganglion) neurons in the periphery (Zigman et al., 2006). Besides its higher expression in the CNS, GHSR is also expressed in the peripheral organs including pancreas, spleen, adrenal glands, thyroid glands, thymus, T cells, and myocardium (Gnanapavan et al., 2002).

1.3 Ghrelin

1.3.1 Ghrelin history and function

Ghrelin is the only known peripherally released orexigenic peptide hormone that acts directly in the hypothalamus and brainstem (Kojima et al., 1999).

In 2000, Tschöp et al. discovered that ghrelin positively regulates food intake, adiposity, and body weight (Tschöp et al., 2000). Afterwards, other central actions of ghrelin were described (Figure 2), such as suppression of brown fat thermogenesis (Yasuda et al., 2003), modulation of sleep (Szentirmai et al., 2006), and modulation of stress and anxiety (Lutter et al., 2008). Ghrelin also plays a significant role in regulating various peripheral functions, such as the regulation of glucose metabolism (Date et al., 2002b), stimulation of gut motility and gastric acid secretion (Masuda et al., 2000), taste sensation (Cai et al., 2013), anti-inflammatory properties (Hattori, 2009), protection against muscle atrophy (Porporato et al., 2013), and improvement of cardiovascular function (Rizzo et al., 2013).

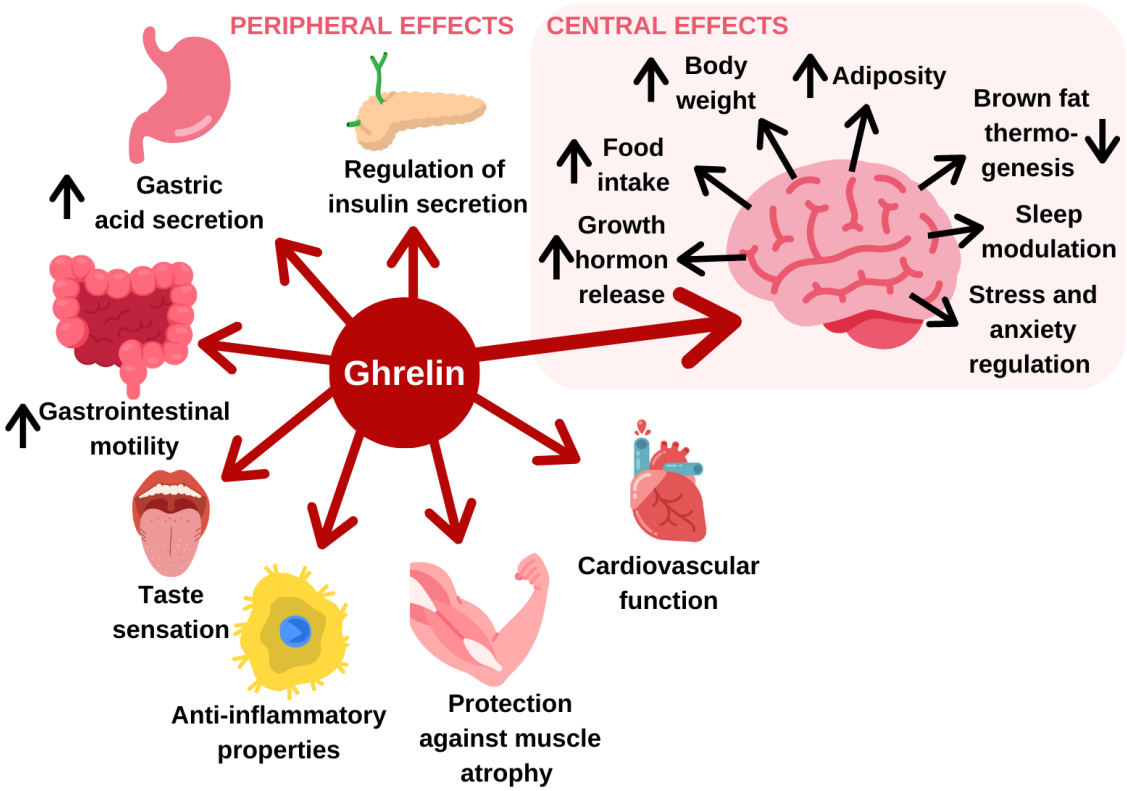

Figure 2: Main central and peripheral effects of ghrelin

1.3.2 Ghrelin structure

Ghrelin consists of 28 amino acids, and it is unusual among peptidic hormones because its Ser^3 is n-octanoylated by non-stable ester bond (Figure 3). This modification is essential for ghrelin biological activity (Sato et al., 2012). The octanoyl residue is post-

translationally attached to the peptide by ghrelin O-acyl-transferase (GOAT). Ghrelin without the acyl group (des-acyl ghrelin) is biologically inactive (Kojima et al., 1999). Des-acyl ghrelin is also present in significant amounts in both blood and stomach, but des-acyl ghrelin can neither bind to GHSR nor exhibit GH release (Sato et al., 2012).

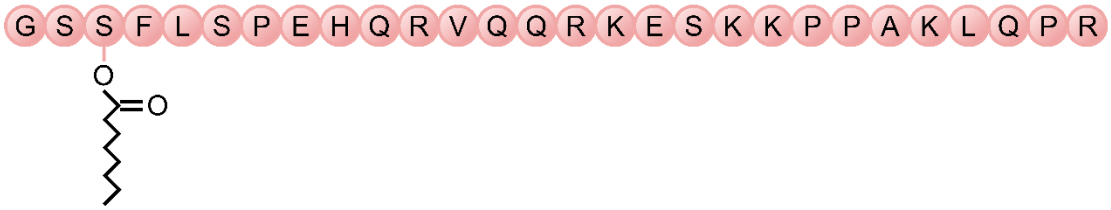

Figure 3: Ghrelin structure

Ghrelin is derived from its preprohormone called preproghrelin, a 117 amino-acid precursor that is produced in gastric X/A-like endocrine cells (Stengel et al., 2010). A signal peptide is cleaved from preproghrelin to form proghrelin (Taylor et al., 2012). Proghrelin is octanoylated by GOAT and then cleaved into ghrelin and obestatin by prohormone convertase 1/3 (PC1/3). Although it was believed that ghrelin and obestatin were antagonistic and obestatin had opposing effect on food intake regulation and decrease of body weight, jejunal motility, and food intake (Zhang et al., 2005, Lacquaniti et al., 2011), other studies disapproved it (Bassil et al., 2007, Gourcerol et al., 2007).

Ghrelin is highly conserved among species including human, rat, mouse, rhesus monkey, Mongolian gerbil, cow, pig, goat, sheep, and dog. Specifically, N-terminal peptide is identical, thus it is assumed that this region is important for ghrelin activity (Sato et al., 2012).

1.3.3 Ghrelin O-acyl-transferase (GOAT)

GOAT post-translationally attaches octanoyl residue of medium-chain fatty acid (MCFA) to the side chain of Ser^3 at proghrelin. The unique post-translation modification occurs before the peptide is processed by PC1/3, stored, and secreted. Although the origin of MCFAs has not been determined, it was found that orally ingested MCFAs are directly utilized for the acyl-modification of ghrelin (Sato et al., 2012).

GOAT was identified in 2008 (Yang et al., 2008) as a member of the membrane-bound O-acyl transferase (MBOAT) family. MBOAT is a group of integral membrane proteins that are localized within the membrane of the endoplasmic reticulum (Mohan et al., 2016).

MBOAT family members have a critical role in lipid biosynthesis, sterol acylation, and acyl modification of secreted proteins, including ghrelin (Khatib et al., 2015).

GOAT requires fatty acid substrates as high-energy fatty acid CoA thioesters and peptides with the amino acid sequence GXSFX, where G corresponds to unblocked amino-terminal glycine, X to any amino acid, S to serine, and F to phenylalanine. This amino acid motif appears specific only for ghrelin and suggests that ghrelin is the only known peptide substrate for GOAT (Muller et al., 2015).

GOAT structural domains are highly conserved among species. GOAT of zebrafish, rats, and mice have even the capacity to acylate human ghrelin (Gutierrez et al., 2008). Ghrelin and GOAT share a similar tissue distribution in both humans and mice. The highest expression of GOAT occurs in pancreas and stomach in humans and stomach and intestine in mice (Muller et al., 2015).

1.4 LEAP2

1.4.1 LEAP2 history and function

In 2003, LEAP2 peptide was originally isolated from human blood (Krause et al., 2003). LEAP2 was characterized as a liver specific peptide, which is released into blood, and shows antimicrobial properties *in vitro* in micromolar concentration (Krause et al., 2003), however, LEAP2 plasma concentration is in a nanomolar range (Mani et al., 2019). Later, it was demonstrated that LEAP2 is expressed not only in the liver, but also in jejunum, duodenum and ileum (Ge et al., 2018). A significant degree of conservation of LEAP2 amino acid sequence from fish to mammals suggests (Li et al., 2021) that LEAP2 may have other essential physiological function than being only an antimicrobial peptide.

In 2018, Ge et al. tested the LEAP2 activation of 168 known human G-protein coupled receptors in both agonist and antagonist modes. LEAP2 was able to fully inhibit GHSR activation by ghrelin (Ge et al., 2018). Later, M'Kadmi proved that LEAP2 also acts as a GHSR inverse agonist and inhibits high intrinsic activity of GHSR (M'Kadmi et al., 2019).

1.4.2 LEAP2 structure

LEAP2 is a 40-residue cationic peptide (Figure 4) (Krause et al., 2003). In the organism, the peptide is first synthesized as a 77-amino acid precursor that is subsequently processed into the mature peptide consisting of 40 amino acids. NMR-based structure analysis indicates that mature LEAP2 consists of an unstructured, hydrophobic N-terminal region and a compact central region containing two disulfide bridges linked in an I-III, II-IV

pattern (Henriques et al., 2010). The part of LEAP2 responsible for binding to GHSR is located in the first 8 amino acids of the N-terminus, which contains hydrophobic amino acids Met[1], Pro[3], Phe[4], and Trp[5]. However, to achieve maximum potency and effectiveness of the binding, a longer N-terminal sequence containing at least the first 12 amino acids is necessary (M'Kadmi et al., 2019).

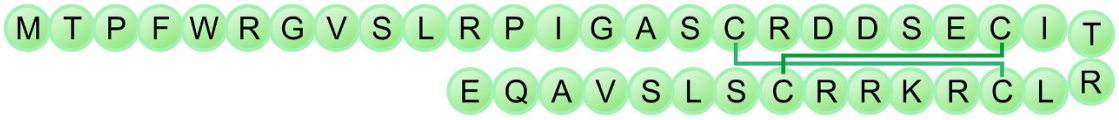

Figure 4: Identical human and mouse LEAP2 structure

1.5 GHSR-ghrelin-LEAP2 interplay

1.5.1 GHSR cell signaling

Naturally, GHSR is activated by its agonist ghrelin and inhibited by its inverse agonist and antagonist LEAP2 (Ge et al., 2018). GHSR has a high constitutive activity independent of ligand, which is nearly 50% of its maximal activation induced by ghrelin secretagogues (Mani and Zigman, 2017).

GHSR is coupled with G protein (Figure 5), specifically $G\alpha_q$ and $G\alpha_{i/o}$. Upon activation of the GHSR-$G\alpha_q$-dependent signaling pathway, phospholipase C (PLC) is recruited to the membrane. PLC catalyzes the enzymatic production of inositol-1,4,5-triphosphate (IP_3) and diacylglycerol (DAG). IP_3 is a second messenger, which initiates Ca^{2+} release from the endoplasmic reticulum (ER). Ca^{2+} interacts with calmodulin (CaM) to activate CaM-dependent protein kinases (CaMK), including CaMKII. CaMKII activation triggers a cascade of signaling events, including the activation of the AMP-activated protein kinase (AMPK) (Andrews, 2011, Ramirez et al., 2019). Both peripheral and central ghrelin administration increased AMPK phosphorylation and activity in the rat hypothalamus (Andersson et al., 2004). Many of the metabolic effects of ghrelin-induced GHSR activation are linked to the presence of pAMPK (Abizaid and Hougland, 2020).

GHSR-$G\alpha_{i/o}$-dependent signaling pathway activates phosphoinositide 3-kinase (PI3K) to regulate the activation of protein kinase A (PKA), protein kinase C ε (PKCε), and serine/threonine-specific protein kinase (AKT) (Ramirez et al., 2019).

G-protein–independent signaling occurs through the β-arrestin scaffolding protein to activate extracellular signal-regulated kinase 1/2 (ERK1/2) and AKT (Ramirez et al., 2019).

Ghrelin signaling is very complex, as well as its biological functions. Thus, it is believed, that certain signaling pathways are connected with particular functions. It was proposed, that β-arrestin signaling is linked to intracellular lipid storage and mitogenic activity (Santos-Zas et al., 2013), whereas the $G\alpha_q$ pathway is associated with GH release (Osterstock et al., 2010) and orexigenic effects of ghrelin (Mende et al., 2018).

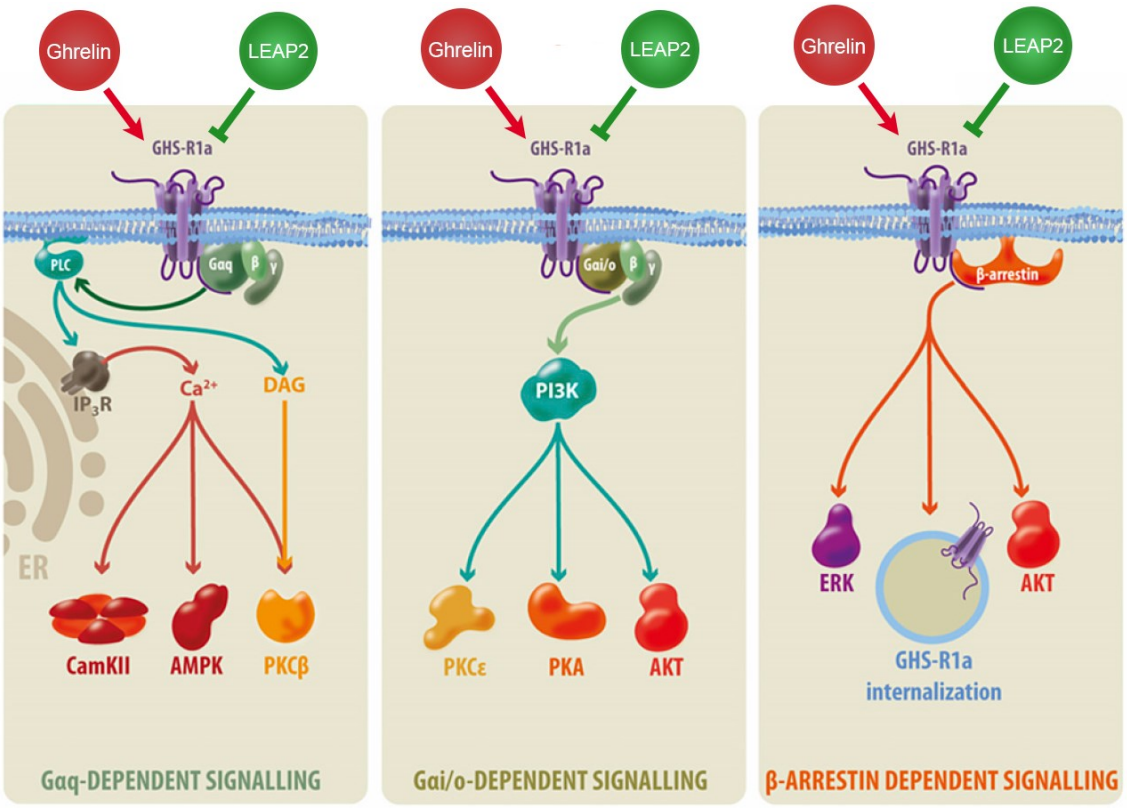

Figure 5: Ghrelin and LEAP2 signaling at GHSR. Ghrelin and LEAP2 bind to GHSR. Ghrelin is a GHSR agonist and activates various signaling pathways. The $G\alpha_q$ activation leads to the activation of phospholipase C (PLC), which leads to an increase in intracellular Ca^{2+} levels from the endoplasmic reticulum (ER). Ca^{2+} activates several proteins including protein kinase C β (PKCβ), calcium calmodulin-dependent protein kinase II (CaMKII), and 5' AMP-activated protein kinase (AMPK). The activation of $G\alpha_{i/o}$ produces the activation of phosphoinositide 3-kinase (PI3K), which produces the activation of protein kinase C ε (PKCε), protein kinase A (PKA), and serine/threonine-specific protein kinase (AKT). In the β-arrestin–dependent pathway, the binding of ghrelin ligand to GHSR activates extracellular signal-regulated kinase (ERK) and AKT that ultimately leads to receptor internalization (Ramirez et al., 2019).

1.5.2 Ghrelin and LEAP2 in food intake regulation

The regulation of energy metabolism is a homeostatic system, with the brain acting as the central coordinator. Body weight stays usually well balanced because the brain is informed about the metabolic status of the individual through hormonal signals reflecting the availability and demand for energy (Woods and D'Alessio, 2008).

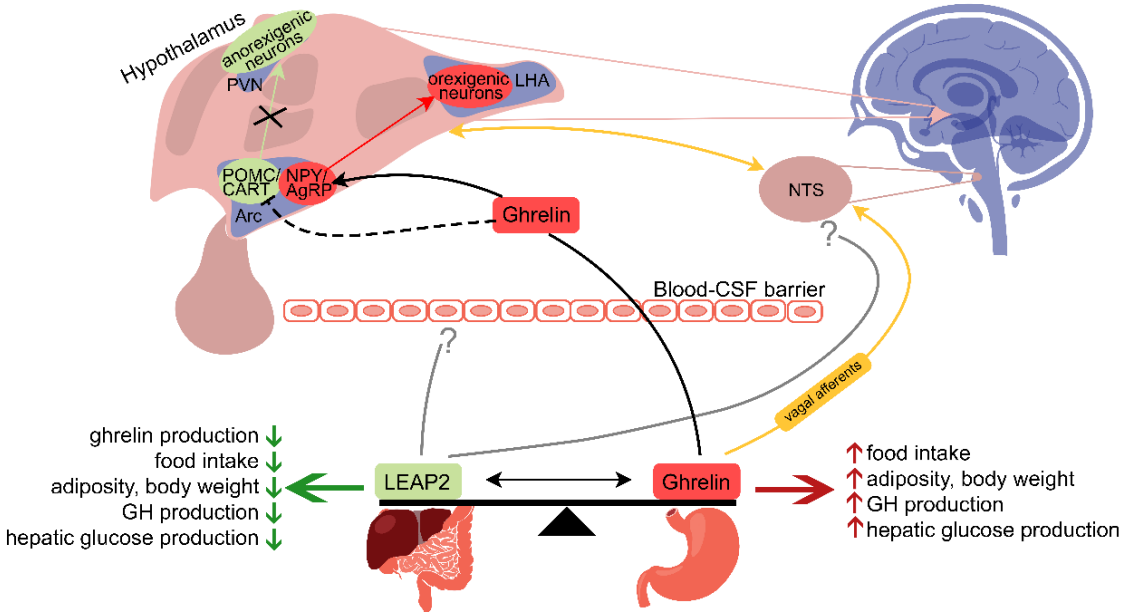

Figure 6:Action of ghrelin and LEAP2 in the organism. Ghrelin is synthesized in the stomach and reaches the *arcuate nucleus* (ARC) via the bloodstream and is transported across the blood-cerebrospinal fluid (CSF) barrier. Ghrelin also stimulates through its vagal afferents, and vagal connection reaches the *nucleus tractus solitarius* (NTS) in the brainstem, which communicates with the hypothalamus. In the hypothalamus, ghrelin activates orexigenic neurons agouti-related peptide/neuropeptide Y (AgRP/NPY), which inhibit anorexigenic neurons pro-opiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART). They are connected to second-order neurons in the paraventricular nucleus (PVN) and the orexigenic neurons in the lateral hypothalamic area (LHA). Liver-expressed antimicrobial peptide 2 (LEAP2) is synthesized in the liver and acts as a counterbalance to ghrelin in organism. GH – growth hormone.

Ghrelin enters the brain through the blood-cerebrospinal fluid (CSF) barrier formed by the choroid plexus and the hypothalamic tanycytes (Uriarte et al., 2021). Ghrelin induced stimulating of food intake occurs in the hypothalamic ARC (Figure 6), which is a part of mediobasal hypothalamus, alongside the third ventricle. ARC contains two neurochemically distinct sets of neurons involved in appetite regulation. The first neuron group is orexigenic and expresses neuropeptide Y (NPY) and Agouti-related protein (AgRP). Increasing NPY release or activation of these neurons leads to increased food intake and decreased energy expenditure. The second neuron group is anorexigenic and expresses cocaine- and amphetamine-regulated transcript (CART) and neuropeptide precursor proopiomelanocortin (POMC), which is processed in hypothalamus into several peptides, such as α-melanocyte-stimulating hormone (α-MSH) (Schwartz et al., 2000). Unlike NPY/AgRP neurons, activation of POMC/CART neurons decreases food intake and increases energy expenditure (Broberger, 2005).

In the ARC, GHSR is expressed in more than 90% of all NPY/AgRP neurons, but it is expressed in less than 8 % of POMC neurons. Consistent with the GHSR expression, ghrelin induces feeding by stimulating NPY and AgRP neuronal activity. At the same time, POMC neuronal activity is suppressed *via* inhibitory γ-aminobutyric acid (GABA) inputs from active NPY/AgRP neurons (Cowley et al., 2003). Chen et al. (2004) showed that double NPY/AgRP knockout mice do not increase food intake in response to ghrelin (Chen et al., 2004). Genetic ablation of AgRP in adulthood abolishes the orexigenic effect of ghrelin (Luquet et al., 2007).

Additionally, ghrelin may increase food intake by acting on the vagus nerve. The ghrelin receptors have been identified in the afferent neurons of the rat and human nodose ganglion, which shows that the vagus nerve may transport ghrelin signals from the stomach to the brain (Sakata et al., 2003). Ghrelin-induced eating is suppressed in rats when the vagal afferent route is blocked either through vagotomy or by applying capsaicin, a specific afferent neurotoxin (Date et al., 2002a). Thus, through the activation of GHSR on the vagal afferent to the stomach, the signal from the vagal afferents may reach the *nucleus tractus solitarius* (NTS), which communicates with the hypothalamus to increase appetite (Delporte, 2013).

LEAP2 acts as both antagonist and inverse agonist of GHSR. LEAP2 antagonist activity can inhibit the ghrelin-induced activation of NPY neurons. LEAP2 inhibitory effects are specific for GHSR; LEAP2 does not inhibit NPY-induced food intake (Islam et al., 2020). However, it is not known yet, how LEAP2 enters the brain and affects NPY neurons, although Mani et al. described the inverse agonist action of LEAP2 at GHSR in hypothalamus, which caused hyperpolarization of NPY neurons and prevented acyl-ghrelin from activating them (Mani et al., 2019).

1.5.3 Ghrelin and LEAP2 in different states of metabolism

The plasma levels of ghrelin and LEAP2 display opposite patterns during fasting and feeding/refeeding. In both humans and mice, plasma LEAP2 rises with higher levels of body weight, body fat, blood sugar, food intake, serum triglycerides (TAG), visceral adiposity, and intrahepatic lipid content (Mani et al., 2019). Plasma LEAP2 decreases after a 24-hour fast and weight loss brought on by a vertical sleeve gastrectomy or a Roux-en-Y gastric bypass (Mani et al., 2019). Ghrelin levels follow an inverse trajectory; thus, the LEAP2-to-ghrelin ratio could be considered a marker of obesity (Mani et al., 2019).

Ghrelin levels in mice with diet-induced obesity (DIO) do not rise when the animals fast (Perreault et al., 2004). In contrast to lean individuals, obese human plasma ghrelin levels do not decrease postprandially (English et al., 2002). Furthermore, peripherally injected ghrelin has no effect after acute administration to agouti mice with adult-onset obesity (Martin et al., 2004), or DIO mice (Perreault et al., 2004). Resistance to ghrelin is observed also in DIO mice after its chronic administration (Gardiner et al., 2010). Ghrelin resistance develops due to several reasons. AgRP/NPY neurons are ineffectively activated, which reduces the orexigenic action of ghrelin in people with obesity (Briggs et al., 2010). It has been demonstrated that feeding mice high-fat (HF) diet for 12 weeks causes a reduction in plasma ghrelin, *GHRL* mRNA in the stomach, and *GHSR* mRNA in the hypothalamus, which indicates the suppression of the ghrelin neuroendocrine axis. Ghrelin does not increase food intake, *NPY* and *AgRP* mRNA expression, or NPY and AgRP peptide secretion in DIO mice, regardless it is delivered peripherally or centrally. However, NPY administered intracerebroventricularly (ICV) stimulates food intake in both DIO and lean mice, demonstrating that obesity has no impact on downstream ghrelin signalling (Briggs and Andrews, 2011).

A low-calorie diet helps obese people to lose weight and restores ghrelin sensitivity, but a following increase of ghrelin plasma levels in the blood promotes weight gain after ending the diet (Briggs et al., 2013). It was suggested that ghrelin resistance is a mechanism created to maintain a greater body weight set point during periods of food availability, maximizing energy reserves during periods of food scarcity (Zigman et al., 2016). This could be the cause of limited efficacy of ghrelin antagonists in the treatment of obesity to date (Vodnik et al., 2016).

Although ghrelin resistance occurs in obese individuals, targeting GHSR could still be a way to develop an anti-obesity drug using LEAP2 analogs. LEAP2 analogs might be able to stop rebound weight gain after switching to a low-calorie diet (Andrews, 2019). In less severe states of obesity, LEAP2 does not show a compensatory effect, thus further increasing LEAP2 may decrease food intake and body weight gain (Mani et al., 2019). Additionally, people who have lost weight through lifestyle changes are vulnerable to regaining it due to an increase in plasma ghrelin, so LEAP2 therapies may be helpful (Gupta et al., 2021).

1.6 GHSR system as a pharmacological target

1.6.1 GHSR agonists

The ghrelin system can be a valuable target for the development of treatment to fight anorexia and cachexia. The orexigenic and lipogenic effect of ghrelin or its analogs could enhance white adipose tissue mass under pathological conditions such as anorexia nervosa, sarcopenia, and cancer-associated cachexia.

GHSR non-peptidic agonist Ibutamoren (MK-0677) (Figure 7A) was developed by Merck in the USA (Patchett et al., 1995). Ibutamoren increased GH secretion and fat-free mass in randomized clinical trial (Nass et al., 2008), however, the further development was discontinued due to increased risk of heart failure in patients with hip fracture (Adunsky et al., 2011).

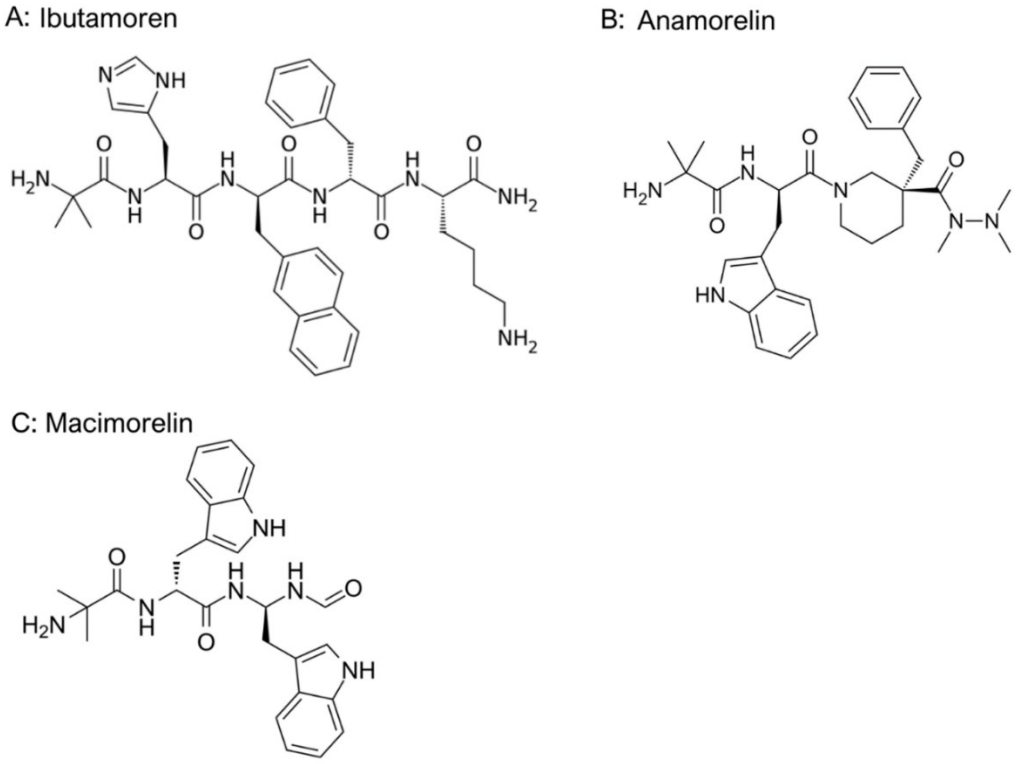

Figure 7: Structures of GHSR non-peptidic agonists Ibutamoren (A) (Patchett et al., 1995), Anamorelin (B) (Pietra et al., 2014), and Macimorelin (C) (Guerlavais et al., 2003)

Another GHSR non-peptidic agonist Anamorelin (Figure 7B) was developed by Helsinn Therapeutics in New Jersey, USA. Anamorelin increased body weight and GH plasma levels in rats (Pietra et al., 2014). Due to these promising effects, Anamorelin advanced to Phase III clinical trials to assess its safety and effectiveness in patients with cachexia induced by non-small cell lung carcinoma. The application for approval was rejected in

2017 by EMA (Temel et al., 2016, Currow et al., 2017), however, it has been recently approved for cancer cachexia treatment in Japan (Wakabayashi et al., 2021).

