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The dietary regulation of LEAP2 depends on meal composition in mice

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Abstract
Ghrelin represents a key hormone regulating energy balance. Upon activation of the growth hormone secretagogue receptor (GHSR), ghrelin increases blood glucose levels, food intake, and promotes weight gain. The liver-expressed antimicrobial peptide 2 (LEAP2) acts as an endogenous antagonist of the GHSR. While the regulation of LEAP2 and its effect on the GHSR likely occur in an opposite pattern to that of ghrelin, the dietary regulation of LEAP2 remains to be described. We, therefore, examined the regulation of LEAP2 by different acute meal challenges (glucose, mixed meal, olive, lard, and fish oil) and diets (chow vs. high-fat) in C57BL/6 male mice. In addition, the effect of specific fatty acids (oleic, docosahexaenoic, and linoleic acid) on LEAP2 was assessed in murine intestinal organoids. While only mixed meal increased liver Leap2 expression, all meal challenges except fish oil increased jejunal Leap2 expression compared to water. Leap2 expression correlated with levels of hepatic glycogen and jejunal lipids. Lipid versus water dosing increased LEAP2 levels in the systemic circulation and portal vein where fish oil was associated with the smallest increase. In line with this, oleic acid, but not docosahexaenoic acid increased Leap2 expression in intestinal organoids. Feeding mice with high-fat versus chow diet not only

Abbreviations: Acadl, long-chain acyl-CoA dehydrogenase; AMPK, AMP-activated protein kinase; Cpt1a, carnitine palmitoyltransferase 1A; Dgat2, diacylglycerol O-acyltransferase 1; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EEC, enteroendocrine cells; EPA, eicosapentaenoic acid; FFA, free fatty acids; Fxr, farnesoid X receptor; HFD, high-fat diet; GHSR, growth hormone secretagogue receptor; GPCR, G protein-coupled receptor; LEAP2, liver-expressed antimicrobial peptide; Lxr, liver X receptor; Pparα, peroxisome proliferator-activated receptor alpha; PUFAs, polyunsaturated fatty acids; Srebp1, sterol regulatory element-binding protein 1.

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1 | INTRODUCTION

Ghrelin is a 28-amino-acid peptide hormone that is predominantly secreted from the stomach and serves as an endogenous ligand for the growth hormone secretagogue receptor (GHSR).1,2 In the brain, GHSR activation in the pituitary gland results in growth hormone secretion whereas activation of GHSR in the hypothalamic arcuate nucleus promotes food intake and weight gain.1,3,4 In the pancreas, ghrelin inhibits and stimulates the secretion of insulin and glucagon, respectively. Rather than acting directly on pancreatic α-cells and β-cells, this effect is likely mediated indirectly through stimulation of δ-cells expressing GHSR that secrete somatostatin, or through GHSR activation in the arcuate nucleus (glucagon secretion only).5

Ghrelin levels are to a large degree regulated by metabolic and feeding status where they increase with a negative energy balance. This is reflected by a pre-prandial rise in plasma ghrelin levels that declines after a meal.2,6 Furthermore, ghrelin levels show a negative correlation with the body mass index (BMI) and are lower in individuals with obesity.7–9

The liver-expressed antimicrobial peptide (LEAP2) has been reported to act as an endogenous antagonist of GHSR by both blocking ghrelin action and reducing constitutive GHSR activity, achieved by its ability to both act as a competitive antagonist and inverse agonist of GHSR.7,10-13 Accordingly, systemic and intracerebroventricular LEAP2 administration has been reported to inhibit ghrelin function in both lean and obese rodents.10,14,15 Conversely, neutralizing antibodies that block LEAP2 function and LEAP2 deletion enhance ghrelin action in mice.10,16 In humans, exogenous LEAP2 has recently been reported to lower postprandial plasma glucose excursions, suppress ad libitum food intake, and have insulinoergic and antilipolytic properties during fasting.17

