Aus der Medizinischen Klinik mit Schwerpunkt Psychosomatik

der Medizinischen Fakultät Charité – Universitätsmedizin Berlin

DISSERTATION

# Ghrelin-*O*-Acyltransferase und Dipeptidylpeptidase-4: zwei mögliche neue Targets für die Therapie der Adipositas

zur Erlangung des akademischen Grades

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von

Pauline Teuffel

aus Filderstadt

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**Abkürzungen**

| | |
|---|---|
| BMI | *Body Mass Index* |
| DPP-4 | Dipeptidylpeptidase-4 |
| EDTA | Ethylendiamintetraacetat |
| ELISA | *Enzyme Linked Immunosorbent Assay* |
| GLP-1 | *Glucagon-like Peptide-1* |
| GOAT | Ghrelin-*O*-Acyltransferase |
| HbA1c | Glykohämoglobin |
| ICD-10 | *International Statistical Classification of Diseases and Related Health Problems*, Ausgabe 10 |
| ip | intraperitoneal |
| PP | Pankreatisches Polypeptid |
| PYY | Peptid YY |
| T2DM | Diabetes mellitus Typ 2 |
| *vs.* | versus |
| WHO | Weltgesundheitsorganisation |

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# 1. Zusammenfassung

***Einleitung.*** Angesichts der rapide zunehmenden Prävalenz der Adipositas und fehlenden pharmakologischen Therapiekonzepten ist ein besseres Verständnis der Nahrungsaufnahme-Regulierung wichtig. Ghrelin ist der einzige bekannte peripher produzierte und zentral aktive Stimulator der Nahrungsaufnahme, die Ghrelin-*O*-Acyltransferase (GOAT) ist das einzige bekannte Ghrelin-aktivierende Enzym. Tierexperimentelle Studien zur Rolle von GOAT bei der Nahrungsaufnahme-Regulation sind widersprüchlich, zu Vorkommen und Funktion beim Menschen liegen kaum Daten vor. Die Dipeptidylpeptidase-4 (DPP-4) spaltet mehrere an der Hunger/Sättigungs-Regulation beteiligte Peptide, hierbei werden vorrangig anorexigene Peptide deaktiviert. Die Regulation von DPP-4 bei chronisch verändertem *Body Mass Index* (BMI) ist unbekannt. ***Methodik.*** In der ersten Studie wurden Nahrungsaufnahme, Körpergewicht und Verhalten von Ratten in den Spezialkäfigen eines neuen Messsystems erhoben. Mithilfe dieses Systems wurde die Nahrungsaufnahme-Mikrostruktur nach peripherer GOAT-Inhibition untersucht (Inhibitor: GO-CoA-Tat; Dosen: 32, 96, 288 µg/kg; $n$=9-11 Ratten/Gruppe). Für die zweite Studie wurde GOAT im humanen Blut gemessen und ihre BMI-abhängige Expression untersucht (BMI-Gruppen: <17,5, 18,5-25, 30-40, 40-50 und >50 kg/m², $n$=9/Gruppe). Für die dritte Studie wurde Patienten (BMI-Spanne von 9 bis 85 kg/m², $n$=75) Blut entnommen und DPP-4 gemessen. ***Ergebnisse.*** In der ersten Studie zeigten die Tiere eine rasche Adaptation in 2-3 Tagen an die Spezialkäfige zur mikrostrukturellen Analyse der Nahrungsaufnahme. Eine GOAT-Hemmung führte zur dosisabhängigen Reduktion der Nahrungsaufnahme (effektive Dosis: 96 µg/kg; -27% in Stunde 2 *vs*. Kontrolltiere; $p<0,05$). Diese Reduktion wurde über eine verringerte Häufigkeit der Mahlzeiten (-15% *vs*. Kontrolltiere; $p<0,05$) bei gleichbleibender Mahlzeitgröße vermittelt und war mit niedrigeren Acyl-Ghrelin-Spiegeln assoziiert (-57% in Stunde 2 *vs*. Kontrolltiere; $p<0,05$). GOAT konnte auch im menschlichen Plasma nachgewiesen werden und war BMI-abhängig exprimiert, was sich in einer positiven Korrelation mit dem BMI widerspiegelte ($r=0,71$, $p<0,001$). In der dritten Studie war auch zirkulierende DPP-4 positiv mit dem BMI korreliert ($r=0,34$, $p=0,004$). ***Schlussfolgerung.*** Eine Methode zur Messung der Nahrungsaufnahme-Mikrostruktur, welche die genaue Charakterisierung nahrungsregulatorischer Peptide erlaubt, wurde für den Einsatz bei Ratten etabliert. Periphere GOAT-Inhibition bewirkte eine Reduktion der Nahrungsaufnahme über gesteigerte *satiety* (späterer Beginn der nächsten Mahlzeit). GOAT ist beim Menschen vorhanden und wird BMI-abhängig exprimiert. Möglicherweise trägt die veränderte Regulierung von GOAT bei Über- (mehr GOAT) bzw. Untergewicht (weniger GOAT) zur Erhaltung des Krankheitszustandes bei. Auch DPP-4 korreliert positiv mit dem BMI mit erhöhten Konzentrationen bei Adipositas. Da DPP-4 vor allem anorexigene Peptide deaktiviert,

könnte sich auch dies ungünstig auf die Gewichtsregulation auswirken. Diese Ergebnisse könnten zu neuen medikamentösen Strategien der Adipositas-Therapie beitragen.

## 1. Abstract

***Introduction.*** In light of the rapidly increasing prevalence of obesity and lack of pharmacological treatment options, a better understanding of feeding regulation is needed. Ghrelin is the only known peripherally produced and centrally acting feeding stimulator, while ghrelin-*O*-acyltransferase (GOAT) is the only known ghrelin-activating enzyme. Animal studies regarding its role in feeding regulation are contradictory and few data is published on the occurrence of GOAT and function in humans. Dipeptidyl peptidase 4 (DPP4-) cleaves several peptides involved in the feeding regulation, mainly deactivating anorexigenic peptides. The regulation of DPP-4 by chronically altered body mass index (BMI) is unknown. ***Methods.*** In the first study, food intake, body weight and behavior of rats housed in the special cages of a new food intake monitoring device were measured. With this device, the microstructure of feeding after peripheral GOAT inhibition was analyzed (inhibitor: GO-CoA-Tat; doses: 32, 96 and 288 µg/kg; $n$=9-11/group). For the second study, GOAT was measured in human blood and its BMI-dependent expression was examined (groups by BMI: <17.5, 18.5-25, 30-40, 40-50, >50 kg/m², $n$=9/group). For the third study, blood samples of subjects with a range of BMI from 9 to 85 kg/m² ($n$=75) were taken and DPP-4 was measured. ***Results.*** In the first study, animals adapted rapidly within 2-3 days to the cages of the feeding monitoring system. GOAT inhibition led to dose-dependent reduction of food intake (effective dose: 96 µg/kg; -27% in 2$^{nd}$ hour *vs*. controls; $p<0.05$). This reduction was caused by a decrease in meal frequency (-15% *vs*. controls; $p<0.05$) with no change in meal size and was associated with decreased acyl ghrelin levels. In the second study, GOAT was detected in the human circulation and showed a positive correlation with BMI ($r=0.71$, $p<0.001$). In the third study, DPP-4 was also positively correlated with BMI ($r=0.34$, $p<0.005$). ***Conclusions.*** A method for the microstructural analysis of feeding, which allows a detailed characterization of feeding regulatory peptides, was established for the use in rats. Peripheral GOAT inhibition in rats caused a decrease in food intake by stimulating satiety (later onset of the next meal). GOAT is present in humans and expressed BMI-dependently. Possibly, an altered expression of GOAT in over- (more GOAT) and underweight (less GOAT), respectively, contributes to the maintenance of these metabolic states. DPP-4 positively correlates with BMI with obese patients showing elevated concentrations. As DPP-4 mainly deactivates anorexigenic peptides, this may also have an adverse effect on body weight regulation. These findings might help to develop new treatment strategies for the drug therapy of obesity.

## 2. Einführung

„*Obesity is one of today's most blatantly visible – yet most neglected – public health problems. […] An escalating global epidemic of overweight and obesity – "globesity" – is taking over many parts of the world. If immediate action is not taken, millions will suffer from an array of serious health disorders.*" – Weltgesundheitsorganisation (WHO) (1)

***Adipositas.*** Die WHO schätzt, dass im Jahr 2014 weltweit über 1,9 Milliarden Erwachsene mit einem *Body Mass Index* (BMI) von ≥25 kg/m² übergewichtig waren, über 600 Millionen davon – etwa 13% der Weltbevölkerung – waren adipös, hatten demnach einen BMI von ≥30 kg/m² (Abb. 1). Damit hat sich die Adipositas seit 1980 mehr als verdoppelt (2). Adipositas ist als verursachender und/oder aggravierender Faktor mit zahlreichen Erkrankungen assoziiert, u.a. mit der Arteriosklerose und der Dyslipidämie (3), arterieller Hypertonie (3), kardiovaskulären Komplikationen (4), Diabetes mellitus Typ 2 (T2DM) (3), Schlafapnoe (5), degenerativen Gelenkerkrankungen (6), psychischen Störungen (7, 8) und diversen Malignomen (9, 10). Massives Übergewicht ist also ein folgenschweres gesundheitliches Problem für betroffene Patienten und eine sozioökonomische Belastung für die Gesellschaft (11). Dennoch gibt es bisher noch keine befriedigenden konservativen Therapiekonzepte. Die Behandlung ist häufig auf die Veränderung der Ernährung und Bewegung beschränkt, wobei das Resultat meist nicht ausreichend ist. Weiterhin sind medikamentöse Behandlungsoptionen sehr limitiert (12). Die derzeit effektivste Therapie ist die bariatrische Chirurgie, ein invasives Verfahren, welches lebenslange Nachsorge erforderlich macht (13). Vor diesem Hintergrund ist ein besseres Verständnis der Mechanismen, die Hunger und Sättigung regulieren, erforderlich. Die vorliegende Arbeit verfolgt das Ziel, einen Beitrag zur genaueren Charakterisierung der Nahrungsaufnahme-Regulation sowie der Körpergewichtshomöostase zu leisten.

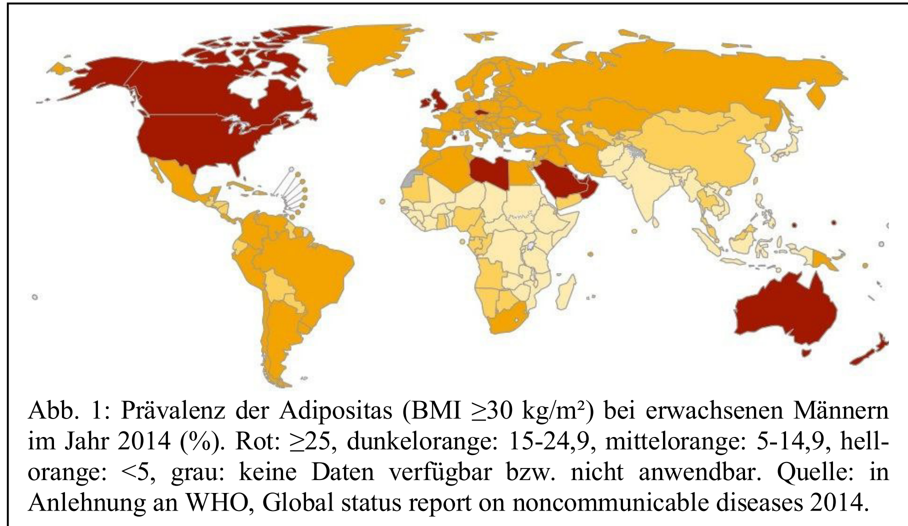

Abb. 1: Prävalenz der Adipositas (BMI ≥30 kg/m²) bei erwachsenen Männern im Jahr 2014 (%). Rot: ≥25, dunkelorange: 15-24,9, mittelorange: 5-14,9, hellorange: <5, grau: keine Daten verfügbar bzw. nicht anwendbar. Quelle: in Anlehnung an WHO, Global status report on noncommunicable diseases 2014.

***Mikrostruktur der Nahrungsaufnahme.*** In der tierexperimentellen Erforschung der Hunger- und Sättigungsregulation ist die Erhebung der Nahrungsaufnahme-Mikrostruktur *State of the Art* (14). Sie umfasst Parameter wie die aufgenommene Nahrungsmenge pro Zeit, Häufigkeit, Größe und Dauer von Mahlzeiten und das Intervall zwischen zwei Mahlzeiten. Diese Parameter können

verwendet werden, um zwei Einflussfaktoren des Fressverhaltens zu unterscheiden: *satiation* (diejenigen Mechanismen, welche zur Beendigung einer Mahlzeit führen) und *satiety* (diejenigen Mechanismen, welche nach einer abgeschlossenen Mahlzeit dazu führen, dass die nächste Mahlzeit später begonnen wird) (15, 16). Diese Detailinformationen ermöglichen den Rückschluss auf mögliche Pathomechanismen bei veränderter Nahrungsaufnahme, daher ist die mikrostrukturelle Untersuchung des Fressverhaltens für die Erforschung der Nahrungsaufnahme heute unverzichtbar. Die traditionelle manuelle Messung der Nahrungsaufnahme ist hierbei nicht anwendbar, da sie ausschließlich über die in definierten Intervallen aufgenommene Futtermenge Aufschluss gibt und zudem eine Verfälschung der Daten durch die Anwesenheit des Untersuchers möglich ist.
Wir haben in der ersten Publikation daher ein Gerät zur Messung des Fressverhaltens ungestörter Tiere eingesetzt, welches im Tierexperiment mit Ratten (17, 18) und Mäusen (19) die kontinuierliche Überwachung der Nahrungsaufnahme und insbesondere ihrer Mikrostruktur erlaubt. Für Mäuse ist dieses System bereits validiert worden (20), für Ratten stand die Validierung bisher noch aus. Wir haben untersucht, ob sich diese Methode für die Arbeit mit Ratten eignet.
***Ghrelin-O-Acyltransferase.*** Hunger und Sattheit werden von einem komplexen Transmitternetzwerk reguliert. Anorexigene Peptide bremsen die Nahrungsaufnahme, während orexigene sie stimulieren. Zu letzteren gehört Ghrelin, welches nach heutigem Kenntnisstand unter den Orexigenen das einzige ist, welches in der Peripherie – vorwiegend im Magen (21, 22) – produziert wird, seine Wirkung aber zentral entfaltet (23, 24). Entscheidend für die Rezeptorbindung und somit für die orexigene Wirkung ist eine einzigartige Fettsäuren-Modifikation (21, 25). Das für diese Acylierung verantwortliche, also Ghrelin aktivierende, Enzym wurde kürzlich in Mäusen und Menschen identifiziert und Ghrelin-*O*-Acyltransferase (GOAT) genannt (26, 27).
GOAT sind verschiedene Wirkungen im Bereich der Glukosehomöostase (28), dem enterohepatischen Kreislauf (29) und der Geschmacksempfindung zugeschrieben worden (30). Der Effekt von GOAT auf Hunger und Sättigung hingegen wurde bisher nur in wenigen Studien untersucht. Das Enzym scheint in den Genussaspekt des Essens involviert zu sein (31). Bei Ghrelin- und GOAT-überexprimierenden Mäusen, die ein mit mittelkettigen Triglyzeriden angereichertes Futter erhielten, wurde eine Gewichtszunahme bei gleichzeitig unveränderter Nahrungsaufnahme nachgewiesen (32). Auch GOAT-Knockout-Mäuse zeigten keine Veränderungen in der Nahrungsaufnahme (28, 32). Eine Studie an Hamstern stellte allerdings ein reduziertes Fressverhalten nach GOAT-Hemmung fest (33). Diese zum Teil widersprüchlichen Ergebnisse könnten den unterschiedlichen experimentellen Protokollen und möglicherweise einsetzenden Kompensationsmechanismen geschuldet sein, sie könnten aber auch mit der ausschließlichen Erhebung der aufgenommenen Futtermenge zusammenhängen, während eine Analyse der Nahrungsaufnahme-

Mikrostruktur bislang nicht erfolgte. In der ersten hier vorgestellten Publikation haben wir daher untersucht, ob der GOAT-Inhibitor GO-CoA-Tat bei *ad libitum* gefütterten Ratten die Nahrungsaufnahme und deren Mikrostruktur, das Verhalten und die Ghrelin-Konzentration verändert.
Während GOAT-mRNA bei Mäusen in Ghrelin-produzierenden Zellen gefunden wurde (34), gab es zur Proteinexpression von GOAT bisher kaum Untersuchungen. Kürzlich wurde bei Mäusen und Ratten die gastrische GOAT-Proteinexpression und im Blut zirkulierende GOAT nachgewiesen, wobei gefastete Tiere erhöhte Plasmaspiegel aufwiesen (35). Da bei Hunger der Acyl-Ghrelin-Spiegel ebenfalls steigt (36), könnte Ghrelin möglicherweise auch extrazellulär acyliert werden und GOAT hierbei regulatorisch agieren. Wir haben in der zweiten Studie untersucht, ob GOAT auch beim Menschen vorkommt. Da Ghrelin negativ mit dem BMI korreliert (37, 38), haben wir zusätzlich den Zusammenhang von GOAT mit dem metabolischen Status untersucht.
***Dipeptidylpeptidase-4.*** In der dritten vorgelegten Publikation haben wir uns mit der Serin-Exopeptidase Dipeptidylpeptidase-4 (DPP-4) beschäftigt. Von denjenigen die Nahrungsaufnahme regulierenden Hormonen, die peripher produziert werden und zentral wirken (39), vermittelt Ghrelin als einziges Hunger, während eine Vielzahl anderer an der Signalisierung von Sattheit beteiligt sind, z.B. Peptid YY (PYY), Pankreatisches Polypeptid (PP) und *Glucagon-like Peptide-1* (GLP-1) (40). Diese drei Hormone sind Substrate der DPP-4, welche PP und GLP-1 spaltet und inaktiviert, während für PYY eine veränderte Rezeptorbindung beschrieben wurde (40).
DPP-4 spielt in vielen Regelkreisen im Körper eine Rolle, etwa bei Inflammation, Körperabwehr, Glukosehomöostase und psychischen Prozessen (40). Bislang ist DPP-4 klinisch vor allem in der Therapie des T2DM relevant, wo DPP-4-Inhibitoren eingesetzt werden, deren Wirkung auf dem reduzierten Abbau von GLP-1 beruht (41), z.B. Sitagliptin und Vildagliptin. Als günstigen Nebeneffekt bei Adipositas-assoziiertem T2DM reduzieren manche dieser DPP-4-Inhibitoren moderat das Körpergewicht (42). Trotz der weit verbreiteten klinischen Anwendung von DPP-4-Inhibitoren ist kaum etwas darüber bekannt, wie sich chronisch verändertes Körpergewicht, insbesondere Adipositas, auf die Regulierung von DPP-4 auswirkt. Zudem wird meist die Enzymaktivität, nicht aber die Proteinkonzentration der DPP-4 gemessen. Eine Studie berichtet von erniedrigter DPP-4-Aktivität im Serum von Anorexia Nervosa-Patienten (43), eine andere Studie kommt zum gegenteiligen Ergebnis (44). Bei gesunden Individuen und bei adipösen Kindern war die Enzymaktivität der DPP-4 im Plasma positiv mit dem BMI korreliert (45, 46), was eventuell aus höherer DPP-4-Expression in Fettgewebe resultieren könnte (47). Wir haben die Konzentration zirkulierender DPP-4 und die Enzymaktivität bei Patienten über eine große BMI-Spanne (9-85 kg/m²) untersucht. Um die Auswirkung einer veränderten DPP-4-Konzentration zu untersuchen, wurden die Plasmaspiegel von PP und GLP-1 gemessen.

# 3. Methodik

## *3.1.* Publikation 1: *Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats*

***Tiere.*** Es wurden erwachsene männliche *Sprague-Dawley* Ratten verwendet. Die Tiere wurden bei kontrollierten Licht- und Temperaturverhältnissen zunächst gruppenweise, später in Einzelkäfigen gehalten und hatten freien Zugang zu Standardfutter und Trinkwasser. Den Tieren wurde eine initiale Eingewöhnungszeit von einer Woche gewährt, anschließend wurden sie täglich an den Untersucher und die experimentellen Handgriffe gewöhnt, um den Stress während der Experimente zu reduzieren (*Handling*). Das Körpergewicht der Tiere als Indikator ihres Wohlbefindens wurde täglich überprüft.

***Präparat.*** Der verwendete GOAT-Inhibitor GO-CoA-Tat wurde bei -80 °C in Pulverform gelagert und kurz vor den Experimenten in steriler Kochsalzlösung gelöst.

***Manuelle Messung der Nahrungsaufnahme.*** Durch Wiegen des Futters vor und nach dem betreffenden Zeitraum konnte die aufgenommene Futtermenge errechnet werden.

***Automatisierte Messung der Nahrungsaufnahme.*** Die mikrostrukturelle Analyse des Fressverhaltens wurde mithilfe eines kommerziellen Nahrungsaufnahme-Messsystems durchgeführt. Dieses ermöglicht durch eine elektronische Mikrowaage das automatische, kontinuierliche Wiegen des Futters im Sekundentakt mit einer Genauigkeit von 0,1 mg. Die Tiere sitzen hierbei in einem Spezialkäfig, der wie reguläre Einzelkäfige Einstreu, Anreicherungsmaterial und gängige Trinkflaschen enthält. Jede Interaktion des Tiers mit dem Futter wird erfasst und mit Zeitpunkt des Beginns, Dauer und aufgenommener Futtermenge von einem angeschlossenen Computer aufgezeichnet. Aus diesen Daten lässt sich eine Vielzahl von Parametern berechnen, wie etwa die Latenzzeit zur ersten Mahlzeit, Häufigkeit, Größe und Dauer der Mahlzeiten, Zeit zwischen zwei Mahlzeiten und Fressgeschwindigkeit. Da die Messung der Nahrungsaufnahme kontinuierlich erfolgt, kann retrospektiv ein beliebiger Zeitraum zur Analyse ausgewählt werden.

***Gewöhnung an automatisiertes Messsystem und Vergleich mit manueller Datenerhebung.*** Das Experiment erfolgte in drei Schritten: In den ersten fünf Tagen wurden Nahrungsaufnahme und Körpergewicht bei gruppenweise gehaltenen Ratten täglich manuell erhoben. Anschließend wurden die Ratten in Einzelkäfige gesetzt, wobei die Tiere weiterhin in Blick- und Geruchskontakt standen. Das Futter wurde, wie zuvor in den Gruppenkäfigen, auf dem Gitterdeckel platziert. Nahrungsaufnahme und Körpergewicht wurden über drei Tage weiterhin täglich manuell gemessen. Zuletzt wurde das Futter über die Futterraufe des Nahrungsaufnahme-Messsystems angeboten und das Körpergewicht weiterhin täglich manuell, die Nahrungsaufnahme nun aber automati-

siert gemessen. Anhand dieser Daten wurde die Gewöhnung der Tiere an die Spezialkäfige untersucht und die beiden Methoden miteinander verglichen.

***Analyse der Nahrungsaufnahme nach GOAT-Hemmung.*** Naiven Ratten wurde zu Beginn der Dunkelphase – ihrer physiologischen Wachphase (48) – intraperitoneal (ip) ein GOAT-Hemmer in unterschiedlichen Dosen (32, 96 und 288 µg/kg), der Kontrollgruppe unter denselben Bedingungen 300 µl sterile Kochsalzlösung injiziert. Im Anschluss wurde die Nahrungsaufnahme automatisiert gemessen. Die Dosis und der Zeitraum, bei denen die deutlichste Reduktion der Nahrungsaufnahme auftrat, wurden allen anschließenden Analysen und späteren Experimenten zugrunde gelegt.

***Bestimmung von Acyl- und Gesamt-Ghrelin nach GOAT-Hemmung.*** Nach intraperitonealer Injektion des GOAT-Inhibitors (96 µg/kg, ip) oder Vehikel (300 µl) zu Beginn der Dunkelphase hatten die Tiere keinen Zugang zum Futter. Die Ratten wurden anästhesiert und Blut kardial zu den Zeitpunkten entweder 0, 1, 2 oder 3 Stunden nach Injektion gewonnen. Die EDTA und Aprotinin enthaltenden, gekühlten Röhrchen wurden unverzüglich zentrifugiert, der Plasmaüberstand abgenommen und bei -80 °C gelagert. Acyl- und Gesamt-Ghrelin wurden mittels ELISA gemessen. Desacyl-Ghrelin wurde als Differenz dieser beiden Werte errechnet.

***Verhaltensbeobachtung in Spezialkäfigen.*** In der ersten Stunde der Dunkelphase wurden Nahrungsaufnahme, Putzverhalten, Lokomotion und Ruhen von *ad libitum* gefütterten Ratten, die zuvor in das Nahrungsaufnahme-Messsystem eingewöhnt worden waren, gemessen.