Macimorelin (JMV 1843) (Figure 7C) was developed by the team of J. Martinez (CNRS, Montpellier, France) and clinical trial was performed by Aeterna Zentaris in Canada. Macimorelin stimulated GH secretion in young dogs (Guerlavais et al., 2003) and in healthy men (Piccoli et al., 2007). A single SC injection of Macimorelin increased food intake in fed mice (Holubova et al., 2013). Macimorelin was approved by U.S. Food and Drug Administration (FDA) in 2017 and EMA in 2019 as a method to diagnose GH deficiency but has not been examined in repeated doses or the cancer cachexia setting (Herodes et al., 2023).

1.6.2 GHSR antagonists

Compounds, that antagonize GHSR can be used for the development of anti-obesity treatment.

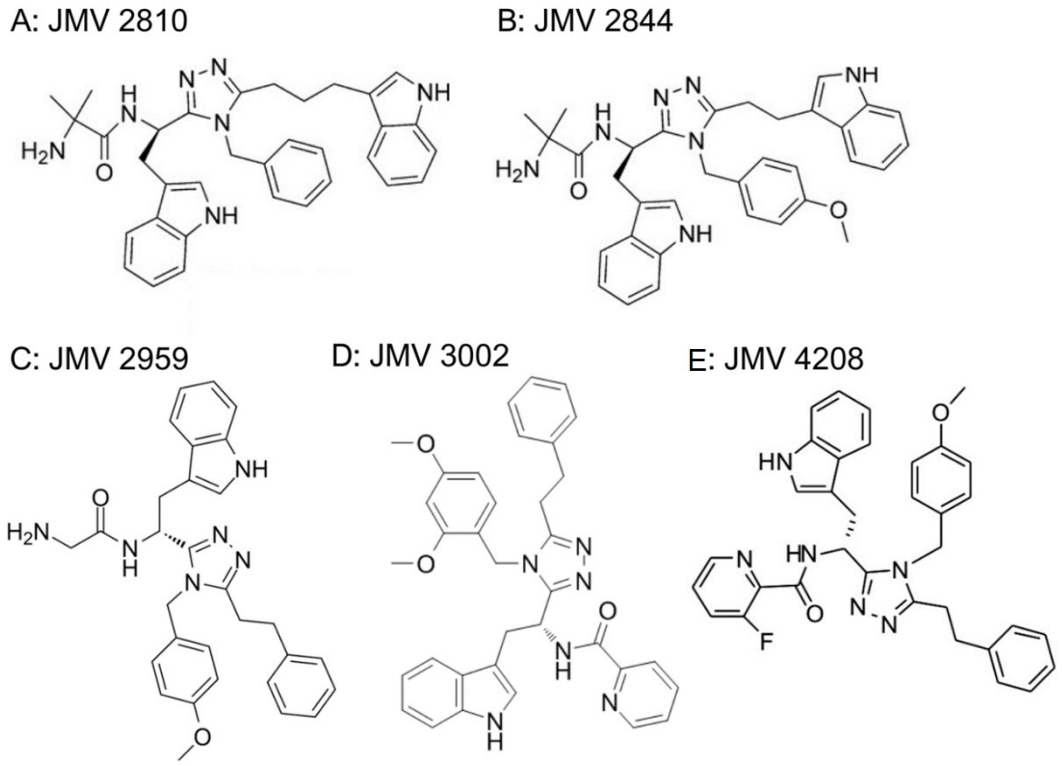

Figure 8: Structures of GHSR antagonists JMV 2810 (A), JMV 2844 (B) (Demange et al., 2007); JMV 2959 (C) (Moulin et al., 2007), JMV 3002 (D) (Salome et al., 2009), and JMV 4208 (E) (Holubova et al., 2014).

GHS analog [D-Lys^3]-GHRP-6 (His-D-Trp-D-Lys-Trp-D-Phe-Lys-NH_2), derived from GHSR agonist GHRP-6, acted as a weak partial antagonist and decreased food intake in lean mice, in DIO mice and leptin-deficient ob/ob mice (Asakawa et al., 2003). Long-term

administration of [D-Lys^3]-GHRP-6 decreased GH secretion and body fat in mice. [D-Lys^3]-GHRP-6 decreased food intake in ovariectomized mice fed HF diet, a model of postmenopausal obesity (Maletinska et al., 2011). However, side effects such as reduced insulin in plasma and increased blood glucose led to insulin intolerance (Mosa et al., 2018), thus excluding [D-Lys^3]-GHRP-6 from suitable candidates for ghrelin signaling inhibition.

Other GHSR antagonists derived from met-enkephalin were synthesized with a 1,2,4-triazole structure (Figure 8). SC injection of JMV2810 inhibited GHRP-6-induced food intake (Demange et al., 2007). The most potent compounds JMV2844 (SC), JMV2959 (ICV, SC, intraperitoneal), and JMV3002 (ICV) reduced food intake in rats (Demange et al., 2007, Moulin et al., 2007, Salome et al., 2009). JMV 4208 and 3002 (SC) decreased food intake in both fasted lean mice and DIO mice (Holubova et al., 2014).

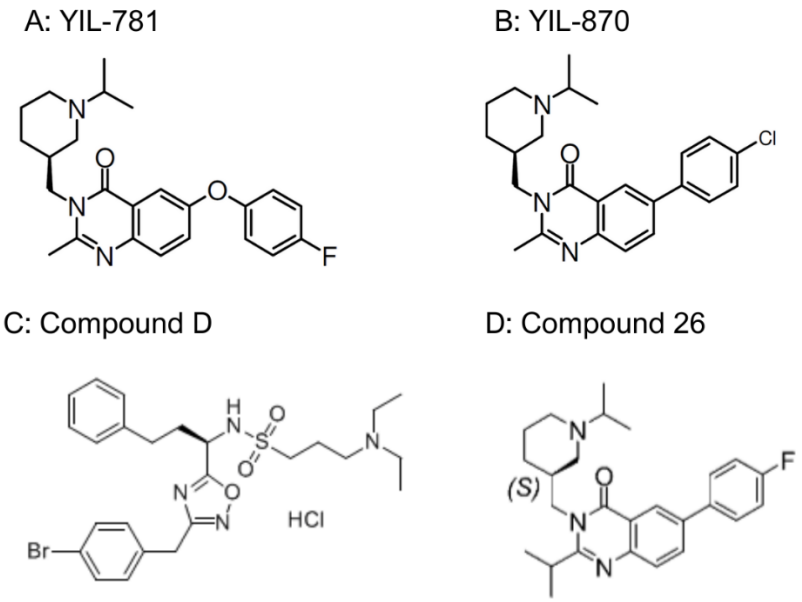

Figure 9: Structures of GHSR antagonists YIL-781 (A), YIL-870 (B) (Esler et al., 2007), Compound D (C) (Longo et al., 2011), Compound 26 (D) (Rudolph et al., 2007)

Other compounds that are promising GHSR antagonists are non-peptide small molecules, YIL-781 and YIL-870 (Figure 9 A, B), which after oral administration decreased body weight, food intake, and fat mass in DIO mice (Esler et al., 2007). Compound D (Figure 9 C) decreased food intake and compound B reduced body weight due to a reduction of white adipose tissue with improved glucose disposal and insulin sensitivity in DIO mice (Longo et al., 2011). Compound 26 (Figure 9 D) derived from piperidine-substituted quinazolinone reduced food intake and body weight along with glucose-lowering effects in mice (Rudolph et al., 2007).

1.6.3 GHSR inverse agonists

Due to the reported high constitutive activity of the GHSR receptor (Holst et al., 2003), GHSR inverse agonists might be superior candidates for anti-obesity therapeutics compared to competitive antagonists. The first described inverse agonist of GHSR is [D-Arg^{1}, D-Phe^{5}, D-Trp$^{7, 9}$, Leu^{11}]-substance P (SPa) (Table 1). Although substance P causes pain, stress, and anxiety, its derivative SPa is considered to act as an inverse agonist of GHSR with a low antagonist action (Holst et al., 2009). However, Maletínská et al. (Maletinska et al., 2011) did not prove the inverse agonist effect of SPa at GHSR, which might be caused by the non-selective activity of SPa, which binds not only to GHSR but also to the neurokinin (Djanani et al., 2003) and bombesin (MacKinnon et al., 2001) receptors.

Table 1: Sequences of Substance P and SPa

Compound	Sequence
Substance P	Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met
SPa	D-Arg-Pro-Lys-Pro-D-Phe-Gln-D-Trp-Phe-D-Trp-Leu-Leu-NH_2

Compound 33 (Figure 10A), 2-alkylamino nicotinamide derivative, suppressed weight gain in obese rats (Takahashi et al., 2015). Artificial small molecules GHSR-IA1 and GHSR-IA2 reduced food intake in lean mice. Moreover, chronically administered GHSR-IA2 reduced food intake and body weight in DIO mice (Abegg et al., 2017).

PF-5190457 (Figure 10B), a spiroazetidino-piperidine derivative, is the first GHSR inverse agonist tested in clinical trials. It increased insulin secretion, decreased postprandial glucose, and attenuated secretion of the GH in humans (Denney et al., 2017). Moreover, PF-5190457 was well tolerated and safe in Phase I in healthy individuals (Denney et al., 2017, Lee et al., 2020).

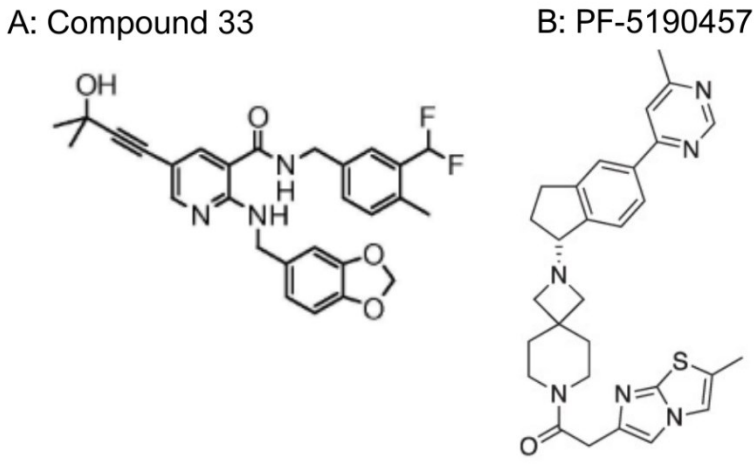

Figure 10: Structures of GHSR inverse agonists Compound 33 (A) (Takahashi et al., 2015), and PF-5190457 (B) (Denney et al., 2017)

1.6.3.1 LEAP2 as a potential anti-obesity drug

The anorexigenic effect of LEAP2 was first described in 2018 (Ge et al., 2018) and it was stated that LEAP2 SC administration attenuated fasting-induced food intake in rats and mice (Mani et al., 2019, Lugilde et al., 2022). Similarly, LEAP2 was reported to inhibit ghrelin-induced effects on food intake independent of diet in mice and rats (Ge et al., 2018, Lugilde et al., 2022). Chronic ICV administration of LEAP2 was shown to centrally reduce food intake in calorie-restricted mice (Islam et al., 2020). However, effect of long-term peripheral administration of LEAP2 on food intake and body weight still needs to be tested.

The mechanism and potential therapeutic applications of LEAP2 have been well-established, but further investigation is needed in preclinical studies. Hagemann et al. went a step further in their new study, giving LEAP2 infusion or placebo to 20 healthy men (Hagemann et al., 2022). Men who received the LEAP2 intravenous intervention had reduced *ad libitum* food consumption and meal duration. There were no significant safety issues found during this pilot observation.

1.7 Structure-activity study of ghrelin and LEAP2

Peptides are increasingly being recognized as potential therapeutics due to their beneficial properties such as high affinity to natural receptors and low toxicity for the organism. However, their low stability and short half-life present significant challenges (Goodwin et al., 2012). To maximize their pharmacological potential, it is important to find effective ways to modify peptides to make their syntheses feasible and preserve their biological activity and increase their stability.

1.7.1 Lipidization

Lipidization of peptides is a potential tool to increase stability and overcome the inability to cross the blood-brain barrier (Zhang and Bulaj, 2012). Moreover, this modification is approved by FDA, thus it is desirable to lipidize peptides to obtain optimal pharmacokinetic and pharmacodynamic characteristics (Menacho-Melgar et al., 2019). Different types of lipids can be used for lipidization, such as isoprenoids, glycosylphosphatidylinositol, phosphatidylethanolamine, cholesterol, or diverse fatty acids (Hanna et al., 2022).

The method that is commonly used in our laboratory is fatty acylation. Diverse fatty acids are covalently attached to the peptide/protein. Short, medium, or long fatty acids can be used, however, the most commonly used fatty acids are: octanoic acid (C8), myristic acid (C14), palmitic acid (C16), and stearic acid (C18) (Maletinska et al., 2015). The properties of lipidized peptides are influenced not only by the choice of fatty acid but also by the bond that attaches fatty acid to protein. While the amide bond is strong and irreversible, ester bonds formed by O-esterification and S-esterification, and disulfide bonds are reversible and weak (Zhang and Bulaj, 2012).

1.7.2 LEAP2 analogs

Synthesis of natural LEAP2 is extremely difficult because of its two S-S bonds between cysteines. However, the entire sequence of LEAP2 is not required for its effects. Specifically, the N-terminal part is responsible for binding to the receptor and exerting its activity. M'Kadmi et al. proved, that the N-terminal segment of LEAP2 acts as an inverse agonist and competitive antagonist of GHSR *in vitro* (M'Kadmi et al., 2019). Moreover, the N-terminal part of LEAP2 can effectively reduce ghrelin-induced food intake in mice. For maximum potency and effectiveness, the N-terminal sequence containing at least the region 1–12 is needed. The N-terminal sequence containing the region 1-14 has similar potency and efficacy (Figure 11). N-terminal methionine residue is easily oxidized; therefore, methionine can be substituted for norleucine to avoid oxidation.

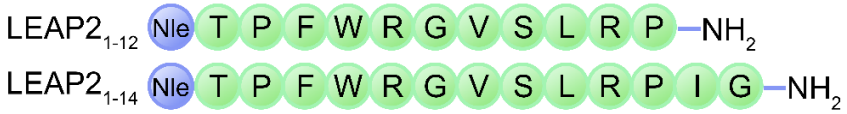

Figure 11: Structures of LEAP2$_{1-12}$ and LEAP2$_{1-14}$

1.7.3 Ghrelin stable analog [Dpr^3]Ghrelin

The biological stability of ghrelin is highly limited, because of its ester bond in Ser^3-O-octanoyl, which is an easy target for hydrolysis (Holubova et al., 2018). Octanoyl-ghrelin hydrolysis can be overcome by replacing Ser^3 with diaminopropionic acid (Dpr). Octanoic acid, which is essential for the biological activity of the peptide, is anchored to the [Dpr^3]Ghrelin (Figure 12) chain by stable amid bond (Bednarek et al., 2000, Maletinska et al., 2012).

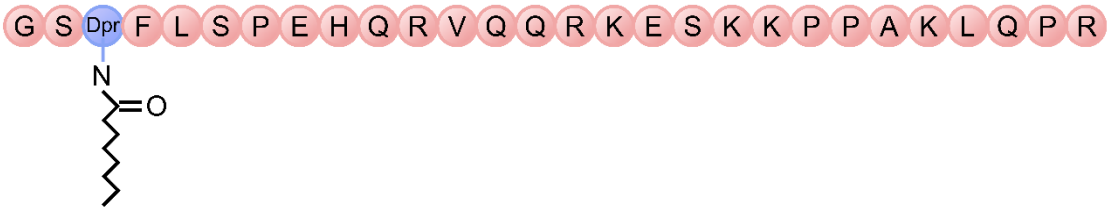

Figure 12: Structure of [Dpr^3]Ghrelin

2 AIMS OF THE THESIS

- **Investigation of *in vitro* characteristics of LEAP2(1-14) and its lipidized analogs compared to natural LEAP2.**

LEAP2 is a potent inverse agonist of GHSR. Its N-terminal LEAP2(1-14) analog was modified with various fatty acids because N-terminal fragment LEAP2 is crucial for LEAP2 biological activity. The first aim of the thesis was to select the most stable and bioavailable lipidized analog of LEAP2(1-14) by analyzing affinity and inverse agonist and antagonist activity at GHSR overexpressed in U2OS cell line.

- **Study of LEAP2(1-14) analogs in short-term *in vivo* experiments.**

The second aim was to study the ability of lipidized and non-lipidized LEAP2(1-14) analog to suppress the orexigenic actions of high endogenous ghrelin in fasted mice as well as of exogenous stable ghrelin analog [Dpr^3]Ghrelin in free-fed mice.

- **Research of the long-term effect of high-fat diet on progress of obesity regarding the development of ghrelin and LEAP2 resistance.**

The third aim was to study the long-term effects of high-fat diet on the interplay of ghrelin and LEAP2 in mice and the development of obesity and ghrelin and LEAP2 resistance.

- **Observation of palm-LEAP2(1-14) ability to prevent development of HF diet-induced obesity**

The fourth aim was to find out if palm-LEAP2(1-14) has the potential to minimize the effects of high-fat diet and normalize morphometric and metabolic parameters which are associated with obesity.

3 METHODS

3.1 Peptide synthesis

All peptide structures are shown in table 2.

Table 2: Overview of peptide sequences

Compound	Structure
Ghrelin	GSS(O-octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR
[Dpr^3]Ghrelin	GS-Dpr-(N-octanoyl)FLSPEHQKAQQRKESKKPPAKLQPR
LEAP2	MTPFWRGVSLRPIGAS**C**RDDSE**C**ITRL**C**RKRR**C**SLSVAQE Disulfide bridges: C17-C28 & C23-C33
LEAP2(1-14)	Nle-TPFWRGVSLRPIG-β-Ala-Lys-NH_2
myr-LEAP2(1-14)	Nle-TPFWRGVSLRPIG- β-Ala-Lys(myr)-NH_2
palm-LEAP2(1-14)	Nle-TPFWRGVSLRPIG- β-Ala-Lys(palm)-NH_2
stear-LEAP2(1-14)	Nle-TPFWRGVSLRPIG- β-Ala-Lys(stear)-NH_2

Myr: myristoyl, palm: palmitoyl, stear: stearoyl, Nle: norleucine.

LEAP-2(38-77) (#075-40) was purchased from Pheonix Pharmaceuticals (Burlingame, CA USA).

3.1.1 Ghrelin and [Dpr^3]Ghrelin synthesis

Ghrelin and its stable analog [Dpr^3]Ghrelin (Table 2) were synthesized at the Institute of Organic Chemistry and Biochemistry of the Czech Academy of Sciences (IOCB, CAS, Prague, Czech Republic) by the solid-phase synthesis according to previously described protocol (Maixnerova et al., 2007) using the Fmoc strategy on an ABI 433A synthesizer (Applied Biosystems, Foster City, CA, USA). Lipidization with corresponding fatty acid (myristic, palmitic, or stearic) was performed on the fully protected peptide before cleaving the peptide from the resin as previously described (Maletínská et al., 2012). The process of purifying and identifying the peptides was completed using liquid chromatography (LC) in combination with mass spectrometry (MS). The peptides had a purity level of over 95%. Peptide synthesis was performed by Miroslava Blechová, MSc., Drug Discovery, IOCB.

Ghrelin was used in the *in vitro* experiments and [Dpr^3]Ghrelin was used in the *in vivo* experiments.

3.1.2 LEAP2(1-14) and lipidized LEAP2(1-14) analogs

We used a LEAP2 fragment containing the initial 14 residues of N-terminal LEAP2 and a β-Ala-Lys-NH_2 linker (hereafter named LEAP2(1-14)) employed for the subsequent lipidization with myristic, palmitic, and stearic acid (Table 2).

LEAP2(1-14) and its lipidized analogs were synthesized at the Institut des Boimolécules Max Mousseron, University of Montpellier (Montpellier, France) (IBMM) using solid-phase peptide synthesis, starting with Agilent Amphisphere 40 RAM resin (0.36 mmol/g) 1100 mg resin, 0.4 mmol. The Fmoc chemistry and HATU/N,N-diisopropylethylamine (DIEA) were used for coupling, along with piperidine/dimethylformamide (DMF) for deprotection. Coupling steps were performed twice for 10 min. Subsequently, the Alloc group of Lys^{16} was deprotected in dichloromethane (DCM) using $Pd(PPh_3)_4$ 0.25 eq. and $PhSiH_3$ 25 eq. twice for 30 min. The peptidyl resin was then washed, dried, and divided into four parts. Three parts were lipidized by coupling them with the corresponding acids using BOP reagent and DIEA, while the fourth part was directly deprotected. Final deprotection was done using trifluoroacetic acid (TFA)/triisopropylsilane (TIS)/H_2O mixture. The peptide was purified using Gilson PLC 2250 Preparative RP-HPLC system with a preparative column in the gradient mode.

The LC/MS system used in the analysis consisted of an HPLC-ZQ (Waters) with an electrospray ionization (ESI) source. The Phenomenex Kinetex column (C18, 100 Å, 100×2.1 mm, 2.6 µm) was used in the analysis. The flow rate was set at 0.5 mL/min and a gradient of 0-100% B over 5 min was used, with eluent A being water/0.1% HCOOH and eluent B being acetonitrile/0.1% HCOOH. Positive-ion electrospray mass spectra were acquired from 100 to 1500 m/z with a scan time of 0.2 s. For both the nebulizing and drying gas, nitrogen was utilized. The peptides that were created had a purity level of over 95%. Peptide synthesis was performed by the team of Sonia Cantel, PhD, and Jean-Alain Fehrentz, PhD, IBMM.

3.1.3 Peptide iodination

Ghrelin was iodinated at His^9 with $Na^{125}I$ (Izotop, Budapest, Hungary) using Iodo-Gen (Pierce, Rockford, IL, USA) according to the previously described protocol (Maletinska et al., 2012). The peptides' identity was confirmed by a MALDI-TOF Reflex IV mass spectrometer (Bruker Daltonics, Billerica, MA, USA), and the ^{125}I-ghrelin's specific activity was approximately 2000 Ci/mmol. After being evaporated and aliquoted, the purified ^{125}I-ghrelin was kept at -20 °C and used in binding studies within a month. Peptide iodination was performed by the team of Ing. Aleš Marek, PhD at IOCB Prague.

3.2 *In vitro* testing of LEAP2(1-14) analogs

3.2.1 Cell culture

T-REx™ Tango™ GHSR-bla U2OS cells, which overexpress GHSR and have a β-lactamase reporter gene controlled by an upstream activation site response element, were provided by Thermo Fisher Scientific Inc. (Waltham, MA, USA). The cells were grown in a humidified incubator at 37°C with 5% CO_2 in McCoy's 5A medium, which was supplemented with 10% dialyzed fetal bovine serum, 0.1 mM nonessential amino acids, 25 mM HEPES buffer (pH 7.3), 1 mM sodium pyruvate, 1% streptomycin/penicillin, 200 µg/mL zeocin, 50 µg/mL hygromycin, and 100 µg/mL geneticin based on the guidelines provided by Thermo Fisher.

3.2.2 Competitive and saturation binding experiments

Competitive binding experiments were performed according to Motulsky and Neubig (Motulsky and Neubig, 2002a). T-REx™ Tango™ GHSR-bla U2OS cells were seeded in 24-well plates at a density of 20,000 cells/well. The cells were allowed to grow for 3 days. Doxycycline was added 16 hours before the experiment at a final dosage of 1.25 ng/mL. Competitive binding studies were conducted in 200 µl of binding buffer (50 mM Tris-Cl pH 7.4, 118 mM NaCl, 5 mM $MgCl_2$, 4.7 mM KCl, 0.1% BSA, and 2 g/L glucose), 25 µl of 0.1 nM ^{125}I-ghrelin, and peptide analogs at final concentrations from 10^{-12} to 10^{-5} M. Saturation experiments were conducted in the same buffer as competitive experiments with increasing concentration of ^{125}I-ghrelin from 0.05 to 2.5 nM and non-labeled ghrelin at concentration 10^{-5} M.

At both competitive and saturation experiments, cells were incubated for 60 minutes at 23°C and subsequently rinsed with wash buffer (20 mM Tris pH 7.4, 118 mM NaCl, 4.7 mM KCl, and 5 mM $MgCl_2$), once in case of competitive binding experiments, or three times in case of saturation binding experiments, and lysed in 0.1 M NaOH. Radioactivity was measured by a γ-counter Wizard 1470 Automatic Gamma Counter (Perkin Elmer, Waltham, MA, USA). The experiments were carried out in duplicate at least three times.

3.2.3 Beta-lactamase-dependent fluorescence resonance energy transfer (FRET) assay

The agonist/inverse agonist and antagonist properties of peptides were observed using T-REx™ Tango™ GHSR-bla U2OS cells. The cells were seeded in 384-well plates at a density of 10,000 cells/well in FreeStyle™ 293 Expression Medium supplemented with doxycycline at the final concentration of either 1.25 ng/mL (agonist and antagonist assay

mode) or 100 ng/mL (inverse agonist assay mode). The assay was performed according to Thermo Fisher's protocol and according to our previous study (Holubova et al., 2018). Agonists and inverse agonists of GHSR were tested at the final concentration from 10^{-12} to 10^{-5} M of peptides. Antagonist assay mode was performed with LEAP2 analogs at the final concentration from 10^{-8} to 10^{-6} M and ghrelin at final concentrations ranging from 10^{-12} to 10^{-5} M. The FlexStation 3 fluorescent plate reader (Molecular Devices, Sunnyvale, CA, USA) was used to measure fluorescence at an excitation wavelength of 409 nm, and emission wavelengths of 460 nm and 530 nm. The experiments were carried out in duplicate at least three times.

3.2.4 Stability of selected LEAP2(1-14) analogs in rat plasma

The stability of selected LEAP2(1-14) analogs was measured at the University of Chemistry and Technology by prof. RNDr. Dr. David Sýkora.

To prepare stock solutions of LEAP2(1-14) and palm-LEAP2(1-14), the compounds were dissolved in 0.1% HCOOH/H_2O to a final concentration of 1 mg/mL. Blank rat plasma was placed in 1.5 mL Protein LoBind Tubes (Eppendorf, USA), thermostated (Incubator NB-T205, N-Biotek, Korea) at 37 °C, and spiked with 10 µL of appropriate peptide solution. The samples were incubated for specified time intervals (0, 1, 2, 4, 8, and 24 h). Endogenous proteins were precipitated with 400 µL of 0.1% HCOOH in acetonitrile/H_2O 8/2 (v/v) solution. After centrifugation (5 min, 14,000×*g,* 4 °C, Micro Star R17, VWR, Germany), the supernatant was transferred to 1.8 mL glass vials for LC/MS. The experiments were performed in triplicates.

UltiMate 3000 (Thermo, USA) consisting of a DGP-3600SD pump, a WPS-3000SL autosampler, and a TCC-3000SD column compartment was used to perform LC. LC eluate was analyzed with a Q-TRAP 3200 mass spectrometer (AB Sciex, Canada). The LC/MS method utilized a 2.1x50 mm XBridge Premier BEH C18 column (Waters, USA) with 2.5 µm (VanGuard Fit) particles. Mobile phase A composed of 0.1% HCOOH in water, while mobile phase B was made up of 0.1% HCOOH in acetonitrile. The gradient time profile was as follows: 0-5 minutes - 100% A to 100% B, 5-7 minutes - 100% B, 7-7.2 minutes - 100% B to 100% A, and 7.2-12 minutes - 100% A. The flow rate was 0.200 mL/min, and the column temperature was kept at 25°C, while the autosampler temperature was set at 15°C. The injection volume was 2 µL of the sample. The data was analyzed using the software Analyst version 1.6 (AB Sciex). The peptides were analyzed using multiple reaction monitoring with a turbo-V ion source equipped with electrospray ionization (ESI)

probe in the positive mode. The ion spray voltage was set to 5500 V, curtain gas at 15 psig, source temperature at 450 °C, ion source gas (1) at 50 psig, and ion source gas (2) at 60 psig.