***Verhaltensbeobachtung nach GOAT-Hemmung.*** Den Tieren wurde zu Beginn der Dunkelphase peripher der GOAT-Inhibitor injiziert (96 µg/kg, ip) und das Verhalten (Nahrungsaufnahme, Putzverhalten, Lokomotion und Ruhen) in der zweiten Stunde nach Injektion – dem Zeitpunkt der maximalen Nahrungsaufnahme-Reduktion – in den gewohnten Käfigen im abgedunkelten Raum protokolliert.

***Statistische Auswertung.*** Die Normalverteilung wurde mittels Kolmogorov-Smirnov-Test untersucht, Unterschiede zwischen den Gruppen wurden mittels T-Test, *one-way* ANOVA mit anschließendem Tukey *Post-hoc*-Test oder *two-way* ANOVA mit anschließendem Holm-Sidak-Test bestimmt. Als signifikant galt $p<0,05$.

### 3.2. Publikation 2: *The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index*

***Probanden.*** Es wurden 45 stationär behandelte Patienten der psychosomatischen Klinik der Charité-Universitätsmedizin Berlin rekrutiert und anhand des BMI in fünf gleich große Gruppen aufgeteilt: BMI <17,5 kg/m² (Anorexia Nervosa), BMI 18,5-25 kg/m² (Normalgewicht), und drei

unterschiedliche Ausprägungsgrade der Adipositas (BMI 30-40, 40-50 und >50 kg/m², *n*=9/Gruppe). Anorexie und Adipositas wurden nach ICD-10-Kriterien diagnostiziert. Ausschlusskriterien waren ein bestehendes Cushing-Syndrom bei den adipösen Probanden, gastrointestinale Eingriffe in der Vorgeschichte (ausgenommen Appendektomie und Cholezystektomie) und relevante organische Erkrankungen bei den normalgewichtigen Patienten.

***Probengewinnung.*** Den nüchternen Patienten wurde am Morgen des zweiten oder dritten Tages nach stationärer Aufnahme venöses Blut abgenommen. Die gekühlten, Aprotinin enthaltenden EDTA-Röhrchen und gekühlten Serumröhrchen wurden unverzüglich zentrifugiert. Der Überstand wurde abgenommen und bei -80 °C gelagert. Körpergröße und -gewicht wurden zum selben Zeitpunkt erhoben.

***Messungen.*** Zur Identifikation von GOAT wurden für alle Proben jeweils drei Western Blots mit polyklonalem Anti-GOAT-Primärantikörper und Anti-*rabbit*-Sekundärantikörper durchgeführt. Bei den entwickelten Membranen wurde die Pixelintensität der entsprechenden Bande (50 kDa) analysiert. Die Gesamt-Ghrelin-Konzentrationen wurde mittels ELISA, Blutglukose und Serumalbumin im Routinelabor gemessen.

***Statistische Auswertung.*** Die Unterschiede zwischen den Gruppen wurden mittels *one-way* ANOVA, gefolgt vom Tukey *Post-hoc*-Test untersucht. Als signifikant galt $p<0,05$. Die Korrelationen wurden mittels linearer Regression ermittelt.

### 3.3. Publikation 3: *Obese patients have higher circulating protein levels of dipeptidyl peptidase IV*

***Probanden.*** Es wurden 75 stationäre Patienten der psychosomatischen Klinik der Charité-Universitätsmedizin Berlin rekrutiert, welche anhand des BMI in fünf gleich große Gruppen (*n*=15/Gruppe) eingeteilt wurden: BMI <17,5 kg/m² (Anorexia Nervosa), BMI 18,5-25 kg/m² (Normalgewicht), und drei unterschiedliche Ausprägungsgrade der Adipositas (BMI 30-40, 40-50 und >50 kg/m²). Anorexie und Adipositas wurden nach ICD-10-Kriterien diagnostiziert. Ausschlusskriterien waren ein bestehendes Cushing-Syndrom, gastrointestinale Eingriffe in der Vorgeschichte (außer Appendektomie und Cholezystektomie) und relevante organische Erkrankungen bei den normalgewichtigen Patienten. Keiner der Patienten erhielt DPP-4-Hemmer oder Medikamente, welche das Körpergewicht beeinflussen.

***Probengewinnung.*** Nüchternen Patienten wurde am Morgen des zweiten oder dritten Tages nach stationärer Aufnahme venöses Blut abgenommen und die gekühlten EDTA- beziehungsweise Serumröhrchen unverzüglich zentrifugiert. Die Serumproben wurden weder mit dem DPP-4-Inhibitor Diprotin A noch mit Aprotinin, für das eine Interferenz mit der DPP-4-

Enzymaktivität nicht ausgeschlossen werden kann, versetzt. Der Überstand wurde bei -80 °C gelagert. Körpergröße und -gewicht wurden zum selben Zeitpunkt erhoben.

***Messungen.*** Für die Western Blots zur Detektion von DPP-4 wurden ein polyklonaler Anti-DPP-4-Primärantikörper und Anti-*goat*-Sekundärantikörper verwendet. Die Western Blots aller 75 Proben wurden dreifach durchgeführt und die Pixeldichte der erwarteten Bande (110 kDa) sowie der stärksten Bande (50 kDa), welche am ehesten ein Fragment aus zwei schweren Ketten repräsentiert, analysiert. Die DPP-4-Aktivität im Serum wurde photometrisch aus der erhöhten Extinktion bei 405 nm mithilfe des DPP-4-Substrats Gly-Pro-*p*-Nitroanilid abgeleitet. Das Verhältnis der Konzentration/Aktivität wurde für jede Probe individuell errechnet. Pankreatisches Polypeptid (PP) und GLP-$1_{(7\text{-}36)}$-Amid wurden mittels ELISA gemessen. Gesamtprotein und Serumalbumin wurden im Routinelabor bestimmt.

***Statistische Auswertung.*** Die Normalverteilung wurde mittels Kolmogorov-Smirnov-Test geprüft, Unterschiede zwischen den Gruppen mittels T-Test, *one-way* ANOVA, gefolgt vom Tukey *Post-hoc*-Test bestimmt. Als signifikant galt $p<0{,}05$. Die Korrelationen wurden mittels Pearson- oder Spearman-Methode untersucht.

## 4. Ergebnisse

### 4.1. Publikation 1: *Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats*

***Kontinuierliche Gewichtszunahme und rasche Adaptation an die Haltung im Nahrungsaufnahme-Messsystem.*** Über alle Phasen der Eingewöhnung in die Spezialkäfige des Nahrungsaufnahme-Messsystems zeigten die Tiere eine kontinuierliche Zunahme des Körpergewichts (Abb. 2). Zwischen der manuellen und der automatisierten Messung der Nahrungsaufnahme konnte weder in der Dunkel- (manuell *vs.* automatisiert: 18,8 ± 0,4 *vs.* 17,8 ± 0,7 g/200 g Körpergewicht), noch in der Hellphase (manuell *vs.* automatisiert: 1,5 ± 0,3 *vs.* 1,9 ± 0,7 g/200 g Körpergewicht) ein Unterschied festgestellt werden ($p$=0,43).

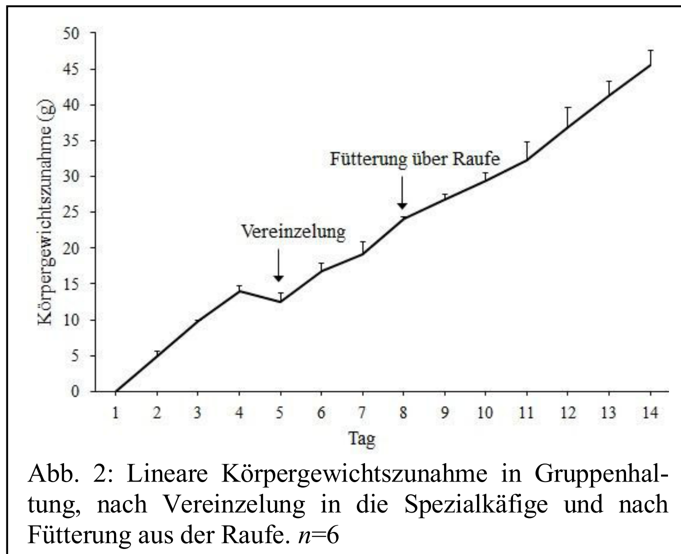


Abb. 2: Lineare Körpergewichtszunahme in Gruppenhaltung, nach Vereinzelung in die Spezialkäfige und nach Fütterung aus der Raufe. $n$=6

***Informationen über Nahrungsaufnahme-Mikrostruktur ungestörter Ratten.*** Mithilfe des Nahrungsaufnahme-Messsystems wurde die Mikrostruktur der Nahrungsaufnahme bei ungestörten Ratten erhoben. Die Ratten zeigten eine 9,1-mal höhere Nahrungsaufnahme in der Dunkel- im Vergleich zur Hellphase ($p$<0,001, $n$=9/Gruppe). Die Steigerung der Nahrungsaufnahme war mit häufigeren Mahlzeiten (8,9-fach, $p$<0,001) assoziiert, wohingegen die Größe der Mahlzeit (1,3-fach, $p$=0,13) im Vergleich zur Hellphase nicht signifikant verändert war.

***Physiologisches Verhalten in Spezialkäfigen.*** Die *Behavioral Satiety Sequence*, eine Abfolge von Verhaltensmustern (Nahrungsaufnahme – Lokomotion – Fellpflege – Ruhen), welche als Kennzeichen physiologischen Verhaltens gut etabliert ist (49), konnte in den Spezialkäfigen beobachtet werden, sodass von einer physiologischen Nahrungsaufnahme ausgegangen wird.

***Reduzierte Nahrungsaufnahme nach GOAT-Hemmung.*** Die Injektion des GOAT-Inhibitors (Dosen: 32, 96 und 288 µg/kg in 300 µl Kochsalzlösung, ip) verursachte

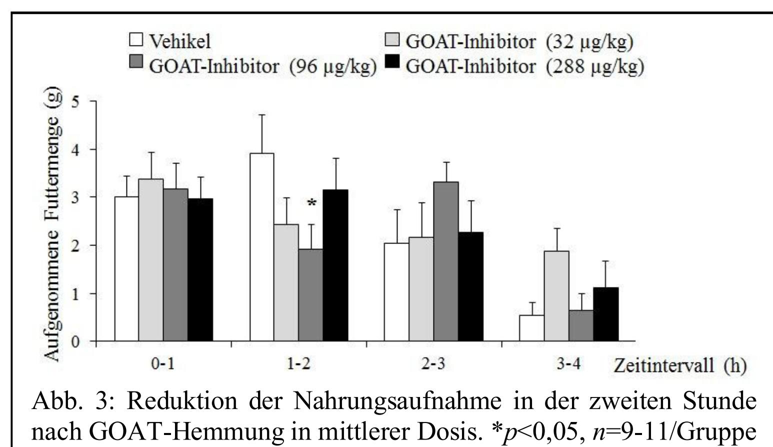


Abb. 3: Reduktion der Nahrungsaufnahme in der zweiten Stunde nach GOAT-Hemmung in mittlerer Dosis. *$p$<0,05, $n$=9-11/Gruppe

im Vergleich zum Vehikel eine dosisabhängige Reduktion der Nahrungsaufnahme, wobei die Dosis von 96 µg GOAT-Inhibitor/kg Körpergewicht den größten Effekt erzielte, weshalb diese Dosis für alle weiteren Analysen verwendet wurde. Dieser war in der zweiten Stunde nach Injektion (-27%, $p$<0,05; Abb. 3) zu beobachten und führte zu einer Reduktion der kumulativen Nahrungsaufnahme über zwei Stunden ($p$<0,05). Aus diesem Grund wurde die Nahrungsaufnahme-Mikrostruktur in den ersten zwei Stunden nach Injektion betrachtet. Der GOAT-Inhibitor reduzierte die Nahrungsaufnahme über eine reduzierte Häufigkeit der Mahlzeiten, wohingegen Größe und Dauer der Mahlzeiten im Vergleich zu Vehikel nicht signifikant verändert waren (Abb. 4). Dies weist auf eine erhöhte *satiety* nach GOAT-Hemmung hin.

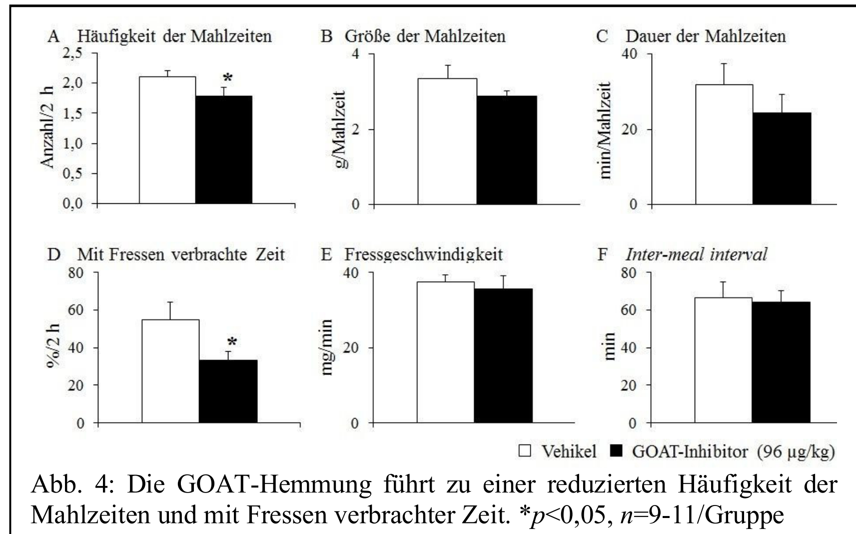


Abb. 4: Die GOAT-Hemmung führt zu einer reduzierten Häufigkeit der Mahlzeiten und mit Fressen verbrachter Zeit. *$p$<0,05, $n$=9-11/Gruppe

***Verhinderter Anstieg von Acyl-Ghrelin nach GOAT-Hemmung.*** Die Injektion von GOAT-Inhibitor (96 µg/kg, ip) führte zu einer Abschwächung des erwarteten Anstiegs von Acyl-Ghrelin während der Dunkelphase im Vergleich zur Kontrollgruppe (-57% nach zwei Stunden, $p$<0,05), wohingegen die Desacyl-Ghrelin-Spiegel zu keinem Zeitpunkt (1, 2, 3 h) verändert waren ($p$<0,27, Abb. 5).

***Veränderung bestimmter Verhaltensparameter nach GOAT-Hemmung.*** In der zweiten Stunde nach GOAT-Hemmung (96 µg/kg, ip) war das Putzverhalten im Vergleich zur Kontrollgruppe reduziert (-60%, $p$<0,01), wohingegen Fress- (jede Interaktion mit der Nahrung, -0,3%), Trink- (+23%), und Lokomotionsverhalten (-2,4%, $p$=0,89) unverändert waren. Abnormales Verhalten wurde nicht beobachtet (Daten nicht gezeigt).

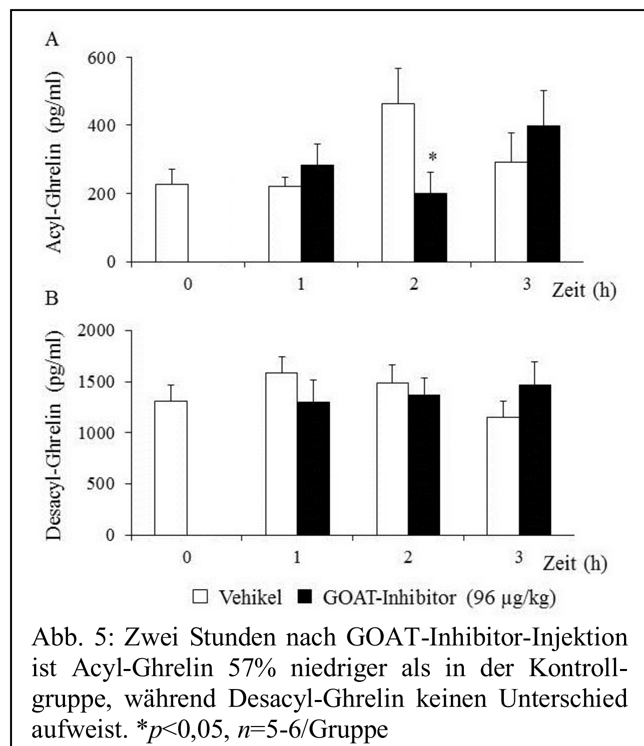


Abb. 5: Zwei Stunden nach GOAT-Inhibitor-Injektion ist Acyl-Ghrelin 57% niedriger als in der Kontrollgruppe, während Desacyl-Ghrelin keinen Unterschied aufweist. *$p$<0,05, $n$=5-6/Gruppe

4.2. Publikation 2: *The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index*

***Nachweis von GOAT im menschlichen Plasma und Abhängigkeit vom BMI.*** Im Western Blot wurde GOAT im Serum an der erwarteten Stelle (50 kDa) nachgewiesen (Abb. 6). Die semiquantitative Untersuchung ergab eine signifikant verringerte GOAT-Konzentration bei den anorektischen Patientinnen (-42 %, $p<0{,}01$) und eine erhöhte GOAT-Konzentration im Serum bei Patienten mit einem BMI >50 kg/m² (+34 %, $p<0{,}05$) im Vergleich zu den normalgewichtigen Kontrollen. Aufgrund dieser Veränderungen konnte eine positive Korrelation von GOAT-Protein und dem BMI beobachtet werden (Abb. 7). Weder Geschlecht noch Alter hatten einen Einfluss auf die GOAT-Konzentration (Daten nicht gezeigt).

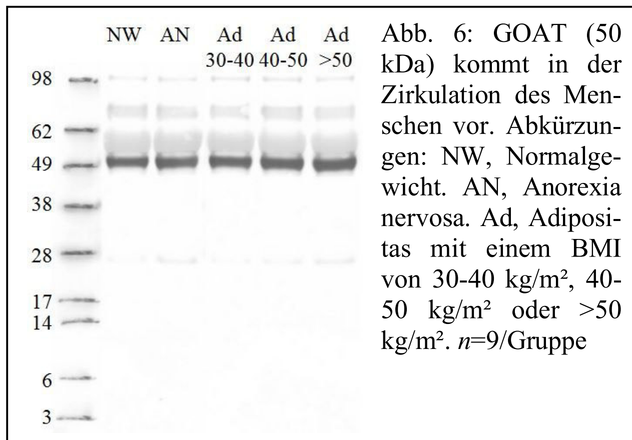


Abb. 6: GOAT (50 kDa) kommt in der Zirkulation des Menschen vor. Abkürzungen: NW, Normalgewicht. AN, Anorexia nervosa. Ad, Adipositas mit einem BMI von 30-40 kg/m², 40-50 kg/m² oder >50 kg/m². $n$=9/Gruppe

Die Konzentration zirkulierenden Ghrelins zeigte eine gegenläufige Kurve mit erhöhten Werten in der Anorexia Nervosa-Gruppe im Vergleich zu allen anderen Gruppen ($p<0{,}001$). Dies spiegelte sich auch in einer negativen Korrelation zwischen Ghrelin und GOAT wider ($r=-0{,}60$, $p<0{,}001$).

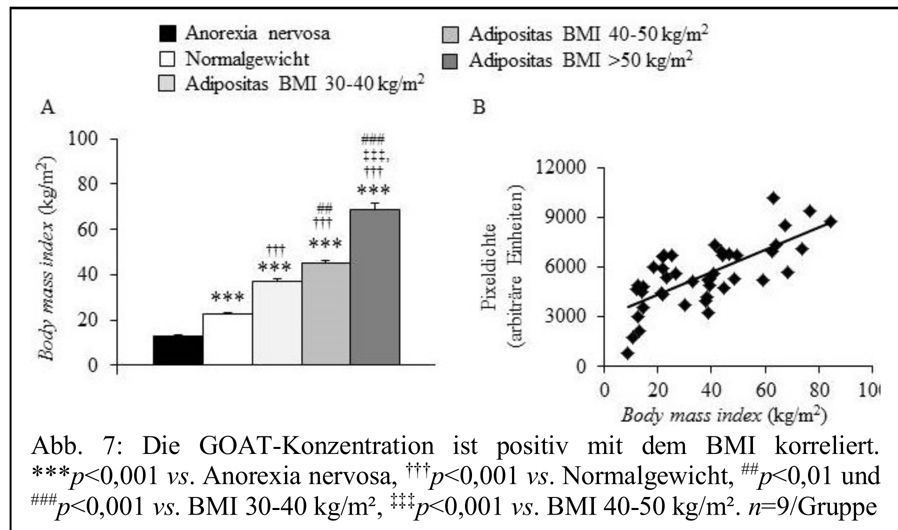


Abb. 7: Die GOAT-Konzentration ist positiv mit dem BMI korreliert. $^{***}p<0{,}001$ *vs*. Anorexia nervosa, $^{\dagger\dagger\dagger}p<0{,}001$ *vs*. Normalgewicht, $^{\#\#}p<0{,}01$ und $^{\#\#\#}p<0{,}001$ *vs*. BMI 30-40 kg/m², $^{\ddagger\ddagger\ddagger}p<0{,}001$ *vs*. BMI 40-50 kg/m². $n$=9/Gruppe

4.3. Publikation 3: *Obese patients have higher circulating protein levels of dipeptidyl peptidase IV*

***Positive Korrelation von DPP-4-Konzentration und BMI.*** Im Western Blot zeigten sich zwei prominente Banden an der für DPP-4 erwarteten Stelle bei 110 kDa und bei 50 kDa, welches wahrscheinlich ein Fragment des Enzyms, bestehend aus zwei schweren Ketten, darstellt. Das vollständige DPP-4-Protein und das Fragment waren stark positiv korreliert ($r=0{,}44$, $p<0{,}001$). Allen weiteren Analysen wurde die 110 kDa-Bande zugrunde gelegt. Die semiquantitative Analyse der DPP-4-Konzentration ergab eine Reduktion in der Anorexia Nervosa-Gruppe im Ver-

gleich zu allen anderen Gruppen, wohingegen die adipösen Gruppen erhöhte Spiegel aufwiesen (Abb.8A). Dies resultierte in einer positiven Korrelation von DPP-4 mit dem BMI ($r$=0,34, $p$<0,01 Abb. 8B).

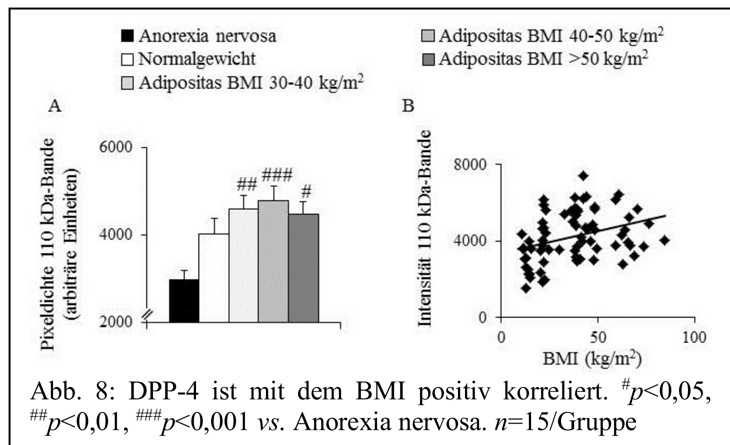


Abb. 8: DPP-4 ist mit dem BMI positiv korreliert. $^{\#}p<0{,}05$, $^{\#\#}p<0{,}01$, $^{\#\#\#}p<0{,}001$ *vs*. Anorexia nervosa. $n$=15/Gruppe

***Korrelationen von DPP-4-Enzymaktivität und der Konzentration/Aktivitäts-Ratio mit dem BMI.*** Trotz der BMI-abhängigen DPP-4-Konzentration war die Enzymaktivität von DPP-4 in allen Gruppen vergleichbar. Es wurde keine Korrelation mit dem BMI beobachtet ($r$=-0,18, $p$=0,13). Das Verhältnis von DPP-4-Konzentration zu DPP-4-Enzymaktivität war bei den adipösen Patienten höher als bei den anderen Gruppen, wobei nur der Unterschied zu den anorektischen Patientinnen statistisch signifikant war ($p$<0,05). Dies ergab eine positiven Korrelation der Konzentration/Aktivitäts-Ratio und dem BMI ($r$=0,31, $p$<0,01, Abb. 9).

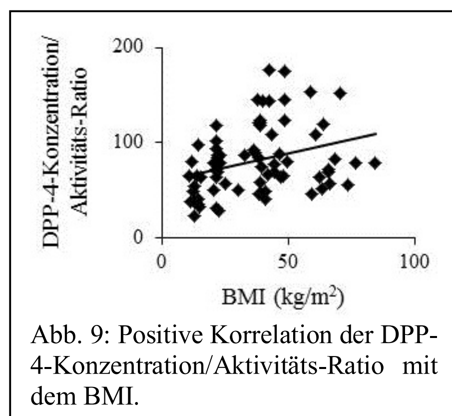


Abb. 9: Positive Korrelation der DPP-4-Konzentration/Aktivitäts-Ratio mit dem BMI.

***Zusammenhänge zwischen PP und GLP-1 mit DPP-4 und dem BMI.*** Im nächsten Schritt wurden Zielmoleküle der DPP-4 untersucht. Das PP zeigte bei Patientinnen mit Anorexia nervosa die höchsten Konzentrationen im Vergleich zu allen anderen Gruppen ($p$<0,05, $n$=15/Gruppe). Dies hatte eine negative Korrelation von PP und BMI zur Folge ($r$=-0,44, $p$<0,001, Abb. 10). Auch die DPP-4-Konzentration und die DPP-4-Konzentration/Aktivitäts-Ratio zeigten eine negative Korrelation mit PP, wohingegen die DPP-4-Enzymaktivität keinen signifikanten Zusammenhang mit der PP-Konzentration erkennen ließ. Im Gegensatz dazu waren die GLP-1-Spiegel in allen Gruppen ähnlich ($p$>0,05), ein Zusammenhang mit DPP-4-Konzentration, -Enzymaktivität oder DPP-4-Konzentration/Aktivitäts-Ratio wurde nicht beobachtet (Daten nicht gezeigt).

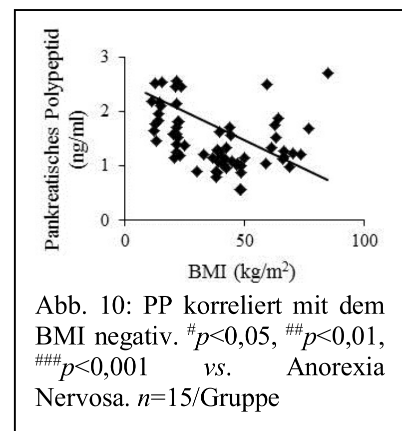


Abb. 10: PP korreliert mit dem BMI negativ. $^{\#}p<0{,}05$, $^{\#\#}p<0{,}01$, $^{\#\#\#}p<0{,}001$ *vs*. Anorexia Nervosa. $n$=15/Gruppe

## 5. Diskussion

***Etablierung einer neuen Methode zur Untersuchung der Nahrungsaufnahme-Mikrostruktur.***

In der ersten Publikation der vorliegenden Arbeit (50) wurde im ersten Schritt ein neues System zur kontinuierlichen und automatischen Erhebung der Nahrungsaufnahme und ihrer Mikrostruktur etabliert. Nahrungsaufnahme ist ein im Tierexperiment häufig erhobener Parameter und spielt eine besondere Rolle für die Untersuchung der Hunger- und Sättigungsregulierung, welche wiederum für das Verständnis der Pathomechanismen von Essstörungen essenziell ist. Die von uns vorgestellte Methode stellt einen großen Fortschritt im Vergleich zur klassischen manuellen Vorgehensweise dar, da bei letzterer die Verzerrung der erhobenen Daten durch die mögliche Störung der Tiere durch den anwesenden Untersucher und eine Lichtquelle während der Dunkelphase nicht ausgeschlossen werden kann. Außerdem kann bei der manuellen Messung lediglich die aufgenommene Futtermenge in Intervallen, nicht kontinuierlich, dargestellt werden, wodurch potenzielle Gegenregulationsmechanismen möglicherweise übersehen werden könnten. Über die Mikrostruktur der Nahrungsaufnahme kann mit der traditionellen Herangehensweise keine Aussage gemacht werden, während das von uns in dieser Studie validierte Messsystem eine Vielzahl einzelner Parameter erhebt, die Aufschluss darüber geben können, welche physiologischen Regelkreise im Einzelfall verändert sind. Im Gegensatz zu früheren Versuchen der automatisierten Messung der Nahrungsaufnahme-Mikrostruktur (51-53) hat das von uns verwendete System die Vorteile der gewohnten Umgebung der Tiere, und vor allem der Verwendung von Standardfutter. Dies schließt eine Verfälschung der Daten durch unphysiologische Futterformen wie Pulver, Mikropellets oder Flüssignahrung und daraus resultierend einen veränderten Sättigungsmechanismus aus.

Obwohl bereits Studien veröffentlicht wurden, welche diese Methode anwenden (54-56), stand die Validierung des Systems für die Arbeit mit Ratten bislang aus. Zu diesem Zweck haben wir untersucht, wie sich die Haltung in den Spezialkäfigen des Nahrungsaufnahme-Messsystems auf das Verhalten der Ratten auswirkt. Wir konnten hierbei eine rasche Gewöhnung der Tiere an die Umgebung und das Fressen aus der speziellen Vorrichtung des Systems zeigen, was sich sowohl an der kontinuierlichen Körpergewichtszunahme als auch an der im Vergleich zu manuellen Bedingungen unveränderten Futtermenge zeigte. Hervorzuheben ist, dass die Ratten in den Spezialkäfigen die normale *Behavioral Satiety Sequence* zeigten, eine gut etablierte Abfolge von postprandialem Verhalten als Zeichen einer physiologischen Nahrungsaufnahme (49). Somit ergab sich kein Anhalt für Stress durch die Haltung in den Spezialkäfigen. Dies wird durch die gewohnte Umgebung mit Einstreu, Rückzugsort und Anreicherungsmaterial und durch den auch nach Vereinzelung sichergestellten Sicht- und Geruchskontakt der Tiere unterstützt.

Mithilfe der automatisierten Messung war eine Analyse der Mikrostruktur zuerst unter basalen Bedingungen möglich. Hierbei zeigte sich, dass die vermehrte Nahrungsaufnahme während der Dunkelphase, der physiologischen Fressphase der Ratten (48), durch eine vermehrte Anzahl der Mahlzeiten vermittelt wird, während die Mahlzeitgröße im Vergleich zur Hellphase unverändert ist. Dies weist auf eine verringerte *satiety* während der Dunkelphase hin, wohingegen die *satiation* (15, 16) während Hell- und Dunkelphase ähnlich zu sein scheint.

Daten über die Eignung dieser neuen Methode unter verschiedenen experimentellen Bedingungen, etwa bei Anwendung unterschiedlicher Futterarten oder bei Tieren mit chronischer zentraler Kanülierung, sind für zukünftige Experimente erforderlich. Wir haben hierzu bereits Untersuchungen durchgeführt, die Ergebnisse wurden bislang allerdings noch nicht veröffentlicht. Zusammenfassend ist das hier vorgestellte System eine zuverlässige Methode zur Analyse der Nahrungsaufnahme-Mikrostruktur bei Ratten.

***Verminderte Nahrungsaufnahme bei Ratten durch Steigerung der satiety nach GOAT-Hemmung.*** Nach Etablierung des Nahrungsaufnahme-Messsystems für den Einsatz bei Ratten wurde die Nahrungsaufnahme-Mikrostruktur nach Hemmung des Ghrelin-acylierenden Enzyms GOAT untersucht (50). Die Untersuchung dieser Parameter ist deshalb von Interesse, da GOAT als bislang einzig bekanntes Ghrelin-aktivierendes Enzym (26, 27) möglicherweise ein Zielmolekül für die medikamentöse Behandlung krankhaft veränderter Körpergewichtszustände sein könnte. Die intraperitoneale Injektion des GOAT-Inhibitors GO-CoA-Tat führte zu einer dosisabhängigen Reduktion der Nahrungsaufnahme während der Dunkelphase, wobei der maximale Effekt bei einer Dosis von 96 µg/kg beobachtet wurde, also interessanterweise nicht bei der höchsten Dosis. Diese U-förmige Dosis-Wirkungs-Beziehung könnte auf eine agonistische Wirkung in höheren Dosierungen des GOAT-Hemmers hindeuten. Wir beobachteten außerdem einen verzögerten Wirkungseintritt mit der deutlichsten Reduktion der Nahrungsaufnahme in der zweiten Stunde nach Inhibitor-Gabe. Dies hängt wahrscheinlich mit dem Zeitpunkt der Injektion zu Beginn der Dunkelphase zusammen, der physiologischen Wachphase der Ratten, in welcher sie dementsprechend auch die meiste Nahrungsaufnahme zeigen (33). Die Konzentration von Ghrelin ist zu diesem Zeitpunkt bereits erhöht (57). In Anbetracht der Halbwertszeit von Ghrelin von etwa 30 Minuten (58) ist eine verzögerte Wirkung einer Hemmung der Ghrelin-Acylierung auf die Nahrungsaufnahme gut erklärbar. Passend zu dieser Vermutung waren auch die Acyl-Ghrelin-Spiegel zwei Stunden nach Injektion um 50% im Vergleich zur Kontrollgruppe reduziert.

Die Mikrostruktur der Nahrungsaufnahme in den ersten zwei Stunden nach Injektion des GOAT-Inhibitors zeigte eine Reduktion der Häufigkeit der Mahlzeiten bei gleichbleibender Mahlzeit-

größe. Dieses Muster weist auf eine erhöhte *satiety* (diejenigen Mechanismen, die den Zeitpunkt des Einsetzens der nächsten Mahlzeit bestimmen) hin, während die *satiation* (Mechanismen, die zur Beendigung einer Mahlzeit führen) unverändert war (15, 16). Dies lässt sich nur teilweise mit den Ergebnissen von Tabarin *et al.* in Einklang bringen, die nach intraperitonealer Injektion eines Ghrelin-Agonisten bei Mäusen eine Erhöhung der Mahlzeitgröße und -häufigkeit beobachteten (59). Mögliche Gründe für die abweichenden Ergebnisse sind die untersuchten Spezies (Ratten *vs*. Mäuse), unterschiedliche Futterarten (Mikropellets *vs.* Standard-Rattenfutter) sowie pharmakologische Besonderheiten des Ghrelin-Agonisten (*vs*. Hemmung von endogenem Ghrelin).

Um eine mögliche Auswirkung der GOAT-Inhibition auf das Verhalten zu untersuchen, wurden die Tiere in der zweiten Stunde nach Injektion beobachtet. Hier zeigte sich eine Reduktion des Putzverhaltens im Vergleich zur Kontrollgruppe, während Lokomotion, Fressverhalten (dies umfasst Fressen selbst, aber auch Schnuppern und Lecken am Futter, ohne Futter aufzunehmen) und Trinkverhalten in beiden Gruppen gleich waren. Das verminderte Putzverhalten könnte mit der Reduktion der Nahrungsaufnahme zusammenhängen, da die *Behavioral Satiety Sequence* eine Transition von Fressverhalten über Putzverhalten zum Ruheverhalten beschreibt (49). Sie könnte allerdings auch eine direkte Folge der GOAT-Inhibition sein, da Acyl-Ghrelin das Putzverhalten bei Ratten steigert (60). Aufgrund der unveränderten Lokomotion ist eine unspezifische Hemmung der Bewegung der Ratten durch den GOAT-Hemmer (z.B. durch Induktion von Übelkeit) nicht wahrscheinlich. Die interessante Beobachtung, dass der GOAT-Hemmer zwar die Nahrungsaufnahme reduziert (-21% im Vergleich zur Kontrollgruppe), das Fressverhalten (sämtliche Interaktion mit dem Futter) jedoch nicht beeinflusst, legt nahe, dass die reine Interaktion der Ratte mit der Nahrung während der Dunkelphase nicht allein durch Ghrelin vermittelt wird, beziehungsweise kompensatorische Mechanismen rasch eingreifen. Auch eine inkomplette Hemmung von Ghrelin (-50% Reduktion der Acyl-Ghrelin-Spiegel im Vergleich zur Kontrolle wurden beobachtet) könnte hierzu beitragen.

Zusammenfassend konnten wir in dieser Studie mithilfe des zuvor etablierten Nahrungsaufnahme-Messsystems zeigen, dass eine periphere GOAT-Hemmung die Nahrungsaufnahme über eine Reduktion von Acyl-Ghrelin vermindert, ein Effekt, der mit einer gesteigerten *satiety* einhergeht.

***Nachweis der BMI-abhängigen Expression von Ghrelin-O-Acyltransferase in der menschlichen Zirkulation.*** In der zweiten Publikation wurde das Ghrelin-acylierende Enzym GOAT erstmals im menschlichen Blut nachgewiesen (61). Es ist zu vermuten, dass GOAT im Gastrointestinaltrakt produziert wird (62) und von dort ins Blut gelangt. Des Weiteren konnten wir zeigen, dass GOAT in Abhängigkeit vom BMI exprimiert wird, da bei anorektischen Patientinnen

niedrigere, bei schwer adipösen Patienten (BMI >50 kg/m²) höhere GOAT-Konzentrationen als bei normalgewichtigen Individuen gemessen wurden. Dies kann nicht auf eine insgesamt höhere Proteinexpression zurückgeführt werden, da sich die Gesamtprotein-Konzentration bei verschiedenen metabolischen Zuständen nicht unterschied. Die Regulation von GOAT abhängig vom chronisch veränderten Körpergewicht könnte sich auf die weitere Gewichtsentwicklung auswirken. Da Ghrelin als bedeutender Stimulator der Nahrungsaufnahme alleinig durch GOAT aktiviert werden kann (26), könnten niedrigere GOAT-Spiegel bei Anorexia Nervosa zu einer Reduktion der Nahrungsaufnahme beitragen. Umgekehrt könnten die erhöhten GOAT-Spiegel bei der Adipositas per magna eine weitere Hyperphagie begünstigen und somit die krankhaft veränderte metabolische Situation verstärken.

Obwohl die Unterschiede der gemessenen GOAT-Werte zwischen den fünf Patientengruppen signifikant waren, sind die Ergebnisse dieser Studie vor dem Hintergrund der relativ kleinen Zahl eingeschlossener Studienteilnehmer (*n*=9/Gruppe) vorsichtig zu betrachten. Einen weiteren limitierenden Faktor unserer Daten stellt die geschlechtlich und altersmäßig unterschiedliche Zusammensetzung der Gruppen dar. Dies ist der Epidemiologie der Anorexia Nervosa mit einem vorwiegenden Vorkommen bei jungen Frauen geschuldet. Die statistische Auswertung ergab allerdings keinen Einfluss von Alter oder Geschlecht auf die GOAT-Expression. Des Weiteren sollte in zukünftigen Studien neben der Proteinkonzentration auch die GOAT-Enzymaktivität berücksichtigt werden.

Bei den anorektischen Patientinnen haben wir, in Übereinstimmung mit anderen Studien, (37, 63) erhöhte Ghrelin-Werte detektiert. Bislang wurden diese Ergebnisse als Reaktion auf das dramatisch verringerte Körpergewicht und den Versuch der Gegenregulation interpretiert. Allerdings zeigten auf der anderen Seite in unserer Studie die adipösen Patienten keinen Unterschied in der Gesamt-Ghrelin-Konzentration im Vergleich zu den normalgewichtigen Probanden. Dieses Ergebnis steht im Gegensatz zu vormals publizierten Daten (38, 64, 65) und könnte mit der hohen Rate an Patienten mit T2DM (37%) in der Adipositas-Gruppe zusammenhängen. Die erhöhten Glukosespiegel bzw. die erfolgte antidiabetische Therapie könnte hierbei den Zusammenhang von Ghrelin mit dem BMI maskieren.

Zusammenfassend wurde in dieser Studie GOAT in der Zirkulation beim Menschen detektiert und eine GOAT-Hemmung als neuer Therapieansatz für die medikamentöse Behandlung der Adipositas postuliert.

***Erhöhte Konzentrationen von Dipeptidylpeptidase-4 bei Adipositas.*** In der dritten Publikation konnten wir eine Korrelation der DPP-4-Konzentration mit dem BMI nachweisen, wobei die adipösen Probanden eine erhöhte Konzentration aufwiesen und die anorektischen Patientinnen

die niedrigsten DPP-4-Spiegel zeigten (66). Da Albumin in allen fünf BMI-Gruppen gleich war, ist eine generelle Veränderung der Proteinexpression bei chronisch verändertem BMI als mögliche Ursache der unterschiedlichen DPP-4-Spiegel unwahrscheinlich. Gleichzeitig war interessanterweise die Enzymaktivität von DPP-4 in allen Gruppen gleich groß, womit die aktuellen Daten abweichende Ergebnisse zu früheren Studien beschreiben, die eine positive Korrelation der DPP-4-Enzymaktivität mit dem BMI bei adipösen Kindern (45) und gesunden jungen Menschen (46) festgestellt hatten. Diese Diskrepanz könnte eventuell darin begründet liegen, dass wir der vorliegenden Studie ein sehr breites Spektrum des Körpergewichts mit einer BMI-Spanne von 9 bis 85 kg/m² zugrunde gelegt haben und dadurch in der Lage waren, ein umfassenderes Bild darlegen zu können.

Neben der Bestimmung der Konzentration und Aktivität allein haben wir die Konzentration/Aktivitäts-Ratio bestimmt. Hierbei zeigten adipöse Patienten eine hohe, Patienten mit Anorexia Nervosa eine erniedrigte Ratio im Vergleich zu Normalgewichtigen. Dies könnte, wie kürzlich postuliert (47), mit einer bei Adipositas verstärkten Expression von DPP-4 im Fettgewebe zusammenhängen, welche dann zu höheren zirkulierenden DPP-4-Spiegeln führt, könnte aber auch auf eine relativ niedrigere Enzymaktivität bei Adipösen hindeuten, welche dann durch verstärkte Enzymbildung kompensiert wird.

In die Regulation der Blutzucker-Homöostase wie auch der Nahrungsaufnahme sind die Hormone GLP-1 und PP involviert, welche gleichzeitig DPP-4-Substrate sind. Während unsere Ergebnisse zeigten, dass GLP-1 in allen fünf BMI-Gruppen gleich war, wies die Anorexia Nervosa-Gruppe höhere PP-Spiegel als alle anderen Gruppen auf. Die negative Korrelation von PP mit dem BMI ist im Einklang mit den Ergebnissen von Uhe *et al.* (67), während bei Adipositas-Patienten entweder unveränderte PP-Spiegel (68) oder eine verminderte PP-Expression (69) beschrieben wurden. Die von uns gezeigte negative Korrelation von PP sowohl mit der DPP-4-Konzentration als auch mit dem Konzentration/Aktivitäts-Verhältnis von DPP-4 könnte möglicherweise ein Hinweis darauf sein, dass DPP-4 bei Adipositas zu einem verstärkten Abbau von PP führt, was wiederum in abgeschwächten Sättigungssignalen in der Regulierung von Hunger und Sättigung resultieren und somit die Hyperphagie unter diesen Bedingungen weiter verschärfen könnte. Gestützt wird die Hypothese, dass DPP-4 auf die Vermittlung von Sättigung einen hemmenden Einfluss haben könnte, von den Ergebnissen von Conarello *et al.*, die bei DPP-4-Knockout-Mäusen eine Unempfänglichkeit für ernährungsbedingte Adipositas (*diet-induced obesity*) beschrieben (70). Weiterhin wurde von einer abgeschwächten DPP-4-Aktivität nach bariatrischer Operation berichtet (71), was zur postoperativen Gewichtsabnahme beitragen könnte. Neben einer Beeinflussung zentraler Sättigungsmechanismen könnte DPP-4 auch an der Ak-

tivierung braunen Fettgewebes beteiligt sein (72). Diese interessante Hypothese sowie der Zusammenhang mit den nahrungsregulatorischen Hormonen sollte in künftigen Studien weiter untersucht werden.
Zusammenfassend haben wir in der dritten Publikation erhöhte DPP-4-Spiegel und ein erhöhtes Konzentration/Aktivitäts-Verhältnis der DPP-4 bei adipösen Patienten beschrieben. Da gleichzeitig PP negativ mit dem BMI korreliert war, könnte DPP-4 bei adipösen Patienten möglicherweise zu einer Hemmung der Nahrungsaufnahme-inhibierenden Mechanismen führen und somit zu einer weiteren Erhöhung des krankhaft veränderten Körpergewichts beitragen.

***Zusammenfassung.*** Die drei in dieser Schrift beschriebenen Studien liefern neue Informationen über die an der Nahrungsaufnahme-Regulation beteiligten Enzyme Ghrelin-*O*-Acyltransferase und Dipeptidylpeptidase-4. Sowohl GOAT als auch DPP-4 könnten potenzielle Zielmoleküle in der medikamentösen Behandlung chronisch veränderten Körpergewichts sein. In diesem Zusammenhang und vor dem Hintergrund der weltweit zunehmenden Prävalenz der Adipositas stellen unsere Publikationen möglicherweise einen weiteren Schritt auf dem Weg zu neuen konservativen therapeutischen Konzepten dar.

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**Eidesstattliche Versicherung**

Ich, Pauline Teuffel, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: „Ghrelin-*O*-Acyltransferase und Dipeptidylpeptidase-4: zwei mögliche neue Targets für die Therapie der Adipositas“ selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung (siehe „Uniform Requirements for Manuscripts (URM)“ des ICMJE – www.icmje.org) kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen) entsprechen den URM (s.o.) und werden von mir verantwortet.

Meine Anteile an den ausgewählten Publikationen entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem Betreuer angegeben sind. Sämtliche Publikationen, die aus dieser Dissertation hervorgegangen sind und bei denen in Autor bin, entsprechen den URM (s.o.) und werden von mir verantwortet.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum　　　　　　　　　　　　　　　　Unterschrift

**Anteilserklärung an den erfolgten Publikationen**

Pauline Teuffel hatte folgenden Anteil an den folgenden Publikationen:

1. Publikation 1: Teuffel P, Wang L, Prinz P, Goebel-Stengel M, Scharner S, Kobelt P, Hoffmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats. Journal of Physiology and Pharmacology. 2015;66:493-503.
    - Betreuung der Versuchstiere: tägliches *Handling* und Gewichtskontrolle
    - Handhabung und tägliche Wartung des Nahrungsaufnahme-Messsystems
    - Planung, Vorbereitung und Durchführung der Experimente:
        - Etablierung des Nahrungsaufnahme-Messsystems (Erhebung der Futtermengen, des Körpergewichtsverlaufs und des Verhaltens)

- Nahrungsaufnahme-Mikrostruktur-Analyse nach GOAT-Hemmung (Vorbereitung und Injektion des GOAT-Inhibitors, Messung der Nahrungsaufnahme-Mikrostruktur mithilfe des automatischen Messsystems)
    - Verhaltensbeobachtung nach GOAT-Hemmung (Vorbereitung und Injektion des GOAT-Inhibitors, Erhebung der Verhaltensparameter)
    - Blutentnahme nach GOAT-Hemmung zur Probengewinnung für Acyl-und Gesamt-Ghrelin-ELISA (Vorbereitung und Injektion des GOAT-Inhibitors, Narkotisierung, Blutentnahme), Durchführung des ELISA
- Mitarbeit an der Datenauswertung
- Schreiben der ersten Version des Papers

2. Publikation 2: Goebel-Stengel M, Hofmann T, Elbelt U, <u>Teuffel P</u>, Ahnis A, Kobelt P, Lambrecht NWG, Klapp BF, Stengel A. The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index. Peptides. 2013;43:13-9.
    - Patienteneinschluss
    - Durchführung der Blutentnahmen
    - Durchführung der GOAT-Western Blots
    - Mitarbeit an der Datenauswertung
    - Mitarbeit am Paper

3. Publikation 3: Stengel A, Goebel-Stengel M, <u>Teuffel P</u>, Hofmann T, Buße P, Kobelt P, Rose M, Klapp BF. Obese patients have higher circulating protein levels of dipeptidyl peptidase IV. Peptides. 2014;61:75-82.
    - Patienteneinschluss
    - Durchführung der Blutentnahmen
    - Durchführung der DPP-4-Western Blots
    - Mitarbeit an der Datenauswertung
    - Mitarbeit am Paper

Datum ____________________ Unterschrift

**Lebenslauf Pauline Teuffel**

Mein Lebenslauf wird aus datenschutzrechtlichen Gründen in der elektronischen Version meiner Arbeit nicht veröffentlicht.

**Publikationen**

1. Treatment with the ghrelin-O-acyltransferase (GOAT) inhibitor GO-CoA-Tat reduces food intake by reducing meal frequency in rats.
   Teuffel P, Wang L, Prinz P, Goebel-Stengel M, Scharner S, Kobelt P, Hoffmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A.
   Journal of Physiology and Pharmacology. 2015;66:493-503.
   Impact Factor: 2,39

2. The dopamine antagonist flupentixol does not alter ghrelin-induced food intake in rats.
   Engster KM, Wismar J, Kroczek AL, Teuffel P, Nolte S, Rose M, Stengel A, Kobelt P.
   Neuropeptides. 2015. (Epub ahead of print)
   Impact Factor: 2,64

3. Obese patients have higher circulating protein levels of dipeptidyl peptidase IV.
   Stengel A, Goebel-Stengel M, Teuffel P, Hofmann T, Buße P, Kobelt P, Rose M, Klapp BF.
   Peptides. 2014;61:75-82.
   Impact Factor: 2,62

4. The ghrelin activating enzyme ghrelin-O-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index.
   Goebel-Stengel M, Hofmann T, Elbelt U, Teuffel P, Ahnis A, Kobelt P, Lambrecht NW, Klapp BF, Stengel A.
   Peptides. 2013;43:13-9.
   Impact Factor: 2,62

**Abstracts**

1. Inhibition of ghrelin-*O*-acyltransferase (GOAT) reduces food intake by increasing satiety in rats.
   Teuffel P, Wang L, Prinz P, Goebel-Stengel M, Kobelt P, Hofmann T, Rose M, Klapp BF, Reeve JR Jr, Stengel A.
   Gastroenterology. 2015;148:S-387.