3.3 *In vivo* experiments

3.3.1 Experimental animals

Male C57Bl/6J (for short-term experiments) or C57Bl/6N (for long term experiments with HF diet) mice (Charles River, Sulzfeld, Germany) were housed in the animal facility under standard conditions at temperature of 23 ± 2 °C and a daily cycle of 12 h light and dark (light from 6:00 AM). Mice had free access to water and either standard (St) diet (ssniff R/M-H cat. no. V1534; Spezialdiäten GmbH, Soest, Germany), which contained 8% kcal from fat, 21% kcal from protein, 71% kcal from carbohydrates or high fat (HF) diet, which contained 60% kcal from fats, 13% kcal from proteins and 27% kcal from carbohydrates (Maletinska et al., 2015).

All animal experiments followed the ethical guidelines of the EU (86/609/EU) for work with animals, and the Act of the Czech Republic law 246/1992, and were approved by the Committee for Experiments with Laboratory Animals of the CAS.

3.3.2 Short-term *in vivo* testing of LEAP2(1-14) analogs

3.3.2.1 Acute food intake after SC administration of LEAP2(1-14) analogs to mice

12-week-old mice (n=5) were kept in individual cages and provided with St diet and water *ad libitum*. The anorexigenic effect of LEAP2 analogs was tested in overnight fasted (17 h) mice. At 8:00 a.m. on the day of the experiment, mice were SC injected with 150 µl of either saline, which was used as a control, or LEAP2 analog, which was dissolved in saline to a final dose of 5 mg/kg of body weight. Mice were given pre-weighted pellets 30 minutes after the peptide SC injection. Food intake was monitored every 30 min for at least 7 hours.

The ability of LEAP2 analogs to suppress the orexigenic effect of [Dpr^3]Ghrelin was tested in free-fed mice. At 8:00 a.m. on the day of the experiment, mice were SC injected with 150 µl of either saline or LEAP2 analog, which was dissolved in saline to a final dose of 5 mg/kg of body weight. 15 minutes after the first injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight. Mice were given pre-weighted pellets 30 minutes after the first injection. Food intake was monitored every 30 min for at

least 7 hours. The mice had free access to water during the experiment. The results were presented as the amount of food consumed in grams.

The data were calculated using GraphPad 8 Prism software. A 2-way analysis of variance (ANOVA), followed by a Bonferroni post hoc test, was used as described in the figure legends. The significance level of $P<0.05$ was considered to be statistically significant.

3.3.2.2 GH release after acute SC administration of [Dpr^3]Ghrelin and selected LEAP2(1-14) analogs to mice

8-week-old mice (n=5-8) were placed in individual cages and provided with St diet and water *ad libitum*. Effect of LEAP2(1-14) and palm-LEAP2(1-14) on [Dpr^3]Ghrelin-induced GH release was tested. At 8:00 a.m. on the day of the experiment, mice were SC injected with 200 µl of either saline or LEAP2 analog which was dissolved in saline to a final dose of 10 mg/kg of body weight. 15 minutes after the first injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight. Mice were sacrificed by decapitation 30 minutes after the beginning of the experiment. Blood was collected in tubes with EDTA, plasma was separated and stored at -80°C until the next use. The concentration of GH in plasma samples was measured using a kit for rat/mouse GH enzyme-linked immunosorbent assay (ELISA) (cat. no. EZRMGH-45K; Merck-Millipore, Burlington, Massachusetts, USA) according to the instructions provided by the manufacturer.

The data were calculated using GraphPad 8 Prism software. A one-way ANOVA, followed by a Bonferroni post hoc test, was used when appropriate as described in the tables and figure legends. The significance level of $P<0.05$ was considered to be statistically significant.

3.3.3 Experimental designs of long-term *in vivo* experiments

3.3.3.1 Long-term *in vivo* study 1: Progress of obesity at HF diet feeding

The experimental design is presented in Figure 13. Mice were separated into 12 groups (n=8) and housed in groups of four individuals per cage when they reached 8 weeks of age. The mice's body weights were monitored every week, and they were sacrificed at various time points, as shown in Figure 13. An oral glucose tolerance test (OGTT) was performed on each group after a 6-hour fasting one week before sacrificing. On the day of sacrificing, free-fed mice were anesthetized with pentobarbital. Blood from heart was collected in tubes with EDTA and plasma was separated and stored at -20°C. Plasma for ELISA detection of active and total ghrelin was pre-treated with Pefabloc® (Carl Roth, Karlsruhe,

Germany) and acidified using HCl. Mice were then perfused with heparinized saline (at a concentration of 20 U/ml) and dissected.

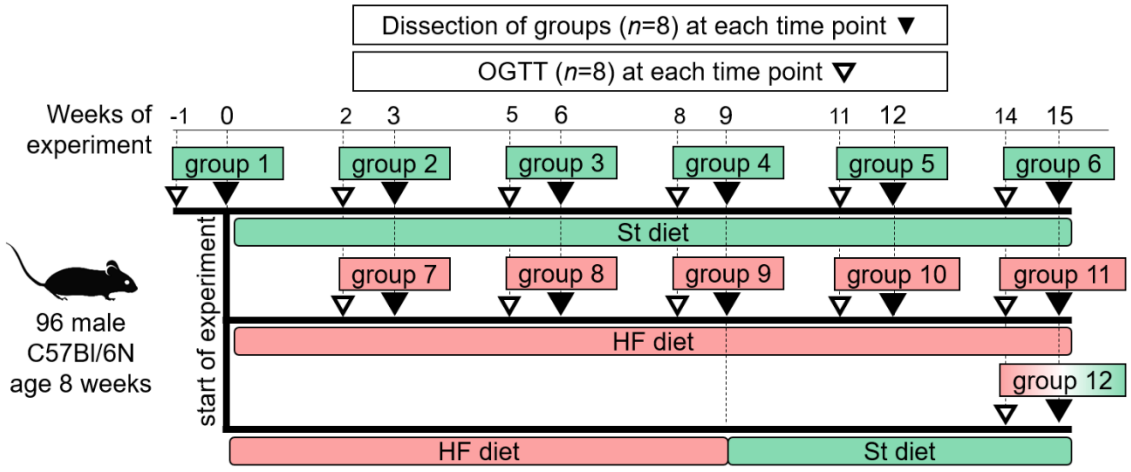

Figure 13: Experimental design of long-term *in vivo* study 1: 96 male C57Bl/6N mice were separated into 12 groups. The first group was used as a control and sacrificed at the beginning of the experiment. Every three weeks, one group of mice that were fed a standard (St) diet (groups 2-6) and one group fed a high-fat (HF) diet (groups 7-11) were sacrificed until week 15. The last group (group 12) was fed a HF diet for the first 9 weeks, then switched to a St diet for the following 6 weeks before being sacrificed at week 15. The white triangles indicate the week of the experiment when the OGTT was performed. The black triangles indicate the week of the experiment when the dissection was performed.

The liver, and epididymal white adipose tissue (eWAT) were weighted. Hypothalamus, eWAT and part of liver were frozen in liquid nitrogen and stored at -80°C. One liver lobe was fixed in 4% paraformaldehyde (PFA) for liver histology. Morphometric and biochemical analyses, and mRNA analyses of tissues were performed. The results were analyzed using two-way ANOVA with Bonferroni's post hoc test or one-way ANOVA with Tukey's test or multiple *t*-tests with Bonferroni-Dunn's method for multiple comparisons as described in the figure legends. The significance level of $P<0.05$ was considered to be statistically significant.

3.3.3.1.1 Oral glucose tolerance test

OGTT was performed at each experimental group one week before sacrificing. Blood samples were collected from the tail vein of the animals after 6 hours of fasting, at 12:00 p.m. (designated as time point 0) to measure insulin, cholesterol, and TAG levels. After this, the animals were perorally loaded with a glucose at a dose of 2 g/kg body weight. Blood glucose levels were measured in whole blood at 15, 30, 60, 120, and 180 minutes after the glucose gavage using a glucometer (LifeScan, Inc., Milpitas, CA, USA).

3.3.3.2 Long-term *in vivo* study 2: Long-term effect of HF diet on development of [Dpr^3]Ghrelin and palm-LEAP2(1-14) resistance

The experimental design is presented in Figure 14. Mice were separated into 5 groups (n=8) and housed in separate cages when they reached 8 weeks of age. The body weights were monitored every week. Two groups were provided with a St diet, while three groups were given a HF diet from the age of 8 weeks. To examine the impact of peripheral ghrelin and palm-LEAP2(1-14) on feeding, the sensitivity to ghrelin and LEAP2 was evaluated in mice that were allowed to feed freely at weeks 0, 2, 4, 8, 10, and 12 of the experiment. At week 8, groups fed with HF diet were switched to a St diet.

The food intake experiment was performed similarly to part 3.3.2.1. At 8:00 a.m. on the day of the experiment, mice were SC injected with 150 µl of either saline or palm-LEAP2(1-14) analog, which was dissolved in saline to a final dose of 5 mg/kg of body weight. 15 minutes after the first injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight. Mice were given pre-weighted pellets 30 minutes after beginning of the experiment. Food intake was monitored every 30 min for at least 6 hours. The mice had free access to water during the experiment. The results were presented as the amount of food consumed in grams. The results were analyzed using two-way ANOVA with Bonferroni's post hoc test or *t*-test as described in the figure legends. The significance level of $P<0.05$ was considered to be statistically significant.

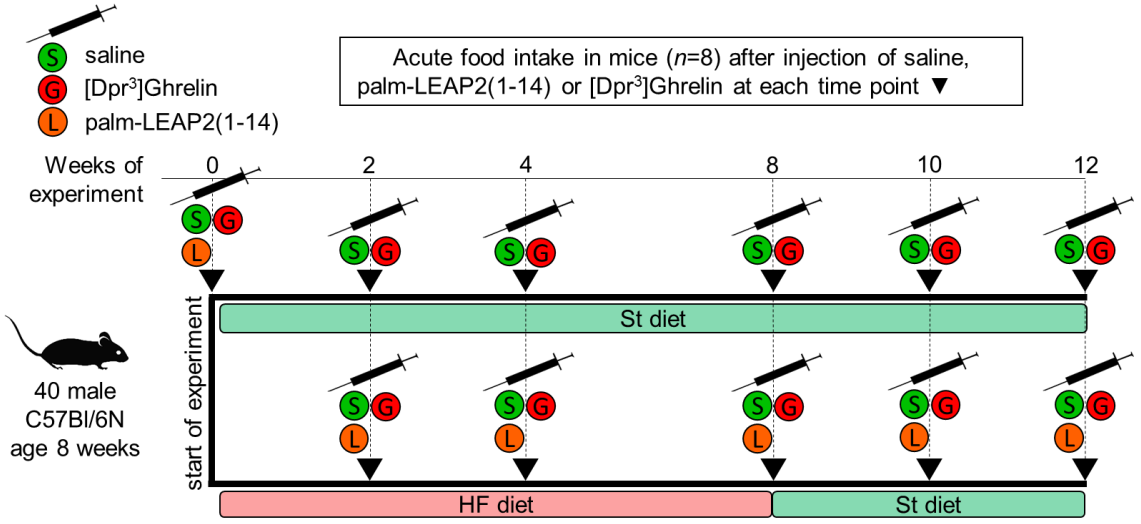

Figure 14: Experimental design of long-term *in vivo* study 2: 40 male C57Bl/6N mice were separated into 5 groups (n=8). Out of these, 2 groups were given a standard (St) diet, while the remaining 3 groups were given a high-fat (HF) diet. In the 8th week of the experiment, groups fed with HF diet were switched to a St diet. The amount of food consumed was recorded after the SC administration of either a saline, palm-LEAP2(1-14), or [Dpr^3]Ghrelin.

3.3.3.3 Long-term *in vivo* study 3: Ability of palm-LEAP to prevent development of HF diet-induced obesity

The experimental design is presented in Figure 15. Mice were separated into 4 groups (n=10) and housed in separate cages when they reached 8 weeks of age. The body weights were monitored every week. One group was provided with a St diet, while three groups were given a HF diet from the age of 8 weeks.

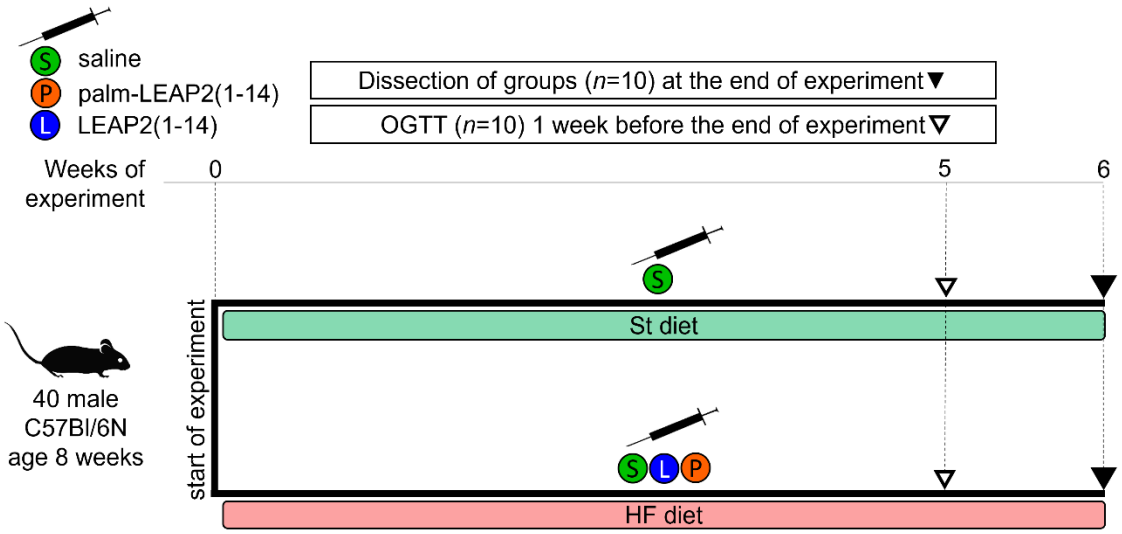

Figure 15: Experimental design of long-term *in vivo* study 3: 40 male C57Bl/6N mice were separated into 4 groups. Out of these, 1 group was given a standard (St) diet, while the remaining 3 groups were given a high-fat (HF) diet. Oral glucose tolerance test (OGTT) was performed at the week 5 of the experiment. Mice were sacrificed at the week 6 of the experiment.

The mice's body weights were monitored three times a week, and they were sacrificed at the week 6 of the experiment. OGTT was performed on each group after a 6-hour fasting one week before sacrificing. On the day of sacrificing, free-fed mice were anesthetized with pentobarbital. Blood from heart was collected in tubes with EDTA and plasma was separated and stored at -20°C. Plasma for ELISA detection of ghrelin was pre-treated with Pefabloc® (Carl Roth, Karlsruhe, Germany) and acidified using HCl. Mice were then perfused with heparinized saline (at a concentration of 20 U/ml) and dissected.

The liver and eWAT were weighted. Morphometric and biochemical analyses were performed. The results were analyzed using one-way or two-way ANOVA with Bonferroni's post hoc test as described in the figure legends. The significance level of $P<0.05$ was considered to be statistically significant.

3.3.4 Methods used in long-term *in vivo* experiments

3.3.4.1 Biochemical parameters in plasma

Plasma from free-fed anesthetized mice was collected to measure leptin, total ghrelin, active ghrelin (Millipore, St. Charles, MI, USA), and C-reactive protein (CRP) with mouse ELISA kits (Thermo Fisher Scientific, Waltham, MA USA). Fasted plasma was used to measure TAG and cholesterol using colorimetric assays (Erba Lachema, Brno, Czech Republic) and insulin on the Sensitive Rat Insulin RIA kit (MilliporeSigma, Burlington, MA, USA).

3.3.4.2 Oxidative stress in liver

Liver samples were homogenized using the Bullet Blender® tissue homogenizer (Next Advance, Inc., Averill Park, NY, USA) in ice-cold lysis buffer (62.5 mM Tris-HCl buffer with pH 6.8, 1% deoxycholate, 1% Triton X-100, 50mM NaF, 1mM Na_3VO_4 and complete protease inhibitor (Roche Applied Science, Mannheim, Germany)). The lysates were sonicated for a minute and centrifuged for 15 minutes at 13,500 × g at 4°C. The protein concentration of the resulting lysates was measured by Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Subsequently, the lysates were diluted to a final concentration of 10 µg/µL with lysis buffer. H_2O_2 concentration was analyzed by the Amplex™ Red Hydrogen Peroxide/Peroxidase Assay Kit (Thermo Fisher Scientific, Waltham, MA USA) according to manufacturer's protocol.

3.3.4.3 Hematoxylin and eosin staining of the liver

The right lobe of the liver was fixed in 4 % PFA overnight and embedded in paraffin blocks. The livers were cut into 5 µm thick slices (n=3) with the Leica ASP200S Tissue Processor (Leica Biosystems, Buffalo Grove, IL, USA) and processed as previously described (Prazienkova et al., 2021). After staining liver slices with hematoxylin and eosin, the slices were covered with a DPX mounting medium (MilliporeSigma, Burlington, MA, United States). Photomicrographs of the stained liver slices were taken with the Olympus IX83 inverted microscope (Olympus Europa SE & Co. KG, Hamburg, Germany). Liver staining was performed by Martina Kojecká, MSc. at IOCB Prague.

3.3.4.4 Determination of mRNA expression

Samples of the hypothalamus and liver for mRNA determination were analyzed by Miloslava Čechová at the Institute for Clinical and Experimental Medicine (IKEM) as written previously (Maletinska et al., 2015).

Samples were homogenized by MagNA Lyser Instrument with MagNA Lyser Green Beads (Roche Diagnostics GmbH, Mannheim, Germany). The total RNA was extracted using MagNA Pure Compact RNA Isolation (Tissue) kit (Roche Diagnostics GmbH, Mannheim, Germany). The mRNA expression of the genes of interest was determined by the ABI PRISM® 7500 instrument (Applied Biosystems, Foster City, CA, USA). Data were normalized to the expression of the reference genes glyceraldehyde3-phosphate dehydrogenase (GAPDH) or beta-2-microglobulin (B2m). Genes of interest in the hypothalamus: AgRP – agouti-related peptide, CART – cocaine- and amphetamine-regulated transcript, GHSR – growth hormone-secretagogue receptor, NPY – neuropeptide Y, POMC – pro-opiomelanocortin. Gene of interest in the liver: LEAP2 – liver expressed antimicrobial peptide 2.

3.1 Statistical analysis

The data are presented as mean ± SEM.

The competitive binding experiments were analyzed by GraphPad 8 Software according to Motulsky (Motulsky and Neubig, 2002b). The dissociation constant (K_D) was obtained from saturation experiments by GraphPad 8 Software (San Diego, CA, USA). Data in figure are expressed as % of maximal total binding.

$$K_D = \frac{[ligand] \times [receptor]}{[ligand - receptor]}$$

The half-inhibitory concentration IC_{50} was determined from the competitive binding experiments by GraphPad 8 Software using a single binding site model and non-linear regression. The value of the inhibition constant of the unlabeled ligand (K_i) was calculated using the Cheng and Prusoff equation (Cheng and Prusoff, 1973). Data in figure are expressed as % of maximal ghrelin binding.

$$K_i = \frac{IC_{50}}{1 + \frac{[radioligand]}{K_d}}$$

The beta-lactamase assay results were analyzed by non-linear regression as log agonist versus response using GraphPad 8 software. The EC_{50} values were established by identifying the peptide concentration that produced 50% of the maximum response. Data in figures are expressed as % of maximal ghrelin-induced GHSR stimulation.

The data from *in vivo* experiments were analyzed using GraphPad Prism 8 software. Food intake and GH release experiments were calculated with one-way ANOVA, followed by a Bonferroni post hoc test as described in the table and figure legends. Long-term experiments were evaluated by two-way ANOVA with Bonferroni's post hoc test or one-way ANOVA with Tukey's test or multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons as described in the figure legends. Outliers were identified by Grubbs test. $P<0.05$ was considered statistically significant.

4 Results

4.1 *In vitro* testing of LEAP2(1-14) analogs

The findings presented in chapter 4.1. were published in (Hola et al., 2022).

4.1.1 Binding affinities of LEAP2(1-14) analogs for GHSR

We designed a series of new N-terminal LEAP2 analogs to choose the most stable and bioavailable inverse agonist of GHSR. The N-terminal section of LEAP2 is crucial for receptor binding, according to M'Kadmi et al. (M'Kadmi et al., 2019). Thus, biologically active LEAP2 analog with β-Ala-Lys linker, LEAP2(1-14), was used and lipidized with different fatty acid residues (myristoyl, palmitoyl, or stearoyl) at its C-terminus.

We performed a saturation binding experiments on U2OS cells overexpressing GHSR to establish K_D values of ^{125}I-ghrelin. The K_D value ± SEM obtained from the saturation binding experiments was (0.38 ± 0.2) nM (Figure 16A).

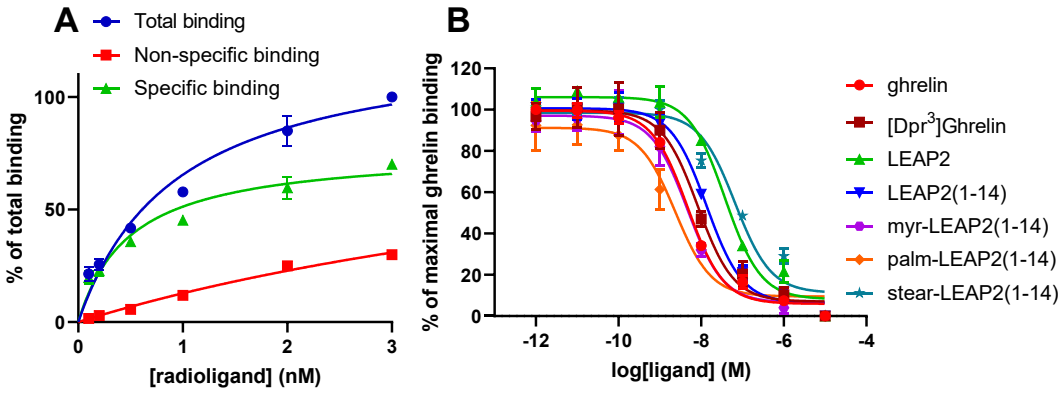

Figure 16: Saturation binding (A) and competitive binding (B) experiments. Data are presented as the mean ± SEM. Data analyzed in Graph-Pad Software were performed in 3–5 independent experiments in duplicates.

In a competitive binding experiment (Table 3 and Figure 16B), ghrelin and LEAP2 analogs competed with ^{125}I-ghrelin for binding to GHSR on U2OS cells. The K_i values for each analog were calculated by Cheng and Prusoff equation (Cheng and Prusoff, 1973) using IC_{50} values obtained from competitive binding experiments (model of one binding site) and the K_D value obtained from saturation binding experiments. Ghrelin and [Dpr^3]Ghrelin had a K_i in a nanomolar range as shown in Table 3. LEAP2 and its N-terminal analog LEAP2(1-14) competed with ^{125}I-ghrelin for binding to GHSR with K_i falling within the 10^{-8} M range. The affinity of LEAP2(1-14) for GHSR was increased by C-terminal palmitoylation and myristoylation, while stearoylation decreased it.

Table 3: Binding affinities and agonist and inverse agonist effect of ghrelin and LEAP2 analogs to GHSR.

Compound	Competitive binding assay K_i (nM)	GHSR agonist potency EC_{50} (nM)	GHSR inverse agonist potency EC_{50} (nM)
Ghrelin	3.35 ± 0.35	3.10 ± 0.47	-
[Dpr^3]Ghrelin	6.63 ± 0.42	5.28 ± 0.63	-
LEAP2	29.97 ± 1.59	-	46.46 ± 5.38
LEAP2(1-14)	11.01 ± 0.96	-	152.54 ± 19.24
myr-LEAP2(1-14)	3.64 ± 0.39	-	45.38 ± 7.41
palm-LEAP2(1-14)	1.91 ± 0.09	-	52.43 ± 7.95
stear-LEAP2(1-14)	53.62 ± 7.88	-	51.11 ± 7.88

Data are presented as the mean ± SEM. K_i values were calculated using the Cheng-Prusoff equation (Cheng and Prusoff, 1973). Data analyzed in Graph-Pad Software were performed in 3–5 independent experiments in duplicates.

4.1.2 Inverse agonist and antagonist properties of LEAP2 analogs at GHSR

A T-REx™ Tango™ GHSR-bla U2OS Cell-based Assay was used to detect the activation of GHSR. Both ghrelin and [Dpr^3]Ghrelin were strong GHSR agonists and activated GHSR with EC_{50} at nanomolar levels (Table 3 and Figure 17A). On the other hand, natural LEAP2 and lipidized LEAP2(1-14) analogs showed a strong inverse agonist activity and suppressed the constitutive activity of GHSR with EC_{50} levels in the 10^{-8} M range. Non-lipidized LEAP2(1-14) is a weaker GHSR inverse agonist with an EC_{50} value about three times higher than the lipidized analogs or natural LEAP2.

To observe the antagonist properties of all LEAP2 analogs, dose-response curves of ghrelin in the absence or presence of increasing concentrations of LEAP2 analogs were prepared. Figure 17B shows that increasing the natural LEAP2 concentration up to 1 μM resulted in more than a ten-fold increase in the EC_{50} of ghrelin. However, non-lipidized LEAP2(1-14) exhibited lower antagonist activity than lipidized analogs as seen in Figure 17C. Lipidization enhanced the antagonist activity of LEAP2(1-14) analogs. All lipidized LEAP2(1-14) analogs (Figure 17D-F) showed an increase in the EC_{50} of ghrelin by more than a thousand times, upon increasing their concentration up to 1 μM.

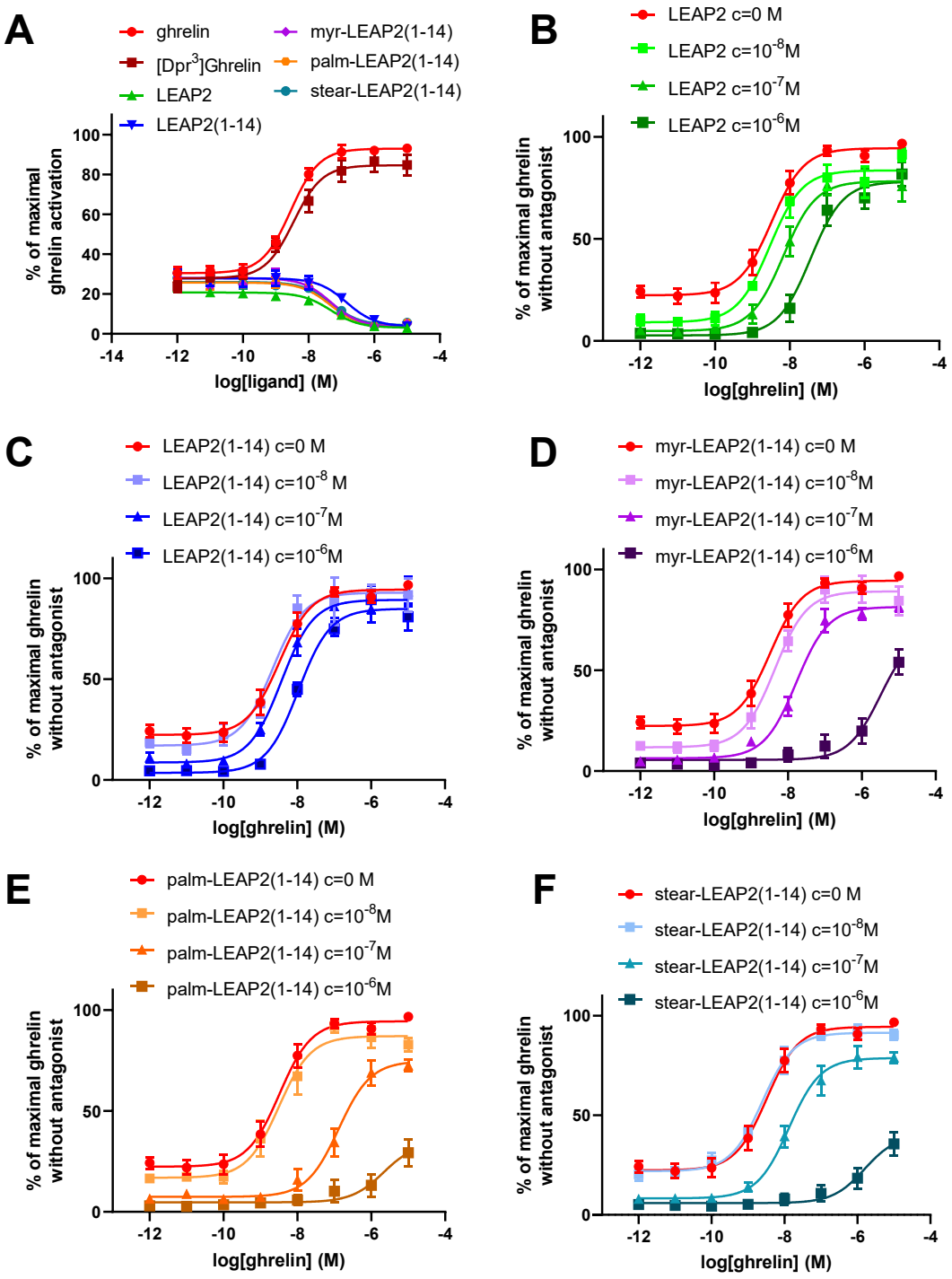

Figure 17: Inverse agonist and antagonist properties of LEAP2(1-14) analogs. Effect of LEAP2 compounds on GHSR activation in inverse agonist mode assay (A). Effect of LEAP2 (B), LEAP2(1-14) (C), myr-LEAP2(1-14) (D), palm-LEAP2(1-14) (E) and stear-LEAP2(1-14) (F) on GHSR activation in antagonist mode assay. The maximum ghrelin-induced GHSR activation in U2OS cells overexpressing GHSR was set as the standard value of 100%. Data are presented as the mean ± SEM. The experiments were performed in duplicates and repeated at least three times and analyzed using non-linear regression.