Das Fragment Nesfatin-1$_{30\text{-}59}$ induziert nach intrazerebroventrikulärer Injektion über eine Reduktion der Mahlzeitgröße eine lang anhaltende Hemmung der Nahrungsaufnahme bei Ratten.

Goebel-Stengel M, Teuffel P, Lembke V, Kobelt P, Hofmann T, Rose M, Klapp BF, Stengel A.

2. Zeitschrift für Gastroenterologie 2014;52.

Etablierung eines automatisierten Messsystems zur Bestimmung der Nahrungsaufnahmemikrostruktur für feste Nahrung bei Ratten.

Goebel-Stengel M, Teuffel P, Wang L, Kobelt P, Rose M, Klapp BF, Reeve JR Jr, Stengel A.

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Das Ghrelin-acylierende Enzym GOAT ist beim Menschen im Plasma detektierbar und abhängig vom Body Mass Index exprimiert.

Hofmann T, Goebel-Stengel M, Elbelt U, Teuffel, P, Ahnis A, Kobelt P, Lambrecht NW, Klapp BF, Stengel A.

6. Zeitschrift für Gastroenterologie 2013;51.

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## Original articles

P. TEUFFEL[1], L. WANG[2], P. PRINZ[1], M. GOEBEL-STENGEL[3], S. SCHARNER[1], P. KOBELT[1], T. HOFMANN[1], M. ROSE[1], B.F. KLAPP[1], J.R. REEVE Jr.[2], A. STENGEL[1]

# TREATMENT WITH THE GHRELIN-*O*-ACYLTRANSFERASE (GOAT) INHIBITOR GO-COA-TAT REDUCES FOOD INTAKE BY REDUCING MEAL FREQUENCY IN RATS

[1]Charite Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charite-Universitaetsmedizin Berlin, Campus Mitte, Berlin, Germany; [2]CURE/Digestive Diseases Research Center, Center for Neurobiology of Stress, Department of Medicine, Digestive Diseases Division at the University of California Los Angeles, and Veterans Affairs Greater Los Angeles Health Care System, CA, USA;
[3]Department of Internal Medicine and Institute of Neurogastroenterology, Martin-Luther-Krankenhaus, Berlin, Germany

The ghrelin acylating enzyme ghrelin-*O*-acyltransferase (GOAT) was recently identified and implicated in several biological functions. However, the effects on food intake warrant further investigation. While several genetic GOAT mouse models showed normal food intake, acute blockade using a GOAT inhibitor resulted in reduced food intake. The underlying food intake microstructure remains to be established. In the present study we used an automated feeding monitoring system to assess food intake and the food intake microstructure. First, we validated the basal food intake and feeding behavior in rats using the automated monitoring system. Afterwards, we assessed the food intake microstructure following intraperitoneal injection of the GOAT inhibitor, GO-CoA-Tat (32, 96 and 288 μg/kg) in freely fed male Sprague-Dawley rats. Rats showed a rapid habituation to the automated food intake monitoring system and food intake levels were similar compared to manual monitoring (P = 0.43). Rats housed under these conditions showed a physiological behavioral satiety sequence. Injection of the GOAT inhibitor resulted in a dose-dependent reduction of food intake with a maximum effect observed after 96 μg/kg (–27%, P = 0.03) compared to vehicle. This effect was delayed in onset as the first meal was not altered and lasted for a period of 2 h. Analysis of the food intake microstructure showed that the anorexigenic effect was due to a reduction of meal frequency (–15%, P = 0.04), whereas meal size (P = 0.29) was not altered compared to vehicle. In summary, pharmacological blockade of GOAT reduces dark phase food intake by an increase of satiety while satiation is not affected.

Key words: *automated food intake monitoring system, behavior, behavioral satiety sequence, food intake pattern, ghrelin,*

## INTRODUCTION

Ghrelin was discovered more than a decade ago and is the endogenous ligand of the growth hormone secretagogue receptor 1a (GHS-R1a) (1), later renamed ghrelin receptor (2). Ghrelin is predominantly produced in the stomach (1, 3) and so far the only known peripherally produced and centrally acting hormone that stimulates food intake (4, 5). In addition, ghrelin is involved in several local effects directly in the stomach such as mucosal healing (6) and may also play a role in gastric carcinogenesis (7). A unique feature of ghrelin is the fatty acid residue on the third amino acid, a prerequisite for binding to the ghrelin receptor (1). The enzyme that catalyzes this acylation was unknown for a long time but identified in 2008 as member of the membrane-bound *O*-acyltransferases (MBOATs) by two independent groups and named ghrelin-*O*-acyltransferase (GOAT) (8, 9). GOAT protein was detected in ghrelin-containing cells of the rodent stomach (10) but also in the peripheral circulation of rodents (10) and humans (11). This may point towards an acylation of ghrelin outside of the stomach.

Several effects of GOAT have been reported, namely an involvement in glucose homeostasis (12), bile acid reabsorption (13) and responsiveness for salty and lipid taste (14). However, only few studies have investigated an effect of GOAT on food intake. GOAT seems to be involved in the hedonic aspect of feeding as mice lacking GOAT show a reduced hedonic feeding response compared to their wild type littermates (15). Interestingly, mice overexpressing ghrelin and GOAT showed an increase in body weight when fed a medium-chain triglyceride-enriched diet while food intake was not altered (16). Similarly, mice lacking GOAT also did not display alterations in food intake (12, 16). One study in Siberian hamsters reported that intraperitoneal (i.p.) injection of the GOAT inhibitor, GO-CoA-Tat reduced food intake, food foraging and hoarding compared to vehicle (17). These partly inconsistent findings may be due to the time course of the studies with compensatory mechanisms becoming more important over time but may also be related to the assessment of overall food intake, while a detailed analysis of the food intake microstructure is lacking.

The food intake microstructure encompasses parameters such as latency to a meal, eating rate, meal frequency, meal size, meal duration and the inter-meal interval. These parameters can be used to distinguish two major characteristics of a condition or a compound influencing food intake: satiation (mechanisms

causing meal termination) and satiety (mechanisms causing a later onset of the next meal after one meal is completed) (18, 19).

In the present study we used an automated episodic food intake monitoring device that allows for continuous monitoring of food intake and the food intake microstructure in undisturbed rats (20-22) and mice (23). Although this system has been validated for mice (24), the validation is still lacking for rats. Therefore, we first validated this system for rats under different experimental conditions. We also manually monitored the behavioral satiety sequence (a progression of behaviors following food intake in rats encompassing 'feeding' itself, 'grooming' and exploration/'locomotion' towards 'resting' (25)) to assess the occurrence of physiological behavior under these conditions. Afterwards, we investigated whether the GOAT inhibitor, GO-CoA-Tat alters food intake and the food intake microstructure in *ad libitum* fed rats during the dark phase, the photoperiod when rats show their greatest food intake (26). We also investigated whether inhibition of GOAT would affect circulating ghrelin levels and alter behavior in addition to food intake.

## MATERIALS AND METHODS

### *Animals*

Adult male Sprague-Dawley rats (Harlan-Winkelmann Co., Borchen, Germany and Harlan, San Diego, CA, USA) weighing 220 – 300 g were group housed under controlled illumination (6:00 AM to 6:00 PM) and temperature (21 – 23°C). Animals had free access to standard rodent diet (Altromin™, Lage, Germany) unless otherwise specified, and tap water. Animal care and experimental procedures followed institutional ethic guidelines and conformed to the requirements of the state authority for animal research (#G 0131/11 and #01001-13).

### *Compound*

The GOAT inhibitor, GO-CoA-Tat (Peptides International Inc., Louisville, KY, USA) was kept in powder form at –80°C and dissolved in pyrogen-free saline before the experiments.

### *Monitoring*

#### *1. Manual food intake monitoring*

Rats were handled daily to become accustomed to the investigators and the experimental procedures. This included removal of the rat from the cage to measure food intake and light hand restraint for body weight monitoring. This daily routine was performed at the same time each day. Food intake was monitored by providing rats with pre-weighed rat chow and weighing of food after defined time intervals (directly after lights on and off, respectively). Food intake was corrected for spillage and expressed as g/200 g body weight (b.w.).

#### *2. Automated food intake monitoring*

The microstructural analysis of feeding behavior was conducted using the BioDAQ episodic food intake monitoring system for rats (BioDAQ, Research Diets, Inc., New Brunswick, NJ, USA), which allows for continuous monitoring of meal patterns in undisturbed rats with minimal human interference as recently described for the use in mice (24). The system consists of a low spill food hopper placed on an electronic balance. Both are mounted on a regular rat single housing cage containing environmental enrichment and bedding material. Water was provided *ad libitum* from regular water bottles. Rats were kept on regular rodent diet unless otherwise specified since it did not cause much spillage. The "bridging phenomenon", that occurs when a pile of retained food spillage underneath the gate can cause erroneous measurements, was observed very rarely.

The food intake monitoring system weighs the hopper with food (± 0.01 g) second by second and detects 'not eating' as weight stable and 'eating' as weight unstable. Every interaction of the rat with the food hopper is recorded. Feeding bouts (changes in stable weight before and after a bout) are recorded with a start time, duration and amount consumed. Bouts are separated by an inter-bout interval (IBI), and meals consist of one or more bouts separated by an inter-meal interval (IMI). The minimum IMI was defined as 15 min, the minimum meal amount as 0.1 g as described in our previous study (21). Based on this definition, food intake was considered as one meal when the feeding bouts occurred within 15 min of the previous response and their sum was equal to or greater than 0.1 g. When bouts of feeding were longer than 15 min apart, they were considered as a new meal. Meal parameters extracted from the software (BioDAQ Monitoring Software 2.3.07) for these studies encompassed the latency to the first meal, meal frequency, meal size, meal duration, inter-meal interval, time spent in meals and the rate of ingestion. Since food intake data were collected continuously, periods of interest could be chosen freely afterwards for the data analysis. Data could be viewed either in the Data Viewer (BioDAQ Monitoring Software 2.3.07) or Excel (Microsoft) for analysis.

#### *3. Behavioral monitoring of satiety sequence*

Rats were acclimated to the BioDAQ system for 1 week. The behavior was monitored in the 1st hour of the dark phase under conditions of dimmed red light by two experienced investigators and consisted of feeding (biting and chewing food), grooming (scratching, licking or biting the fur, limbs or genitals), locomotion (movements involving all four limbs; walking, jumping or circling) and resting (sitting or lying in a relaxed position) as described before (27). Eight rats were monitored at the same time once per min and 5 s per rat. The behavior counts were grouped in 12 × 5 min time bins.

#### *4. Behavioral monitoring following treatment*

Rats were acclimated to the BioDAQ system for 1 week. *Ad libitum* fed rats were treated with vehicle or GOAT inhibitor directly before the onset of the dark phase as described below and placed in their home cage with a paper grid under the cage divided into six equal squares. Behavior was monitored during the 2nd hour post injection during the dark phase. Behavior was assessed manually and simultaneously in 3 rats/investigator as described in our previous studies using a time-sampling technique (21, 28). Briefly, during the 2nd hour post injection behaviors including eating (eating as well as food approach consisting of sniffing and licking food), drinking (drinking and water approach), grooming (washing, licking, and scratching) and locomotor activity (defined as at least one rat paw crossing the boundary of one square, the total number of squares crossed was counted) were assessed by two investigators who sat motionless in front of the cages with a dim light for a period of 1 h. Each behavior was counted again when it lasted > 5 s. Food intake was assessed at the same time. In pilot experiments we established that the inter-investigator variability was < 5%.

### *Measurement of acyl and total ghrelin levels*

Group housed rats were handled for a period of 1 week. *Ad libitum* fed rats were treated with vehicle or GOAT inhibitor

directly before the onset of the dark phase as described below and food was removed. Blood was obtained at 0 h (before injection) or 1, 2 or 3 h post injection by cardiac puncture. Therefore, rats were anesthetized with a mixture of ketamine (75 mg/kg i.p.; Fort Dodge Laboratories, Fort Dodge, IA, USA) and xylazine (5 mg/kg i.p.; Mobay, Shawnee, KS, USA). Afterwards, the thoracic cavity was quickly opened and 1 ml of cardiac blood was collected in chilled syringes rinsed with ethylene diamine tetraacetic acid (EDTA) and transferred into cooled tubes containing 10 µl EDTA (7.5%, Sigma, St. Louis, MO, USA) and aprotinin (1.2 Trypsin Inhibitory Unit per 1 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, USA) for peptidase inhibition. Tubes were placed back on ice and immediately (within 3 min) centrifuged at 4°C for 10 min at 3000 × g. Plasma was separated and stored at –80°C until further processing.

Rat acyl (# EZRGRA-90K, Millipore, Billerica, MA, USA) and total (#EZRGRT-91K, Millipore) ghrelin levels were assessed using commercial ELISA kits following the manufacturer's instructions. Desacyl ghrelin was calculated as the difference of total minus acyl ghrelin for each individual sample. All samples were processed in one batch. The intra-assay variability was < 5% for acyl and < 2% for total ghrelin.

*Experimental protocols*

*1. Habituation to automated food intake monitoring system and comparison with manual assessment*

After an initial habituation period of seven days, rats continued to be group-housed (3 – 4/cage) and food intake and body weight were monitored daily. After five days, rats were separated into single housing cages which were placed adjacent to each other so the animals could stay in eye and odor contact. Food was provided from the top of the cage and the manual monitoring of food intake and body weight was continued. After another three days, food was provided from the hopper and food intake measured by the automated food intake monitoring system. Body weight was monitored daily throughout this period. Food intake assessed by the automated food intake monitoring system was compared between different time points of the habituation period (days 1 and 2 versus days 5 and 6) and also to the manual assessment. The food intake microstructure was compared between the light and the dark phase.

*2. Monitoring of behavior in the automated food intake monitoring system*

To assess the occurrence of physiological behavior in rats single housed in cages connected to the automated food intake monitoring system, the behavior was monitored manually in *ad libitum* fed naïve rats during the first hour of the dark phase.

*3. Food intake microstructure in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor*

*Ad libitum* fed naïve rats were habituated to the system and injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (32, 96 or 288 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake was monitored using the automated food intake monitoring system. The medium dose was based on a recent study investigating the effect of GOAT inhibition on the hypothalamic-pituitary-adrenal axis in rats (29). The dose inducing the most pronounced reduction in food intake was selected for analysis of the food intake microstructure.

*4. Acyl and desacyl ghrelin levels in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor*

*Ad libitum* fed naive rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (96 µg/kg in 300 µl saline, the dose that induced the most pronounced reduction of food intake) directly at the beginning of the dark phase. Food was removed and blood obtained before injection (0 h) or at 1, 2 and 3 h post injection and acyl as well as total ghrelin levels assessed by ELISA. Desacyl ghrelin was calculated as the difference of total minus acyl ghrelin.

*5. Monitoring of behavior in rats injected intraperitoneally with ghrelin-O-acyltransferase inhibitor*

*Ad libitum* fed naive rats were habituated to the system and on the day of the experiment the amount of bedding was reduced and a paper grid dividing the cage into 6 squares was placed underneath the cage. Directly before the dark phase started rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor GO-CoA-Tat (96 µg/kg in 300 µl saline, the dose that induced the most pronounced reduction of food intake). Behavior was monitored during the 2$^{nd}$ h post injection, the period when GOAT inhibition showed the maximum reduction of food intake.

*Statistical analysis*

Data are expressed as mean ± S.E.M. Distribution of the data was determined by using the Kolmogorov-Smirnov test. Differences between two groups were assessed using the t-test, one-way ANOVA followed by all pair-wise multiple comparison procedures (Tukey post hoc test) or two-way ANOVA followed by Holm-Sidak method. Differences were considered significant when $P < 0.05$ (SigmaStat 3.1., Systat Software, San Jose, CA, USA).

## RESULTS

*Rats show normal body weight gain when housed individually and quickly adapt to the automated food intake monitoring system*

Naive, group-housed rats showed a linear body weight gain during the first four days (3.1 ± 1.5 g/day, *Fig. 1*). On the day of separation, there was a slight decrease in body weight (–1.5 ± 0.8 g). This quickly faded and rats housed individually and fed from the cage tops again showed a linear body weight gain of 3.6 ± 1.3 g/day (*Fig. 1*). After providing food from the food hopper instead of the top of the cage, the linear body weight gain was also observed (2.7 ± 0.1 g/day; P = 0.71 compared to previous time points; *Fig. 1*).

We next compared the food intake of naive rats housed in individual cages and assessed manually with food intake assessed by the automated food intake monitoring system. Neither the dark phase (18.8 ± 0.4 vs. 17.8 ± 0.7 g/200 g b.w.), light phase (1.5 ± 0.3 vs. 1.9 ± 0.7 g/200 g b.w.) nor the total 24-h food intake (20.3 ± 0.5 vs. 19.7 ± 0.3 g/200 g b.w.) differed between the two methods of assessment (P = 0.43). Likewise, when assessed at different time points after providing food from the feeding hopper (days 1 and 2 compared to days 5 and 6 of the habituation period), no differences of dark phase (17.5 ± 0.7 vs. 17.8 ± 0.7 g/200 g b.w., P = 0.79), light phase (1.8 ± 0.4 vs. 1.9 ± 0.7 g/200 g b.w., *P* = 0.94) and total 24-h food intake (19.3 ± 0.5 vs. 19.7 ± 0.3 g/200 g b.w., P = 0.59) were observed.

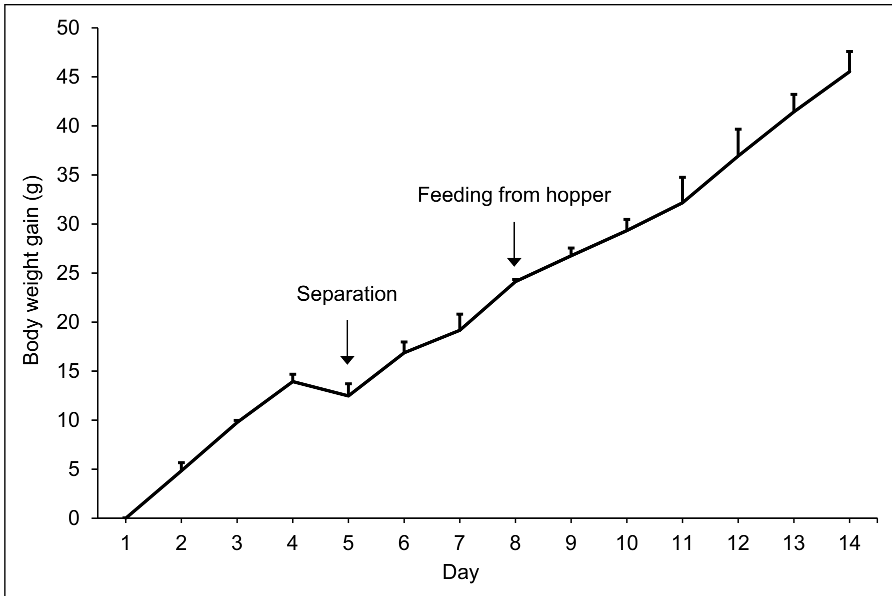


*Fig. 1.* Body weight gain in rats before and after separation. Rats were housed in groups of three and then on day five separated in single housing cages with eye and odor contact. Food was provided from the top of the cage and on day eight from the hopper of the automated feeding monitoring system. Body weight was assessed daily and expressed as body weight gain. Data are presented as mean ± S.E.M., n = 6.

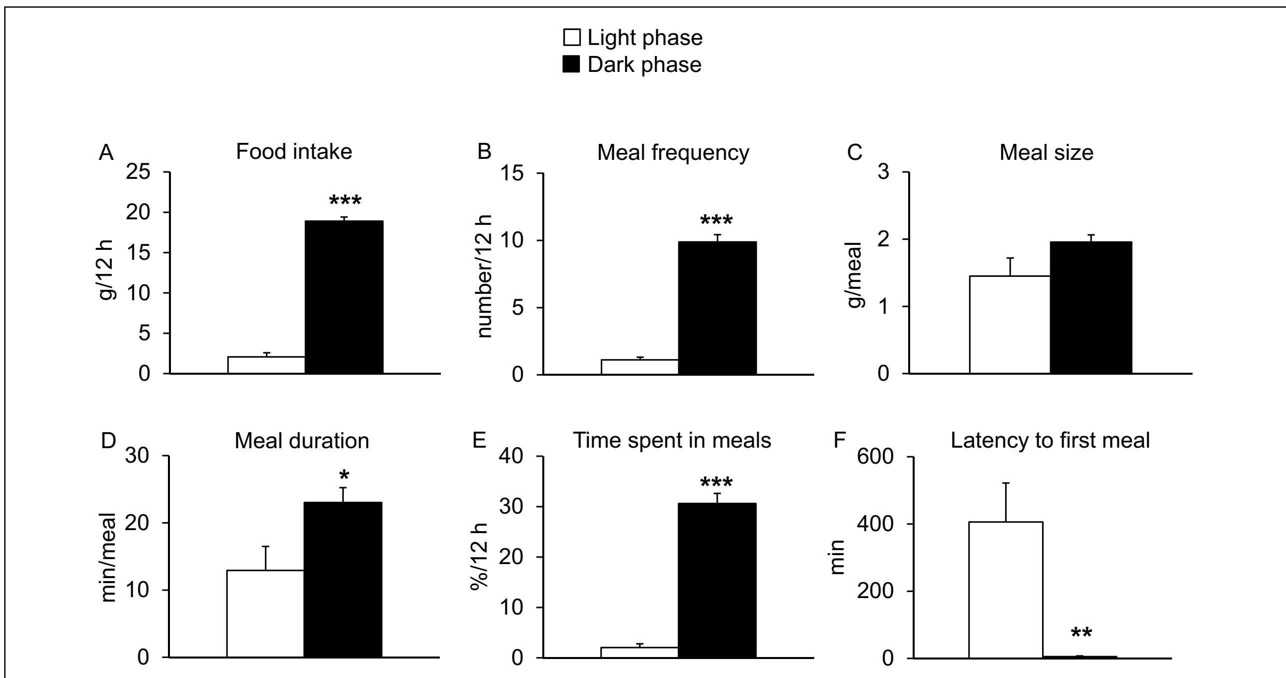


*Fig. 2.* Food intake microstructure during the light and dark photoperiod. Food intake (A) and the underlying food intake microstructure encompassing meal frequency (B), meal size (C), meal duration (D), time spent in meals (E) and the latency to the first meal (F) were assessed over a period of 24 h and the parameters compared for light (6:00 AM to 6:00 PM) versus dark phase (6:00 PM to 6:00 AM). Each bar represents the mean ± S.E.M. of 9 rats/group. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs. light phase.

*Undisturbed rats show a greater food intake at night compared to the light phase which is associated with a higher meal frequency and longer duration but not meal size*

We investigated the food intake microstructure for dark and light phase meals in individually housed undisturbed rats fed normal rat chow and habituated to the food intake monitoring system. At night, rats showed a 9.1-times greater food intake compared to light phase intake ($P < 0.001$; *Fig. 2A*). This increase was associated with a higher meal frequency (8.9-times, $P < 0.001$; *Fig. 2B*), longer meal duration (1.8-times, $P < 0.05$; *Fig. 2D*) and more time spent in meals (15.0-times, $P < 0.001$; *Fig. 2E*), whereas the meal size was not significantly larger compared to the light phase (1.3-times, $P = 0.13$; *Fig. 2C*). Also the latency to the first meal was shorter (75-times) in the dark compared to the light phase ($P < 0.01$; *Fig. 2F*).

*A physiological behavioral satiety sequence is observed in rats housed in automated food intake monitoring cages*

The behavioral satiety sequence was investigated manually at the beginning of the dark phase in rats housed in cages of the automated food intake monitoring system. Feeding behavior initially increased up to a maximum

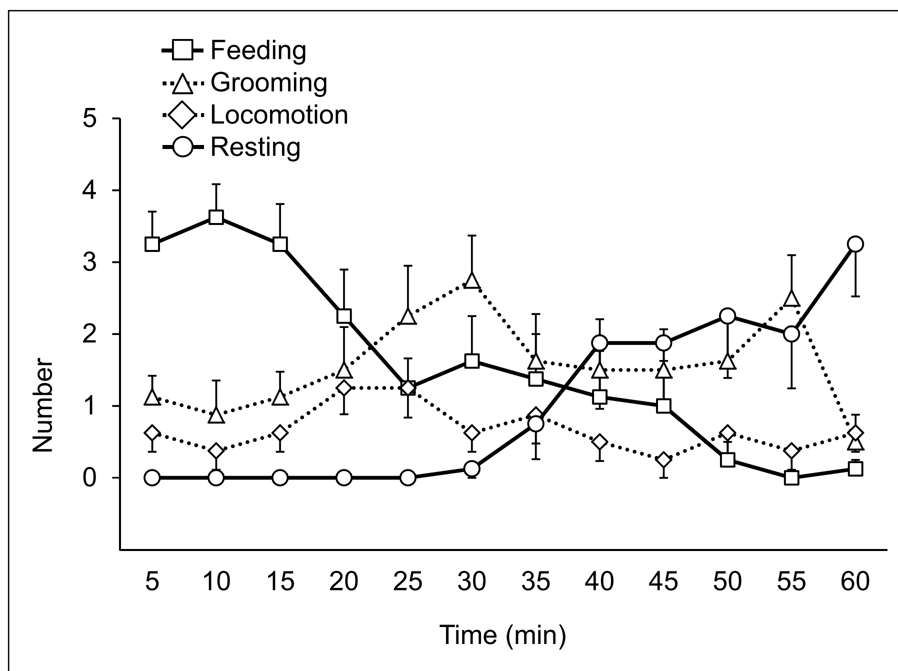


*Fig. 3.* The behavioral satiety sequence observed in rats housed in cages of the automated feeding monitoring system. Rats were single-housed in regular cages connected to the automated food intake monitoring system. While food intake was measured automatically, the behavior consisting of feeding, grooming, locomotion and resting was monitored manually at the beginning of the dark phase (6:00 PM to 7:00 PM) over a period of one hour. The physiological behavioral satiety sequence was observed with a decrease of dark phase feeding behavior and an increase in grooming, locomotion and particularly resting. Each line represents the mean ± S.E.M. of 8 rats/group.

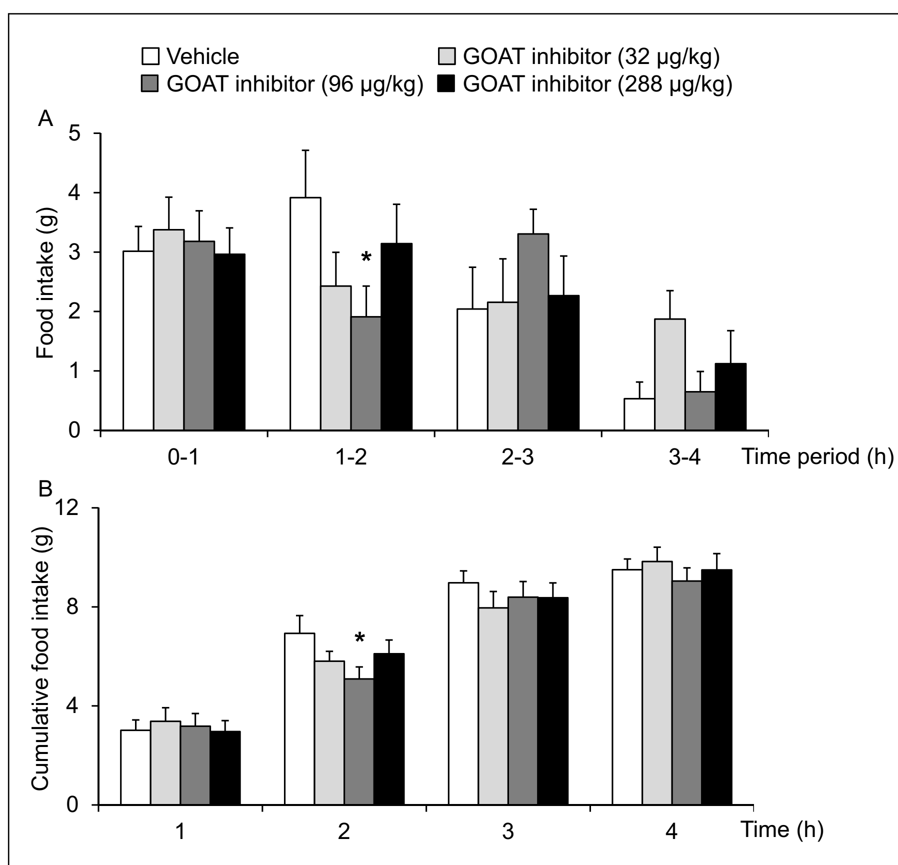


*Fig. 4.* Dark phase food intake in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (32, 96 or 288 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake was monitored using the automated food intake monitoring system and expressed as hourly (A) or cumulative (B) food intake. Each bar represents the mean ± S.E.M. of 9 – 11 rats/group. * $P < 0.05$ vs. vehicle.

observed at 10 min (3.6 ± 0.5) and then gradually decreased reaching a nadir at 60 min (0.1 ± 0.1; *Fig. 3*). Grooming behavior showed the opposite pattern with low values at the beginning (1.1 ± 0.3) and a gradual increase until 30 min (2.8 ± 0.6). Afterwards, a temporary decrease was observed at 35 min (1.6 ± 0.7) followed by an increase reaching 2.5 ± 0.6 at 55 min and a decrease at 60 min (0.5 ± 0.4, *Fig. 3*). Locomotion remained fairly stable over the 1-h observation period (e.g. 30 min: 0.6 ± 0.3, *Fig. 3*). Resting behavior was absent at the beginning (5 min: 0.0 ± 0.0) and gradually increased reaching a maximum at 60 min (3.3 ± 0.7, *Fig. 3*). The lines of feeding and resting behavior crossed between 35 and 40 min (*Fig. 3*). No abnormal behavior was observed during this experiment.

*Table 1.* Food intake in rats fed *ad libitum* and injected with vehicle or GOAT inhibitor intraperitoneally before the dark phase.

| **Food intake (g)** | **Group Vehicle** (n = 10) | **GOAT inhibitor** (32 µg/kg, n = 11) | **GOAT inhibitor** (96 µg/kg, n = 9) | **GOAT inhibitor** (288 µg/kg, n = 10) |
|---|---|---|---|---|
| **Food intake per period** | | | | |
| 0–4 h | 9.5 ± 0.4 | 9.8 ± 0.6 | 9.0 ± 0.5 | 9.5 ± 0.7 |
| 4–8 h | 7.6 ± 0.7 | 6.1 ± 0.6 | 8.3 ± 0.5 | 6.6 ± 0.6 |
| 8–12 h | 3.7 ± 0.9 | 3.4 ± 0.9 | 2.1 ± 0.7 | 3.8 ± 0.7 |
| 12–16 h | 0.5 ± 0.3 | 0.4 ± 0.3 | 0.4 ± 0.3 | 0.3 ± 0.2 |
| 16–20 h | 0.3 ± 0.2 | 0.5 ± 0.2 | 0.1 ± 0.1 | 0.4 ± 0.3 |
| 20–24 h | 2.7 ± 0.3 | 2.3 ± 0.4 | 3.3 ± 0.4 | 2.2 ± 0.4 |
| **Cumulative food intake** | | | | |
| 4 h | 9.5 ± 0.4 | 9.8 ± 0.6 | 9.0 ± 0.5 | 9.5 ± 0.7 |
| 8 h | 17.1 ± 0.8 | 16.0 ± 0.7 | 17.3 ± 0.7 | 16.1 ± 0.6 |
| 12 h | 20.9 ± 0.6 | 19.4 ± 0.5 | 19.4 ± 0.9 | 19.8 ± 0.6 |
| 16 h | 21.3 ± 0.5 | 19.8 ± 0.5 | 19.8 ± 0.7 | 20.1 ± 0.5 |
| 20 h | 21.6 ± 0.4 | 20.3 ± 0.4 | 19.9 ± 0.7 | 20.5 ± 0.5 |
| 24 h | 24.4 ± 0.5 | 22.6 ± 0.6 | 23.3 ± 0.6 | 22.7 ± 0.5 |

Mean ± S.E.M. No significant differences were observed.

*Table 2.* Food intake microstructure of the first meal in rats fed *ad libitum* and injected with vehicle or GOAT inhibitor intraperitoneally before the dark phase.

| **Parameter** | **Vehicle** (n = 10) | **GOAT inhibitor** (96 µg/kg, n = 9) |
|---|---|---|
| Latency to first meal (min) | 4.0 ± 1.1 | 4.9 ± 1.3 |
| Size of first meal (g) | 2.8 ± 0.4 | 2.7 ± 0.3 |
| Duration of first meal (min) | 25.9 ± 5.3 | 21.2 ± 4.8 |
| Eating rate of first meal (mg/min) | 38.3 ± 5.7 | 28.6 ± 3.3 |
| Inter-meal interval (min) | **52.4 ± 6.9** | **76.9 ± 5.9*** |
| Satiety ratio after first meal (min/g food eaten) | **21.8 ± 3.6** | **30.3 ± 3.1*** |

Mean ± S.E.M. Significant differences are shown in bold. * P < 0.05.

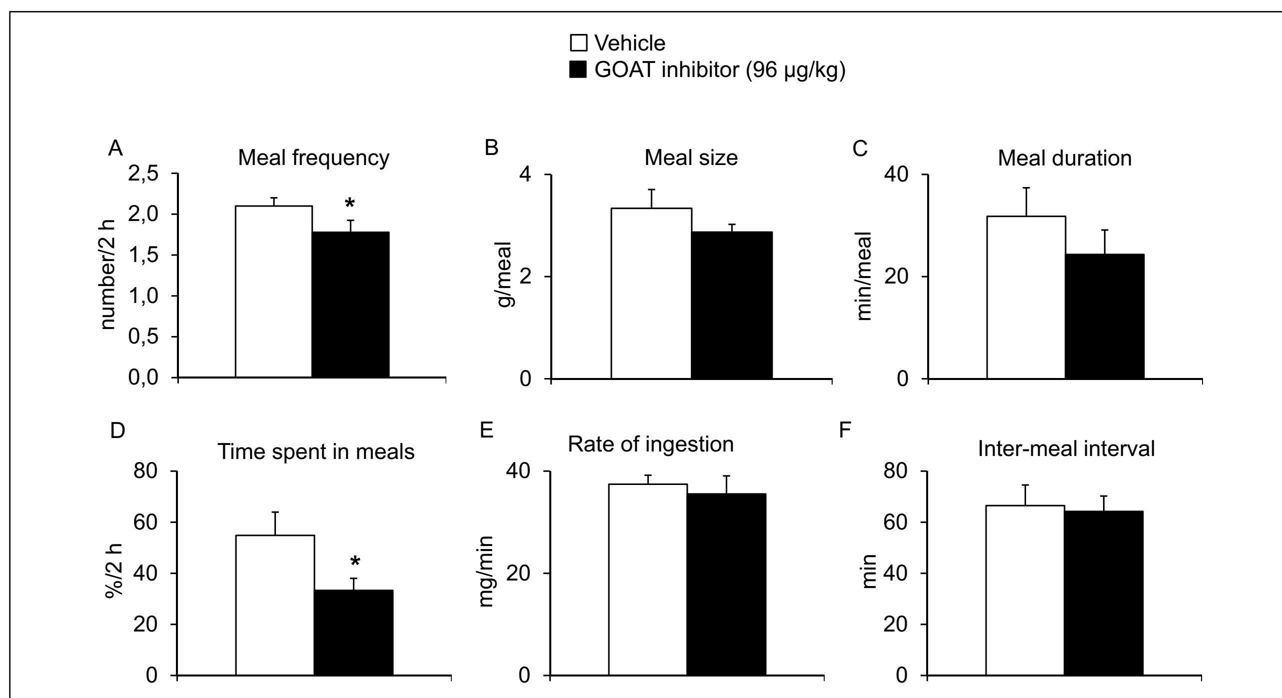


*Fig. 5.* Food intake microstructure in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase and food intake microstructure encompassing meal frequency (A), meal size (B), meal duration (C), time spent in meals (D), rate of ingestion (E) and inter-meal interval (F) was assessed using the automated food intake monitoring system and analyzed for the first 2 h post injection. Each bar represents the mean ± S.E.M. of 9 – 10 rats/group. * P < 0.05 vs. vehicle.

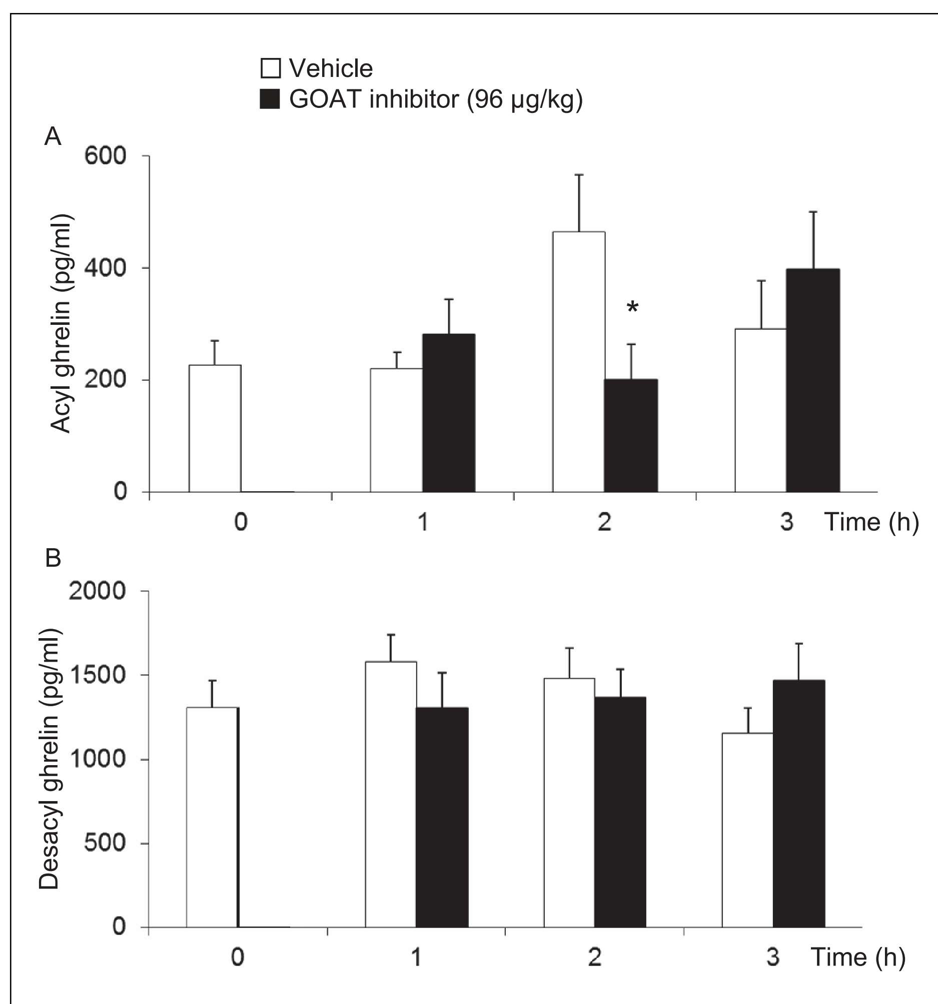


*Fig. 6.* Circulating acyl and desacyl ghrelin levels in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase. Food was removed but rats had access to water. Blood was obtained at 0, 1, 2 or 3 h post injection and acyl as well as total ghrelin levels measured by ELISA. Desacyl ghrelin levels were calculated by subtracting total minus acyl ghrelin levels for each rat. Each bar represents the mean ± S.E.M. of 5 – 6 rats/group. * P < 0.05 vs. vehicle.

*The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat reduces dark phase food intake by a reduction of meal frequency while meal size is not altered*

Injection of the GOAT inhibitor at the beginning of the dark phase led to a dose dependent reduction of food intake compared to vehicle (*Fig. 4A*). The reduction was delayed in onset and observed during the second hour post injection, and the dose response of the GOAT inhibitor seems to be U-shaped with a maximum effect at 96 µg/kg (–27%, P = 0.03; *Fig. 4A*). This resulted in a reduction of the 2-h cumulative food intake (P = 0.03; *Fig. 4B*). Two way ANOVA indicated a significant influence of time ($F_{3,159}$ = 10.7, P < 0.001). After 4 h, no significant differences were observed between rats injected with GOAT inhibitor or vehicle (P > 0.05; *Table 1*).

Based on these data the dose of 96 µg/kg and the period of 2 h were used for the analysis of the food intake microstructure. The GOAT inhibitor led to a reduction of meal frequency (–15%, P = 0.04; *Fig. 5A*) and the time spent in meals (–39%, P = 0.03; *Fig. 5D*), whereas meal size (P = 0.29; *Fig. 5B*), meal duration (P = 0.33; *Fig. 5C*), rate of ingestion (P = 0.63; *Fig. 5E*) and the inter-meal interval (P = 0.83; *Fig. 5F*) were not altered during the 2-h period compared to vehicle. However, when analyzing the food intake microstructure of the first meal, the interval following the first meal was prolonged after injection of the GOAT inhibitor (+47%, P = 0.02) leading to an increased satiety ratio compared to vehicle (+39%, P < 0.05; *Table 2*).

*The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat prevents the increase of acyl ghrelin levels during the dark phase while desacyl ghrelin is not altered*

Baseline levels of acyl ghrelin at the beginning of the dark phase were 226.2 ± 43.8 pg/ml (*Fig. 6A*). At 1 h post injection, no significant differences were observed between rats injected with vehicle vs. the GOAT inhibitor group (P = 0.39; *Fig. 6A).* At 2 h post injection, rats injected with GOAT inhibitor displayed a –57% reduction of acyl ghrelin levels compared to vehicle injected rats (P = 0.03), while after 3 h no significant difference was observed (P = 0.45; *Fig. 6A).* Two way ANOVA indicated a significant interaction of treatment × time ($F_{(2,29)}$ = 3.6, P = 0.04).

Baseline levels of desacyl ghrelin at the beginning of the dark phase were 1305.9 ± 160.1 pg/ml (*Fig. 6B*). No significant differences were observed at either time point between rats injected with vehicle or GOAT inhibitor (P > 0.27; *Fig. 6B).* Two way ANOVA indicated no significant impact of treatment ($F_{(1,30)}$ = 0.03, P = 0.88), time ($F_{(2,30)}$ = 0.24, P = 0.78) or an interaction of treatment × time ($F_{(2,30)}$ = 1.1, P = 0.34).

*The ghrelin-O-acyltransferase inhibitor GO-CoA-Tat reduces grooming behavior while locomotion is not altered*

Rats injected with the GOAT inhibitor, GO-CoA-Tat showed a –21% reduction of 2-h food intake compared to vehicle treated rats (data not shown). Behavioral assessment during the 2$^{nd}$ h post injection, the period where rats had shown the maximum

*Fig. 7.* Behavior in rats intraperitoneally injected with the GOAT inhibitor. *Ad libitum* fed rats were injected intraperitoneally with vehicle (pyrogen-free saline, 300 µl) or the GOAT inhibitor, GO-CoA-Tat (96 µg/kg in 300 µl saline) directly at the beginning of the dark phase. Single housed rats with paper divided into six equal squares that was placed under their home cage had *ad libitum* access to food and water throughout the experiment. During the 2nd hour post injection behaviors, including eating (including food approach, A), drinking (including water approach, B), grooming behavior (washing, licking, and scratching; C) and locomotor activity (total number of squares crossed; D) were monitored manually for 1 h by two observers. Each behavior was counted again when lasting > 5 s. Bars indicate means ± S.E.M. of 6 rats/group. ** P < 0.01 vs. vehicle.

reduction of food intake, indicated that eating behavior (including food approach, *Fig. 7A*) and drinking behavior (including water approach, *Fig. 7B*) were not different between the two groups. Injection of the GOAT inhibitor reduced grooming behavior (–60%, P < 0.01; *Fig. 7C*), while locomotor activity was not altered compared to vehicle (–2.4%, P = 0.89; *Fig. 7D*). No signs of abnormal behavior were observed following treatment with GO-CoA-Tat (data not shown).

## DISCUSSION

Using an automated food intake monitoring device in the present study we show that the GOAT inhibitor, GO-CoA-Tat reduces early dark phase food intake. By analyzing the underlying food intake microstructure, this reduction is due to a decrease in meal frequency, while meal size is not significantly altered.

Food intake is often assessed in animal experiments and the interest is steadily growing in light of the increasing prevalence of human obesity (30, 31) and the consecutive need for a better understanding of the mechanisms regulating hunger and satiety. The manual measurement of food intake is the classical approach; however, this assessment might disturb the animals and does not provide information on the underlying food intake microstructure. Early on, measurement techniques were developed to gain insight into the food intake microstructure including the measurement of consumed liquid (32, 33), powder (34, 35) or micropelleted food (36, 37). However, all these formulations of food do not represent the physiological type of food used in most studies where food intake is assessed manually. Therefore, systems for the assessment of the food intake microstructure using regular solid rat chow have been developed (38, 39). In the present study we used an automated episodic food intake monitoring device to monitor the food intake microstructure of solid food in undisturbed rats. Although the system has been used in rats before (20-22) and validated for mice (24), the validation was lacking for rats. Therefore, the first step was to validate the system.

Rats showed a rapid habituation to the episodic food intake monitoring system as indicated by the linear continuation of body weight gain despite the single housing and feeding out of a food hopper. Moreover, the system shows good concordance to manual food intake monitoring providing the same amounts of food ingested in either photoperiod. In addition, the system allows for assessment of the underlying food intake microstructure which provides detailed insight into the mechanisms involved in the modulation of food intake under the respective experimental condition without any disturbance of the animals by the investigator or a light source.

It is important to note that rats maintained in the BioDAQ system showed a physiological behavior following food intake, which was assessed using the behavioral satiety sequence, a parameter established several decades ago (25, 40). The behavioral satiety sequence represents a consecutive progression of behaviors following food intake in rats encompassing feeding itself, grooming, exploration and resting. The behavioral satiety sequence is considered physiological if two major requirements are met: the final item 'resting' is observed and there is a lack of abnormal behavior during the test (41). In the present study we assessed the occurrence of the behavioral satiety sequence manually in rats housed in cages of the automated food intake monitoring device and observed an initial surge of feeding behavior, a period of grooming and a transition towards a predominant occurrence of resting behavior. The lines of feeding and resting behavior crossed between 35 and 40 min indicating the occurrence of satiety around that time as described before (42-45). No abnormal behavior or signs of sickness were observed. These findings indicate the occurrence of physiological satiety under the present housing conditions.

After these initial experiments we investigated the modulation of food intake using the GOAT inhibitor, GO-CoA-Tat that was introduced by Barnett and colleagues showing an inhibition of GOAT in cell lines stably expressing GOAT and preproghrelin as well as *in vivo* in mice (46). Intraperitoneal injection of the GOAT inhibitor reduced dark phase food intake in freely fed rats. Interestingly, this dose-dependent reduction showed a U-shaped relationship with a maximum effect at 96 µg/kg. Whether higher doses have additional agonistic or unspecific effects needs to be further investigated. The reduction of food intake by GO-CoA-Tat was delayed in onset and observed mainly in the second hour post injection. This is likely due to the fact that circulating ghrelin is already up-regulated at the beginning of the dark phase (47), the phase rats usually eat (26). Considering the half-life of ghrelin of around 30 min (48), an inhibition of GOAT should result in measurable effects of reduced ghrelin signaling with a lag phase in line with the delay observed in the present study. The effect on food intake was short lasting and only observed during the first 2 h, likely due to the clearance of the GOAT inhibitor, GO-CoA-Tat. These hypotheses are corroborated by the alterations of acyl ghrelin observed. While no change of acyl ghrelin levels is detected at 1 h post injection, treatment with GO-CoA-Tat prevents the dark phase related increase of acyl ghrelin which results in a more than 50% difference compared to saline treated rats at 2 h likely underlying the reduction of food intake observed. Interestingly, no modulation of desacyl ghrelin is observed giving rise to a specific effect on the acylation of ghrelin.

Analysis of the food intake microstructure of the first 2 h post injection showed that inhibition of GOAT decreases food intake by a reduction of meal frequency and a prolongation of the interval after the first meal, while meal size is not altered. In addition, the satiety ratio was also increased following inhibition of GOAT. These data give rise to an induction of satiety (mechanisms causing a later onset of the next meal after one meal is completed) (18, 19), while satiation (mechanisms causing meal termination) is not affected. Partly corresponding to these data, Tabarin *et al*. reported an increase of meal size and meal frequency in mice following intraperitoneal injection of the ghrelin agonist, BIM-28131 (49). The differential effects of GOAT inhibition in the present (alteration of satiety while satiation is not affected) and stimulation of ghrelin signaling in the study using the ghrelin agonist, BIM-28131 (alteration of satiation and satiety) may be due to species differences (rats *versus* mice), the assessment method of food intake (micropellet *versus* regular solid rat chow) or reflect additional pharmacological properties of the ghrelin agonist, BIM-28131.

To exclude unspecific effects of GOAT inhibition on behavior and to investigate additional behavioral alterations besides food intake, these were measured manually. Interestingly, although inhibition of GOAT in this experiment reduced food intake by 21% in the first 2 h post injection, behavioral analysis during the 2nd hour, the period when the greatest reduction of food intake was observed before, showed that eating behavior which included eating itself but also food approach (sniffing and licking food) was not different between the two groups. This indicates that, although food intake is reduced, the overall interaction with the food is not altered by GOAT inhibition. Whether this is due to an incomplete blockade of ghrelin acylation or a compensatory effect of other hormones will have to be further investigated. Similar to the effect on eating behavior, also drinking behavior (including water approach) was not different between the two groups. Also locomotor activity was not reduced pointing towards the absence of unspecific sickness and nausea induced by the compound. Interestingly, GOAT inhibition reduced grooming behavior compared to vehicle which may be a subsequent effect due to the reduced food intake as the physiological satiety sequence progresses from food intake to grooming behavior (25, 40). On the other hand, it may also indicate a direct effect as acyl ghrelin was shown to increase grooming behavior in rats (50). Overall, injection of the GOAT inhibitor does not seem to induce sickness or abnormal behaviors, further pointing towards a specific effect on ghrelin acylation.