4.1.3 Stability of LEAP2(1-14) and palm-LEAP2(1-14) in rat plasma

The stability of LEAP2(1-14) analogs was tested by prof. RNDr. Dr. David Sýkora (ICT, Prague). As shown in Figure 18, palm-LEAP2(1-14) has significantly higher stability in blood plasma compared to its non-lipidized analog. This means that palm-LEAP2(1-14) remains active in rat plasma for an extended period of time, resulting in a prolonged effect, which is highly desirable.

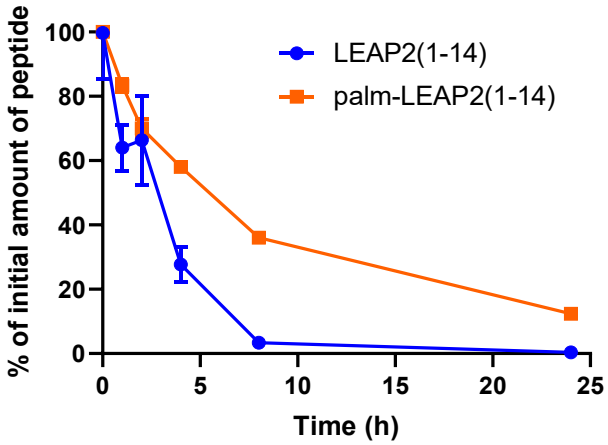

Figure 18: Stability of LEAP2(1-14) and palm-LEAP2(1-14) in rat plasma. Data are presented as the mean ± SEM. The experiments were performed in triplicates.

4.2 Short-term *in vivo* testing of LEAP2(1-14) analogs

4.2.1 Acute food intake in mice after SC administration of LEAP2(1-14) analogs

The anorexigenic effect of LEAP2(1-14) analogs was tested in overnight fasted mice (Figure 19). The cumulative food intake was recorded for 7 hours after the SC administration of LEAP2(1-14) analogs. Palm-LEAP2(1-14) significantly reduced the cumulative food intake in fasted mice compared to control group, whereas non-lipidized LEAP2(1-14) as well as myr-LEAP2(1-14) and stear-LEAP2(1-14) showed only a weak anorexigenic effect.

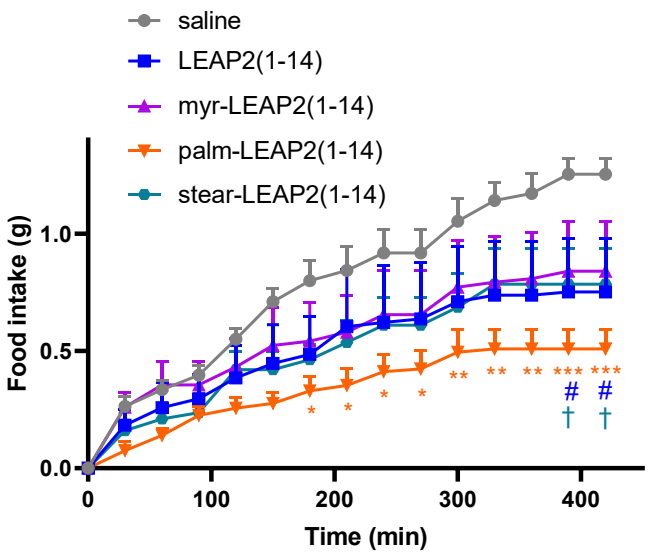

Figure 19: Acute food intake in fasted mice after SC administration of LEAP2(1-14) analogs. All the lipidized and non-lipidized LEAP2(1-14) analogs were SC injected at a dose of 5 mg/kg to overnight fasted mice and the food intake was monitored every 30 minutes for 7 hours. The data were analyzed using 2-way ANOVA followed by Bonferroni post hoc test. The results are expressed as means ± SEM. The significance levels are *P<0.05; **P<0.01; ***P<0.001 palm-LEAP2(1-14) vs saline-treated group, #P<0.05, ##P<0.01, ###P<0.001 LEAP2(1-14) vs saline-treated group; †P<0.05, ††P<0.01, †††P<0.001 stear-LEAP2(1-14) vs saline-treated group (n=5).

The antagonistic effect of LEAP2(1-14) and its lipidized analogs on the orexigenic effects of [Dpr^3]Ghrelin was evaluated in free-fed mice (Figure 20A-D). All the peptides caused a significant decrease in [Dpr^3]Ghrelin-induced food intake. Moreover, palm-LEAP2(1-14) completely suppressed the orexigenic action of [Dpr^3]Ghrelin and reduced food intake even below the food intake of saline-treated mice.

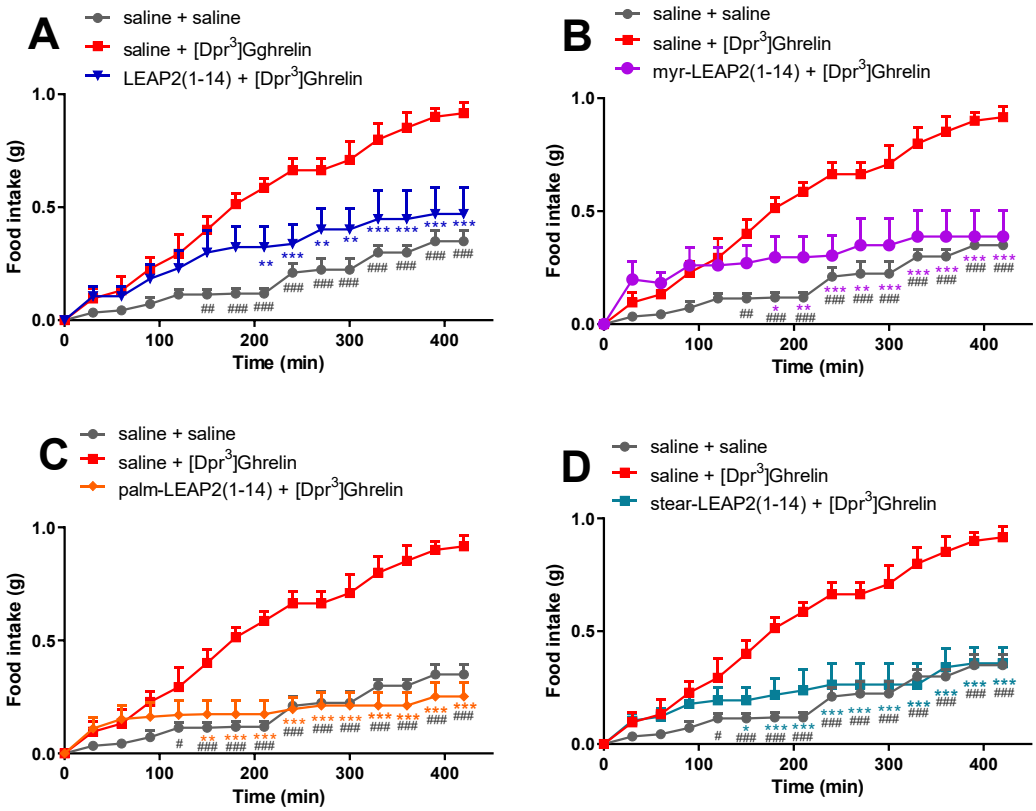

Figure 20: Acute food intake in free-fed mice after SC administration of [Dpr^3]Ghrelin and LEAP2(1-14) analogs. Effect of LEAP2(1-14) (A), myr-LEAP2(1-14) (B), palm-LEAP2(1-14) (C), and stear-LEAP2(1-14) (D). All the lipidized and non-lipidized LEAP2(1-14) analogs were SC injected at a dose of 5 mg/kg, 15 minutes after the first injection, the mice were injected with [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight or saline. The food intake was monitored every 30 minutes for 7 hours. The data were analyzed using 2-way ANOVA followed by Bonferroni post hoc test. The results are expressed as means ± SEM. The significance levels are *P<0.05; **P<0.01; ***P<0.001, LEAP2(1-14) analog vs [Dpr^3]Ghrelin-treated group, #P<0.05, ##P<0.01, ###P<0.001 saline vs [Dpr^3]Ghrelin-treated group (n=5).

4.2.2 Effect of LEAP2(1-14) and palm-LEAP2(1-14) on [Dpr^3]Ghrelin-induced GH release

The effect of LEAP2(1-14) and the most potent lipidized analog palm-LEAP2(1-14) on GH release was tested in young 8-week-old mice (Figure 21). SC injection of neither LEAP2(1-14) nor palm-LEAP2(1-14) affected the plasma level of GH. Since the level of GH in plasma is naturally low, we tested the ability of LEAP2(1-14) analogs to inhibit GH release induced by [Dpr^3]Ghrelin. It was found that the palm-LEAP2(1-14) significantly reduced the release of GH induced by [Dpr^3]Ghrelin. However, this effect was not observed when the non-lipidized form of LEAP2(1-14) was administered.

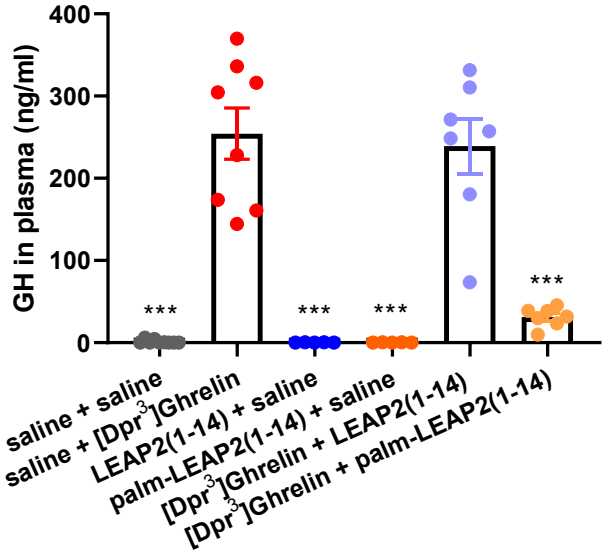

Figure 21: Effect of LEAP2(1-14) and palm-LEAP2(1-14) on [Dpr^3]Ghrelin-induced GH release in 8-weeks-old mice. Mice were SC injected with 200 µl of saline or LEAP2 analog at a dose of 10 mg/kg of body weight. 15 minutes after injection, mice were SC injected with saline or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight. Blood was collected 30 minutes after beginning of the experiment. The concentration of GH in plasma samples was measured using a commercially available ELISA kit. The data were analyzed using 1-way ANOVA followed by Bonferroni post hoc test. The results are expressed as means ± SEM. The significance levels are *P<0.05; **P<0.01; ***P<0.001, compared to the saline + [Dpr^3]Ghrelin treated group (n=5).

4.3 Progress of obesity at HF diet feeding

The results obtained in this chapter are after major revision in Journal of Molecular Endocrinology.

4.3.1 Long-term effect of HF diet on body weight and eWAT weight

The scheme of the experiment is shown in Figure 13. Starting from week 0 of the experiment (age of mice 8 weeks), mice were given a HF diet. The mice's body weight was monitored every week for the following 15 weeks.

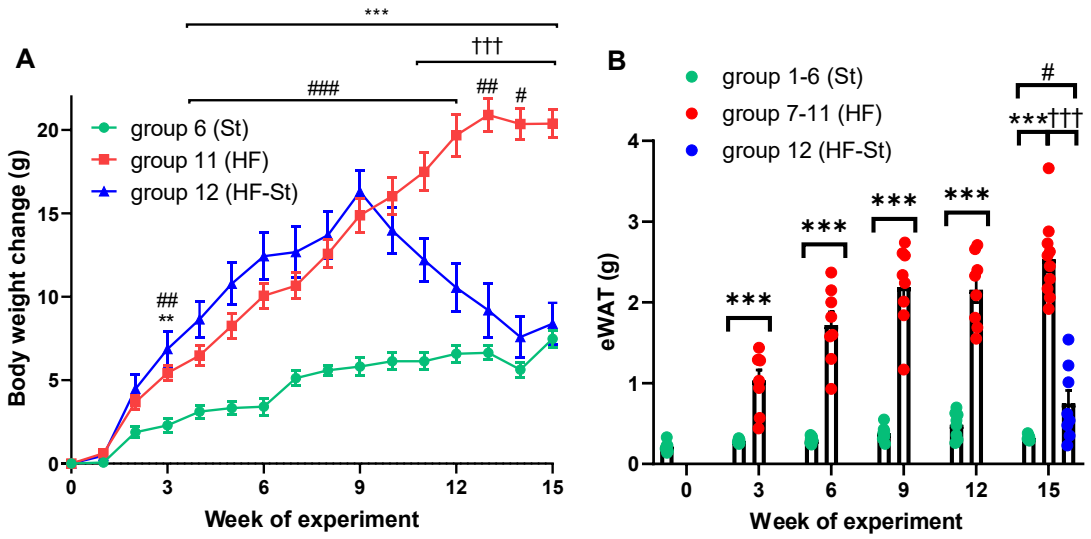

Figure 22: Effect of HF diet and switch of HF diet to St diet on body weight (A) and eWAT weight (B) in mice. Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). Data are presented as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with Bonferroni's post hoc test (A) and multiple t-tests with Bonferroni-Dunn's method for multiple comparisons (B). Significance is *P<0.05, **P<0.01, ***P<0.001 HF vs St; #P<0.05, ##P<0.01, ###P<0.001 HF-St vs St; †P<0.05, ††P<0.01, †††P<0.001 HF-St vs HF (n=8).

Feeding mice with a HF diet resulted in a continuous increase in both body weight and eWAT weight (Figure 22) compared to a St diet. These differences became noticeable as early as 3 weeks after beginning of the HF diet feeding. Significant decrease in body weight was observed in mice that were switched to a St diet after 9 weeks on a HF diet already after two weeks of St diet feeding and their final body weight was comparable to the control group fed a St diet for 15 weeks. 6 weeks of St diet feeding after 9 weeks of HF diet feeding did not result in the complete reduction of eWAT, however the eWAT weight was significantly lower than in the group fed exclusively a HF diet for 15 weeks (Figure 22B).

4.3.2 Long-term effect of HF diet on glucose tolerance

Glucose tolerance was evaluated using the OGTT. Already after 3 weeks of consuming a HF diet, plasma glucose levels increased significantly after glucose gavage (Figure 23). Switching from HF diet feeding at week 9 to a St diet feeding decreased the glucose levels to the level comparable to mice fed exclusively a St diet for 15 weeks. Moreover, already at week 3 of the experiment, the fasted glucose was significantly increased in mice fed a HF diet compared to those on St diet (Figure 24A); the glucose decreased to level of exclusively St diet-fed mice after switch to St diet from HF diet at week 9. Area under the curve (AUC) values of OGTT showed increased glucose intolerance of mice fed HF diet compared to St diet fed mice from the 6th week of HF diet feeding (Figure 24 B).

Mice that were fed a HF diet for 9 weeks had notably higher fasted plasma insulin levels (Figure 24 C) compared to those fed a St diet. There was no significant difference in plasma insulin levels between the group that had been fed a 9-week HF diet and then switched to a 6-week St diet and the group that had been fed exclusively a St diet for 15 weeks.

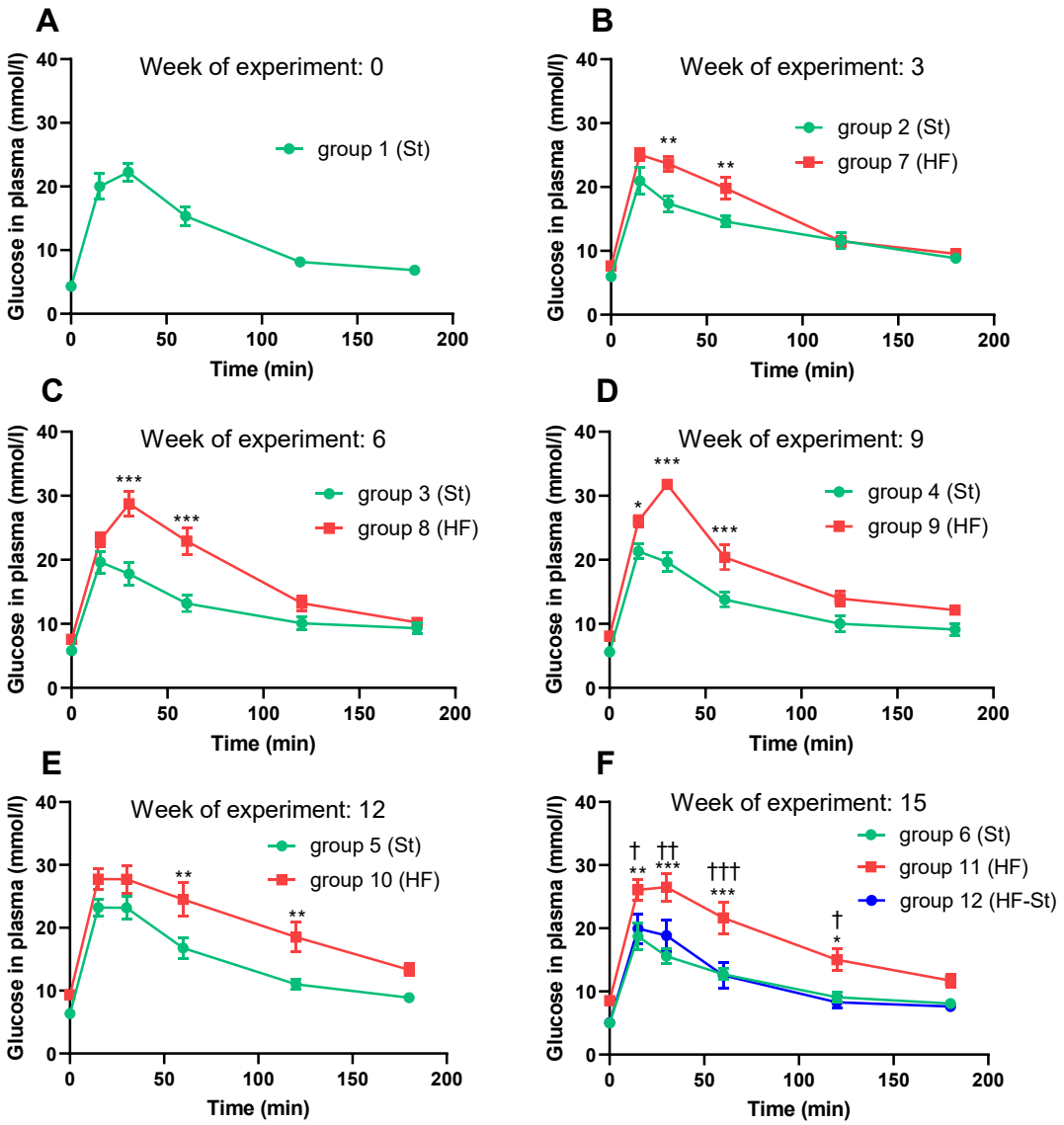

Figure 23: Effect of HF diet and switch of HF diet to St diet on blood glucose levels after oral glucose gavage, dose 2 g/kg of body weight. Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). OGTT at 0 weeks (A), 3 weeks (B), 6 weeks (C), 9 weeks (D), 12 weeks (E), and 15 weeks of the experiment. Data are expressed as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with Bonferroni's post hoc test. Significance is **P*<0.05, ***P*<0.01, ****P*<0.001 HF vs St; †*P*<0.05, ††*P*<0.01, †††*P*<0.001 HF-St vs HF (*n*=8).

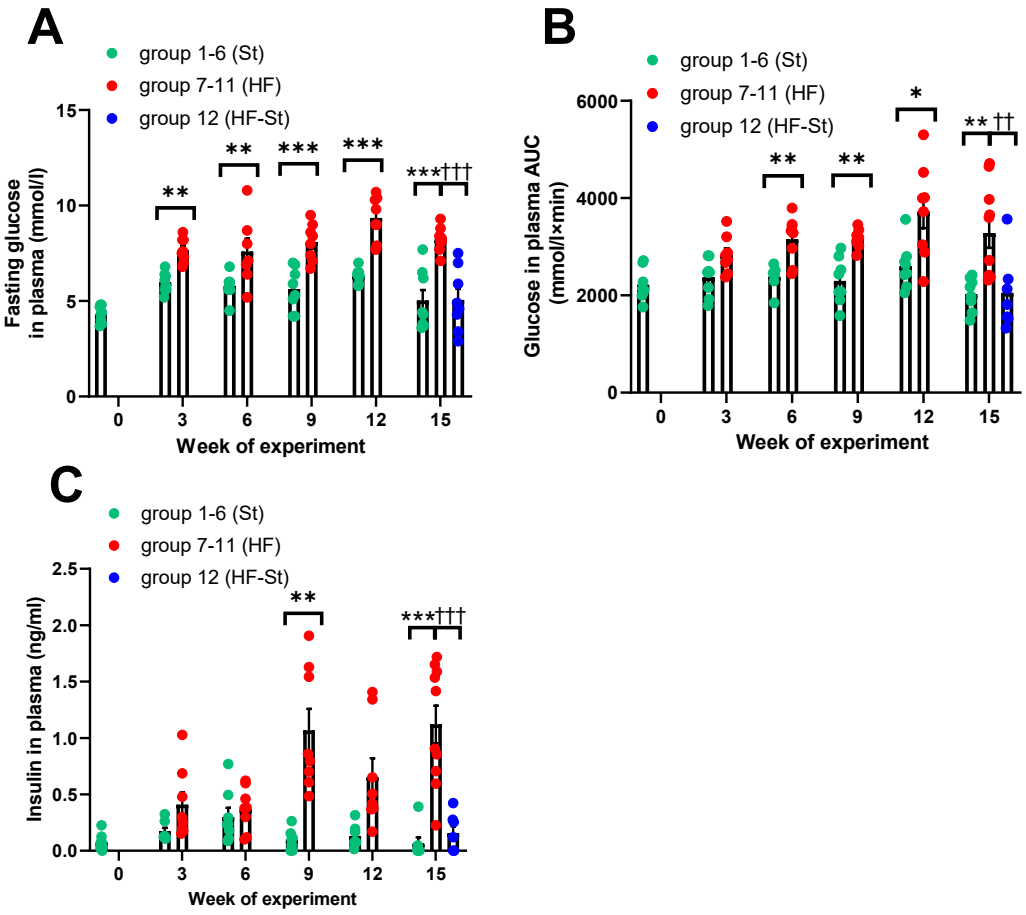

Figure 24: Effect of HF diet and switch of HF diet to St diet on plasma levels of fasting glucose (A), AUC of curves of blood glucose levels (B), and insulin plasma levels (C) after oral glucose gavage (dose 2 g/kg). Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). Data are expressed as mean ± SEM. Statistical analysis was conducted using multiple t-test with Bonferroni-Dunn's method for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 HF vs St; †P<0.05, ††P<0.01, †††P<0.001 HF-St vs HF (n=8).

4.3.3 Long-term effect of HF diet on ghrelin and leptin levels in plasma and *LEAP2* mRNA in liver

The levels of leptin in the blood (Figure 25A) followed the level of eWAT; it increased in HF diet fed groups and significantly decreased in mice switched to St diet at week 9 to the level observed in mice fed St diet for all 15 weeks of experiment. Similarly to leptin, *LEAP2* mRNA (Figure 25B) expressed in the liver increased in mice fed HF diet and decreased in the group that was given a HF diet for 9 weeks and then switched to a St diet for 6 weeks.

Active (Figure 25C) and total ghrelin (Figure 25D) levels in plasma had the opposite trend than leptin in plasma and *LEAP2* mRNA in the liver. Mice that were given a HF diet had lower levels of both active and total ghrelin compared to those that were given a St diet.

Mice that were fed HF diet for 9 weeks and St diet for 6 weeks had higher active ghrelin level than those fed HF diet; their active ghrelin was similar to the level found in the group that was only given a St diet for 15 weeks. It is interesting that the mice that were given a HF diet for 9 weeks and then switched to a St diet for 6 weeks had the same total ghrelin levels as the mice that were only given the HF diet for 15 weeks.

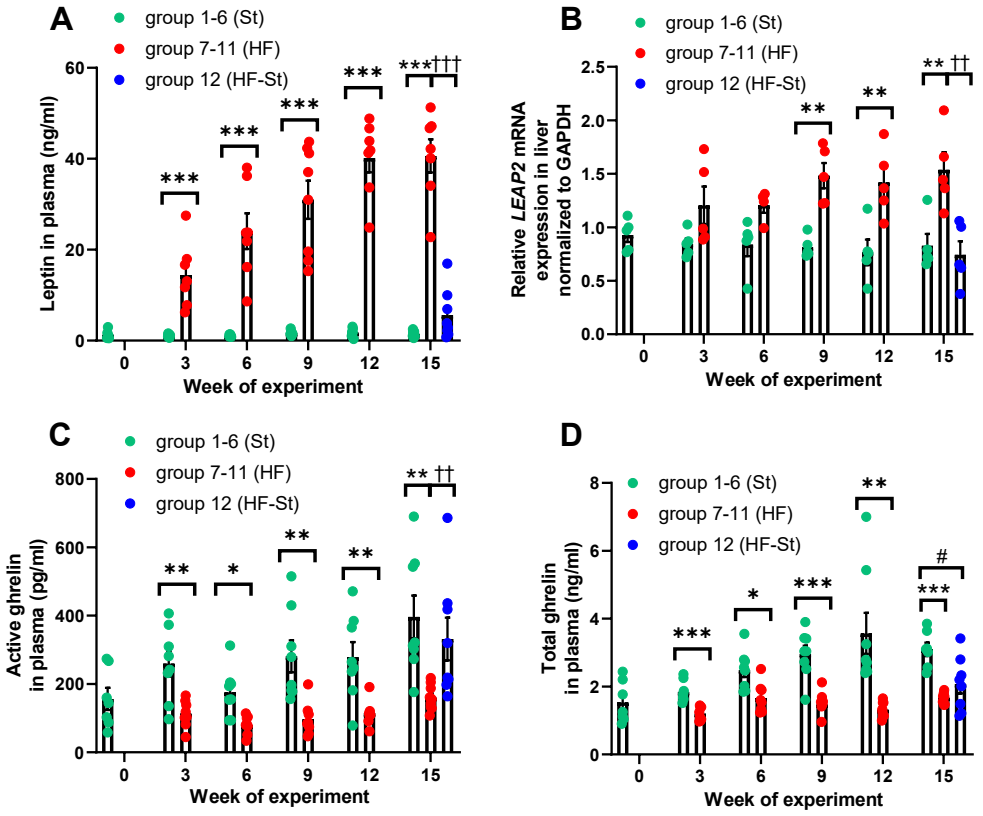

Figure 25: Effect of HF diet and switch of HF diet to St diet on the level of leptin in plasma (A), *LEAP2* mRNA in the liver (B), and active (C) and total (D) ghrelin in plasma. Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). Data are presented as mean ± SEM. Statistical analysis was performed using the multiple *t*-test with Bonferroni-Dunn's method for multiple comparisons. Significance is **P*<0.05, ***P*<0.01, ****P*<0.001 HF vs St; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 HF-St vs St; †*P*<0.05, ††*P*<0.01, †††*P*<0.001 HF-St vs HF (*n*=8).