In summary, in the present study we validated an automated food intake monitoring system for the assessment of food intake microstructure of regular rat chow in undisturbed rats. Importantly, rats housed in these cages show a normal feeding behavior as indicated by a physiological behavioral satiety sequence. Using this system we showed that pharmacological peripheral inhibition of GOAT *via* a reduction of acyl ghrelin levels reduces dark phase food intake with a delayed onset and short duration by an increase of satiety, while satiation is not affected.

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Author's address: Dr. Andreas Stengel, Charite Universitaetsmedizin Berlin, Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, 30 Hindenburgdamm Street, 12200 Berlin, Germany
E-mail: andreas.stengel@charite.de

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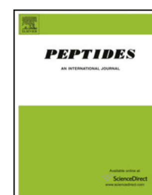

# The ghrelin activating enzyme ghrelin-*O*-acyltransferase (GOAT) is present in human plasma and expressed dependent on body mass index

Miriam Goebel-Stengel[a,1], Tobias Hofmann[b,1], Ulf Elbelt[c], Pauline Teuffel[b], Anne Ahnis[b], Peter Kobelt[b], Nils W.G. Lambrecht[d], Burghard F. Klapp[b], Andreas Stengel[b,*]

[a] *Department of Internal Medicine, Institute of Neurogastroenterology and Motility, Martin-Luther Hospital, Academic Teaching Institution of Charité-Universitätsmedizin Berlin, Berlin, Germany*
[b] *Charité Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charité-Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany*
[c] *Division of Endocrinology, Diabetes and Nutrition, Charité-Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany*
[d] *Gastrointestinal Endocrinology, Veterans Affairs Long Beach Healthcare System, Long Beach, CA, USA*

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ABSTRACT

Ghrelin is the only known peripherally produced and centrally acting peptide hormone stimulating food intake. The acylation of ghrelin is essential for binding to its receptor. Recently, the ghrelin activating enzyme ghrelin-*O*-acyltransferase (GOAT) was identified in mice, rats and humans. In addition to gastric mucosal expression, GOAT was also detected in the circulation of rodents and its expression was dependent on metabolic status. We investigated whether GOAT is also present in human plasma and whether expression levels are affected under different conditions of body weight. Normal weight, anorexic and obese subjects with body mass index (BMI) 30–40, 40–50 and >50 were recruited ($n = 9$/group). In overnight fasted subjects GOAT protein expression was assessed by Western blot and ghrelin measured by ELISA. GOAT protein was detectable in human plasma. Anorexic patients showed reduced GOAT protein levels (−42%, $p < 0.01$) whereas obese patients with BMI > 50 had increased concentrations (+34%) compared to normal weight controls. Ghrelin levels were higher in anorexic patients compared to all other groups (+62–78%, $p < 0.001$). Plasma GOAT protein expression showed a positive correlation with BMI ($r = 0.71$, $p < 0.001$) and a negative correlation with ghrelin ($r = -0.60$, $p < 0.001$). Summarized, GOAT is also present in human plasma and GOAT protein levels depend on the metabolic environment with decreased levels in anorexic and increased levels in morbidly obese patients. These data may indicate that GOAT counteracts the adaptive changes of ghrelin observed under these conditions and ultimately contributes to the development or maintenance of anorexia and obesity as it is the only enzyme acylating ghrelin.

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## 1. Introduction

Hunger and satiety are regulated by a complex network of transmitters. In opposition to a multitude of food intake suppressing (anorexigenic) hormones, ghrelin is the only known peripherally produced and centrally acting hormone that stimulates food intake (orexigen) [16]. Ghrelin is a 28 amino acid peptide with a unique fatty acid modification on the serine in position 3 [5] essential for binding to the ghrelin receptor [5,6] and consequently for the orexigenic action. The enzyme catalyzing the acylation of ghrelin, unknown for almost a decade, was recently identified in mice and humans as a member of the membrane-bound *O*-acyltransferases (MBOATs), MBOAT4 and then renamed ghrelin-*O*-acyltransferase (GOAT) [3,19]. Localization studies identified GOAT mRNA expression in ghrelin immunoreactive cells in mice [12]. However, GOAT protein expression studies were limited so far due to the restricted availability of specific antibodies. Recently, we reported GOAT protein expression in mouse and rat stomach with an exclusive distribution in ghrelin-producing X/A-like cells in mice and with additional occurrence in histamine secreting enterochromaffin-like cells (ECL cells) in rats [15]. Surprisingly, GOAT protein was also detected in the circulation of mice and rats and levels increased during fasting [15], a condition when acyl ghrelin levels also increase to stimulate feeding [9]. This led to the hypothesis of an extracellular acylation of ghrelin and a regulatory role of GOAT under different metabolic conditions. However, the situation in humans is not clear. Therefore, we investigated whether GOAT also occurs in the circulation of humans. In addition, we tested whether circulating GOAT is dependent on metabolic status in subjects with different body mass

* Corresponding author at: Charité Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charitéplatz 1, 10117 Berlin, Germany. Tel.: +49 30 450 553 002; fax: +49 30 450 553 900.
*E-mail address:* andreas.stengel@charite.de (A. Stengel).
[1] These authors contributed equally to this work.

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index (BMI), namely in anorexic subjects known to have increased circulating ghrelin levels [11] and in obese subjects described to display low ghrelin levels [18].

For correlation, ghrelin levels were assessed in these subjects.

## 2. Materials and methods

### 2.1. Subjects

All patients were hospitalized in the Division of Psychosomatic Medicine at Charité-Universitätsmedizin Berlin and gave written informed consent. Blood collection was performed on day 2 or 3 after hospital admission before the onset of changes due to dietary treatment to increase or reduce body weight, respectively. All parameters were assessed on the same morning. The protocol was approved by the local ethics committee for human research (protocol number EA1/114/10). A total of 45 subjects participated in this study and were divided in three groups: normal weight (BMI 18.5–25 kg/m$^2$), anorexia nervosa (BMI < 17.5 kg/m$^2$) and different stages of obesity (BMI 30–40 kg/m$^2$, BMI 40–50 kg/m$^2$ and BMI > 50 kg/m$^2$, $n$ = 9/group). Anorexic and obese patients were diagnosed according to the International Classification of Diseases-10 (ICD-10). In obese patients hypercortisolism was excluded by assessment of urinary free cortisol excretion in a 24 h sample or – in case of clinical suspicion – with a dexamethasone suppression test. Subjects had no history of gastrointestinal surgery except for appendectomy or cholecystectomy. All normal weight patients were exclusively hospitalized due to functional bodily symptoms (no patients with functional dyspepsia or irritable bowel syndrome) without relevant somatic disorders.

### 2.2. Blood collection

After an overnight fast, venous blood was collected between 07:00 and 08:00 am in chilled EDTA tubes containing aprotinin (0.6 trypsin inhibitor/0.5 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, USA) or serum tubes which were immediately centrifuged at 3000 rpm for 10 min at 4 °C. Plasma or serum, respectively, was separated and aliquots stored at −80 °C until further processing.

### 2.3. Measurements

#### 2.3.1. Western blot for GOAT

Crude protein fractions of plasma were prepared as described before [14,15]. Briefly, protein concentrations from plasma were determined using a BCA protein assay according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA). Gel samples were prepared by mixing protein samples with sample buffer (4% sodium dodecyl sulfate [SDS], 0.05% bromophenol blue [w/v], 20% glycerol, 1% mercaptoethanol [v/v] in 0.1 Tris buffer, pH 6.8). Samples were boiled for 1 min before gel electrophoresis and equal amounts of protein (20 μg/lane) were loaded on a 4–12% SDS polyacrylamide gel (SDS-PAGE, NuPage; Invitrogen, Carlsbad, CA, USA) and run in 2-(N-morpholino)ethanesulphonic acid buffer. Then, proteins were transferred by electrophoresis to nitrocellulose membranes (BioPlot-NC; Costar, Cambridge, MA, USA) for 1 h at 4 °C. Membranes were washed in distilled water and stained in Ponceau-S in 3% trichloroacetic acid solution and images were taken. Membranes were washed twice with Tween–Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, and 0.05% Tween [v/v]) and incubated in Tween-TBS containing 5% (w/v) nonfat milk (Carnation, Nestlé, Glendale, CA, USA). After 60 min, membranes were incubated in anti-GOAT polyclonal antibody (Catalog No. H-032-11, Phoenix Pharmaceuticals, Burlingame, CA, USA) diluted 1:1000 in Tween-TBS. This antibody was raised against amino acids 181–199 of human GOAT (manufacturer's information) and specificity was established by immunohistochemistry in human gastric tissue that showed staining of cells with an endocrine phenotype which colocalized with ghrelin (unpublished data). After 1 h, membranes were washed 5 times with Tween-TBS and incubated with the secondary antibody solution (anti-rabbit IgG conjugated to alkaline phosphatase; Promega, Madison, WI, USA) diluted 1:2000 in Tween-TBS. After 1 h, membranes were washed 3 times again before color development in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$ [pH 9.5]) containing 0.3% nitroblue tetrazolium solution (v/v) and 0.15% 5-bromo-4-chloro-3-indolyl-L-phosphate solution (v/v) for 5–10 min according to the manufacturer's instructions. Western blots for all samples ($n$ = 9/group) were repeated twice and pixel intensity (area under the curve) of each lane at the expected size of GOAT (50 kDa) analyzed using NIH Image J version 1.45. GOAT protein concentrations were normalized to circulating albumin.

#### 2.3.2. ELISA for ghrelin

Total ghrelin (catalog #EK-031-30) plasma levels were measured using a commercial enzyme-linked immunosorbent assay (human ghrelin ELISA, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA). The kit recognizes human ghrelin but also cross-reacts with rat ghrelin. Both forms of ghrelin, acyl as well as desacyl ghrelin are recognized by the antibody (manufacturer's information). Samples were processed in two batches; the intra-assay variability was 5% and the inter-assay variability 8%.

#### 2.3.3. Glucose and albumin

Blood glucose and serum albumin levels were assessed by routine laboratory procedures in the central hospital laboratory of Charité – Universitätsmedizin Berlin.

#### 2.3.4. Height and body weight

Body weight and height were assessed in overnight fasted subjects wearing underwear only and body mass index (BMI) calculated as kg/m$^2$.

### 2.4. Statistical analysis

Data are expressed as mean ± standard error of mean (SEM) and were analyzed by ANOVA followed by all pair-wise multiple comparison procedures (Tukey *post hoc* test). Correlations were determined by univariate linear regression. $p < 0.05$ was considered significant.

## 3. Results

### 3.1. GOAT is detectable in human plasma and dependent on body mass index

A Western blot of human plasma proteins stained with anti-GOAT antibody indicated one prominent band at 50 kDa corresponding to the molecular weight of the GOAT enzyme (Fig. 1) indicating that full length GOAT is also present in the human blood circulation.

Western blots of plasma from normal weight, anorexic and obese subjects with different stages of obesity suggested lower levels of GOAT protein in anorexic patients and higher levels in obese subjects with very high BMI of >50 kg/m$^2$ (Fig. 2A). Semi-quantitative analysis indicated a significantly lower GOAT protein concentration in anorexic patients (−42%, $p < 0.01$) compared to normal weight controls and higher plasma GOAT protein levels in obese subjects with BMI > 50 kg/m$^2$ (+34%, $p < 0.05$, Fig. 2B). Interestingly, obese patients with BMI 30–40 also had slightly lower

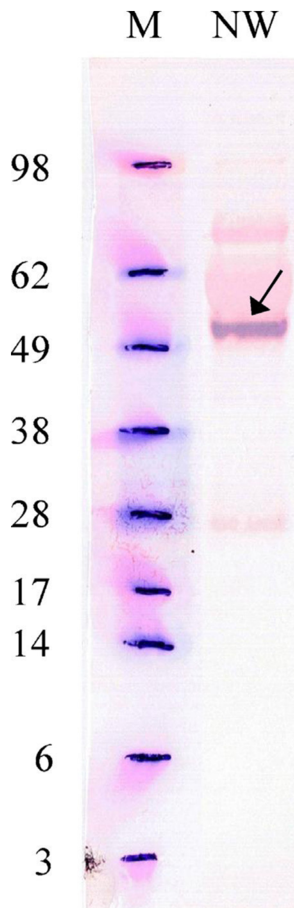


**Fig. 1.** Western blot for GOAT in human plasma. Lane 1 contains the molecular weight standard, lane 2 contains human plasma. The antibody recognizes a prominent band at the expected size of 50 kDa (arrow). *Abbreviations*: M, marker; NW, normal weight.

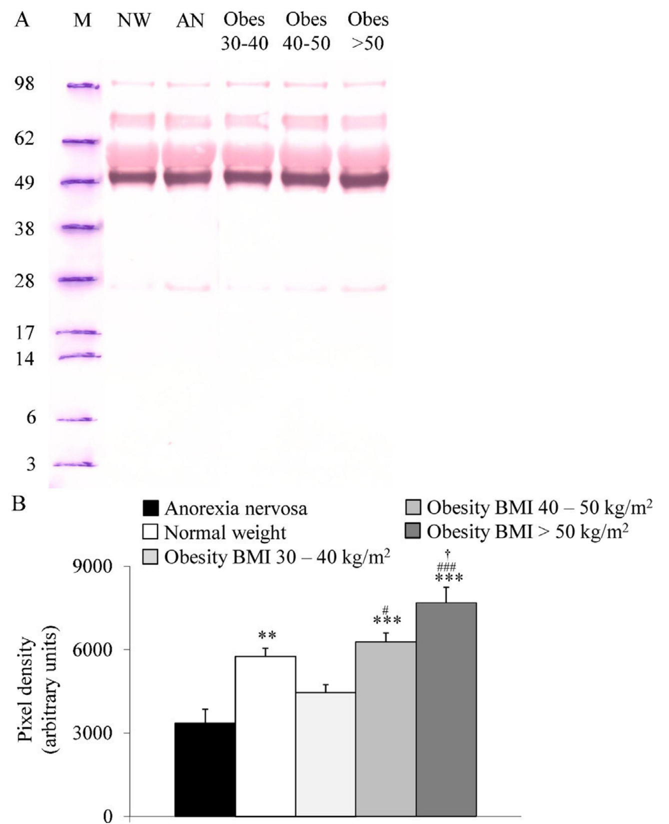


**Fig. 2.** GOAT in plasma of normal weight, anorexic and obese subjects with increasing body mass index. Lane 1 contains the molecular weight standard, lane 2 contains pooled plasma of normal weight (NW) subjects, lane 3 contains plasma of anorexia nervosa (AN) patients, lane 4 contains plasma of obese subjects with BMI between 30 and 40 kg/m$^2$ (Obes 30–40), lane 5 contains plasma of obese subjects with BMI between 40 and 50 kg/m$^2$ (Obes 40–50) and lane 6 contains plasma of obese subjects with BMI > 50 kg/m$^2$ (Obes > 50, A). Semi-quantitative analysis shows less GOAT protein in plasma of anorexic subjects compared to normal weight controls and an increased concentration in plasma of obese subjects with BMI > 50 kg/m$^2$ ($n$ = 9/group, B). $^{\dagger}p < 0.05$ *vs.* normal weight; $^{**}p < 0.01$ and $^{***}p < 0.001$ *vs.* AN; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ *vs.* Obes 30–40. *Abbreviation*: M, marker.

GOAT protein concentrations (−23%, $p = 0.01$) whereas GOAT protein levels of patients with BMI 40–50 were slightly higher (+9%, $p = 0.96$) compared to normal weight controls without reaching statistical significance (Fig. 2B).

While the anorexic group consisted of female patients only, normal weight (4 female, 5 male), the obese BMI 30–40 (5 female, 4 male), BMI 40–50 (5 female, 4 male) and BMI > 50 kg/m$^2$ groups (4 female, 5 male) encompassed both female and male patients. Neither in the individual groups (data not shown) nor in the whole study population differences in circulating GOAT protein concentrations were observed between female and male patients (5984 ± 360 *vs.* 6190 ± 430, $p = 0.73$).

### *3.2. Plasma GOAT shows a positive correlation with body mass index and a negative correlation with circulating ghrelin*

Normal weight subjects and the three different groups of obese patients did not differ in age whereas the anorexic patients were significantly younger (Fig. 3A). No correlation was observed between age and GOAT protein concentrations ($r = 0.15$, $p = 0.44$; Fig. 3B). Body height was not different in all groups (Fig. 3C) and circulating GOAT protein did not correlate with height ($r = 0.03$, $p = 0.98$; Fig. 3D). As expected, body weight significantly differed in all groups with an increase from anorexia to normal weight and different stages of obesity ($p < 0.01$; Fig. 3E). Univariate regression analysis showed a significant positive correlation of plasma GOAT protein concentration with body weight ($r = 0.70$, $p < 0.001$; Fig. 3F). Also, body mass index differed significantly in all groups ($p < 0.01$, Fig. 4A) and plasma GOAT concentrations showed a positive correlation with BMI ($r = 0.71$, $p < 0.001$; Fig. 4B). Circulating ghrelin levels were significantly higher in anorexic patients compared to all other groups ($p < 0.001$, Fig. 4C) and showed a significant negative correlation with circulating GOAT concentrations ($r = -0.60$, $p < 0.001$; Fig. 4D). Total ghrelin levels were also negatively correlated with BMI ($r = -0.47$, $p < 0.01$; Fig. 4E).

Serum albumin concentrations did not differ between normal weight and anorexic subjects, whereas obese patients with BMI 30–40 and >50 kg/m$^2$ had slightly reduced albumin concentrations compared to patients with anorexia nervosa (−8%, $p < 0.05$; Fig. 5A). No correlation was observed between albumin and GOAT protein concentrations corrected for albumin ($r = -0.19$, $p = 0.85$; Fig. 5B) or not corrected for circulating albumin levels ($r = 0.03$, $p = 0.85$; data not shown). Blood glucose levels were significantly higher in the three obesity groups compared to anorexia nervosa ($p < 0.05$; Fig. 5C). Plasma GOAT protein levels and blood glucose showed a trend toward a correlation without reaching statistical significance ($r = 0.30$, $p = 0.052$; Fig. 5D). Body mass index and blood glucose were positively correlated ($r = 0.48$, $p < 0.001$; Fig. 5E), whereas circulating ghrelin levels showed a negative correlation with blood glucose ($r = -0.38$, $p < 0.05$; Fig. 5F). In the three obesity groups, 37% had diabetes mellitus type 2, whereas normal weight and anorexic patients were devoid of diabetes mellitus. Ghrelin or GOAT data did not differ in patients with or without diabetes mellitus and results

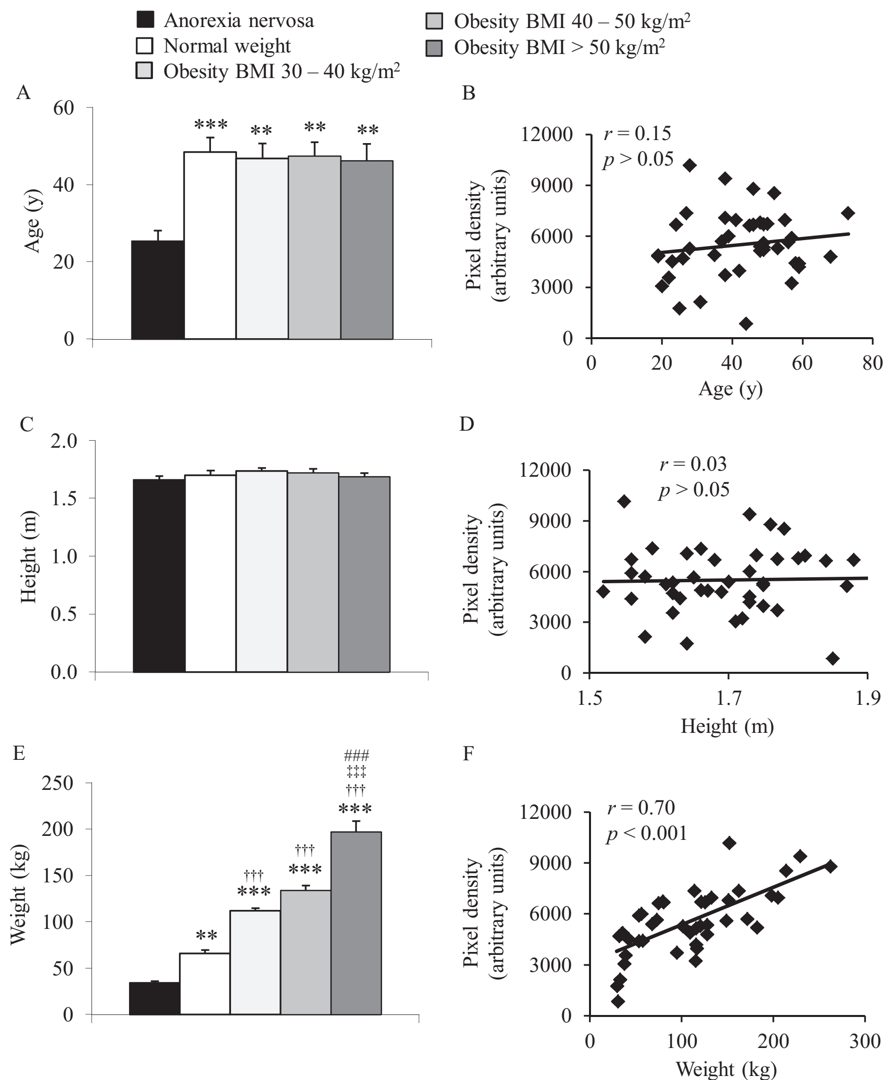


**Fig. 3.** Age, height and weight in the groups with different BMI and the correlation with GOAT protein concentration. Anorexic patients were younger than subjects of all other groups (A). No correlation was observed between GOAT and age (B). Height was not different in all groups (C) and no correlation was observed with GOAT (D). Body weight was different between groups (E) and plasma GOAT protein concentration showed a positive correlation with body weight (F). Values for *r* and *p* are indicated in each correlation graph. †††$p < 0.001$ *vs.* normal weight; **$p < 0.01$ and ***$p < 0.001$ *vs.* anorexia nervosa; ###$p < 0.001$ *vs.* obesity with BMI 30–40 kg/m$^2$; ‡‡‡$p < 0.001$ *vs.* obesity with BMI 40–50 kg/m$^2$.

were not different for ghrelin and GOAT when these patients were excluded from analysis (data not shown).

In the study population, mild-moderate gastritis was present in 1 of 9 patients in the normal weight group, in 2 of 9 anorexic patients, in 2 of 9 obese patients with BMI 30–40, in 1 of 9 of the BMI 40–50 group and in 3 of 9 patients with BMI > 50 kg/m$^2$. In these patients, Helicobacter pylori was detected histologically in one patient in the BMI 30–40 group and in one patient in the group of BMI > 50 kg/m$^2$. Data did not differ between patients with or without mild-moderate gastritis and results were not different for ghrelin and GOAT when these patients were excluded from analysis (data not shown).

## 4. Discussion

In previous studies, GOAT mRNA expression has been detected in various peripheral tissues in animals [3,19] and humans [7]. However, data on protein expression are scarce so far. We previously detected GOAT protein in the blood circulation of rats and mice [15]. In the present study we show that GOAT protein is also present in human plasma indicated by a strong band at the expected size of 50 kDa suggesting that GOAT is released into the circulation, likely derived from stomach and gut where high GOAT mRNA expression levels are known [7].