4.3.4 Long-term effect of HF diet on cholesterol, TAG and CRP plasma levels, liver steatosis, and oxidative stress in the liver

At the end of the experiment, the levels of cholesterol, TAG, and CRP in the blood plasma, were compared between groups fed St diet (group 6), HF diet (group 11), and HF diet switched to St diet (group 12). After being on a HF diet for 15 weeks, there was a significant increase in the level of cholesterol in the blood plasma (Figure 26A). After switching the HF diet to St diet, the levels of cholesterol and as well as CRP (26C)

decreased and became similar to the levels observed in mice that were only given a St diet for 15 weeks. The level of TAG in the blood plasma (Figure 26B) tended to increase in the group that was given HF diet for 15 weeks compared to the other groups.

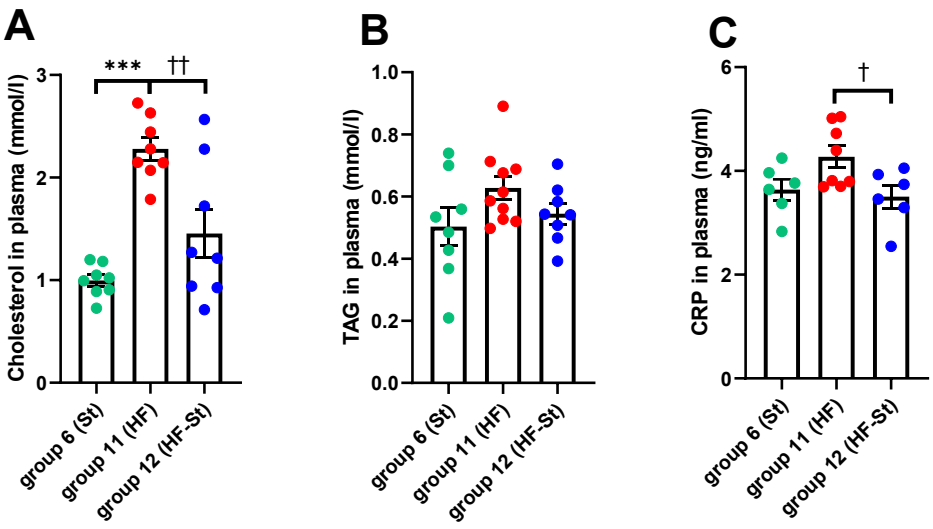

Figure 26: Effect of HF diet and switch of HF diet to St diet on cholesterol (A), TAG (B), and CRP (C) in plasma at the end of the experiment. Mice were fed with St diet (St: group 6), HF diet (HF: group 11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). Data are presented as mean ± SEM. Statistical analysis was performed by 1-way ANOVA with Tukey's method for multiple comparisons. Significance is **P*<0.05, ***P*<0.01, ****P*<0.001 HF vs St; †*P*<0.05, ††*P*<0.01, †††*P*<0.001 HF-St vs HF (n=8).

The level of oxidative stress in the liver was compared among groups 2-12. The concentration of H_2O_2, which is a marker of oxidative stress, was found to be significantly increased in the groups that were given a HF diet for either 9 or 15 weeks (Figure 27A). When the mice switched the HF diet feeding to a St diet feeding, there was a tendency for oxidative stress to decrease. 15 weeks of HF diet feeding resulted in reversible steatosis in the liver (Figure 27C). When the mice were switched from the HF diet to a St diet, the amount of visible lipid droplets in the liver decreased and became similar to the levels observed in mice that were only given a St diet for 15 weeks. The liver weight (Figure 27B) showed a trend towards an increase in the group that was given HF diet for 15 weeks compared to the other groups.

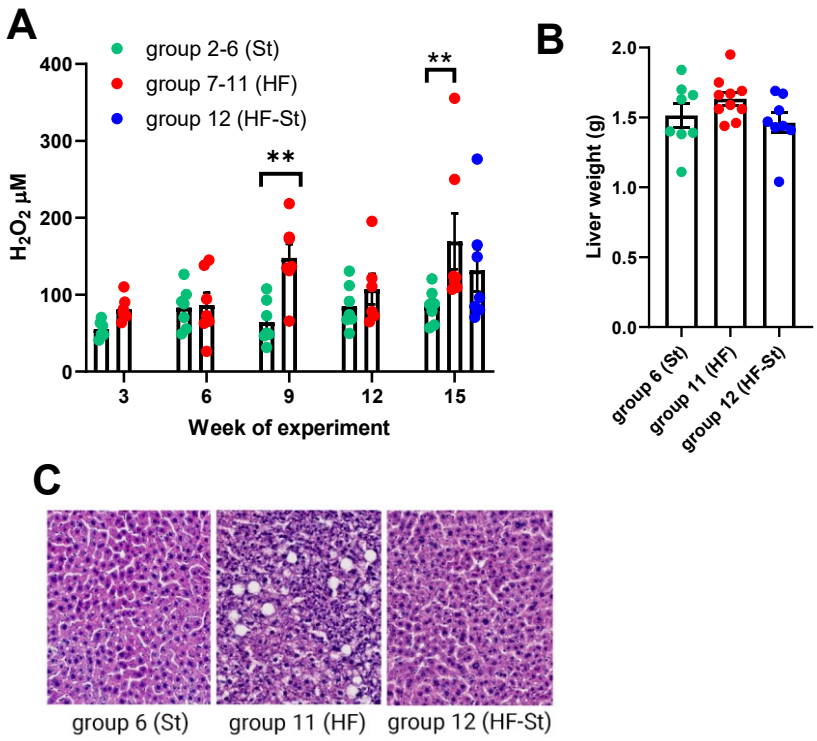

Figure 27: Effect of HF diet and switch of HF diet to St diet on oxidative stress in the liver (A), liver weight (B) and liver steatosis at the end of the experiment (C). Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet at week 9 of the experiment (HF-St: group 12). Data are presented as mean ± SEM. Statistical analysis was performed by multiple t-test with Bonferroni-Dunn's method for multiple comparisons (A) and 1-way ANOVA with Tukey's method for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 [Dpr^3]Ghrelin vs saline (n=8).

4.3.5 Long-term effect of HF diet on hypothalamic mRNA expression of neuropeptides

mRNA expression of neuropeptides in the hypothalamus was compared among groups fed St diet (group 6), HF diet (group 11), and HF diet switched to St diet (group 12) (Figure 28). *AgRP*, *GHSR*, and *NPY* mRNA levels did not change after HF diet feeding. The expression of *POMC* and *CART* mRNA in the hypothalamus showed a trend towards an increase in the group of mice that were fed HF diet for 15 weeks compared to the other groups.

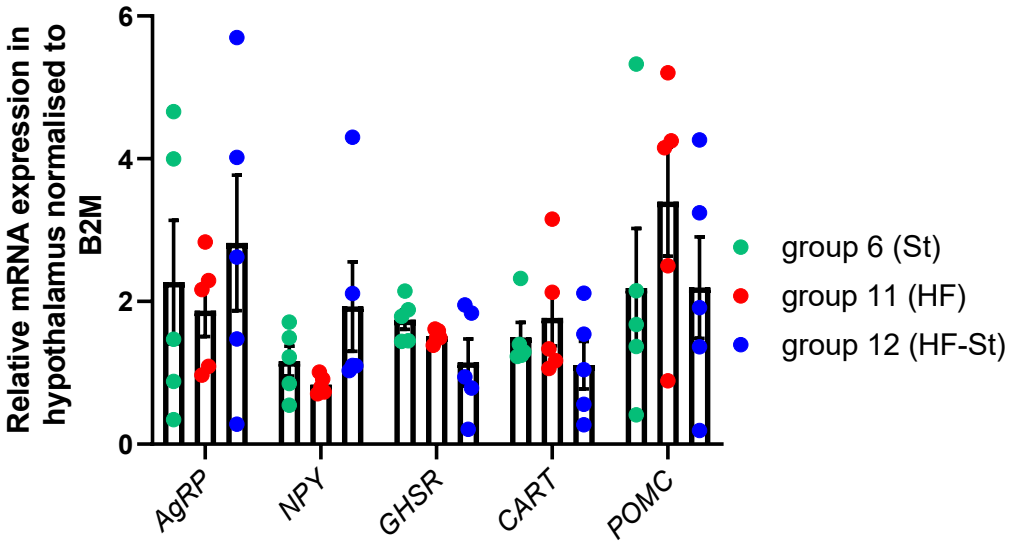

Figure 28: Effect of HF diet and switch of HF diet to St diet on mRNA expression of *AgRP*, *CART*, *GHSR*, *NPY*, and *POMC* in hypothalamus. Mice were fed with St diet (St: group 1-6), HF diet (HF: group 7-11), or switched from HF diet to ST diet (HF-St: group 12) at week 9 of the experiment. Data were normalized to *B2M* and presented as mean ± SEM. Statistical analysis was performed by 1-way ANOVA with Bonferroni's method for multiple comparisons (n=5). AgRP – agouti-related peptide, CART – cocaine- and amphetamine-regulated transcript, GHSR – growth hormone-secretagogue receptor, NPY – neuropeptide Y, POMC – pro-opiomelanocortin.

4.3.6 Long-term effect of HF diet on development of [Dpr^3]ghrelin and palm-LEAP2 resistance

To observe the development of LEAP2 resistance, mice were fed HF diet and SC injected with either [Dpr^3]Ghrelin or palm-LEAP2(1-14) (Figure 29 and 30). Mice fed a St diet were used as the control group. The results in Figure 29 are expressed as cumulative food intake during the entire experiment (360 minutes) and in Figure 30 as cumulative food intake per 270 minutes. Just two weeks after being fed a HF diet, mice had already developed a resistance to the acute effects of ghrelin, meaning that [Dpr^3]Ghrelin was no longer able to increase their food intake. Additionally, food intake had decreased below the baseline level due to the administration of palm-LEAP2(1-14). After being fed a HF diet for 4 weeks, the mice developed a resistance to the effects of palm-LEAP2(1-14), which was no longer able to decrease their basal level of food intake. However, after switching to a St diet for 4 weeks, their sensitivity to [Dpr^3]Ghrelin had been restored, while their sensitivity to palm-LEAP2(1-14) remained unchanged.

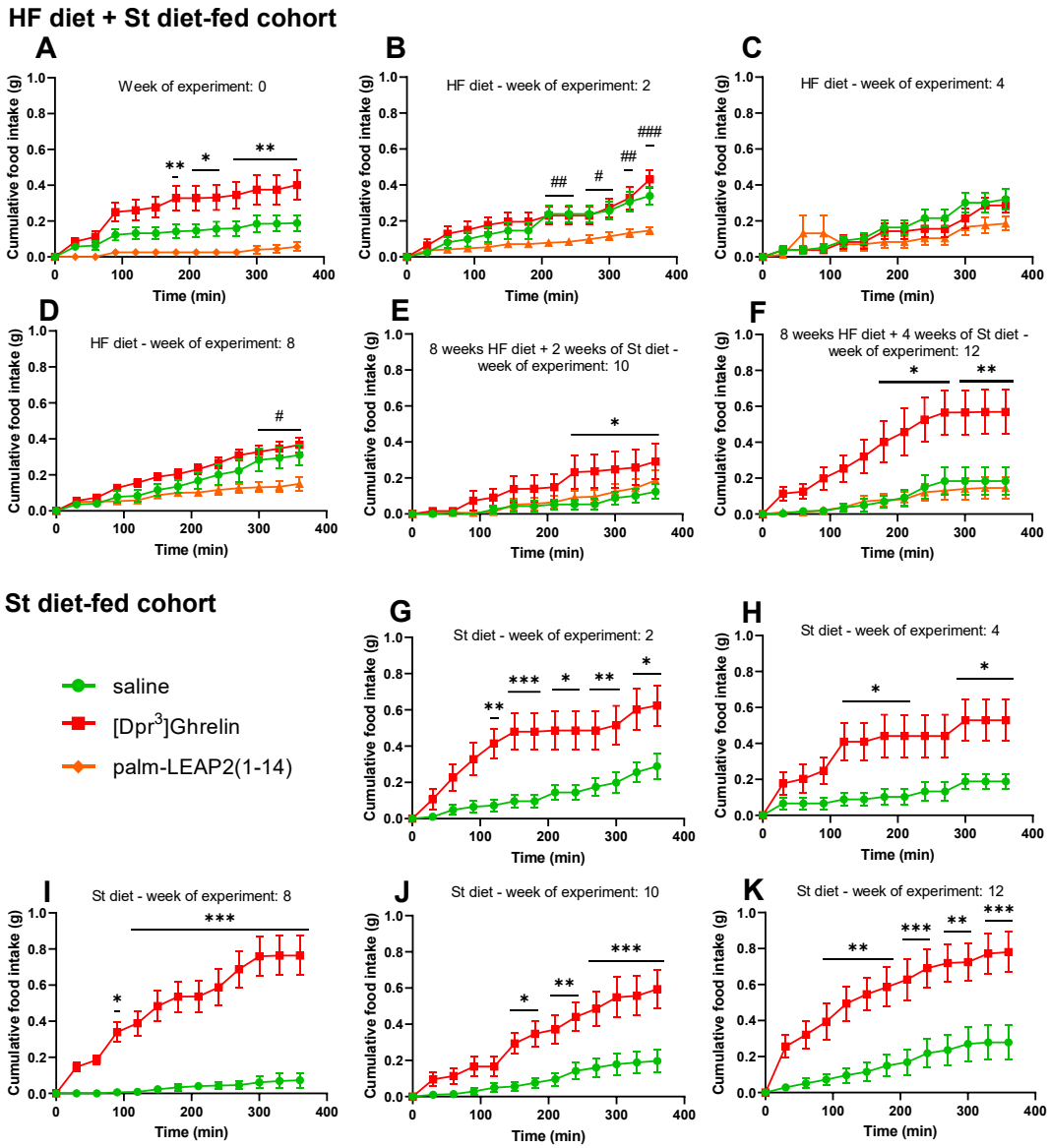

Figure 29: Cumulative food intake after SC administration of palm-LEAP2(1-14) at a dose of 5 mg/kg of body weight or [Dpr^3]Ghrelin at a dose of 1 mg/kg of body weight to mice at the beginning of experiment (A) and after feeding a HF diet for 2 (B), 4 (C), and 8 (D) weeks followed by a St diet only for a further 2 (E) and 4 (F) weeks or exclusively St diet for 2 (G), 4 (H), 8 (I), 10 (J), and 12 (K). Data are presented as mean ± SEM. Statistical analysis was performed by two-way ANOVA with Bonferroni's post hoc test. Significance is **P*<0.05, ***P*<0.01, ****P*<0.001 [Dpr^3]Ghrelin vs saline; #*P*<0.05, ##*P*<0.01, ###*P*<0.001 palm-LEAP2(1-14) vs saline (*n*=6-8).

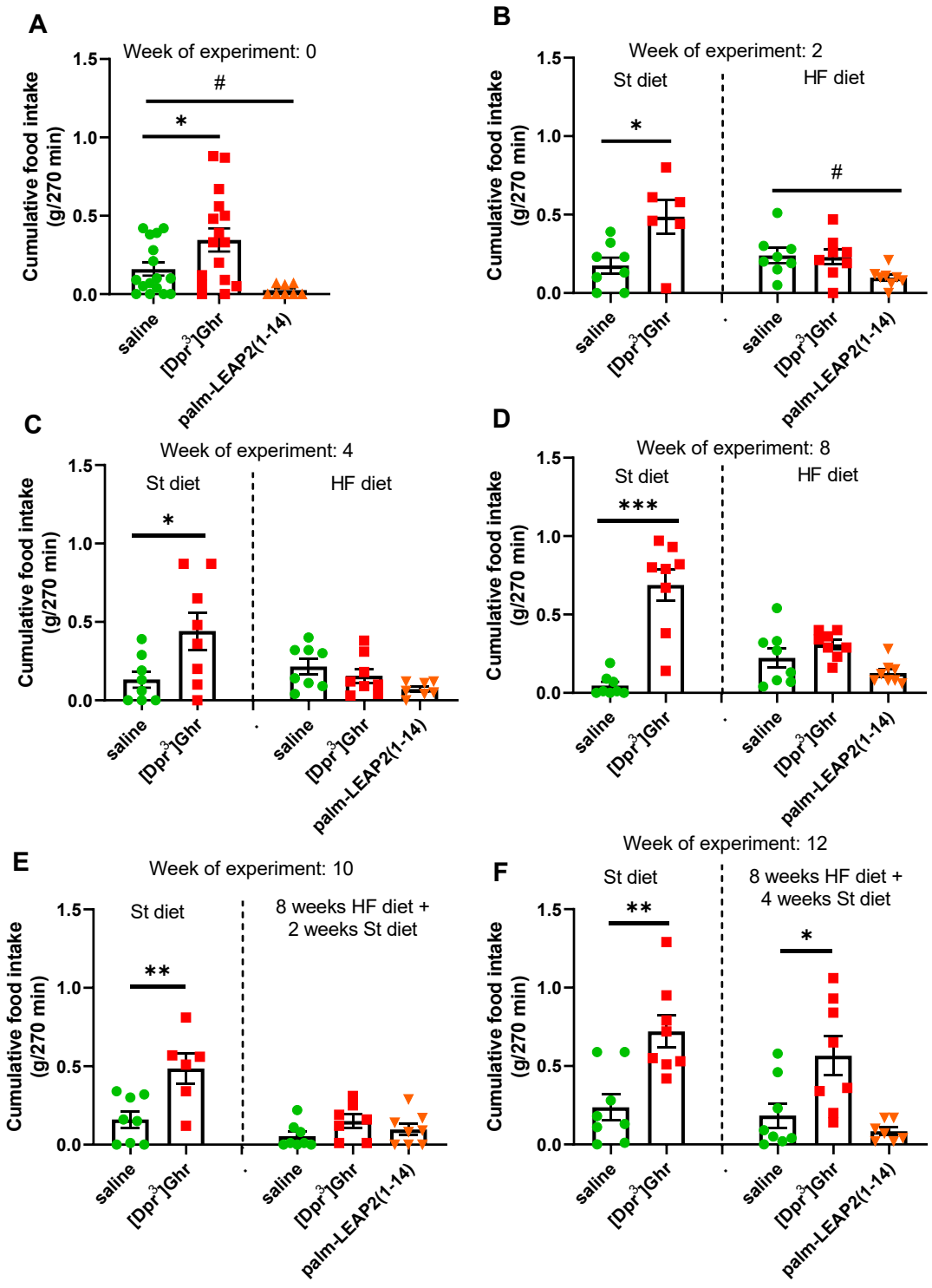

Figure 30: Cumulative food intake 270 minutes after SC [Dpr^3]Ghrelin (1 mg/kg of body weight) or palm-LEAP2(1-14) (5 mg/kg of body weight) administration in mice fed a HF diet or a St diet for 0 (A), 2 (B), 4 (C), and 8 (D) weeks followed by a St diet only for a further 2 (E) and 4 (F) weeks. Data are presented as mean ± SEM. Statistical analysis was performed by t-test. Significance is *P<0.05, **P<0.01, ***P<0.001 [Dpr^3]Ghrelin vs saline; #P<0.05, ##P<0.01, ###P<0.001 palm-LEAP2(1-14) vs saline (n=6-8).

4.4 Ability of palm-LEAP to prevent development of HF diet-induced obesity

4.4.1 Effect of chronic palm-LEAP2(1-14) administration on body weight and eWAT weight in mice fed HF diet

With the beginning of HF diet feeding, the intervention with LEAP2(1-14) or palm-LEAP2(1-14) started. As shown in Figure 31, HF diet feeding resulted in a continuous increase in both body weight and eWAT weight during the whole experiment compared to a St diet fed mice. Non lipidized LEAP2(1-14) intervention did not have any effect on body weight nor eWAT weight, however long-term administration of palm-LEAP2(1-14) slightly reduced body weight gain induced by a HF diet (Figure 31A).

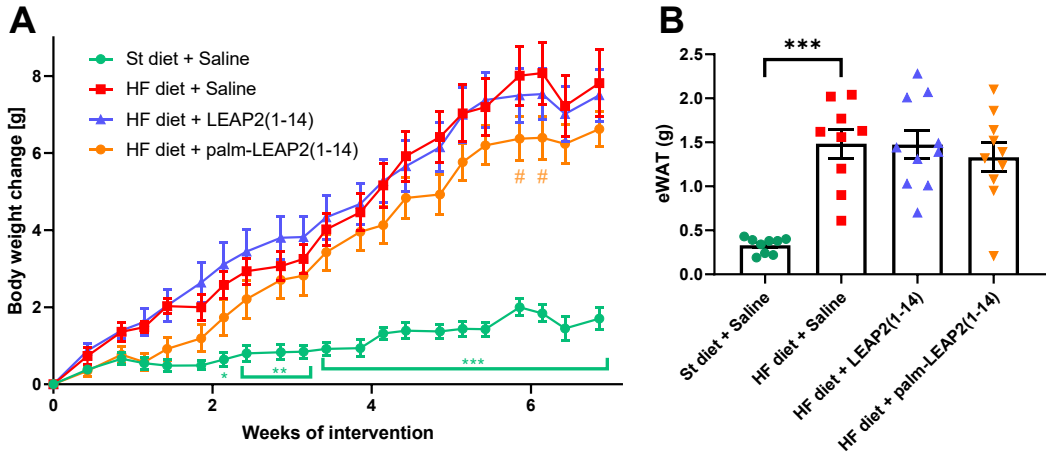

Figure 31: Effect of SC administration of chronic LEAP2(1-14) (10 mg/kg of body weight) and palm-LEAP2(1-14) (10 mg/kg of body weight) on body weight (A) and eWAT weight (B) in mice fed with a HF diet. Data are presented as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with Bonferroni's post hoc test (A) and 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 St diet + saline vs HF diet + saline; #P<0.05, ##P<0.01, ###P<0.001 HF diet + palm-LEAP2(1-14) vs HF diet + saline (n=10).

4.4.2 Effect of chronic palm-LEAP2(1-14) administration on the development of glucose intolerance in mice fed HF diet

Glucose tolerance was evaluated through the OGTT. Compared to mice fed St diet, HF diet fed mice showed increased plasma glucose levels after glucose gavage (Figure 32A), as well as increased fasted glucose plasma levels (Figure 32C). OGTT results were presented also as AUC values, where AUC curves tended to increase after the HF diet feeding (Figure 32B). Mice that were fed a HF diet for six weeks had notably higher fasted plasma insulin levels (Figure 32D) compared to those fed a St diet. However, nor

LEAP2(1-14) neither palm-LEAP2(1-14) prevent increased glucose intolerance induced by HF diet, increased fasted glucose plasma levels or increased levels of insulin.

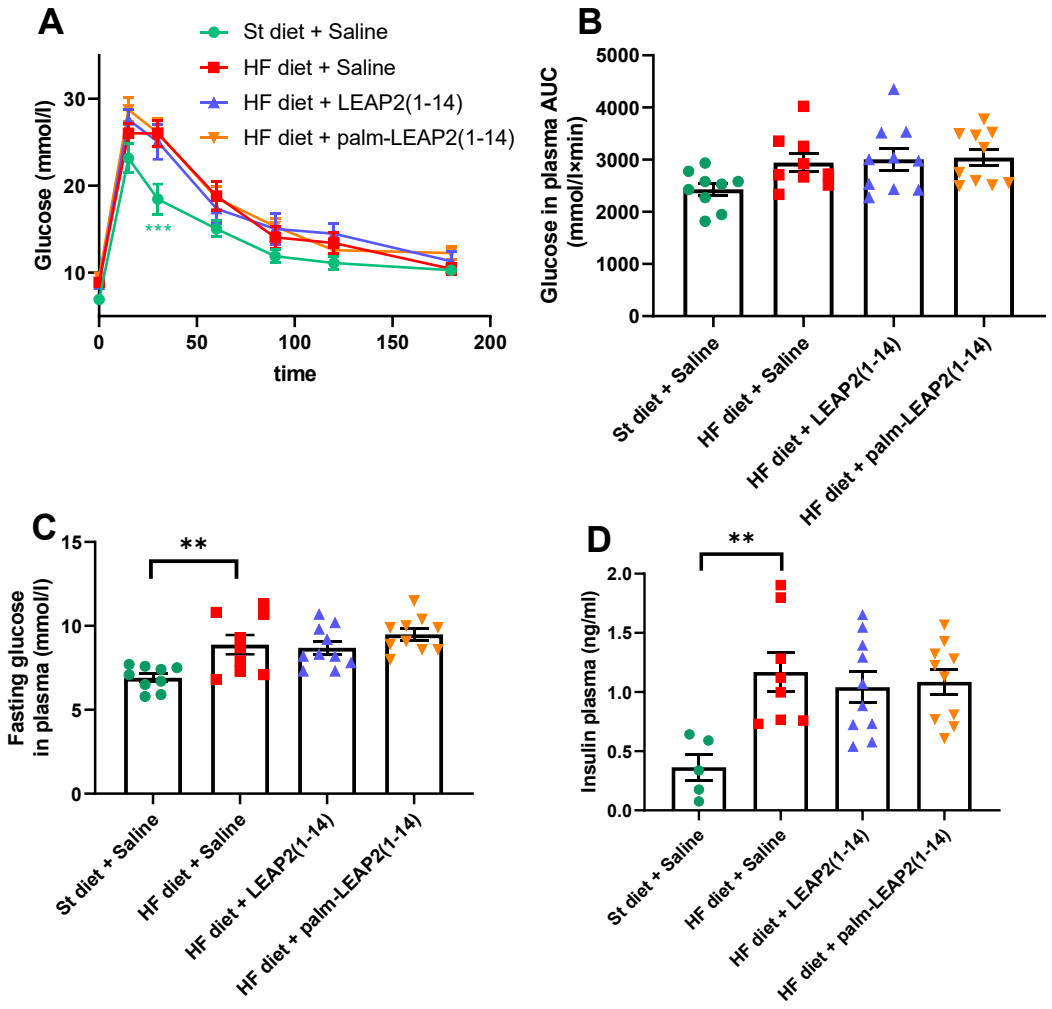

Figure 32: Effect of SC administration of chronic LEAP2(1-14) (10 mg/kg of body weight) and palm-LEAP2(1-14) (10 mg/kg of body weight) on the development of glucose intolerance after oral glucose gavage, dose 2 g/kg of body weight: levels of glucose after glucose gavage (A), AUC of OGTT curves (B), plasma levels of fasting glucose (C), and insulin plasma levels (D). Data are expressed as mean ± SEM. Statistical analysis was conducted using 2-way ANOVA with Bonferroni's post hoc test (A) and 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons (B-D). Significance is *P<0.05, **P<0.01, ***P<0.001 vs HF diet + Saline (n=10).

4.4.3 Effect of chronic palm-LEAP2(1-14) administration on ghrelin, LEAP2 and leptin levels in plasma in mice fed HF diet

No differences were observed in either total or active ghrelin between groups fed the HF or St diet nor after the LEAP2(1-14) analogs interventions (Figure 33 A, B). Six weeks of HF diet induced an increase in leptin (Figure 33C) and LEAP2 (Figure 33D) levels in plasma. LEAP2(1-14) interventions did not have any effect on plasma levels of leptin nor

LEAP2. Long-term administration of palm-LEAP2 significantly decreased plasma leptin in HF diet fed mice.

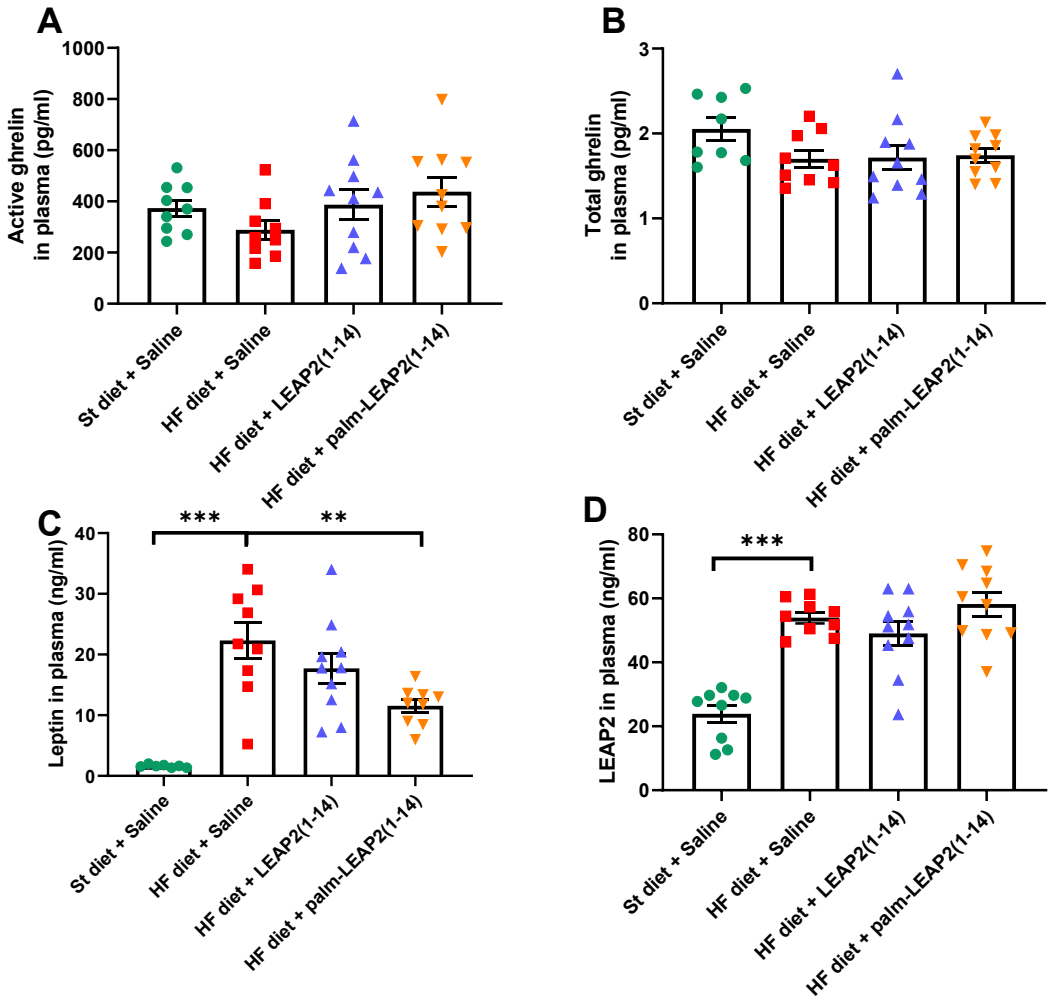

Figure 33: Effect of SC administration of chronic LEAP2(1-14) (10 mg/kg of body weight) and palm-LEAP2(1-14) (10 mg/kg of body weight) on the levels of active ghrelin (A), total ghrelin (B), leptin (C), and LEAP2 (D) in plasma. Data are presented as mean ± SEM. Statistical analysis was performed using the 1-way ANOVA with Bonferroni's post hoc test for multiple comparisons. Significance is *P<0.05, **P<0.01, ***P<0.001 vs HF diet + Saline (n=10).

5 DISCUSSION

Obesity is dangerous due to its association with an increased risk of chronic diseases, such as type 2 diabetes, cardiovascular complications and metabolic dysfunction. Due to the fact that obesity has reached epidemic proportions in recent decades, it is necessary to develop an effective anti-obesity treatment. One promising approach in this field is the utilization of modified endogenous compounds that regulate body weight and energy balance. Ghrelin, that acts through its receptor GHSR, is the only known peripheral peptide that increases food intake (Kojima et al., 1999). Antagonizing of GHSR could be a promising way in anti-obesity research.

Because GHSR has a high constitutive activity (Holst et al., 2004), the research has shifted towards the study of inverse agonists that can effectively reduce the baseline signaling of GHSR. The LEAP2 peptide acts as ghrelin competitive antagonist, that suppresses ghrelin-induced food intake and ghrelin-induced GH release (Ge et al., 2018), but also as GHSR inverse agonist that inhibits the constitutive stimulation of basal food intake (M'Kadmi et al., 2019).

The synthesis of LEAP2 is very complex, and therefore it is preferable to work with analogs that are easier to synthesize, but at the same time have the similar affinity to and activity at GHSR as the natural peptide. In a previous study (M'Kadmi et al., 2019), it was demonstrated that N-terminal fragments of LEAP2 possess inverse agonist properties against GHSR and reduce ghrelin-induced food intake. However, the stability of peptides after peripheral administration is low and therefore, it is important to enhance peptide stability by different strategies. Lipidization of peptides promotes their central action after peripheral administration and increases their stability in the bloodstream by binding to albumin. Thus, lipidized N-terminal analogs of LEAP2 might be good candidates for possible obesity treatment.

In my PhD thesis, lipidized N-terminal LEAP2(1-14) analogs were used for *in vitro* and *in vivo* studies in order to investigate the interplay between ghrelin and LEAP2 analogs.

5.1 *In vitro* testing of LEAP2(1-14) analogs

M'Kadmi recently proved that N-terminal sequence that contains at least the first 12 amino acids is necessary for full GHSR binding and activation (M'Kadmi et al., 2019). We used a peptide containing the initial 14 residues of N-terminal LEAP2 and a β-Ala-Lys-NH_2

linker (hereafter named LEAP2(1-14)) employed for the subsequent lipidization with myristic, palmitic and stearic acid (Table 2).

We verified that truncation of LEAP2 to its N-terminal fragment LEAP2(1-14) does not impair its ability to bind to GHSR compared to native LEAP2. We proved that the lipidization did not impair ability of LEAP2(1-14) to bind to the overexpressed GHSR in U2OS cells. LEAP2(1-14) and its lipidized analogs displayed K_i values in the 10^{-9}-10^{-8} M range that were equivalent to those of natural LEAP2. The highest affinity for GHSR was obtained with the palmitoylated analog. Both palm-LEAP2(1-14) and myr-LEAP2(1-14) had higher affinity for GHSR than the non-lipidized analog. Stearoylation slightly reduced the affinity for GHSR, in comparison with other lipidized analogs, however, the affinity was comparable with natural LEAP2.

We tested the activity of LEAP2(1-14) and its lipidized analogs at GHSR using a β-lactamase reporter gene response assay, which is designed to measure both inverse agonism and antagonism. With an EC_{50} of roughly 10^{-8}-10^{-7} M, all tested LEAP2-derived analogs reduced GHSR constitutive activity, which proves that they are all inverse agonists of GHSR. This finding is consistent with the work of M'Kadmi et al. (M'Kadmi et al., 2019), who disproved Ge's claim that LEAP2 does not have inverse agonist activity (Ge et al., 2018). To verify inverse agonism, M'Kadmi et al. (M'Kadmi et al., 2019) used measurement of the basal level of inositol phosphate 1 (IP1), instead of β-arrestin recruitment used by Ge et al. (Ge et al., 2018). β-arrestin recruitment at GHSR is low under basal conditions and thus can lead to misleading results. The activity of LEAP2(1-14) on the GHSR receptor was slightly lower than that of the natural LEAP2. However, lipidization increased the inverse agonist activity of LEAP2(1-14) analogs at GHSR with EC_{50} comparable to that of natural LEAP2.

In antagonist assay, LEAP2 analogs were able to shift ghrelin-induced activation curves without changing the maximal effect induced by ghrelin. This indicates that LEAP2 acts as a competitive antagonist and shares a common ligand-binding pocket with ghrelin at GHSR which is in accordance with M'Kadmi's et al. and Wang's et al. studies (M'Kadmi et al., 2019, Wang et al., 2019), but contradicts the study of Ge et al. (Ge et al., 2018). LEAP2 dissociates slowly from GHSR (Wang et al., 2019), and therefore incubation times might affect the equilibrium between ghrelin and LEAP2 during the experiment. Ge et al. (Ge et al., 2018) used 30 min pre-incubation of GHSR overexpressing cells with LEAP2 followed 90- or 180-min incubation with agonist. When LEAP2 is pre-incubated with

GHSR1a, it stays attached to the receptor for a longer period of time and exhibits noncompetitive antagonistic properties (Wang et al., 2019). Although we pre-incubated GHSR-overexpressing cells with LEAP2 analogs, the subsequent incubation with ghrelin lasted for 16 hours, so equilibrium could have been established. Similarly to the inverse agonist assay, LEAP2(1-14) exhibited diminished antagonist activity in comparison to natural LEAP2, but myr-LEAP2(1-14), palm-LEAP2(1-14), and stear-LEAP2(1-14) demonstrated even higher levels of antagonist activity comparable to natural LEAP2.

Lipidization is commonly employed to extend the half-life of peptides (Kurtzhals et al., 1995). Palm-LEAP2(1-14) showed the highest affinity to and highest inverse agonist and antagonist activity at GHSR, and therefore it was used to compare stability of lipidized and non-lipidized LEAP2(1-14) analog. Compared to LEAP2(1-14), palm-LEAP2(1-14) had significantly higher stability.

Taken together, all lipidized LEAP2(1-14) analogs showed higher affinity for GHSR-transfected U2OS cells when compared to non-lipidized LEAP(1-14) and preserved ability to inhibit GHSR constitutive activity comparable to natural LEAP2. Novel LEAP2 analog palm-LEAP2(1-14) showed the best *in vitro* properties and high stability in rat plasma among the tested analogs.

5.2 Short term *in vivo* testing of LEAP2(1-14) analogs

In order to examine the antagonistic properties of LEAP2(1-14) analogs *in vivo*, we focused on inhibition of two well-known functions of ghrelin, food intake and GH release.

Ghrelin acts directly in the hypothalamus and stimulates orexigenic neuropeptides which results in increased food intake (Cowley et al., 2003). The blood levels of ghrelin and LEAP2 have opposite trend in fasting and feeding in mice and humans. Ghrelin levels are higher and LEAP2 levels are lower in fasting individuals (Mani et al., 2019). We tested the ability of LEAP2 analogs to suppress either the high level of endogenous ghrelin in fasted mice or the peripherally administered ghrelin analog [Dpr^3]Ghrelin in free fed mice with low endogenous ghrelin levels. [Dpr^3]Ghrelin is stable ghrelin analog with similar affinity to and activity at GHSR used in our previous study (Maletinska et al., 2012).

From all of LEAP2(1-14) analogs, palm-LEAP2(1-14) had the highest anorexigenic activity in fasted mice. Non-lipidized LEAP2(1-14) showed a slight reduction in food intake, which was already proved in previous studies, where N-terminal LEAP2 analog

decreased food intake in fasted mice similarly to natural LEAP2 (M'Kadmi et al., 2019, Fernandez et al., 2022).

LEAP2(1-14) attenuated [Dpr^3]Ghrelin-induced food intake in free-fed mice similarly as described by M'Kadmi et al. (M'Kadmi et al., 2019). Palm-LEAP2(1-14) and stear-LEAP2(1-14) completely attenuated [Dpr^3]Ghrelin-induced food intake in free-fed mice. Conversely, myr-LEAP2(1-14) exhibited relatively low potency in inhibiting [Dpr^3]Ghrelin-induced food intake similar to non-lipidized LEAP2(1-14) analog.

These findings suggest that palm-LEAP2(1-14) effectively inhibits the orexigenic function of both endogenous ghrelin and exogenous [Dpr^3]Ghrelin and it is the most potent of the tested lipidized LEAP2(1-14) analogs. The anorexigenic effect of palm-LEAP2(1-14) is similar to that of natural LEAP2 shown in the previous study (Ge et al., 2018).

Following these findings, we focused on palm-LEAP2(1-14) and non-lipidized LEAP2(1-14) to investigate [Dpr^3]Ghrelin-induced GH release in young mice. The stimulation of the GHSR by ghrelin in pituitary cells was shown to trigger significant GH secretion (Kojima et al., 1999) whereas natural LEAP2 was found to suppress ghrelin-induced GH secretion in mice (Islam et al., 2020, Ge et al., 2018). We compared the ability of LEAP2(1-14) and palm-LEAP2(1-14) to suppress the [Dpr^3]Ghrelin-induced GH release in young mice. While palm-LEAP2(1-14) suppressed [Dpr^3]Ghrelin-induced GH release, LEAP2(1-14) had no effect on it. The reduced ability of LEAP2(1-14) to inhibit [Dpr^3]Ghrelin-induced GH release could potentially be attributed to its lower stability or bioavailability in organisms in comparison to palm-LEAP2(1-14).

In conclusion, novel LEAP2 analog palm-LEAP2(1-14) showed potent anorexigenic effects, and a single SC injection fully inhibited [Dpr^3]Ghrelin-induced food intake and GH release (Figure 34).

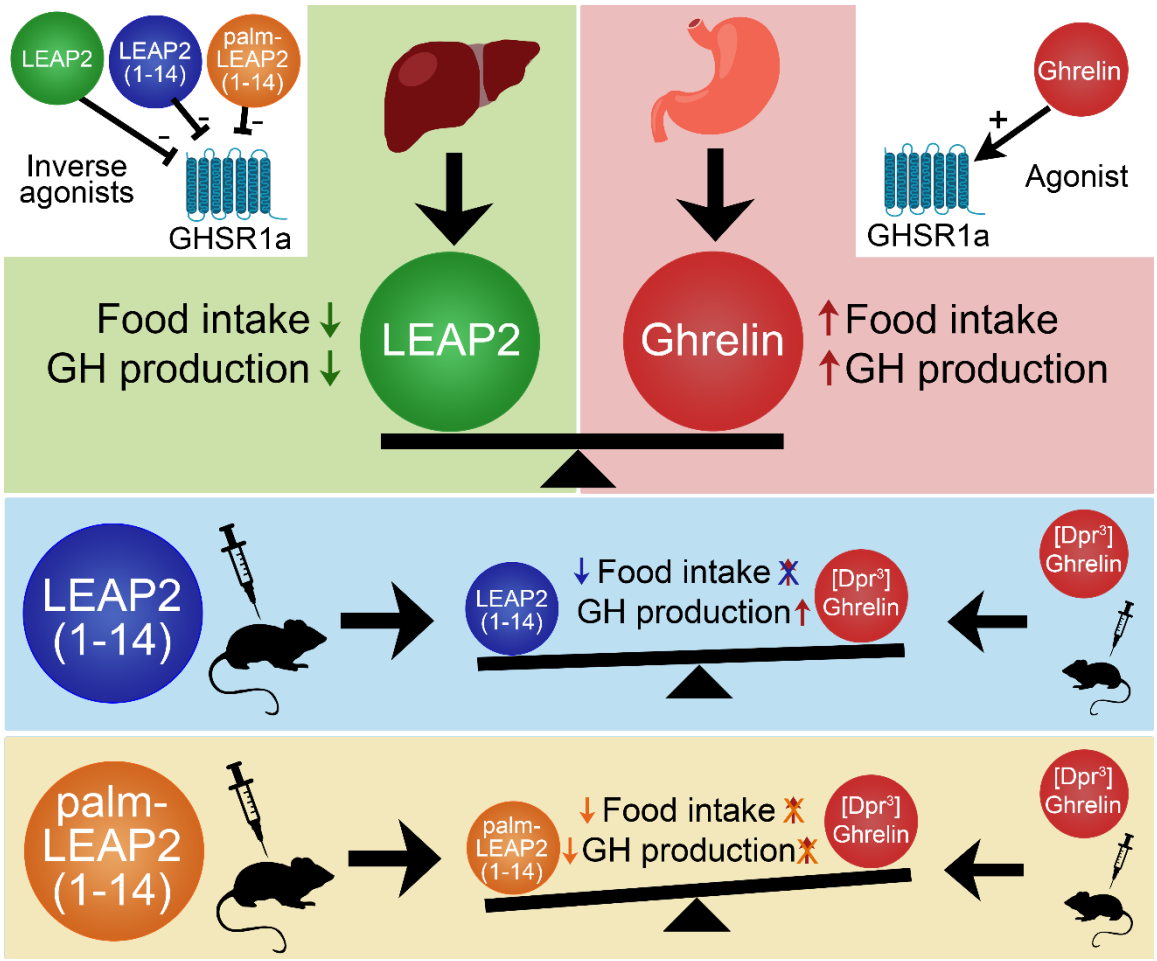

Figure 34: Scheme of *in vitro* and *in vivo* properties of ghrelin, LEAP2 and its novel analogs LEAP2(1-14) and palm-LEAP2(1-14). LEAP2 and its analogs are inverse agonists of ghrelin receptor GHSR. LEAP2 counteracts the effects of ghrelin and reduces food intake and ghrelin-induced growth hormone (GH) release. LEAP(1-14) was able to suppress [Dpr^3]Ghrelin-induced food intake but did not affect [Dpr^3]Ghrelin-induced GH release. Palm-LEAP2(1-14) was able to suppress both [Dpr^3]Ghrelin-induced food intake and [Dpr^3]Ghrelin-induced GH release.

5.3 Progress of obesity at HF diet feeding

In order to be able to monitor the long-term effect of LEAP2 analogs on obese individuals, we first wanted to examine the interplay between endogenous LEAP2 and ghrelin at obesity progression. In obese individuals, sensitivity to ghrelin declines (Briggs et al., 2010). After reduction of the body weight, ghrelin level and sensitivity to ghrelin are increased and metabolic parameters are normalized (Briggs et al., 2013). However, it is still not clear whether obesity affects sensitivity to LEAP2 or how long does it take for HF diet feeding to induce LEAP2 resistance in DIO mice.

Previous research has demonstrated that obesity decreases ghrelin levels in blood. (Cummings et al., 2001, Tschop et al., 2001). Briggs et al. proved that switching the HF diet to the control diet after 12 weeks of HF diet feeding re-increased level of active ghrelin

but did not normalize the total ghrelin level (Briggs et al., 2014). In our experiment, we observed that already 3 weeks of HF diet resulted in decreased levels of active and total ghrelin. After switching the HF diet for St diet at week 9, only active ghrelin levels were restored, as in the previous research (Briggs et al., 2014). We believe that an increase in active ghrelin – rather than total ghrelin – is considerably more significant for ghrelin sensitivity.

LEAP2 has an opposite trend in obesity progression than ghrelin. The plasma LEAP2 is elevated in obese mice and humans (Andrews, 2019) and so is liver *LEAP2* mRNA expression in liver in DIO mice (Holm et al., 2022). On the other hand, plasma LEAP2 is decreased in diet-induced weight loss (Mani et al., 2019). After three weeks of consuming HF diet, we observed a slight rise in liver *LEAP2* mRNA levels in mice. *LEAP2* mRNA expression in liver significantly increased after nine weeks of HF diet feeding and it decreased after switching from HF diet to a St diet to the level seen in animals fed a St diet exclusively.

In agreement with numerous studies describing effect of HF diet feeding of mice and rats (Enriori et al., 2007, Lin et al., 2000, Papathanassoglou et al., 2006, de Git et al., 2018, Williams et al., 2014, Varghese et al., 2020), we observed progressive increase in body weight, resulting from the consumption of a HF diet, increased adiposity accompanied by elevated plasma leptin and cholesterol levels. We are the first who compared a time course of plasma active and total ghrelin, together with *LEAP2* mRNA expression in the livers of mice fed a HF diet, a St diet, or HF diet switched back to St diet at week 9. Similarly, after the switch to a St diet, there was a corresponding rise in active ghrelin levels, but not total ghrelin, and a decrease in *LEAP2* mRNA expression, accompanied by a restoration of body weight, normalization of plasma cholesterol levels, and improved glucose tolerance.

Reynolds et al. demonstrated that feeding mice HF diet for 6 weeks resulted in glucose intolerance that was overcome when the mice were switched to a St diet (Reynolds et al., 2015). In our experiment, we observed the elevated glucose excursions at OGTT already 3 weeks after feeding HF diet; they were attenuated after switching to St diet. Insulin plasma levels were significantly enhanced after 9 weeks of HF diet feeding but were not enough to restore glucose tolerance to normal levels.

Obesity and metabolic syndrome are closely linked to chronic low-level inflammation, which is characterised by increased circulating CRP and permanently increased oxidative

stress (Monteiro and Azevedo, 2010, Brooks and Maklakov, 2010). Increased CRP corresponds with increased levels of LEAP2 (Francisco et al., 2020) and decreased ghrelin in plasma (Riedl et al., 2007, Peracchi et al., 2006). Moreover, decreased ghrelin correlates with increased immunoglobulin production, which is frequently reported in individuals with chronic liver disease (Okamatsu et al., 2009), and also with liver inflammation (Machado et al., 2012). Increased active ghrelin and decreased LEAP2 liver production was associated with a decrease in plasma CRP, as well as decreased oxidative stress and steatosis in the liver after switching of HF diet to a St diet.

In this study, mRNA expression of the orexigenic neuropeptides *AgRP* and *NPY* did not differ in either the St diet-fed group or the HF diet-fed group. Briggs et al. reported that hypothalamic *AgRP* and *NPY* expression in free-fed mice is identical in HF- and control-fed groups, which is consistent with our study. In fasting mice, however, mRNA expression of *AgRP* and *NPY* was higher in the control diet-fed group than in the HF diet-fed group (Briggs et al., 2011). In our work, mRNA expression of *POMC* and *CART* in free fed mice tended to rise after the HF diet feeding, similarly to Kohsaka et al. (Kohsaka et al., 2007).

In previous studies, it was shown that ghrelin resistance developed after 3-4 weeks of HF diet feeding (Briggs et al., 2014, Naznin et al., 2015) and that both peripheral and central administration of ghrelin to DIO mice did not induce food intake (Perreault et al., 2004, Briggs et al., 2010, Gardiner et al., 2010). However, the ghrelin sensitivity could be restored after the diet-induced weight loss (Briggs et al., 2013). To determine whether obese mice would respond to palm-LEAP2(1-14) by reducing food intake, we acutely SC administered palm-LEAP2(1-14) to mice fed a HF diet at several time points of HF diet feeding. Our findings indicate that mice develop [Dpr^3]Ghrelin resistance as early as after 2 weeks feeding a HF diet, while resistance to palm-LEAP2(1-14) develops after 4 weeks. Unlike palm-LEAP2(1-14) resistance, [Dpr^3]Ghrelin resistance was reversible and it was restored 4 weeks after the switching HF diet to St diet.

Taken together, our findings indicate that switching the HF diet for St diet restores plasma levels of active ghrelin to levels observed in mice exclusively fed a St diet as well as the expression of *LEAP2* mRNA in the liver. Furthermore, the switch to a St diet effectively mitigates the adverse effects of HF diet consumption, such as increased body weight, elevated leptin levels, glucose intolerance, and liver steatosis. Additionally, our study

reveals that a HF diet not only induces reversible ghrelin resistance but also resistance to palm-LEAP2(1-14) treatment.

5.4 Ability of palm-LEAP to prevent development of HF diet-induced obesity

We confirmed the previously reported results (Mani et al., 2019), that in obesity, ghrelin levels in plasma decrease and LEAP2 levels in plasma increase. Gupta et al. hypothesizes (Gupta et al., 2021) that therapeutic interventions that raise plasma LEAP2 or further reduce plasma ghrelin, would serve to limit the development of obesity and glucose intolerance. However, we found out that already 4 weeks of HF diet feeding lead to reduced sensitivity to palm-LEAP2(1-14), thus raising LEAP2 level might not have the desired effect on suppressing the development of obesity.

Therefore, we decided to find out if the chronic administration of palm-LEAP2(1-14) can suppress the effect of the HF diet on mouse metabolism before the onset of palm-LEAP2(1-14) resistance. Palm-LEAP2(1-14) was SC administered to mice from the first day of HF diet feeding. Its effect was compared with non-lipidized analog LEAP2(1-14).

The non-lipidized analog had no effect on the metabolomic and morphometric parameters affected by the HF diet. Palm-LEAP2(1-14) tended to decrease body weight gain induced by HF diet feeding, but the difference was mostly not significant. A significant difference between the palm-LEAP2(1-14)-treated group and the control group was observed only in plasma leptin levels, where leptin levels were reduced. Other parameters, such as active and total ghrelin and LEAP2 in plasma did not change after chronic administration of palm-LEAP2(1-14). Likewise, palm-LEAP2(1-14) did not affect glucose tolerance in OGTT testing.

Cui et al. suggest 4 ways of developing ghrelin resistance that could also correspond to LEAP2 resistance: decreased circulating levels of ghrelin, impaired transport of ghrelin to hypothalamic GHSR, reduced expression of GHSR and reduced expression of AgRP and NPY (Cui et al., 2017). The use of other GHSR inverse agonists than LEAP2 and its analogs could help to discover the mechanism of LEAP2 resistance. Small molecule GHSR inverse agonist GHSRIA2 induced decrease in body weight and visceral fat in DIO mice and also improved sensitivity to glucose in OGTT test (Abegg et al., 2017). This would suggest that LEAP2 resistance occurs upstream of LEAP2 binding to the GHSR and is related to either elevated plasma LEAP2 levels or impaired LEAP2 transport to the

hypothalamic GHSR. Therefore, the administration of another LEAP2 analog would not affect the metabolomic and morphometric parameters in DIO mice.

In conclusion, the use of GHSR inverse agonists for the treatment of obesity shows promise; nevertheless, additional study is required to fully understand the mechanisms of ghrelin and LEAP2 resistance and to investigate how to bypass ghrelin and LEAP2 resistance while still targeting GHSR.

6 SUMMARY

There has been a global search for anti-obesity treatments that are both effective and safe, for last decades. LEAP2, which suppress orexigenic effect of ghrelin by decreasing activity of ghrelin receptor GHSR, may represent a promising target for pharmacotherapy in the treatment of obesity and associated conditions. In this study, the interaction between ghrelin and LEAP2 in the regulation of food intake and obesity was explored. The research focused on investigating the potential of modified N-terminal peptide LEAP2(1-14) and its lipidized analogs as anti-obesity treatments, as well as examining the impact of a HF diet on ghrelin and LEAP2 resistance.

A series of N-terminal LEAP2 analogs were designed and tested *in vitro* and *in vivo*, with a particular focus on their binding affinity, activity at GHSR, and their effects on food intake and GH release. In the *in vitro* testing of LEAP2(1-14) analogs, it was found that lipidization did not impair the ability of the analogs to bind to the GHSR. The affinity for GHSR was highest for the palmitoylated analog, followed by the myristoylated analog and the non-lipidized analog. All tested analogs displayed inverse agonist activity, effectively reducing GHSR constitutive activity. The lipidized analogs showed increased inverse agonist activity compared to the non-lipidized analog, with palm-LEAP2(1-14) exhibiting the highest activity. In antagonist assays, the LEAP2(1-14) analogs acted as competitive antagonists as well as natural LEAP2. The lipidized analogs demonstrated higher levels of antagonist activity than both the natural LEAP2 and LEAP2(1-14), with palm-LEAP2(1-14) showing the highest affinity and activity. The stability of palm-LEAP2(1-14) was also significantly higher than that of LEAP2(1-14).

In short-term *in vivo* testing, palm-LEAP2(1-14) exhibited potent anorexigenic effects, effectively suppressing both endogenous ghrelin and exogenous [Dpr^3]Ghrelin-induced food intake in mice. It also suppressed [Dpr^3]Ghrelin-induced GH release, while the non-lipidized analog had no effect on GH release. These findings suggested that palm-LEAP2(1-14) is a promising candidate for inhibiting the orexigenic function of ghrelin and suppressing GH release.

Before long-term administration of LEAP2 to obese mice, the relationship between endogenous ghrelin and LEAP2 in progressive obesity had to be monitored. It was observed that HF diet feeding led to decreased plasmatic levels of active and total ghrelin, while *LEAP2* mRNA in liver increased. Switching from an HF diet to a St diet resulted in

restored active ghrelin levels and decreased *LEAP2* mRNA expression in liver, accompanied by improvements in body weight, plasma cholesterol levels, and glucose tolerance.

Due to the early onset of palm-LEAP2(1-14) resistance, the long-term effect of palm-LEAP2(1-14) on obesity was monitored from the first day of feeding with the HF diet. Palm-LEAP2(1-14) slightly reduced weight gain caused by the HF diet and decreased plasma leptin levels. However, palm-LEAP2(1-14) was unable to fully counteract the effects of the HF diet.

The results show that although palm-LEAP2(1-14) is an effective inverse agonist in both *in vitro* and short-term *in vivo* experiments, it fails to suppress the metabolic and morphometric changes induced by a HF diet in the long-term treatment of obesity.

7 CONCLUSIONS

This study provides insights into the interaction between ghrelin and LEAP2 in the regulation of food intake and obesity. Series of novel N-terminal LEAP2(1-14) analogs, lipidized with different fatty acids was tested for its affinity to and activation of GHSR *in vitro* and *in vivo*.

Palm-LEAP2(1-14) had the highest affinity for GHSR and acted as inverse agonist as well as competitive antagonist of GHSR. *In vivo* experiments proved that palm-LEAP2(1-14) reduced food intake in both fasted mice with high endogenous ghrelin level and free fed mice with exogenous [Dpr^3]Ghrelin. Palm-LEAP2(1-14) inhibited [Dpr^3]Ghrelin-induced GH release and showed increased stability in rat plasma compared to non-lipidized LEAP2(1-14). In order to study long-term effect of palm-LEAP2(1-14) in obese mice, we studied HF diet-induced obesity and the development of ghrelin and LEAP2 resistance in mice. HF diet feeding decreased plasma active and total ghrelin and increased liver *LEAP2* mRNA expression. The switch to a St diet normalized liver *LEAP2* mRNA expression and active ghrelin levels but not total ghrelin. Palm-LEAP2(1-14) and [Dpr^3]Ghrelin resistance developed after HF diet feeding, [Dpr^3]Ghrelin sensitivity restored upon switching to a standard diet. Effect of palm-LEAP2(1-14) on HF diet feeding since the first day of HF diet feeding was studied. Palm-LEAP2(1-14) slightly reduced the body weight gain and decreased plasma leptin level. However, palm-LEAP2(1-14) was not able to suppress the effect of HF diet due to palm-LEAP2(1-14) resistance.