Previously, we have shown that circulating GOAT protein is upregulated in rats and mice during conditions of fasting [15]. This suggested a regulatory role of GOAT under these conditions based on the assumption that GOAT is a regulator of energy balance [4]. Here, we investigated the expression of GOAT protein under conditions of altered body weight in patients with anorexia nervosa or different stages of obesity. GOAT protein levels were lower in anorexic patients and higher in patients with severe obesity (BMI > 50 kg/m$^2$) compared to normal weight controls and obesity with BMI of 30–40 and 40–50 kg/m$^2$. This is not due to an overall alteration in circulating protein levels with albumin being the most abundant, as serum albumin concentrations did not greatly differ between normal weight, anorexic and obese subjects with different stages of obesity. Furthermore, GOAT protein levels were corrected for circulating albumin. The alteration of GOAT plasma protein levels in anorexic and severely obese subjects was also reflected in a positive correlation of plasma GOAT protein expression with BMI suggesting a regulation of GOAT dependent on long term metabolic

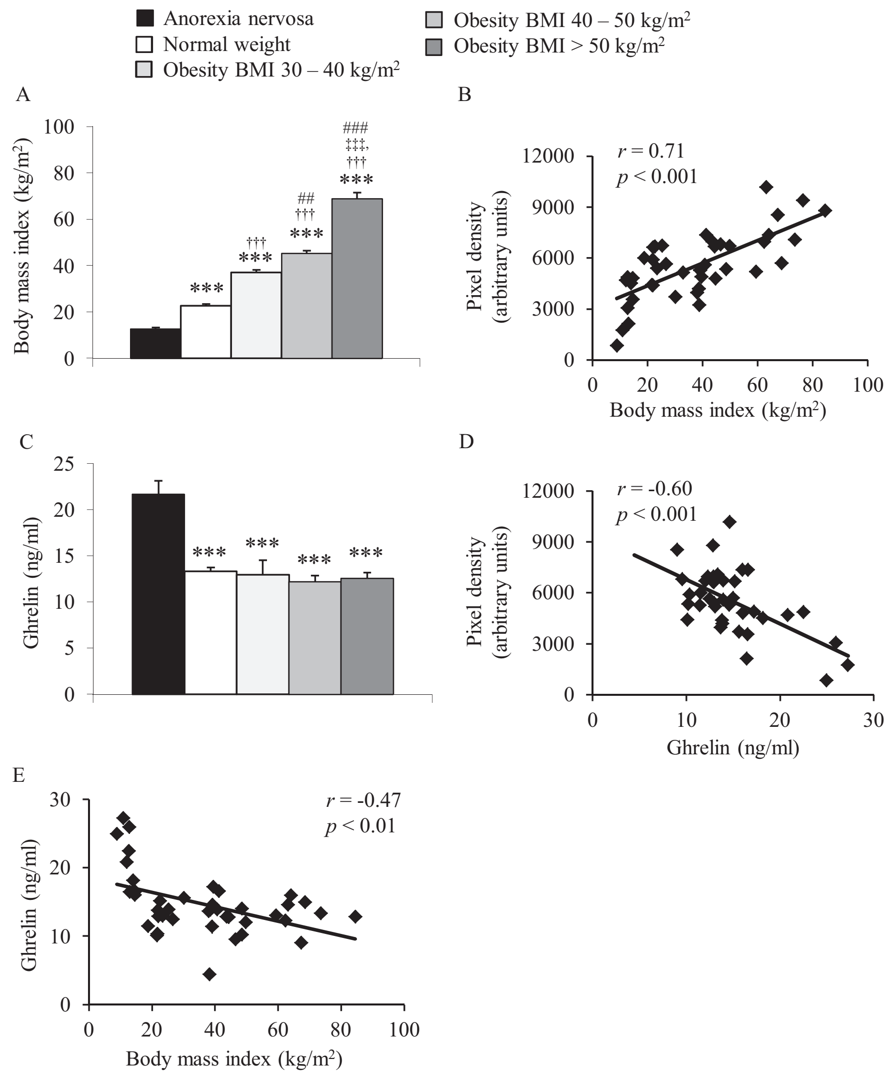


**Fig. 4.** Body mass index and circulating ghrelin levels in the groups with different BMI and the correlation with GOAT protein concentration. Body mass index was different between groups (A) and plasma GOAT protein concentration showed a positive correlation with body weight (B). Anorexic patients had higher ghrelin levels than all other groups (C) and plasma ghrelin showed a negative correlation with plasma GOAT protein concentration (D). Ghrelin also showed a negative correlation with body mass index (E). Values for *r* and *p* are indicated in each correlation graph. †††$p < 0.001$ *vs.* normal weight; ***$p < 0.001$ *vs.* anorexia nervosa; ##$p < 0.01$ and ###$p < 0.001$ *vs.* obesity with BMI 30–40 kg/m$^2$; ‡‡‡$p < 0.001$ *vs.* obesity with BMI 40–50 kg/m$^2$.

status. Since GOAT is the only enzyme known to acylate ghrelin as shown by the complete absence of acylated ghrelin in GOAT knockout mice [3], the changes of GOAT protein expression described here may contribute to the chronic alterations of body weight observed in these patients with reduced signaling under conditions of anorexia nervosa and increased signaling in obese patients.

In patients with anorexia nervosa we observed robustly increased ghrelin levels compared to all other groups. The increase of circulating ghrelin in anorexic patients is in line with previous studies [10,11,17] and thought to represent an adaptive change to stimulate appetite and promote body weight gain. Interestingly, in our study total ghrelin levels were not altered in obese subjects compared to normal weight controls. This finding is different from several previous studies showing a decrease under conditions of obesity [8,13,18]. The reason for this difference is not clear at this point. The three obesity groups had higher blood glucose levels compared to anorexic patients. The occurrence of obesity-associated type 2 diabetes mellitus (in 37% of the present obese study population) may impact on ghrelin levels as well as the related medication as metformin, commonly used to treat type 2 diabetes mellitus in Germany, was shown to significantly increase ghrelin levels by >20% [1]. However, only one patient was on medication with metformin at the time of the blood withdrawal so the influence of metformin on the ghrelin levels observed is less likely. Lastly, one has to note that when expressed as correlation, ghrelin levels still show the negative correlation with BMI in the present study as reported before [18].

Despite the fact that these data provide new insight into alterations of GOAT expression in humans, cautious interpretation is necessary due to the following limitations of the study. First, the number of patients is relatively small (five groups, $n = 9$/group) and despite significant differences between groups this finding should be corroborated in a larger sample. Second, while in obese and normal weight patients both men and women have been investigated only anorexic women were included in the study. This is based on the at least 10-fold higher prevalence of anorexia nervosa in women compared to men. However, no differences in GOAT expression levels between men and women have been observed, neither in the individual groups nor in the overall study population. Third, anorexic patients in this study were younger compared to the normal weight and obese groups which were age-matched. However, no correlation of GOAT expression with

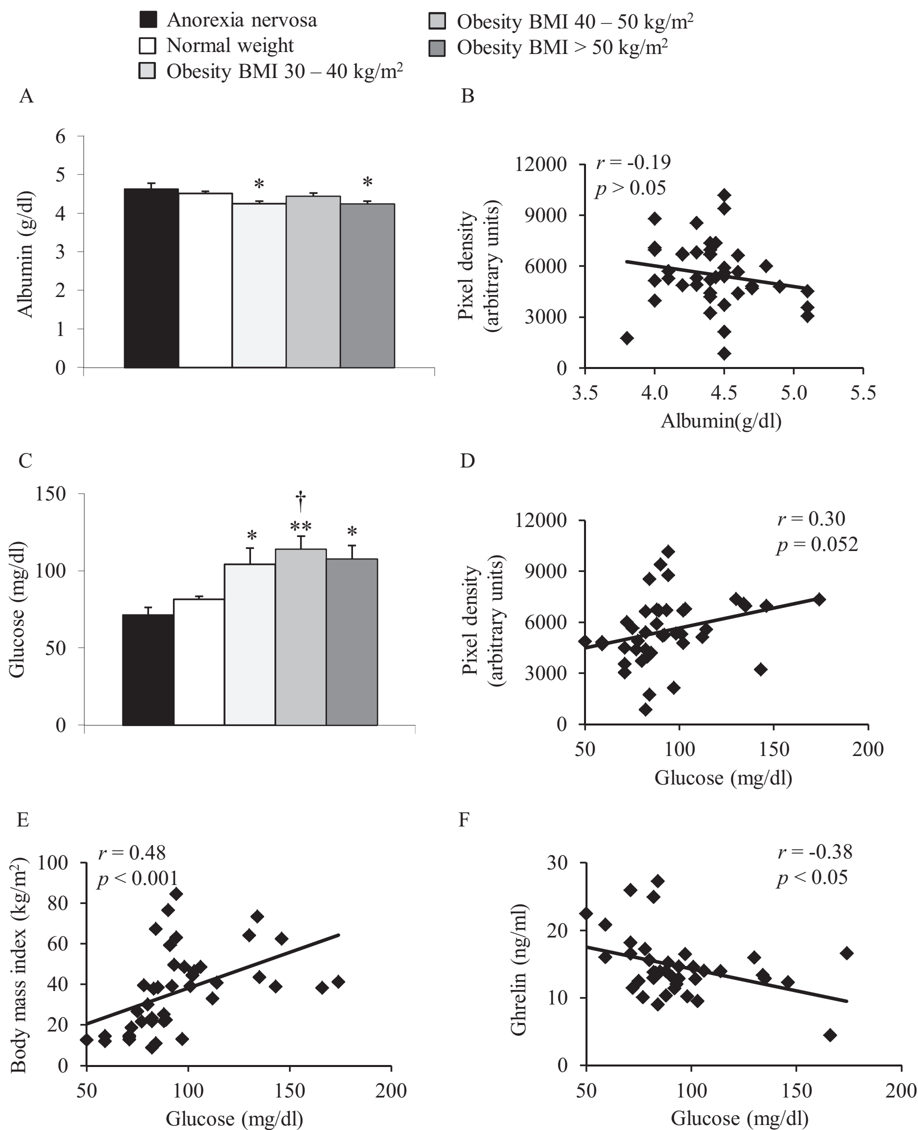


**Fig. 5.** Serum albumin and blood glucose levels in the groups with different BMI and the correlation with GOAT protein concentration, body mass index and circulating ghrelin. Albumin levels were lower in the BMI 30–40 kg/m$^2$ and >50 kg/m$^2$ groups compared to anorexia nervosa (A). No correlation was observed between albumin and plasma GOAT protein concentration (B). Blood glucose was lower in anorexic patients compared to the obesity groups (C). GOAT and glucose showed a trend toward a correlation without reaching statistical significance (D). Body mass index and blood glucose showed a positive correlation (E), whereas plasma ghrelin and blood glucose were negatively correlated (F). Values for *r* and *p* are indicated in each correlation graph. $^{\dagger}p < 0.05$ *vs.* normal weight; $^{*}p < 0.05$ and $^{**}p < 0.01$ *vs.* anorexia nervosa.

age was found in any group (data not shown) or the whole study population investigated here. Lastly, GOAT protein expression has been assessed semi-quantitatively using Western blot since quantitative means of assessment such as radioimmunoassay are not available yet. In addition, we investigated GOAT protein expression, whereas activity was not measured. New techniques such as catalytic assays using enzyme-linked click-chemistry (cat-ELCCA), a method that was recently applied to assess GOAT enzyme activity *in vitro* [2], will help to investigate this in future studies.

In summary, GOAT protein is present in human plasma and its amount dependent on the metabolic state indicated by BMI, with lower levels in anorexic patients and higher levels in severely obese subjects compared to normal weight subjects. The positive correlation of GOAT and BMI and the negative correlation with ghrelin may point toward a counter-regulatory role of GOAT with less acylation under conditions of anorexia nervosa and increased activation of ghrelin in patients with severe obesity and consequently a contributing role of GOAT in the development or maintenance of these diseases.

## Conflict of interest

The authors have nothing to disclose. No conflicts of interest exist.

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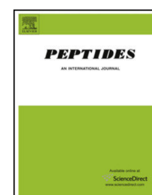

# Obese patients have higher circulating protein levels of dipeptidyl peptidase IV

Andreas Stengel [a,*], Miriam Goebel-Stengel [b], Pauline Teuffel [a], Tobias Hofmann [a], Petra Buße [a], Peter Kobelt [a], Matthias Rose [a], Burghard F. Klapp [a]

[a] *Charité Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charité-Universitätsmedizin Berlin, Campus Benjamin Franklin, Berlin, Germany*

[b] *Department of Internal Medicine, Institute of Neurogastroenterology and Motility, Martin-Luther Hospital, Academic Teaching Institution of Charité-Universitätsmedizin Berlin, Berlin, Germany*

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ABSTRACT

Dipeptidyl peptidase IV (DPPIV) is a protease with broad distribution involved in various homeostatic processes such as immune defense, psychoneuroendocrine functions and nutrition. While DPPIV protein levels were investigated in patients with hypoectic disorders, less is known under conditions of obesity. Therefore, we investigated DPPIV across a broad range of body mass index (BMI). Blood samples from hospitalized patients with normal weight (BMI 18.5–25 kg/m$^2$), anorexia nervosa (BMI <17.5 kg/m$^2$) and obesity (BMI 30–40, 40–50 and >50 kg/m$^2$, $n$ = 15/group) were tested cross-sectionally and DPPIV concentration and total enzyme activity and the DPPIV targets, pancreatic polypeptide (PP) and glucagon-like peptide (GLP-1) were measured. DPPIV protein expression was detected in human plasma indicated by a strong band at the expected size of 110 kDa and another major band at 50 kDa, likely representing a fragment comprised of two heavy chains. Obese patients had higher DPPIV protein levels compared to normal weight and anorexics (+50%, $p < 0.05$) resulting in a positive correlation with BMI ($r = 0.34$, $p = 0.004$). DPPIV serum activity was similar in all groups ($p > 0.05$), while the concentration/activity ratio was higher in obese patients ($p < 0.05$). Plasma PP levels were highest in anorexic patients (~2-fold increase compared to other groups, $p < 0.05$), whereas GLP-1 did not differ among groups ($p < 0.05$). Taken together, circulating DPPIV protein levels depend on body weight with increased levels in obese resulting in an increased concentration/activity ratio. Since DPPIV deactivates food intake-inhibitory hormones like PP, an increased DPPIV concentration/activity ratio might contribute to reduced food intake-inhibitory signaling under conditions of obesity.

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## Introduction

The prevalence of obesity is expanding worldwide in industrialized and also developing countries and therefore the term "globesity" was created (homepage world health organization). Obesity is associated with several diseases such as type 2 diabetes mellitus, dyslipidemia and arteriosclerosis (leading to an increased risk for cardiovascular diseases) [10], sleep apnea [20], degenerative joint diseases [2], mental disorders [21,24] and certain forms of cancer including hepatocellular, colorectal, pancreatic [6] and breast cancer [4]. Therefore, obesity is a major medical problem for the patient as well as a socioeconomic burden for the society [27]. It has been known for a long time that already a modest (5–10%) weight loss leads to an improvement of the patients' metabolic situation [9,30]. However, drug treatment options are very limited so far [14]. Therefore, a better understanding of the mechanisms regulating hunger and satiety is necessary.

Hunger and satiety are regulated by a multitude of peptide and protein hormones that exert their food intake-modulatory actions in the brain but most of them are predominantly produced in the gastrointestinal tract [26]. While ghrelin is the only peripherally produced and centrally acting hormone that stimulates food intake, a number of gastrointestinal hormones are involved in the reduction of food intake, including cholecystokinin, oxyntomodulin,

*Abbreviations:* BMI, body mass index; DPPIV, dipeptidyl peptidase IV; GLP-1, glucagon-like peptide 1; PP, pancreatic polypeptide.

* Corresponding author at: Charité Center for Internal Medicine and Dermatology, Division of General Internal and Psychosomatic Medicine, Charitéplatz 1, 10117 Berlin, Germany. Tel.: +49 30 450 553 002.

*E-mail addresses:* andreas.stengel@charite.de, a.stengel@gmx.de (A. Stengel).

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peptide YY (PYY), pancreatic polypeptide (PP) and glucagon-like peptide 1 (GLP-1) [26]. Interestingly, several of these hormones, namely PYY, PP and GLP-1 are target to dipeptidyl peptidase IV (DPPIV) resulting in cleavage and inactivation of PP and GLP-1, while a feeding-modulatory effect has been described for PYY due to different receptor binding [11].

DPPIV (also known as CD26) is a serine exopeptidase that is widely expressed in the body and exerts pleiotropic functions with an involvement in immune functions, inflammation, glucose homeostasis, regulation of hunger and satiety and psychomodulation [11]. Especially the involvement in glucose control is of clinical interest since several DPPIV inhibitors such as sitagliptin, saxagliptin, linagliptin and alogliptin are on the market as antidiabetic drugs [8]. Besides the beneficial effects on glucose homeostasis, some of these compounds also – although modestly – reduce body weight [5], a favorable effect in obesity-associated diabetes mellitus. Contrasting with the wide clinical use of DPPIV inhibitors, the knowledge on the regulation of DPPIV under conditions of chronically altered body weight, especially obesity is limited. Moreover, mostly DPPIV enzyme activity is assessed, while the protein concentration is not described. One study reported lowered DPPIV enzyme activity in serum of patients with anorexia nervosa compared to normal weight controls [29], whereas another study described the opposite result with an elevation of serum DPPIV activity levels in anorexic compared to healthy subjects [13]. In healthy normal weight subjects, plasma DPPIV enzyme activity was positively correlated with body mass index (BMI) [17], a finding that was also described in obese children [19]. This may be due to higher expression of DPPIV in adipose tissue as recently hypothesized [22].

Therefore, the aim of this study was to investigate circulating DPPIV protein concentrations as well as total enzyme activity across a broad range of BMI (9–85 kg/m$^2$). For this reason, the patient population was divided into groups according to the BMI: anorexia nervosa (BMI <17.5 kg/m$^2$), normal weight (BMI 18.5–25 kg/m$^2$) and three groups of obesity (BMI 30–40, 40–50 and >50 kg/m$^2$). To investigate the possible effect of an altered DPPIV concentration, we also measured circulating levels of PP and GLP-1, two major molecules subject to degradation by DPPIV.

## Materials and methods

### *Subjects*

All patients were hospitalized in the Division of Psychosomatic Medicine at Charité-Universitätsmedizin Berlin and gave written informed consent. Blood collection was performed on day 2 or 3 after hospital admission before the onset of changes due to dietary treatment to increase or reduce body weight, respectively. All parameters were assessed on the same morning. The protocol was approved by the local ethics committee for human research (ethics committee Campus Charité Mitte, protocol number EA1/114/10).

A total of 75 subjects participated in this study and were divided in five groups: normal weight (BMI 18.5–25 kg/m$^2$), anorexia nervosa (BMI < 17.5 kg/m$^2$) and different stages of obesity (BMI 30–40 kg/m$^2$, BMI 40–50 kg/m$^2$ and BMI > 50 kg/m$^2$, $n$ = 15/group). Subjects were devoid of a history for gastrointestinal surgery except for appendectomy or cholecystectomy. All normal weight patients were hospitalized exclusively due to psychosomatic disorders with functional bodily symptoms (functional gastrointestinal symptoms excluded) without relevant somatic disorders. Anorexic and obese patients were diagnosed according to the International Classification of Diseases-10 (ICD-10) and were hospitalized for weight gain or loss therapy, respectively. In obese patients hypercortisolism was excluded by assessment of urinary free cortisol excretion in a 24 h sample or – in case of clinical suspicion – with a dexamethasone suppression test. None of the patients were on DPPIV inhibitor medication or on medication intended to modulate body weight (for weight reduction or increase, respectively).

### *Blood collection*

After an overnight fast, venous blood was withdrawn between 0700 and 0800 h in serum tubes and cooled EDTA tubes containing the peptidase inhibitor aprotinin (inhibiting trypsin, chymotrypsin, plasmin and kallikrein; 0.6 trypsin inhibitor/0.5 ml blood; ICN Pharmaceuticals, Costa Mesa, CA, USA) which were immediately centrifuged at 3000 rpm for 10 min at 4 °C. Since DPPIV protein concentration and enzyme activity was measured, no DPPIV inhibitor such as Diprotin A was used. In addition, since an interference of aprotinin and the DPPIV enzyme activity cannot be ruled out, the serum samples did not contain the peptidase inhibitor aprotinin. Plasma or serum was separated and aliquots stored at −80 °C until further processing.

### *Measurements*

#### *Height and body weight*

Body weight and height were assessed in overnight fasted subjects wearing underwear only and BMI calculated as kg/m$^2$.

#### *Total protein and albumin*

Serum albumin and total protein levels were assessed by routine laboratory procedures in the central hospital laboratory of Charité-Universitätsmedizin Berlin.

#### *Western blot*

Crude protein fractions of plasma were prepared as described before [25]. Briefly, protein concentrations from plasma were determined using a BCA protein assay according to the manufacturer's protocol (Pierce Biotechnology, Rockford, IL, USA). Subsequently, gel samples were prepared by mixing protein samples with sample buffer (4% sodium dodecyl sulfate [SDS], 0.05% bromophenol blue [w/v], 20% glycerol, 1% mercaptoethanol [v/v] in 0.1 Tris buffer, pH 6.8). Samples were boiled for 1 min before gel electrophoresis and equal amounts of protein (20 μg/lane) were loaded on a 4–12% SDS polyacrylamide gel (SDS-PAGE, NuPage; Invitrogen, Carlsbad, CA, USA) and run in 2-(N-morpholino)ethanesulphonic acid buffer. Afterwards, proteins were transferred by electrophoresis to nitrocellulose membranes (BioPlot-NC; Costar, Cambridge, MA, USA) for 1 h at 4 °C. Membranes were washed in distilled water and stained in Ponceau-S in 3% trichloroacetic acid solution and images were taken. Membranes were washed twice with Tween-Tris-buffered saline (TBS; 10 mM Tris, 150 mM NaCl, and 0.05% Tween [v/v]) and incubated in Tween-TBS containing 5% (w/v) nonfat milk (Carnation, Nestlé, Glendale, CA, USA). After 1 h, membranes were incubated in anti-DPPIV polyclonal goat antibody (#ABIN 374762; antibodies-online, Aachen, Germany) diluted 1:5000 in Tween-TBS. After 1 h, membranes were washed five times with Tween-TBS and incubated with the secondary antibody (donkey anti-goat IgG conjugated to alkaline phosphatase, #V1151; Promega, Madison, WI, USA) diluted 1:2000 in Tween-TBS. After 1 h, membranes were washed three times again before color development in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, and 5 mM $MgCl_2$ [pH 9.5]) containing 0.3% nitroblue tetrazolium solution (v/v) and 0.15% 5-bromo-4-chloro-3-indolyl-l-phosphate solution (v/v) for 5–10 min according to the manufacturer's instructions. Western blots for all individual samples (five groups, $n$ = 15/group, 75 samples) were performed three times and pixel intensity (area under the curve) of each lane at the expected size of DPPIV (110 kDa) and at a second

major band of 50 kDa analyzed using Image J version 1.45 (National Institute of Health, Bethesda, MD, USA). Concentrations of DPPIV protein assessed semi-quantitatively were corrected for circulating albumin (DPPIV protein/albumin concentration = corrected DPPIV protein concentration).

*Enzyme activity*

DPPIV activity in serum was assessed using the substrate Gly-Pro p-nitroanilide (Sigma–Aldrich, Seelze, Germany) as previously described [12,13]. Briefly, 20 μl of serum were incubated with 10 μl of 2 mM Gly-Pro p-nitroanilide in 170 μl 0.1 M Tris–HCl (pH 8.0) for 30 min. The reaction was stopped by the addition of 800 μl sodium acetate buffer (1 M, pH 4.5). The DPPIV activity was deduced from the increase of extinction at 405 nm due to the amount of chromogenic substrate metabolized by DPPIV. The concentration/activity ratio was calculated for each individual sample.

*Enzyme-linked immunosorbent assay*

Plasma levels of pancreatic polypeptide (#EK-054-02, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and glucagon-like peptide $1_{(7–36)}$ amide (# EK-028-11, Phoenix Pharmaceuticals, Inc.) were measured using a commercial enzyme-linked immunosorbent assay (ELISA, range 0–100 ng/ml). The samples were processed in two batches; the intra-assay variability was <10% and the inter-assay variability <15%.

*Statistical analysis*

Distribution of the data was determined by using the Kolmogorov–Smirnov test. Data are expressed as mean ± standard error of the mean (SEM). Differences between groups were calculated using one-way analysis of variance (ANOVA) followed by Tukey *post hoc* test. Correlations were determined by Pearson's or Spearman's analysis depending on the distribution of the data. Differences between groups were considered significant when $p < 0.05$. All statistical analyses were conducted using SigmaStat 3.1 (Systat Software, San Jose, CA, USA).

## Results

*Circulating DPPIV concentration is dependent on body mass index*

The Western blot stained for DPPIV showed two prominent bands: one at the expected size of 110 kDa indicating full length DPPIV protein and the other one at 50 kDa which is likely to represent a fragment comprised of two heavy chains of DPPIV (Fig. 1).

Next, we investigated whether the expression of DPPIV would differ dependent on BMI. The study sample was separated into five BMI groups: anorexia nervosa (BMI < 17.5 kg/m$^2$), normal weight (BMI 18.5–25 kg/m$^2$) and three obese groups (BMI 30–40, 40–50 and >50 kg/m$^2$). Per definition, these groups significantly differed in BMI ($p < 0.001$; Fig. 2A). Total circulating protein levels did not differ among groups ($p > 0.05$; Fig. 2B), while DPPIV concentrations (representative picture of pooled samples shown in Fig. 2C) were lower in anorexic subjects compared to all other groups and higher in obese patients when the 110 kDa band was assessed semi-quantitatively ($p < 0.05$; Fig. 2D). The 50 kDa band was similar in normal weight and anorexic patients, while higher protein levels were observed in obese subjects ($p < 0.05$; Fig. 2E). When calculated for each patient, the protein levels of the 50 and 110 kDa band showed a positive correlation ($r = 0.44$, $p < 0.001$; Fig. 2F). Since the 110 kDa band represents full length DPPIV this was used for all

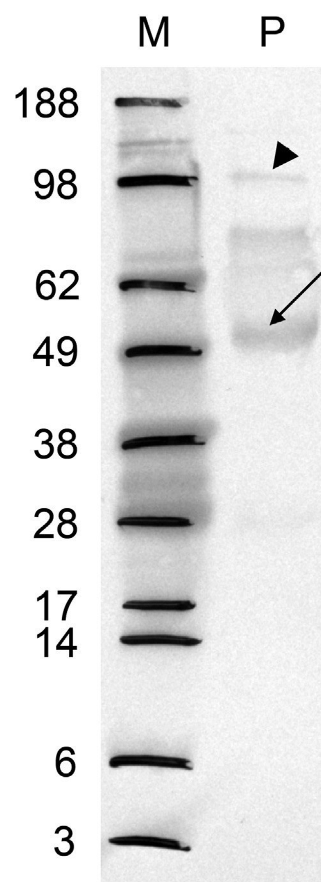


**Fig. 1.** Western blot for DPPIV in human plasma. Lane 1 contains the molecular weight standard, lane 2 contains human plasma. The antibody recognizes two prominent bands: one at the expected size of ~110 kDa (arrowhead) likely representing full length dipeptidyl peptidase IV, the other one at ~50 kDa (arrow) that is likely to represent a fragment comprised of two heavy chains of DPPIV/CD26. Abbreviations: M, marker (molecular weight standard); P, plasma.

further analyses. Circulating DPPIV concentrations showed a significant positive correlation with BMI ($r = 0.34$, $p = 0.004$; Fig. 2G).