In conclusion, the findings contribute to the understanding of obesity and suggest that modified N-terminal peptide LEAP2(1-14) and its lipidized analogs, particularly palm-LEAP2(1-14) holds promise for anti-obesity treatment, but further investigations are necessary to overcome the challenges of long-term treatment and resistance development. Alternative approaches are needed to improve the effectiveness of anti-obesity therapies that target the ghrelin and LEAP2 pathways.

8 REFERENCES

ABEGG, K., BERNASCONI, L., HUTTER, M., WHITING, L., PIETRA, C., GIULIANO, C., LUTZ, T. A. & RIEDIGER, T. 2017. Ghrelin receptor inverse agonists as a novel therapeutic approach against obesity-related metabolic disease. *Diabetes Obes Metab,* 19**,** 1740-1750.

ABIZAID, A. & HOUGLAND, J. L. 2020. Ghrelin Signaling: GOAT and GHS-R1a Take a LEAP in Complexity. *Trends Endocrinol Metab,* 31**,** 107-117.

ADUNSKY, A., CHANDLER, J., HEYDEN, N., LUTKIEWICZ, J., SCOTT, B. B., BERD, Y., LIU, N. & PAPANICOLAOU, D. A. 2011. MK-0677 (ibutamoren mesylate) for the treatment of patients recovering from hip fracture: A multicenter, randomized, placebo-controlled phase IIb study. *Archives of Gerontology and Geriatrics,* 53**,** 183-189.

ANDERSSON, U., FILIPSSON, K., ABBOTT, C. R., WOODS, A., SMITH, K., BLOOM, S. R., CARLING, D. & SMALL, C. J. 2004. AMP-activated protein kinase plays a role in the control of food intake. *J Biol Chem,* 279**,** 12005-8.

ANDREWS, Z. B. 2011. Central mechanisms involved in the orexigenic actions of ghrelin. *Peptides,* 32**,** 2248-55.

ANDREWS, Z. B. 2019. The next big LEAP2 understanding ghrelin function. *J Clin Invest,* 129**,** 3542-3544.

APOVIAN, C. M., ARONNE, L. J., BESSESEN, D. H., MCDONNELL, M. E., MURAD, M. H., PAGOTTO, U., RYAN, D. H., STILL, C. D. & ENDOCRINE, S. 2015. Pharmacological management of obesity: an endocrine Society clinical practice guideline. *J Clin Endocrinol Metab,* 100**,** 342-62.

ASAKAWA, A., INUI, A., KAGA, T., KATSUURA, G., FUJIMIYA, M., FUJINO, M. A. & KASUGA, M. 2003. Antagonism of ghrelin receptor reduces food intake and body weight gain in mice. *Gut,* 52**,** 947-52.

BASSIL, A. K., HAGLUND, Y., BROWN, J., RUDHOLM, T., HELLSTROM, P. M., NASLUND, E., LEE, K. & SANGER, G. J. 2007. Little or no ability of obestatin to interact with ghrelin or modify motility in the rat gastrointestinal tract. *Br J Pharmacol,* 150**,** 58-64.

BEDNAREK, M. A., FEIGHNER, S. D., PONG, S. S., MCKEE, K. K., HRENIUK, D. L., SILVA, M. V., WARREN, V. A., HOWARD, A. D., VAN DER PLOEG, L. H. & HECK, J. V. 2000. Structure-function studies on the new growth hormone-releasing peptide, ghrelin: minimal sequence of ghrelin necessary for activation of growth hormone secretagogue receptor 1a. *J Med Chem,* 43**,** 4370-6.

BOWERS, C. Y., MOMANY, F. A., REYNOLDS, G. A. & HONG, A. 1984a. On the in vitro and in vivo activity of a new synthetic hexapeptide that acts on the pituitary to specifically release growth hormone. *Endocrinology,* 114**,** 1537-45.

BOWERS, C. Y., REYNOLDS, G. A. & MOMANY, F. A. 1984b. New advances on the regulation of growth hormone (GH) secretion. *Int J Neurol,* 18**,** 188-205.

BRIGGS, D. I. & ANDREWS, Z. B. 2011. Metabolic status regulates ghrelin function on energy homeostasis. *Neuroendocrinology,* 93**,** 48-57.

BRIGGS, D. I., ENRIORI, P. J., LEMUS, M. B., COWLEY, M. A. & ANDREWS, Z. B. 2010. Diet-induced obesity causes ghrelin resistance in arcuate NPY/AgRP neurons. *Endocrinology,* 151**,** 4745-55.

BRIGGS, D. I., LEMUS, M. B., KUA, E. & ANDREWS, Z. B. 2011. Diet-induced obesity attenuates fasting-induced hyperphagia. *J Neuroendocrinol,* 23**,** 620-6.

BRIGGS, D. I., LOCKIE, S. H., BENZLER, J., WU, Q., STARK, R., REICHENBACH, A., HOY, A. J., LEMUS, M. B., COLEMAN, H. A., PARKINGTON, H. C., TUPS, A. & ANDREWS, Z. B. 2014. Evidence that diet-induced hyperleptinemia, but not hypothalamic gliosis, causes ghrelin resistance in NPY/AgRP neurons of male mice. *Endocrinology,* 155**,** 2411-22.

BRIGGS, D. I., LOCKIE, S. H., WU, Q., LEMUS, M. B., STARK, R. & ANDREWS, Z. B. 2013. Calorie-restricted weight loss reverses high-fat diet-induced ghrelin resistance, which

contributes to rebound weight gain in a ghrelin-dependent manner. *Endocrinology,* 154**,** 709-17.

BROBERGER, C. 2005. Brain regulation of food intake and appetite: molecules and networks. *Journal of Internal Medicine,* 258**,** 301-327.

BROOKS, R. & MAKLAKOV, A. 2010. Sex differences in obesity associated with total fertility rate. *PLoS One,* 5**,** e10587.

CAI, H., CONG, W. N., DAIMON, C. M., WANG, R., TSCHOP, M. H., SEVIGNY, J., MARTIN, B. & MAUDSLEY, S. 2013. Altered lipid and salt taste responsivity in ghrelin and GOAT null mice. *PLoS One,* 8**,** e76553.

COULTER, A. A., REBELLO, C. J. & GREENWAY, F. L. 2018. Centrally Acting Agents for Obesity: Past, Present, and Future. *Drugs,* 78**,** 1113-1132.

COWLEY, M. A., SMITH, R. G., DIANO, S., TSCHOP, M., PRONCHUK, N., GROVE, K. L., STRASBURGER, C. J., BIDLINGMAIER, M., ESTERMAN, M., HEIMAN, M. L., GARCIA-SEGURA, L. M., NILLNI, E. A., MENDEZ, P., LOW, M. J., SOTONYI, P., FRIEDMAN, J. M., LIU, H., PINTO, S., COLMERS, W. F., CONE, R. D. & HORVATH, T. L. 2003. The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. *Neuron,* 37**,** 649-61.

CUI, H., LOPEZ, M. & RAHMOUNI, K. 2017. The cellular and molecular bases of leptin and ghrelin resistance in obesity. *Nat Rev Endocrinol,* 13**,** 338-351.

CUMMINGS, D. E., PURNELL, J. Q., FRAYO, R. S., MA, M. K., DELLINGER, E. P. & WEIGLE, D. S. 2001. Plasma ghrelin levels are markedly decreased after gastric bypass surgery in humans. *Obesity Research,* 9**,** 73s-73s.

CURROW, D., TEMEL, J. S., ABERNETHY, A., MILANOWSKI, J., FRIEND, J. & FEARON, K. C. 2017. ROMANA 3: a phase 3 safety extension study of anamorelin in advanced non-small-cell lung cancer (NSCLC) patients with cachexia. *Ann Oncol,* 28**,** 1949-1956.

DATE, Y., MURAKAMI, N., TOSHINAI, K., MATSUKURA, S., NIIJIMA, A., MATSUO, H., KANGAWA, K. & NAKAZATO, M. 2002a. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. *Gastroenterology,* 123**,** 1120-8.

DATE, Y., NAKAZATO, M., HASHIGUCHI, S., DEZAKI, K., MONDAL, M. S., HOSODA, H., KOJIMA, M., KANGAWA, K., ARIMA, T., MATSUO, H., YADA, T. & MATSUKURA, S. 2002b. Ghrelin is present in pancreatic alpha-cells of humans and rats and stimulates insulin secretion. *Diabetes,* 51**,** 124-9.

DAVENPORT, A. P., BONNER, T. I., FOORD, S. M., HARMAR, A. J., NEUBIG, R. R., PIN, J. P., SPEDDING, M., KOJIMA, M. & KANGAWA, K. 2005. International Union of Pharmacology. LVI. Ghrelin receptor nomenclature, distribution, and function. *Pharmacol Rev,* 57**,** 541-6.

DE GIT, K. C. G., PETERSE, C., BEERENS, S., LUIJENDIJK, M. C. M., VAN DER PLASSE, G., LA FLEUR, S. E. & ADAN, R. A. H. 2018. Is leptin resistance the cause or the consequence of diet-induced obesity? *Int J Obes (Lond),* 42**,** 1445-1457.

DELPORTE, C. 2013. Structure and physiological actions of ghrelin. *Scientifica (Cairo),* 2013**,** 518909.

DEMANGE, L., BOEGLIN, D., MOULIN, A., MOUSSEAUX, D., RYAN, J., BERGE, G., GAGNE, D., HEITZ, A., PERRISSOUD, D., LOCATELLI, V., TORSELLO, A., GALLEYRAND, J. C., FEHRENTZ, J. A. & MARTINEZ, J. 2007. Synthesis and pharmacological in vitro and in vivo evaluations of novel triazole derivatives as ligands of the ghrelin receptor. 1. *J Med Chem,* 50**,** 1939-57.

DENNEY, W. S., SONNENBERG, G. E., CARVAJAL-GONZALEZ, S., TUTHILL, T. & JACKSON, V. M. 2017. Pharmacokinetics and pharmacodynamics of PF-05190457: The first oral ghrelin receptor inverse agonist to be profiled in healthy subjects. *Br J Clin Pharmacol,* 83**,** 326-338.

DJANANI, A., KANEIDER, N. C., STURN, D. & WIEDERMANN, C. J. 2003. Agonist function of the neurokinin receptor antagonist, [D-Arg1,D-Phe5,D-Trp7,9,Leu11]substance P, in monocytes. *Regulatory Peptides,* 115**,** 123-129.

ENGLISH, P. J., GHATEI, M. A., MALIK, I. A., BLOOM, S. R. & WILDING, J. P. 2002. Food fails to suppress ghrelin levels in obese humans. *J Clin Endocrinol Metab,* 87**,** 2984.

ENRIORI, P. J., EVANS, A. E., SINNAYAH, P., JOBST, E. E., TONELLI-LEMOS, L., BILLES, S. K., GLAVAS, M. M., GRAYSON, B. E., PERELLO, M., NILLNI, E. A., GROVE, K. L. & COWLEY, M. A. 2007. Diet-induced obesity causes severe but reversible leptin resistance in arcuate melanocortin neurons. *Cell Metab,* 5**,** 181-94.

ESLER, W. P., RUDOLPH, J., CLAUS, T. H., TANG, W., BARUCCI, N., BROWN, S. E., BULLOCK, W., DALY, M., DECARR, L., LI, Y., MILARDO, L., MOLSTAD, D., ZHU, J., GARDELL, S. J., LIVINGSTON, J. N. & SWEET, L. J. 2007. Small-molecule ghrelin receptor antagonists improve glucose tolerance, suppress appetite, and promote weight loss. *Endocrinology,* 148**,** 5175-85.

FERNANDEZ, G., CABRAL, A., DE FRANCESCO, P. N., URIARTE, M., REYNALDO, M., CASTROGIOVANNI, D., ZUBIRIA, G., GIOVAMBATTISTA, A., CANTEL, S., DENOYELLE, S., FEHRENTZ, J. A., TOLLE, V., SCHIOTH, H. B. & PERELLO, M. 2022. GHSR controls food deprivation-induced activation of CRF neurons of the hypothalamic paraventricular nucleus in a LEAP2-dependent manner. *Cell Mol Life Sci,* 79**,** 277.

FERRE, G., LOUET, M., SAUREL, O., DELORT, B., CZAPLICKI, G., M'KADMI, C., DAMIAN, M., RENAULT, P., CANTEL, S., GAVARA, L., DEMANGE, P., MARIE, J., FEHRENTZ, J. A., FLOQUET, N., MILON, A. & BANERES, J. L. 2019. Structure and dynamics of G protein-coupled receptor-bound ghrelin reveal the critical role of the octanoyl chain. *Proc Natl Acad Sci U S A,* 116**,** 17525-17530.

FRANCISCO, V., TOVAR, S., CONDE, J., PINO, J., MERA, A., LAGO, F., GONZALEZ-GAY, M. A., DIEGUEZ, C. & GUALILLO, O. 2020. Levels of the Novel Endogenous Antagonist of Ghrelin Receptor, Liver-Enriched Antimicrobial Peptide-2, in Patients with Rheumatoid Arthritis. *Nutrients,* 12.

FRUH, S. M. 2017. Obesity: Risk factors, complications, and strategies for sustainable long-term weight management. *J Am Assoc Nurse Pract,* 29**,** S3-S14.

GARDINER, J. V., CAMPBELL, D., PATTERSON, M., KENT, A., GHATEI, M. A., BLOOM, S. R. & BEWICK, G. A. 2010. The hyperphagic effect of ghrelin is inhibited in mice by a diet high in fat. *Gastroenterology,* 138**,** 2468-76, 2476 e1.

GE, X., YANG, H., BEDNAREK, M. A., GALON-TILLEMAN, H., CHEN, P., CHEN, M., LICHTMAN, J. S., WANG, Y., DALMAS, O., YIN, Y., TIAN, H., JERMUTUS, L., GRIMSBY, J., RONDINONE, C. M., KONKAR, A. & KAPLAN, D. D. 2018. LEAP2 Is an Endogenous Antagonist of the Ghrelin Receptor. *Cell Metab,* 27**,** 461-469 e6.

GNANAPAVAN, S., KOLA, B., BUSTIN, S. A., MORRIS, D. G., MCGEE, P., FAIRCLOUGH, P., BHATTACHARYA, S., CARPENTER, R., GROSSMAN, A. B. & KORBONITS, M. 2002. The tissue distribution of the mRNA of ghrelin and subtypes of its receptor, GHS-R, in humans. *J Clin Endocrinol Metab,* 87**,** 2988.

GOODWIN, D., SIMERSKA, P. & TOTH, I. 2012. Peptides As Therapeutics with Enhanced Bioactivity. *Current Medicinal Chemistry,* 19**,** 4451-4461.

GOURCEROL, G., ST-PIERRE, D. H. & TACHE, Y. 2007. Lack of obestatin effects on food intake: should obestatin be renamed ghrelin-associated peptide (GAP)? *Regul Pept,* 141**,** 1-7.

GUERLAVAIS, V., BOEGLIN, D., MOUSSEAUX, D., OIRY, C., HEITZ, A., DEGHENGHI, R., LOCATELLI, V., TORSELLO, A., GHE, C., CATAPANO, F., MUCCIOLI, G., GALLEYRAND, J. C., FEHRENTZ, J. A. & MARTINEZ, J. 2003. New active series of growth hormone secretagogues. *J Med Chem,* 46**,** 1191-203.

GUPTA, D., OGDEN, S. B., SHANKAR, K., VARSHNEY, S. & ZIGMAN, J. M. 2021. "A LEAP 2 conclusions? Targeting the ghrelin system to treat obesity and diabetes". *Mol Metab,* 46**,** 101128.

GUTIERREZ, J. A., SOLENBERG, P. J., PERKINS, D. R., WILLENCY, J. A., KNIERMAN, M. D., JIN, Z., WITCHER, D. R., LUO, S., ONYIA, J. E. & HALE, J. E. 2008. Ghrelin octanoylation mediated by an orphan lipid transferase. *Proc Natl Acad Sci U S A,* 105**,** 6320-5.

HAGEMANN, C. A., JENSEN, M. S., HOLM, S., GASBJERG, L. S., BYBERG, S., SKOV-JEPPESEN, K., HARTMANN, B., HOLST, J. J., DELA, F., VILSBOLL, T., CHRISTENSEN, M. B., HOLST, B. & KNOP, F. K. 2022. LEAP2 reduces postprandial glucose excursions and ad libitum food intake in healthy men. *Cell Rep Med,* 3**,** 100582.

HANNA, C. C., KRIEGESMANN, J., DOWMAN, L. J., BECKER, C. F. W. & PAYNE, R. J. 2022. Chemical Synthesis and Semisynthesis of Lipidated Proteins. *Angewandte Chemie-International Edition,* 61.

HATTORI, N. 2009. Expression, regulation and biological actions of growth hormone (GH) and ghrelin in the immune system. *Growth Horm IGF Res,* 19**,** 187-97.

HENRIQUES, S. T., TAN, C. C., CRAIK, D. J. & CLARK, R. J. 2010. Structural and functional analysis of human liver-expressed antimicrobial peptide 2. *Chembiochem,* 11**,** 2148-57.

HERODES, M., ANDERSON, L. J., SHOBER, S., SCHUR, E. A., GRAF, S. A., AMMER, N., SALAS, R., MARCELLI, M. & GARCIA, J. M. 2023. Pilot clinical trial of macimorelin to assess safety and efficacy in patients with cancer cachexia. *J Cachexia Sarcopenia Muscle,* 14**,** 835-846.

HOLA, L., ZELEZNA, B., KARNOSOVA, A., KUNES, J., FEHRENTZ, J. A., DENOYELLE, S., CANTEL, S., BLECHOVA, M., SYKORA, D., MYSKOVA, A. & MALETINSKA, L. 2022. A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin. *J Pharmacol Exp Ther,* 383**,** 129-136.

HOLM, S., HUSTED, A. S., SKOV, L. J., MORVILLE, T. H., HAGEMANN, C. A., JORSAL, T., DALL, M., JAKOBSEN, A., KLEIN, A. B., TREEBAK, J. T., KNOP, F. K., SCHWARTZ, T. W., CLEMMENSEN, C. & HOLST, B. 2022. Beta-Hydroxybutyrate Suppresses Hepatic Production of the Ghrelin Receptor Antagonist LEAP2. *Endocrinology,* 163.

HOLST, B., CYGANKIEWICZ, A., JENSEN, T. H., ANKERSEN, M. & SCHWARTZ, T. W. 2003. High constitutive signaling of the ghrelin receptor--identification of a potent inverse agonist. *Mol Endocrinol,* 17**,** 2201-10.

HOLST, B., FRIMURER, T. M., MOKROSINSKI, J., HALKJAER, T., CULLBERG, K. B., UNDERWOOD, C. R. & SCHWARTZ, T. W. 2009. Overlapping binding site for the endogenous agonist, small-molecule agonists, and ago-allosteric modulators on the ghrelin receptor. *Mol Pharmacol,* 75**,** 44-59.

HOLST, B., HOLLIDAY, N. D., BACH, A., ELLING, C. E., COX, H. M. & SCHWARTZ, T. W. 2004. Common structural basis for constitutive activity of the ghrelin receptor family. *J Biol Chem,* 279**,** 53806-17.

HOLST, B. & SCHWARTZ, T. W. 2004. Constitutive ghrelin receptor activity as a signaling set-point in appetite regulation. *Trends Pharmacol Sci,* 25**,** 113-7.

HOLUBOVA, M., BLECHOVA, M., KAKONOVA, A., KUNES, J., ZELEZNA, B. & MALETINSKA, L. 2018. In Vitro and In Vivo Characterization of Novel Stable Peptidic Ghrelin Analogs: Beneficial Effects in the Settings of Lipopolysaccharide-Induced Anorexia in Mice. *J Pharmacol Exp Ther,* 366**,** 422-432.

HOLUBOVA, M., NAGELOVA, V., LACINOVA, Z., HALUZIK, M., SYKORA, D., MOULIN, A., BLAYO, A. L., FEHRENTZ, J. A., MARTINEZ, J., STOFKOVA, A., JURCOVICOVA, J., ZELEZNA, B. & MALETINSKA, L. 2014. Triazole GHS-R1a antagonists JMV4208 and JMV3002 attenuate food intake, body weight, and adipose tissue mass in mice. *Mol Cell Endocrinol,* 393**,** 120-8.

HOLUBOVA, M., SPOLCOVA, A., DEMIANOVA, Z., SYKORA, D., FEHRENTZ, J. A., MARTINEZ, J., STOFKOVA, A., JURCOVICOVA, J., DRAPALOVA, J., LACINOVA, Z., HALUZIK, M., ZELEZNA, B. & MALETINSKA, L. 2013. Ghrelin agonist JMV 1843 increases food intake, body weight and expression of orexigenic neuropeptides in mice. *Physiol Res,* 62**,** 435-44.

HOWARD, A. D., FEIGHNER, S. D., CULLY, D. F., ARENA, J. P., LIBERATOR, P. A., ROSENBLUM, C. I., HAMELIN, M., HRENIUK, D. L., PALYHA, O. C., ANDERSON, J., PARESS, P. S., DIAZ, C., CHOU, M., LIU, K. K., MCKEE, K. K., PONG, S. S., CHAUNG, L. Y., ELBRECHT, A., DASHKEVICZ, M., HEAVENS, R., RIGBY, M., SIRINATHSINGHJI, D. J., DEAN, D. C., MELILLO, D. G., PATCHETT, A. A.,

NARGUND, R., GRIFFIN, P. R., DEMARTINO, J. A., GUPTA, S. K., SCHAEFFER, J. M., SMITH, R. G. & VAN DER PLOEG, L. H. 1996. A receptor in pituitary and hypothalamus that functions in growth hormone release. *Science,* 273**,** 974-7.

CHEN, H. Y., TRUMBAUER, M. E., CHEN, A. S., WEINGARTH, D. T., ADAMS, J. R., FRAZIER, E. G., SHEN, Z., MARSH, D. J., FEIGHNER, S. D., GUAN, X. M., YE, Z., NARGUND, R. P., SMITH, R. G., VAN DER PLOEG, L. H., HOWARD, A. D., MACNEIL, D. J. & QIAN, S. 2004. Orexigenic action of peripheral ghrelin is mediated by neuropeptide Y and agouti-related protein. *Endocrinology,* 145**,** 2607-12.

CHENG, Y. & PRUSOFF, W. H. 1973. Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol,* 22**,** 3099-108.

ISLAM, M. N., MITA, Y., MARUYAMA, K., TANIDA, R., ZHANG, W., SAKODA, H. & NAKAZATO, M. 2020. Liver-expressed antimicrobial peptide 2 antagonizes the effect of ghrelin in rodents. *J Endocrinol,* 244**,** 13-23.

KHATIB, M. N., GAIDHANE, S., GAIDHANE, A. M., SIMKHADA, P. & ZAHIRUDDIN, Q. S. 2015. Ghrelin O Acyl Transferase (GOAT) as a Novel Metabolic Regulatory Enzyme. *J Clin Diagn Res,* 9**,** LE01-5.

KHERA, R., MURAD, M. H., CHANDAR, A. K., DULAI, P. S., WANG, Z., PROKOP, L. J., LOOMBA, R., CAMILLERI, M. & SINGH, S. 2016. Association of Pharmacological Treatments for Obesity With Weight Loss and Adverse Events: A Systematic Review and Meta-analysis. *JAMA,* 315**,** 2424-34.

KOHSAKA, A., LAPOSKY, A. D., RAMSEY, K. M., ESTRADA, C., JOSHU, C., KOBAYASHI, Y., TUREK, F. W. & BASS, J. 2007. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab,* 6**,** 414-21.

KOJIMA, M., HOSODA, H., DATE, Y., NAKAZATO, M., MATSUO, H. & KANGAWA, K. 1999. Ghrelin is a growth-hormone-releasing acylated peptide from stomach. *Nature,* 402**,** 656-60.

KOJIMA, M. & KANGAWA, K. 2005. Ghrelin: structure and function. *Physiol Rev,* 85**,** 495-522.

KRAUSE, A., SILLARD, R., KLEEMEIER, B., KLUVER, E., MARONDE, E., CONEJO-GARCIA, J. R., FORSSMANN, W. G., SCHULZ-KNAPPE, P., NEHLS, M. C., WATTLER, F., WATTLER, S. & ADERMANN, K. 2003. Isolation and biochemical characterization of LEAP-2, a novel blood peptide expressed in the liver. *Protein Sci,* 12**,** 143-52.

KRENTZ, A. J., FUJIOKA, K. & HOMPESCH, M. 2016. Evolution of pharmacological obesity treatments: focus on adverse side-effect profiles. *Diabetes Obes Metab,* 18**,** 558-70.

KURTZHALS, P., HAVELUND, S., JONASSEN, I., KIEHR, B., LARSEN, U. D., RIBEL, U. & MARKUSSEN, J. 1995. Albumin binding of insulins acylated with fatty acids: characterization of the ligand-protein interaction and correlation between binding affinity and timing of the insulin effect in vivo. *Biochem J,* 312 (Pt 3)**,** 725-31.

LACQUANITI, A., DONATO, V., CHIRICO, V., BUEMI, A. & BUEMI, M. 2011. Obestatin: an interesting but controversial gut hormone. *Ann Nutr Metab,* 59**,** 193-9.

LEE, M. R., TAPOCIK, J. D., GHAREEB, M., SCHWANDT, M. L., DIAS, A. A., LE, A. N., COBBINA, E., FARINELLI, L. A., BOUHLAL, S., FAROKHNIA, M., HEILIG, M., AKHLAGHI, F. & LEGGIO, L. 2020. The novel ghrelin receptor inverse agonist PF-5190457 administered with alcohol: preclinical safety experiments and a phase 1b human laboratory study. *Mol Psychiatry,* 25**,** 461-475.

LI, H. Z., SHOU, L. L., SHAO, X. X., LI, N., LIU, Y. L., XU, Z. G. & GUO, Z. Y. 2021. LEAP2 has antagonized the ghrelin receptor GHSR1a since its emergence in ancient fish. *Amino Acids,* 53**,** 939-949.

LI, J., ZHANG, M., WANG, M., WANG, Z., LIU, Y., ZHANG, W. & WANG, N. 2016. GHSR deficiency suppresses neointimal formation in injured mouse arteries. *Biochem Biophys Res Commun,* 479**,** 125-131.

LIN, S., STORLIEN, L. H. & HUANG, X. F. 2000. Leptin receptor, NPY, POMC mRNA expression in the diet-induced obese mouse brain. *Brain Res,* 875**,** 89-95.

LONGO, K. A., GOVEK, E. K., NOLAN, A., MCDONAGH, T., CHAROENTHONGTRAKUL, S., GIULIANA, D. J., MORGAN, K., HIXON, J., ZHOU, C., KELDER, B., KOPCHICK, J. J., SAUNDERS, J. O., NAVIA, M. A., CURTIS, R., DISTEFANO, P. S. & GEDDES, B. J. 2011. Pharmacologic inhibition of ghrelin receptor signaling is insulin sparing and promotes insulin sensitivity. *J Pharmacol Exp Ther,* 339**,** 115-24.

LUGILDE, J., CASADO, S., BEIROA, D., CUNARRO, J., GARCIA-LAVANDEIRA, M., ALVAREZ, C. V., NOGUEIRAS, R., DIEGUEZ, C. & TOVAR, S. 2022. LEAP-2 Counteracts Ghrelin-Induced Food Intake in a Nutrient, Growth Hormone and Age Independent Manner. *Cells,* 11.

LUQUET, S., PHILLIPS, C. T. & PALMITER, R. D. 2007. NPY/AgRP neurons are not essential for feeding responses to glucoprivation. *Peptides,* 28**,** 214-25.

LUTTER, M., SAKATA, I., OSBORNE-LAWRENCE, S., ROVINSKY, S. A., ANDERSON, J. G., JUNG, S., BIRNBAUM, S., YANAGISAWA, M., ELMQUIST, J. K., NESTLER, E. J. & ZIGMAN, J. M. 2008. The orexigenic hormone ghrelin defends against depressive symptoms of chronic stress. *Nat Neurosci,* 11**,** 752-3.

M'KADMI, C., CABRAL, A., BARRILE, F., GIRIBALDI, J., CANTEL, S., DAMIAN, M., MARY, S., DENOYELLE, S., DUTERTRE, S., PERALDI-ROUX, S., NEASTA, J., OIRY, C., BANERES, J. L., MARIE, J., PERELLO, M. & FEHRENTZ, J. A. 2019. N-Terminal Liver-Expressed Antimicrobial Peptide 2 (LEAP2) Region Exhibits Inverse Agonist Activity toward the Ghrelin Receptor. *J Med Chem,* 62**,** 965-973.