*DPPIV total enzyme activity does not depend on body mass index, while the concentration activity ratio is positively correlated with body mass index*

Unlike the DPPIV concentration, the DPPIV enzyme activity was not different between groups ($p > 0.05$; Fig. 3A) and did not show a statistically significant correlation with BMI ($r = -0.18$, $p = 0.13$; Fig. 3B). When the DPPIV concentration/activity ratio was assessed for each subject, the obese groups showed a higher ratio compared to the anorexic and normal weight patients reaching statistical significance for the anorexic subjects ($p < 0.05$; Fig. 3C). This resulted in a positive correlation between the DPPIV concentration/activity ratio and BMI ($r = 0.31$, $p = 0.009$; Fig. 3D).

Since male and female patients were included in the normal weight and obese groups but not in the anorexic group (due to the much higher prevalence of anorexia nervosa in females), we also analyzed the data for possible sex differences. No differences between male and female subjects were observed in any group for DPPIV concentration and enzyme activity ($p > 0.05$; Table 1). The concentration/activity ratio was slightly higher in female compared to male patients in the BMI group of 40–50 kg/m$^2$ only ($p = 0.03$), while in all other groups no differences were observed ($p > 0.05$; Table 1).

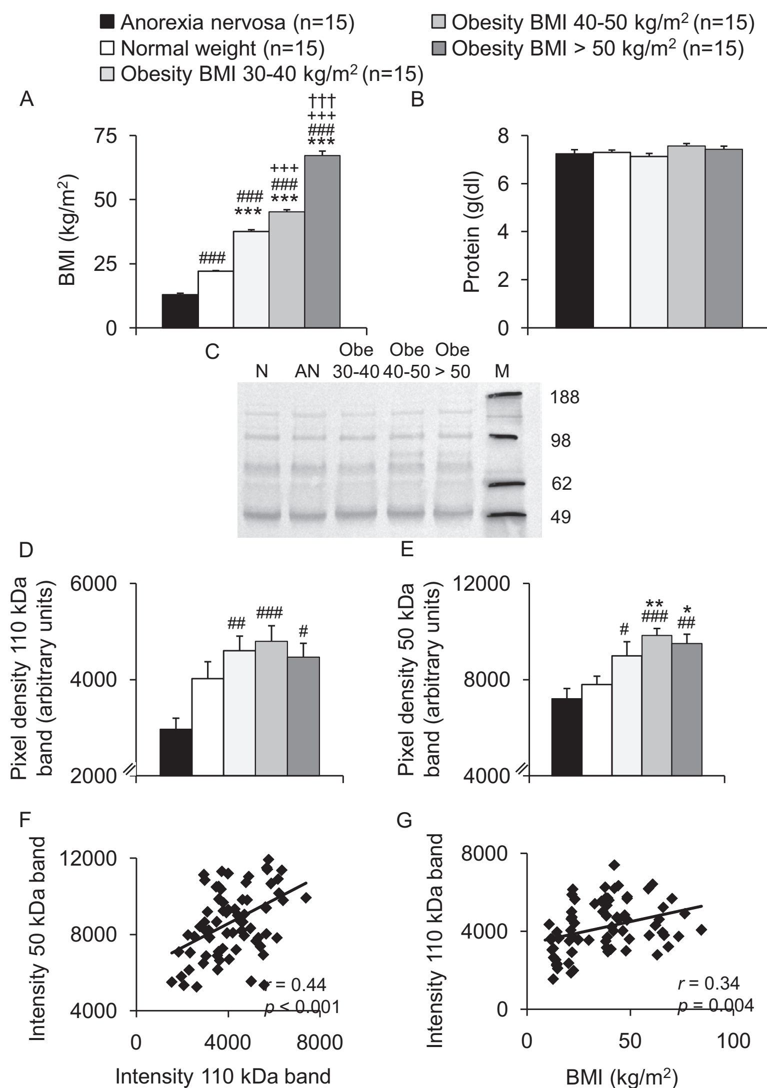


**Fig. 2.** DPPIV in plasma of normal weight, anorexic and obese subjects with increasing body mass index. Per definition, the body mass index (BMI) is significantly different in the five different groups: anorexia nervosa (AN), normal weight (N), and obese subjects with BMI between 30–40 kg/m² (Obe 30–40), 40–50 kg/m² (Obe 40–50) and >50 kg/m² (Obe > 50), (A). Total serum protein does not differ between these groups (B). DPPIV protein levels as measured by semi-quantitative assessment of pixel density show a difference between groups (representative picture of pooled samples shown in C, analysis shown in D and E). Semi-quantitative analysis of the 110 kDa band shows less DPPIV protein in plasma of anorexic subjects compared to all other groups and higher levels in obese subjects (D). For the 50 kDa band, normal weight and anorexic patients show similar levels, while higher levels are observed in obese subjects (E). Protein levels of the 50 and 110 kDa band show a highly significant positive correlation (F). Plasma DPPIV protein concentration (110 kDa band assessed since this represents full length DPPIV) shows a positive correlation with BMI (G). Values for *r* and *p* are indicated in each correlation graph. Data in (A, B, D, E) are expressed as mean ± SEM of $n = 15$ subjects/group. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ *vs.* normal weight; # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ *vs.* anorexia nervosa; +++ $p < 0.001$ *vs.* obese subjects with BMI between 30–40 kg/m² and ††† $p < 0.001$ *vs.* obese subjects with BMI between 40 and 50 kg/m². Abbreviations: M, marker (molecular weight standard).

**Table 1**
Comparison of DPPIV concentration, activity and concentration/activity ratio in male *vs.* female patients of the different body mass index groups.

| Parameter | Group | | | |
|---|---|---|---|---|
| | Normal weight | Obesity BMI 30–40 kg/m² | Obesity BMI 40–50 kg/m² | Obesity BMI > 50 kg/m² |
| DPPIV concentration (arbitrary unit) | | | | |
| Female | 4047 ± 543 | 4446 ± 452 | 5190 ± 425 | 4543 ± 491 |
| Male | 3993 ± 472 | 4784 ± 413 | 4345 ± 433 | 4363 ± 233 |
| DPPIV activity (U/l) | | | | |
| Female | 55.9 ± 3.3 | 52.5 ± 7.7 | 47.1 ± 5.7 | 50.3 ± 4.6 |
| Male | 56.0 ± 3.7 | 54.8 ± 4.4 | 63.8 ± 7.2 | 62.2 ± 5.1 |
| DPPIV concentration/activity ratio | | | | |
| Female | 72.9 ± 9.3 | 93.0 ± 12.1 | 120.7 ± 15.0 | 97.5 ± 15.0 |
| Male | 73.4 ± 9.2 | 93.6 ± 13.9 | 72.2 ± 9.5* | 68.0 ± 5.1 |

Data are expressed as mean ± SEM of $n = 7–8$ subjects/sex/group.
The anorexic group was not included in this analysis as only female anorexic patients were enrolled in this study.
Abbreviations: BMI, body mass index.
* $p < 0.05$.

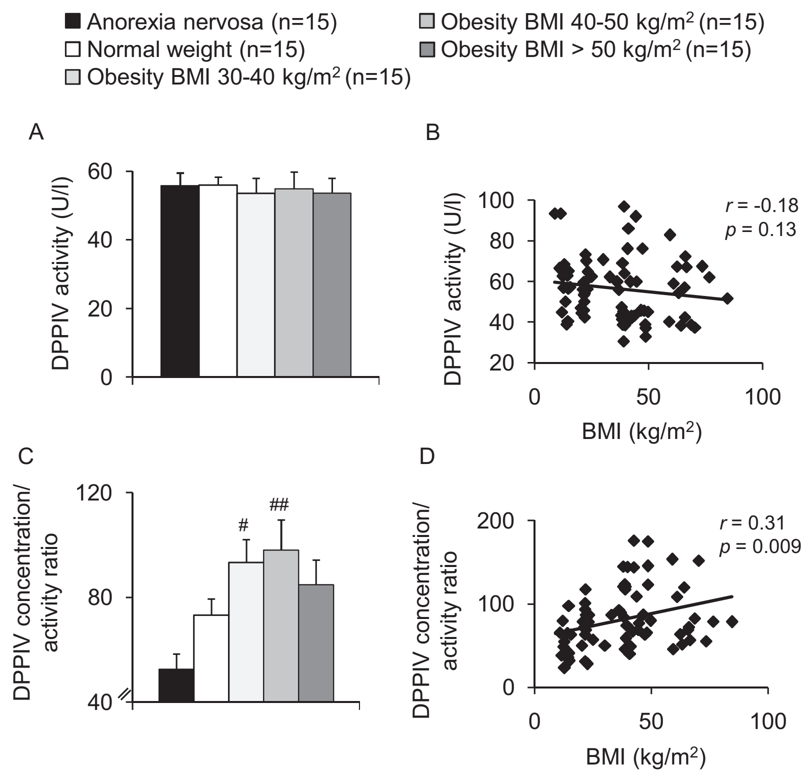


**Fig. 3.** DPPIV activity and concentration/activity ratio in the circulation of normal weight, anorexic and obese subjects with increasing body mass index. DPPIV activity measured using Gly-Pro p-nitroanilide as substrate does not differ between the five groups (A). No correlation is observed between DPPIV activity and BMI (B). The DPPIV concentration/activity ratio was calculated for each subject. This ratio is higher in obese patients reaching significance for the groups of obese subjects with BMI between 30–40 and 40–50 kg/m$^2$ compared to anorexic patients (C). A positive correlation is observed between the DPPIV concentration/activity ratio and BMI (D). Values for $r$ and $p$ are indicated in the correlation graphs. Data in (A) are expressed as mean ± SEM of $n = 15$ subjects/group. # $p < 0.05$ and ## $p < 0.01$ *vs.* anorexia nervosa.

*Circulating pancreatic polypeptide concentrations correlate with body mass index and circulating DPPIV, while glucagon-like peptide 1 levels do not*

Since DPPIV plays a major role in the deactivation of several anorexigenic hormones, we next investigated the plasma levels of two DPPIV substrates, PP and GLP-1. Patients with anorexia nervosa displayed higher PP plasma levels compared to all other groups ($p < 0.05$; Fig. 4A) resulting in a negative correlation with BMI ($r = -0.44$, $p < 0.001$; Fig. 4B). Also the DPPIV concentration ($r = -0.25$, $p = 0.04$; Fig. 4C) and the DPPIV concentration/activity ratio ($r = -0.36$, $p = 0.02$; Fig. 4E) showed a negative correlation with circulating PP levels, while DPPIV activity did not ($r = 0.20$, $p = 0.10$; Fig. 4D).

Unlike PP, GLP-1 levels did not differ between groups ($p > 0.05$; Fig. 5A) and displayed no correlation with BMI ($r = 0.04$, $p = 0.73$; Fig. 5B). Likewise, GLP-1 plasma levels did not correlate with circulating DPPIV concentration ($r = 0.05$, $p = 0.71$; Fig. 5C), DPPIV activity ($r = 0.03$, $p = 0.81$; Fig. 5D) or the DPPIV concentration/activity ratio ($r = 0.06$, $p = 0.61$; Fig. 5E).

## Discussion

In the present study we showed that DPPIV protein concentration is correlated with body mass index with an increased concentration in obese and the lowest concentration in patients with anorexia nervosa. The increased DPPIV concentration is not due to an overall alteration of protein expression as circulating total protein levels were not altered in the five BMI groups of patients. In addition, DPPIV protein concentrations were corrected for serum albumin levels. The Western blot detected two major bands with one at the expected size of 110 kDa indicating full length DPPIV protein and the other one at ~50 kDa that likely represents a fragment comprised of two heavy chains of DPPIV that also contains the epitope as previously described [15]. These data extend previous reports that showed a positive correlation of DPPIV with BMI in healthy subjects [17] and obese children [19]. It is to note that previous studies measured enzyme activity, whereas in the current study circulating DPPIV protein concentration as well as enzyme activity were assessed. Interestingly, no differences were observed for total DPPIV enzyme activity with similar levels across all groups analyzed. The reason for the discrepancy to the two studies mentioned above is not known but may be linked to the difference in the patient population with a very broad BMI spectrum (9–85 kg/m$^2$) analyzed in the current study. In line with the increase in DPPIV protein concentration, the DPPIV concentration/activity ratio also showed a positive correlation with BMI with higher levels in obese and lowest levels in anorexic subjects. Overall, this gives rise to activated DPPIV signaling under conditions of obesity, which may be due to increased DPPIV expression in and release from adipose tissue of obese subjects as recently suggested [22]. Alternatively, circulating DPPIV protein could have less activity in obese subjects which has to be compensated by higher protein expression, a hypothesis to be further investigated.

The current study population consisted of male and female patients with an equal contribution in all groups except for anorexia nervosa, where only women were analyzed. This is due to the at least 10-fold higher prevalence of anorexia nervosa in women compared to men. This is unlikely to influence the current results for the other BMI-defined groups as no statistically significant sex differences were detected for DPPIV concentration or enzyme activity. Merely the female patients with BMI 40–50 kg/m$^2$ showed a slightly higher DPPIV concentration/activity ratio compared to male patients of the same BMI group, a finding that should be

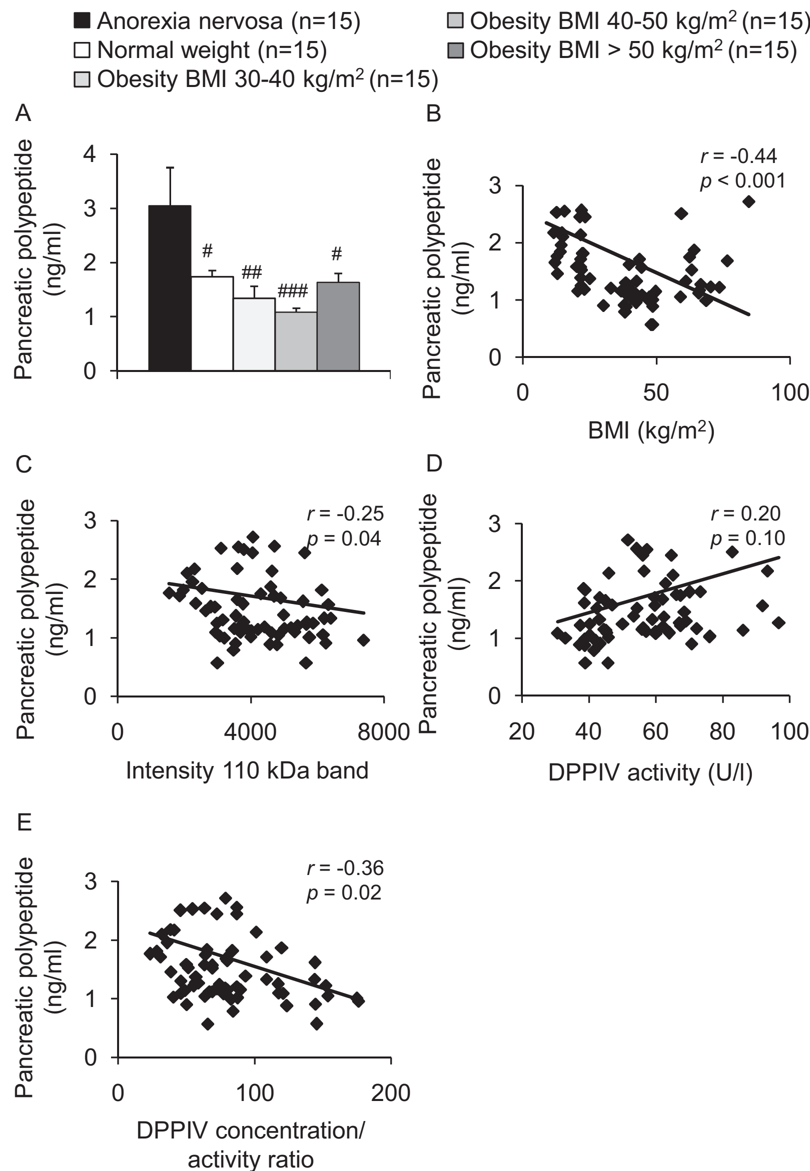


**Fig. 4.** Pancreatic polypeptide in plasma of normal weight, anorexic and obese subjects with increasing body mass index. Pancreatic polypeptide levels in plasma were assessed using ELISA. Patients with anorexia nervosa show higher pancreatic polypeptide levels compared to all other groups, the lowest levels are observed in the group of obese subjects with BMI between 30 and 40 kg/m$^2$ (A). A negative correlation is observed between pancreatic polypeptide plasma levels and BMI (B). Also DPPIV concentration (C) and the DPPIV concentration/activity ratio (E) negatively correlate with pancreatic polypeptide levels, while DPPIV activity does not (D). Values for *r* and p are indicated in each correlation graph. Data in (A) are expressed as mean ± SEM of $n = 15$ subjects/group. # $p < 0.05$, ## $p < 0.01$ and ### $p < 0.001$ *vs*. anorexia nervosa.

followed up in a larger cohort of patients. Lastly, it is to note that a detailed statistical evaluation of these subgroups is not possible due to the low number of females within groups and therefore the possibility of a type 2 error exists.

As expected, the distribution of diabetic subjects was different across groups. While no diabetic subjects were present in the anorexic and normal weight group, six (40%), four (27%) and four (27%) patients with type 2 diabetes mellitus were present in the obese group with BMI > 50 kg/m$^2$ and in the two other obesity groups, respectively. A positive correlation was observed for DPPIV concentration and insulin across all groups ($r = 0.29$, $p = 0.02$), while no association was detected for DPPIV and glucose, HbA1c or the homeostasis model assessment of insulin resistance (HOMA-IR, data not shown). The positive correlation of DPPIV concentration and BMI remained significant after exclusion of the diabetic subjects ($r = 0.34$, $p = 0.008$) indicating that the differences observed for DPPIV are unlikely to be predominantly due to alterations of the glucose homeostasis and diabetes.

None of our patients were on medication intended to modulate body weight. Since also other drugs that potentially influence body weight such as antidepressants, neuroleptics or antidiabetics might influence the results observed, we checked for these confounding variables. While no patients in the anorexic group took any of these drugs, two (13%) in the normal weight group, three (20%) in the obese groups with BMI of 30–40 and 40–50 kg/m$^2$, respectively and four (27%) in the obese group with BMI > 50 kg/m$^2$ were on drugs potentially altering body weight. However, it is unlikely that this influenced the results since after exclusion of these patients the results for DPPIV protein concentration and DPPIV enzyme activity were not significantly different (data not shown; $p > 0.5$).

Since DPPIV is a major enzyme catalyzing the degradation of GLP-1 and PP, two hormones involved in the regulation of food

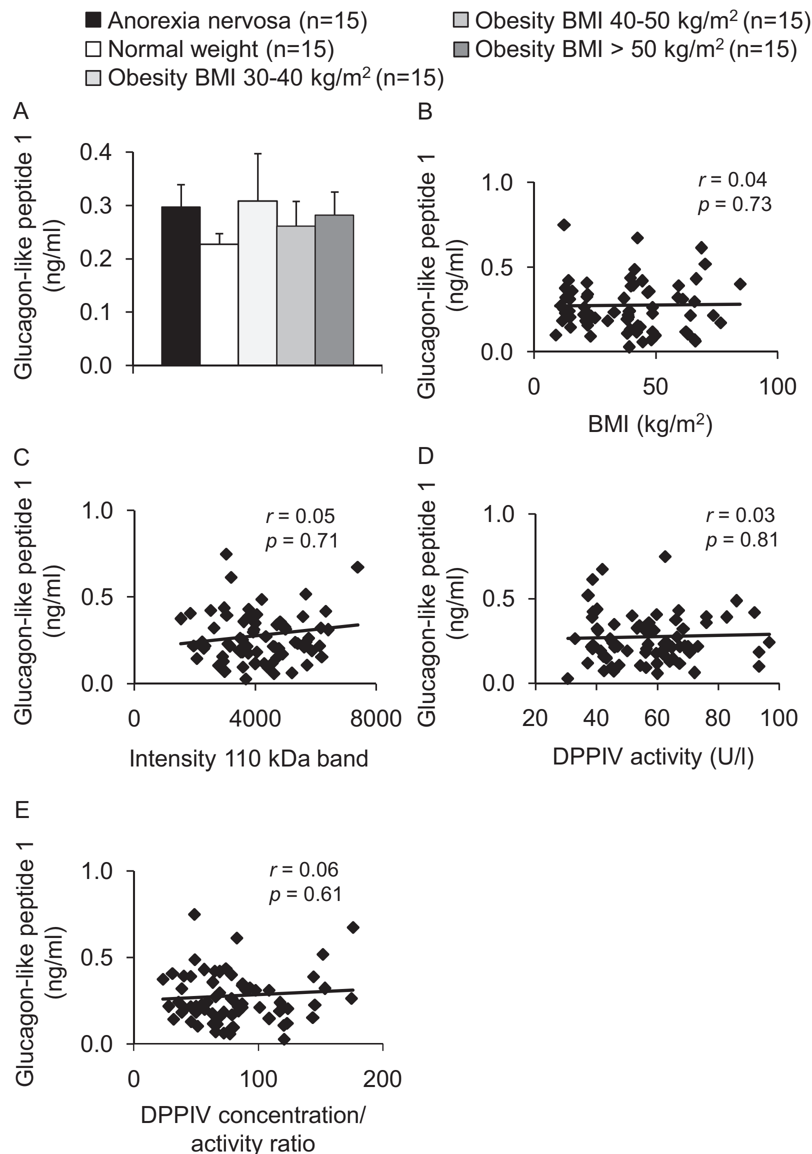


**Fig. 5.** Glucagon-like peptide 1 in plasma of normal weight, anorexic and obese subjects with increasing body mass index. Glucagon-like peptide 1 levels in plasma were assessed using ELISA. No differences in glucagon-like peptide 1 plasma levels are observed among the five groups (A). No correlation is observed between glucagon-like peptide and BMI (B), DPPIV concentration (C), DPPIV activity (D) or the DPPIV concentration/activity ratio (E). Values for $r$ and $p$ are indicated in each correlation graph. Data in (A) are expressed as mean ± SEM of $n = 15$ subjects/group. $p > 0.05$.

intake and glucose control [3,31], we also measured levels of these hormones in our patient population. While no differences across groups were observed for GLP-1, circulating PP levels were higher in the group of anorexia nervosa compared to all other groups and showed a negative correlation with BMI corroborating the results of a previous study showing higher PP levels in anorexic patients compared to normal weight controls [28], while previous results in obese were not consistent with either no changes [16] or a decrease reported [18]. Interestingly, a negative association was observed between PP and DPPIV protein concentration and the DPPIV concentration/activity ratio. This finding might give rise to a role for DPPIV in the enhanced degradation of PP resulting in decreased PP levels and consequently decreased food intake-inhibitory signaling under conditions of obesity which might play a role in the pathogenesis of obesity and/or the perpetuation of the disease. However, this hypothesis should be further corroborated in studies using stimulation of PP in response to a meal and also under conditions of inhibited DPPIV.

Further supporting the hypothesis of an important involvement of DPPIV in the regulation of anorexigenic signaling, a genetic animal model lacking DPPIV shows a protection against the development of diet-induced obesity [7]. Lastly, DPPIV activity was reported to decrease following gastric bypass surgery [1] which may contribute to the effective weight loss following bariatric surgery. Taken together, these beneficial findings of DPPIV inhibition may be due to altered food intake-inhibitory signaling but might also be linked to an activation of brown adipose tissue as recently suggested in an animal study [23].

In summary, circulating DPPIV protein levels as well as the DPPIV concentration/activity ratio are correlated with body mass index with increased levels in obese subjects. While GLP-1 levels did not differ across BMI groups, PP levels were higher in anorexia nervosa and lowest in obese patients. Since DPPIV is involved in the degradation of food intake-inhibitory hormones, an increased DPPIV concentration/activity ratio might contribute to reduced food intake-inhibitory signaling under conditions of obesity.

However, this hypothesis has to be further investigated in stimulation studies measuring PP levels postprandially as well as under conditions of pharmacological DPPIV inhibition.

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