MACKINNON, A. C., WATERS, C., JODRELL, D., HASLETT, C. & SETHI, T. 2001. Bombesin and substance P analogues differentially regulate G-protein coupling to the bombesin receptor. Direct evidence for biased agonism. *J Biol Chem,* 276**,** 28083-91.

MACHADO, M. V., COUTINHO, J., CAREPA, F., COSTA, A., PROENCA, H. & CORTEZ-PINTO, H. 2012. How adiponectin, leptin, and ghrelin orchestrate together and correlate with the severity of nonalcoholic fatty liver disease. *Eur J Gastroenterol Hepatol,* 24**,** 1166-72.

MAIXNEROVA, J., HLAVACEK, J., BLOKESOVA, D., KOWALCZYK, W., ELBERT, T., SANDA, M., BLECHOVA, M., ZELEZNA, B., SLANINOVA, J. & MALETINSKA, L. 2007. Structure-activity relationship of CART (cocaine- and amphetamine-regulated transcript) peptide fragments. *Peptides,* 28**,** 1945-53.

MALETINSKA, L., MATYSKOVA, R., MAIXNEROVA, J., SYKORA, D., PYCHOVA, M., SPOLCOVA, A., BLECHOVA, M., DRAPALOVA, J., LACINOVA, Z., HALUZIK, M. & ZELEZNA, B. 2011. The Peptidic GHS-R antagonist [D-Lys(3)]GHRP-6 markedly improves adiposity and related metabolic abnormalities in a mouse model of postmenopausal obesity. *Mol Cell Endocrinol,* 343**,** 55-62.

MALETINSKA, L., NAGELOVA, V., TICHA, A., ZEMENOVA, J., PIRNIK, Z., HOLUBOVA, M., SPOLCOVA, A., MIKULASKOVA, B., BLECHOVA, M., SYKORA, D., LACINOVA, Z., HALUZIK, M., ZELEZNA, B. & KUNES, J. 2015. Novel lipidized analogs of prolactin-releasing peptide have prolonged half-lives and exert anti-obesity effects after peripheral administration. *Int J Obes (Lond),* 39**,** 986-93.

MALETINSKA, L., PYCHOVA, M., HOLUBOVA, M., BLECHOVA, M., DEMIANOVA, Z., ELBERT, T. & ZELEZNA, B. 2012. Characterization of new stable ghrelin analogs with prolonged orexigenic potency. *J Pharmacol Exp Ther,* 340**,** 781-6.

MALETÍNSKÁ, L., PÝCHOVÁ, M., HOLUBOVÁ, M., BLECHOVÁ, M., DEMIANOVÁ, Z., ELBERT, T. & ŽELEZNÁ, B. 2012. Characterization of new stable ghrelin analogs with prolonged orexigenic potency. *J Pharmacol Exp Ther,* 340**,** 781-6.

MANI, B. K., PUZZIFERRI, N., HE, Z., RODRIGUEZ, J. A., OSBORNE-LAWRENCE, S., METZGER, N. P., CHHINA, N., GAYLINN, B., THORNER, M. O., THOMAS, E. L., BELL, J. D., WILLIAMS, K. W., GOLDSTONE, A. P. & ZIGMAN, J. M. 2019. LEAP2 changes with body mass and food intake in humans and mice. *J Clin Invest,* 129**,** 3909-3923.

MANI, B. K. & ZIGMAN, J. M. 2017. Ghrelin as a Survival Hormone. *Trends Endocrinol Metab,* 28**,** 843-854.

MARTIN, N. M., SMALL, C. J., SAJEDI, A., PATTERSON, M., GHATEI, M. A. & BLOOM, S. R. 2004. Pre-obese and obese agouti mice are sensitive to the anorectic effects of peptide YY(3-36) but resistant to ghrelin. *Int J Obes Relat Metab Disord,* 28**,** 886-93.

MASUDA, Y., TANAKA, T., INOMATA, N., OHNUMA, N., TANAKA, S., ITOH, Z., HOSODA, H., KOJIMA, M. & KANGAWA, K. 2000. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochem Biophys Res Commun,* 276**,** 905-8.

MENACHO-MELGAR, R., DECKER, J. S., HENNIGAN, J. N. & LYNCH, M. D. 2019. A review of lipidation in the development of advanced protein and peptide therapeutics. *Journal of Controlled Release,* 295**,** 1-12.

MENDE, F., HUNDAHL, C., PLOUFFE, B., SKOV, L. J., SIVERTSEN, B., MADSEN, A. N., LUCKMANN, M., DIEP, T. A., OFFERMANNS, S., FRIMURER, T. M., BOUVIER, M. & HOLST, B. 2018. Translating biased signaling in the ghrelin receptor system into differential in vivo functions. *Proceedings of the National Academy of Sciences of the United States of America,* 115**,** E10255-E10264.

MOHAN, H., GASNER, M., RAMESH, N. & UNNIAPPAN, S. 2016. Ghrelin, ghrelin-O-acyl transferase, nucleobindin-2/nesfatin-1 and prohormone convertases in the pancreatic islets of Sprague Dawley rats during development. *J Mol Histol,* 47**,** 325-36.

MONTEIRO, R. & AZEVEDO, I. 2010. Chronic inflammation in obesity and the metabolic syndrome. *Mediators Inflamm,* 2010.

MOSA, R., HUANG, L., LI, H., GRIST, M., LEROITH, D. & CHEN, C. 2018. Long-term treatment with the ghrelin receptor antagonist [d-Lys3]-GHRP-6 does not improve glucose homeostasis in nonobese diabetic MKR mice. *Am J Physiol Regul Integr Comp Physiol,* 314**,** R71-R83.

MOTULSKY, H. & NEUBIG, R. 2002a. Analyzing radioligand binding data. *Curr Protoc Neurosci,* Chapter 7**,** Unit 7.5.

MOTULSKY, H. & NEUBIG, R. 2002b. Analyzing radioligand binding data. *Curr Protoc Neurosci,* Chapter 7**,** Unit 7 5.

MOULIN, A., DEMANGE, L., BERGE, G., GAGNE, D., RYAN, J., MOUSSEAUX, D., HEITZ, A., PERRISSOUD, D., LOCATELLI, V., TORSELLO, A., GALLEYRAND, J. C., FEHRENTZ, J. A. & MARTINEZ, J. 2007. Toward potent ghrelin receptor ligands based on trisubstituted 1,2,4-triazole structure. 2. Synthesis and pharmacological in vitro and in vivo evaluations. *J Med Chem,* 50**,** 5790-806.

MULLER, T. D., BLUHER, M., TSCHOP, M. H. & DIMARCHI, R. D. 2022. Anti-obesity drug discovery: advances and challenges. *Nat Rev Drug Discov,* 21**,** 201-223.

MULLER, T. D., NOGUEIRAS, R., ANDERMANN, M. L., ANDREWS, Z. B., ANKER, S. D., ARGENTE, J., BATTERHAM, R. L., BENOIT, S. C., BOWERS, C. Y., BROGLIO, F., CASANUEVA, F. F., D'ALESSIO, D., DEPOORTERE, I., GELIEBTER, A., GHIGO, E., COLE, P. A., COWLEY, M., CUMMINGS, D. E., DAGHER, A., DIANO, S., DICKSON, S. L., DIEGUEZ, C., GRANATA, R., GRILL, H. J., GROVE, K., HABEGGER, K. M., HEPPNER, K., HEIMAN, M. L., HOLSEN, L., HOLST, B., INUI, A., JANSSON, J. O., KIRCHNER, H., KORBONITS, M., LAFERRERE, B., LEROUX, C. W., LOPEZ, M., MORIN, S., NAKAZATO, M., NASS, R., PEREZ-TILVE, D., PFLUGER, P. T., SCHWARTZ, T. W., SEELEY, R. J., SLEEMAN, M., SUN, Y., SUSSEL, L., TONG, J., THORNER, M. O., VAN DER LELY, A. J., VAN DER PLOEG, L. H., ZIGMAN, J. M., KOJIMA, M., KANGAWA, K., SMITH, R. G., HORVATH, T. & TSCHOP, M. H. 2015. Ghrelin. *Mol Metab,* 4**,** 437-60.

NASS, R., PEZZOLI, S. S., OLIVERI, M. C., PATRIE, J. T., HARRELL, F. E., JR., CLASEY, J. L., HEYMSFIELD, S. B., BACH, M. A., VANCE, M. L. & THORNER, M. O. 2008. Effects of an oral ghrelin mimetic on body composition and clinical outcomes in healthy older adults: a randomized trial. *Ann Intern Med,* 149**,** 601-11.

NAZNIN, F., TOSHINAI, K., WAISE, T. M., NAMKOONG, C., MD MOIN, A. S., SAKODA, H. & NAKAZATO, M. 2015. Diet-induced obesity causes peripheral and central ghrelin resistance by promoting inflammation. *J Endocrinol,* 226**,** 81-92.

NIKOLOPOULOS, D., THEOCHARIS, S. & KOURAKLIS, G. 2010. Ghrelin: A potential therapeutic target for cancer. *Regulatory Peptides,* 163**,** 7-17.

OKAMATSU, Y., MATSUDA, K., HIRAMOTO, I., TANI, H., KIMURA, K., YADA, Y., KAKUMA, T., HIGUCHI, S., KOJIMA, M. & MATSUISHI, T. 2009. Ghrelin and leptin modulate immunity and liver function in overweight children. *Pediatr Int,* 51**,** 9-13.

OSTERSTOCK, G., ESCOBAR, P., MITUTSOVA, V., GOUTY-COLOMER, L. A., FONTANAUD, P., MOLINO, F., FEHRENTZ, J. A., CARMIGNAC, D., MARTINEZ, J., GUERINEAU, N. C., ROBINSON, I. C., MOLLARD, P. & MERY, P. F. 2010. Ghrelin stimulation of growth hormone-releasing hormone neurons is direct in the arcuate nucleus. *PLoS One,* 5**,** e9159.

PAPATHANASSOGLOU, E., EL-HASCHIMI, K., LI, X. C., MATARESE, G., STROM, T. & MANTZOROS, C. 2006. Leptin receptor expression and signaling in lymphocytes: kinetics during lymphocyte activation, role in lymphocyte survival, and response to high fat diet in mice. *J Immunol,* 176**,** 7745-52.

PATCHETT, A. A., NARGUND, R. P., TATA, J. R., CHEN, M. H., BARAKAT, K. J., JOHNSTON, D. B., CHENG, K., CHAN, W. W., BUTLER, B., HICKEY, G. & ET AL. 1995. Design and biological activities of L-163,191 (MK-0677): a potent, orally active growth hormone secretagogue. *Proc Natl Acad Sci U S A,* 92**,** 7001-5.

PERACCHI, M., BARDELLA, M. T., CAPRIOLI, F., MASSIRONI, S., CONTE, D., VALENTI, L., RONCHI, C., BECK-PECCOZ, P., AROSIO, M. & PIODI, L. 2006. Circulating ghrelin levels in patients with inflammatory bowel disease. *Gut,* 55**,** 432-3.

PERREAULT, M., ISTRATE, N., WANG, L., NICHOLS, A. J., TOZZO, E. & STRICKER-KRONGRAD, A. 2004. Resistance to the orexigenic effect of ghrelin in dietary-induced obesity in mice: reversal upon weight loss. *Int J Obes Relat Metab Disord,* 28**,** 879-85.

PICCOLI, F., DEGEN, L., MACLEAN, C., PETER, S., BASELGIA, L., LARSEN, F., BEGLINGER, C. & DREWE, J. 2007. Pharmacokinetics and pharmacodynamic effects of an oral ghrelin agonist in healthy subjects. *J Clin Endocrinol Metab,* 92**,** 1814-20.

PIETRA, C., TAKEDA, Y., TAZAWA-OGATA, N., MINAMI, M., XIA, Y. F., DUUS, E. M. & NORTHRUP, R. 2014. Anamorelin HCl (ONO-7643), a novel ghrelin receptor agonist, for the treatment of cancer anorexia-cachexia syndrome: preclinical profile. *Journal of Cachexia Sarcopenia and Muscle,* 5**,** 329-337.

PORPORATO, P. E., FILIGHEDDU, N., REANO, S., FERRARA, M., ANGELINO, E., GNOCCHI, V. F., PRODAM, F., RONCHI, G., FAGOONEE, S., FORNARO, M., CHIANALE, F., BALDANZI, G., SURICO, N., SINIGAGLIA, F., PERROTEAU, I., SMITH, R. G., SUN, Y., GEUNA, S. & GRAZIANI, A. 2013. Acylated and unacylated ghrelin impair skeletal muscle atrophy in mice. *J Clin Invest,* 123**,** 611-22.

PRAZIENKOVA, V., FUNDA, J., PIRNIK, Z., KARNOSOVA, A., HRUBA, L., KORINKOVA, L., NEPRASOVA, B., JANOVSKA, P., BENZCE, M., KADLECOVA, M., BLAHOS, J., KOPECKY, J., ZELEZNA, B., KUNES, J., BARDOVA, K. & MALETINSKA, L. 2021. GPR10 gene deletion in mice increases basal neuronal activity, disturbs insulin sensitivity and alters lipid homeostasis. *Gene,* 774**,** 145427.

RAMIREZ, V. T., VAN OEFFELEN, W., TORRES-FUENTES, C., CHRUSCICKA, B., DRUELLE, C., GOLUBEVA, A. V., VAN DE WOUW, M., DINAN, T. G., CRYAN, J. F. & SCHELLEKENS, H. 2019. Differential functional selectivity and downstream signaling bias of ghrelin receptor antagonists and inverse agonists. *FASEB J,* 33**,** 518-531.

REYNOLDS, K. A., BOUDOURES, A. L., CHI, M. M., WANG, Q. & MOLEY, K. H. 2015. Adverse effects of obesity and/or high-fat diet on oocyte quality and metabolism are not reversible with resumption of regular diet in mice. *Reprod Fertil Dev,* 27**,** 716-24.

RIEDL, M., MAIER, C., HANDISURYA, A., LUGER, A. & KAUTZKY-WILLER, A. 2007. Insulin resistance has no impact on ghrelin suppression in pregnancy. *J Intern Med,* 262**,** 458-65.

RIZZO, M., RIZVI, A. A., SUDAR, E., SOSKIC, S., OBRADOVIC, M., MONTALTO, G., BOUTJDIR, M., MIKHAILIDIS, D. P. & ISENOVIC, E. R. 2013. A review of the cardiovascular and anti-atherogenic effects of ghrelin. *Curr Pharm Des,* 19**,** 4953-63.

RUDOLPH, J., ESLER, W. P., O'CONNOR, S., COISH, P. D., WICKENS, P. L., BRANDS, M., BIERER, D. E., BLOOMQUIST, B. T., BONDAR, G., CHEN, L., CHUANG, C. Y., CLAUS, T. H., FATHI, Z., FU, W., KHIRE, U. R., KRISTIE, J. A., LIU, X. G., LOWE,

D. B., MCCLURE, A. C., MICHELS, M., ORTIZ, A. A., RAMSDEN, P. D., SCHOENLEBER, R. W., SHELEKHIN, T. E., VAKALOPOULOS, A., TANG, W., WANG, L., YI, L., GARDELL, S. J., LIVINGSTON, J. N., SWEET, L. J. & BULLOCK, W. H. 2007. Quinazolinone derivatives as orally available ghrelin receptor antagonists for the treatment of diabetes and obesity. *J Med Chem,* 50**,** 5202-16.

SAKATA, I., YAMAZAKI, M., INOUE, K., HAYASHI, Y., KANGAWA, K. & SAKAI, T. 2003. Growth hormone secretagogue receptor expression in the cells of the stomach-projected afferent nerve in the rat nodose ganglion. *Neurosci Lett,* 342**,** 183-6.

SALOME, N., HAAGE, D., PERRISSOUD, D., MOULIN, A., DEMANGE, L., EGECIOGLU, E., FEHRENTZ, J. A., MARTINEZ, J. & DICKSON, S. L. 2009. Anorexigenic and electrophysiological actions of novel ghrelin receptor (GHS-R1A) antagonists in rats. *Eur J Pharmacol,* 612**,** 167-73.

SANTOS-ZAS, I., LODEIRO, M., GURRIARAN-RODRIGUEZ, U., BOUZO-LORENZO, M., MOSTEIRO, C. S., CASANUEVA, F. F., CASABIELL, X., PAZOS, Y. & CAMINA, J. P. 2013. beta-Arrestin signal complex plays a critical role in adipose differentiation. *Int J Biochem Cell Biol,* 45**,** 1281-92.

SATO, T., NAKAMURA, Y., SHIIMURA, Y., OHGUSU, H., KANGAWA, K. & KOJIMA, M. 2012. Structure, regulation and function of ghrelin. *J Biochem,* 151**,** 119-28.

SHIIMURA, Y., HORITA, S., HAMAMOTO, A., ASADA, H., HIRATA, K., TANAKA, M., MORI, K., UEMURA, T., KOBAYASHI, T., IWATA, S. & KOJIMA, M. 2020. Structure of an antagonist-bound ghrelin receptor reveals possible ghrelin recognition mode. *Nat Commun,* 11**,** 4160.

SCHALLA, M. A. & STENGEL, A. 2019. Pharmacological Modulation of Ghrelin to Induce Weight Loss: Successes and Challenges. *Curr Diab Rep,* 19**,** 102.

SCHWARTZ, M. W., WOODS, S. C., PORTE, D., SEELEY, R. J. & BASKIN, D. G. 2000. Central nervous system control of food intake. *Nature,* 404**,** 661-671.

SMITH, R. G., CHENG, K., SCHOEN, W. R., PONG, S. S., HICKEY, G., JACKS, T., BUTLER, B., CHAN, W. W., CHAUNG, L. Y., JUDITH, F. & ET AL. 1993. A nonpeptidyl growth hormone secretagogue. *Science,* 260**,** 1640-3.

STENGEL, A., GOEBEL, M., WANG, L. & TACHE, Y. 2010. Ghrelin, des-acyl ghrelin and nesfatin-1 in gastric X/A-like cells: role as regulators of food intake and body weight. *Peptides,* 31**,** 357-69.

SZENTIRMAI, E., HAJDU, I., OBAL, F., JR. & KRUEGER, J. M. 2006. Ghrelin-induced sleep responses in ad libitum fed and food-restricted rats. *Brain Res,* 1088**,** 131-40.

TAK, Y. J. & LEE, S. Y. 2021. Anti-Obesity Drugs: Long-Term Efficacy and Safety: An Updated Review. *World J Mens Health,* 39**,** 208-221.

TAKAHASHI, B., FUNAMI, H., IWAKI, T., MARUOKA, H., SHIBATA, M., KOYAMA, M., NAGAHIRA, A., KAMIIDE, Y., KANKI, S., IGAWA, Y. & MUTO, T. 2015. Orally active ghrelin receptor inverse agonists and their actions on a rat obesity model. *Bioorg Med Chem,* 23**,** 4792-4803.

TAYLOR, M. S., HWANG, Y., HSIAO, P. Y., BOEKE, J. D. & COLE, P. A. 2012. Ghrelin O-acyltransferase assays and inhibition. *Methods Enzymol,* 514**,** 205-28.

TEMEL, J. S., ABERNETHY, A. P., CURROW, D. C., FRIEND, J., DUUS, E. M., YAN, Y. & FEARON, K. C. 2016. Anamorelin in patients with non-small-cell lung cancer and cachexia (ROMANA 1 and ROMANA 2): results from two randomised, double-blind, phase 3 trials. *Lancet Oncology,* 17**,** 519-531.

TSCHÖP, M., SMILEY, D. L. & HEIMAN, M. L. 2000. Ghrelin induces adiposity in rodents. *Nature,* 407**,** 908-13.

TSCHOP, M., WEYER, C., TATARANNI, P. A., DEVANARAYAN, V., RAVUSSIN, E. & HEIMAN, M. L. 2001. Circulating ghrelin levels are decreased in human obesity. *Diabetes,* 50**,** 707-9.

URIARTE, M., DE FRANCESCO, P. N., FERNANDEZ, G., CASTROGIOVANNI, D., D'ARCANGELO, M., IMBERNON, M., CANTEL, S., DENOYELLE, S., FEHRENTZ, J. A., PRAETORIUS, J., PREVOT, V. & PERELLO, M. 2021. Circulating ghrelin crosses

the blood-cerebrospinal fluid barrier via growth hormone secretagogue receptor dependent and independent mechanisms. *Molecular and Cellular Endocrinology,* 538.

VARGHESE, J., JAMES, J. V., ANAND, R., NARAYANASAMY, M., REBEKAH, G., RAMAKRISHNA, B., NELLICKAL, A. J. & JACOB, M. 2020. Development of insulin resistance preceded major changes in iron homeostasis in mice fed a high-fat diet. *J Nutr Biochem,* 84**,** 108441.

VODNIK, M., STRUKELJ, B. & LUNDER, M. 2016. Ghrelin Receptor Ligands Reaching Clinical Trials: From Peptides to Peptidomimetics; from Agonists to Antagonists. *Horm Metab Res,* 48**,** 1-15.

WADDEN, T. A., WEBB, V. L., MORAN, C. H. & BAILER, B. A. 2012. Lifestyle modification for obesity: new developments in diet, physical activity, and behavior therapy. *Circulation,* 125**,** 1157-70.

WAKABAYASHI, H., ARAI, H. & INUI, A. 2021. The regulatory approval of anamorelin for treatment of cachexia in patients with non-small cell lung cancer, gastric cancer, pancreatic cancer, and colorectal cancer in Japan: facts and numbers. *J Cachexia Sarcopenia Muscle,* 12**,** 14-16.

WANG, J. H., LI, H. Z., SHAO, X. X., NIE, W. H., LIU, Y. L., XU, Z. G. & GUO, Z. Y. 2019. Identifying the binding mechanism of LEAP2 to receptor GHSR1a. *FEBS J,* 286**,** 1332-1345.

WILDING, J. P. H., BATTERHAM, R. L., CALANNA, S., DAVIES, M., VAN GAAL, L. F., LINGVAY, I., MCGOWAN, B. M., ROSENSTOCK, J., TRAN, M. T. D., WADDEN, T. A., WHARTON, S., YOKOTE, K., ZEUTHEN, N., KUSHNER, R. F. & GROUP, S. S. 2021. Once-Weekly Semaglutide in Adults with Overweight or Obesity. *N Engl J Med,* 384**,** 989-1002.

WILLIAMS, L. M., CAMPBELL, F. M., DREW, J. E., KOCH, C., HOGGARD, N., REES, W. D., KAMOLRAT, T., THI NGO, H., STEFFENSEN, I. L., GRAY, S. R. & TUPS, A. 2014. The development of diet-induced obesity and glucose intolerance in C57BL/6 mice on a high-fat diet consists of distinct phases. *PLoS One,* 9**,** e106159.

WOODS, S. C. & D'ALESSIO, D. A. 2008. Central control of body weight and appetite. *J Clin Endocrinol Metab,* 93**,** S37-50.

YANG, J., BROWN, M. S., LIANG, G., GRISHIN, N. V. & GOLDSTEIN, J. L. 2008. Identification of the acyltransferase that octanoylates ghrelin, an appetite-stimulating peptide hormone. *Cell,* 132**,** 387-96.

YASUDA, T., MASAKI, T., KAKUMA, T. & YOSHIMATSU, H. 2003. Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. *Neurosci Lett,* 349**,** 75-8.

ZHANG, J. V., REN, P. G., AVSIAN-KRETCHMER, O., LUO, C. W., RAUCH, R., KLEIN, C. & HSUEH, A. J. 2005. Obestatin, a peptide encoded by the ghrelin gene, opposes ghrelin's effects on food intake. *Science,* 310**,** 996-9.

ZHANG, L. & BULAJ, G. 2012. Converting peptides into drug leads by lipidation. *Curr Med Chem,* 19**,** 1602-18.

ZIGMAN, J. M., BOURET, S. G. & ANDREWS, Z. B. 2016. Obesity Impairs the Action of the Neuroendocrine Ghrelin System. *Trends Endocrinol Metab,* 27**,** 54-63.

ZIGMAN, J. M., JONES, J. E., LEE, C. E., SAPER, C. B. & ELMQUIST, J. K. 2006. Expression of ghrelin receptor mRNA in the rat and the mouse brain. *J Comp Neurol,* 494**,** 528-48.

LIST OF MY PUBLICATIONS

Publications related to Ph.D. thesis:

1. Holá, L.; Železná, B.; Karnošová, A.; Kuneš, J.; Fehrentz, JA.; Denoyelle, S.; Cantel, S.; Blechová, M.; Sýkora, D.; Myšková, A.; Maletínská, L. A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin. *J Pharmacol Exp Ther.* 2022, 383(2), 129-136. https://doi.org/ 10.1124/jpet.122.001322. **IF_{2022} = 4.40**
2. Holá, L.; Tureckiová, T.; Kuneš, J.; Železná, B.; A.; Maletínská, L. High-fat diet induces resistance to ghrelin and LEAP2 peptide analogs in mice. *J. Mol. Endocrinol.* 2023. Under major revesion. **IF_{2023} = 4.87**
3. Karnošová, A.; Strnadová, V.; Holá, L.; Železná, B.; Kuneš, J.; Maletínská, L. Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study. *Int. J. Mol. Sci*. 2021, 22, 8904. https://doi.org/10.3390/ijms22168904. **IF_{2021} = 6.21**

Publications not related to Ph.D. thesis:

1. Strnadová, V.; Karnošová, A.; Blechová, M.; Neprašová, B.; Holá, L.; Němcová, A.; Myšková, A.; Sýkora, D.; Železná, B.; Kuneš, J.; Maletínská, L. Search for lipidized PrRP analogs with strong anorexigenic effect: In vitro and in vivo studies. *Neuropeptides*. 2023, 98**,** 102319. https://doi.org/10.1016/j.npep.2022.102319. **IF_{2023} = 3.29**
2. Mráziková, L.; Neprašová B.; Mengr A.; Popelová A.; Strnadová V.; Holá L.; Železná B.; Kuneš J.; Maletínská L. Lipidized Prolactin-Releasing Peptide as a New Potential Tool to Treat Obesity and Type 2 Diabetes Mellitus: Preclinical Studies in Rodent Models. *Front. Pharmacol*. 2021, 12, 779962. https://doi.org/10.3389/fphar.2021.779962. **IF_{2021} = 5.55**
3. Kořínková, L.; Pražienková, V.; Černá, L.; Karnošová, A.; Železná, B.; Kuneš, J.; Maletínská, L. Pathophysiology of NAFLD and NASH in Experimental Models: The Role of Food Intake Regulating Peptides. *Front Endocrinol.* 2020, 11, 597583. https://doi.org/10.3389/fendo.2020.597583. **IF_{2020} = 5.56**
4. Novotná, B.; Holá, L.; Staś, M.; Gutten, O.; Smola, M.; Zavřel, M.; Vavřina, Z.; Buděšínský, M.; Liboska, R.; Chevrier, F.; Dobiaš, J.; Boura, E.; Rulíšek, L.; Birkuš, G. Enzymatic Synthesis of 3′–5′, 3′–5′ Cyclic Dinucleotides, Their Binding Properties to the Stimulator of Interferon Genes Adaptor Protein, and Structure/Activity Correlations. *Biochemistry.* 2021, 60, 48, 3714-3727. https://doi.org/10.1021/acs.biochem.1c00692. **IF_{2021} = 3.32**

SUPPLEMENTS

Supplement 1:

Holá, L.; Železná, B.; Karnošová, A.; Kuneš, J.; Fehrentz, JA.; Denoyelle, S.; Cantel, S.; Blechová, M.; Sýkora, D.; Myšková, A.; Maletínská, L. A Novel Truncated Liver Enriched Antimicrobial Peptide-2 Palmitoylated at its N-Terminal Antagonizes Effects of Ghrelin. *J Pharmacol Exp Ther.* 2022, 383(2), 129-136. https://doi.org/ 10.1124/jpet.122.001322.

Supplement 2:

Holá, L.; Tureckiová, T.; Kuneš, J.; Železná, B.; A.; Maletínská, L. High-fat diet induces resistance to ghrelin and LEAP2 peptide analogs in mice. *J. Mol. Endocrinol.* 2023. Under major revesion.

Supplement 3:

Karnošová, A.; Strnadová, V.; Holá, L.; Železná, B.; Kuneš, J.; Maletínská, L. Palmitoylation of Prolactin-Releasing Peptide Increased Affinity for and Activation of the GPR10, NPFF-R2 and NPFF-R1 Receptors: In Vitro Study. *Int. J. Mol. Sci*. 2021, 22, 8904. https://doi.org/10.3390/ijms22168904.