The highest expression of LEAP2 is found in the small intestine and liver.10 The high abundance of LEAP2 in the jejunum, where it is densely expressed in intestinal epithelial cells,10,18 indicates its potential for regulation in response to nutrients. Accordingly, LEAP2 has been reported to be regulated by both metabolic and feeding status.7,10,19 Plasma LEAP2 levels have for example been shown to decrease with fasting (and exposure to ketone bodies), whereas concentrations increase with glucose administration or subsequent refedding in rodents.7,10,15,19 Decreased Leap2 expression levels in the liver and small intestine have also been reported in fasted versus fed broiler chicks, with expression levels returning to the levels observed in the fed chicks upon refedding, although it should be mentioned that ghrelin exerts opposite functions on food intake in avian species.20 In humans, increased circulating levels of LEAP2 are found in obese individuals, and postprandial increases in LEAP2 have been reported to correlate with BMI.7,19 Plasma LEAP2 levels have also been reported to be increased in ob/ob mice and in mice with diet-induced obesity—an increase that in the latter could be reversed by weight loss.7,15 Finally, systemic LEAP2 levels have been reported to correlate with numerous metabolic parameters of obesity and to be increased in people with type 2 diabetes, where a positive correlation with HbA1c levels was found,7,21 suggesting that LEAP2 may act as a compensatory peptide against overweight and diabetes. This notion is further supported by the increased food intake, weight gain, and hepatic fat accumulation reported to occur in female LEAP2-KO versus wild-type mice.16

Since the changes in LEAP2 usually occur in a manner opposite to that of plasma ghrelin, LEAP2 has been suggested to modulate GHSR activity in concert with ghrelin, with LEAP2 serving as the dominant ligand of the GHSR in the setting of energy surplus (obesity/food intake), while the opposite is expected to occur in states of energy deficit (fasting/weight loss).7 Due to the counter-regulatory effects of LEAP2 and ghrelin on appetite and body metabolism, modulation of the LEAP2/ghrelin molar ratio may represent a future therapeutic target to treat obesity.

Although a role for LEAP2 in the regulation of energy balance has been established, it remains to be described how LEAP2 is regulated in response to different meals/diets and how this regulation occurs in tissues responsible for its release. The main purpose of these studies was, therefore, to assess the LEAP2 regulation in response to acute meal challenges (glucose, mixed meal, or lipid)
and how this regulation is affected by diet composition in mice. Since LEAP2 has been reported to increase with a positive energy balance, we hypothesize that LEAP2 is up-regulated by meal digestion. Furthermore, since the distribution and storage of carbohydrates and lipids differ upon ingestion, the associated regulation of LEAP2 is expected to differ between the liver and intestine.

2 | MATERIALS AND METHODS

2.1 | In vivo studies

All procedures performed in this study have been approved by the Danish Animal Experiment Inspectorate (License no. 2019-15-0201-00289). Male C57BL/6 mice were purchased from Janvier Labs (53 940 Le Genest-Saint-Isle, France) and group-housed in cages enriched with bedding and nesting material (6 mice per cage). The cages were kept at a constant temperature 22 ± 2°C and mice were exposed to a normal 12-h light/dark cycle (from 6:00 AM). Mice had free access to chow diet (Altromin #1310, Brogaarden, Lynge, Denmark) and water at all times unless otherwise specified. All mice were randomly divided into the study groups by simple randomization in all studies (Microsoft Excel, Microsoft Corporation, Washington, USA), thereby minimizing the effect of cage and treatment order on study outcome. All in vivo procedures were performed at 9:00 AM.

Male C57BL/6 mice aged 12–14 weeks were included to assess the effect of an acute meal challenge on the regulation of LEAP2. All mice were fasted for 16 h prior to oral gavage of glucose (2 g/kg body weight), mixed meal (240 kcal/100 mL, carbohydrate [49 kcal%], protein [16 kcal%], fat [35 kcal%]; Nutricia Nutridrink, Alleroed, Denmark), olive oil, lard oil, fish oil (Cas no. 8001-25-0, 8016-28-2, and 8002-50-4 from Sigma-Aldrich, 2860 Soeborg, Denmark) or a dose-matched volume of water (n = 7–9 per group). All mice received a dose of 10 mL/kg body weight. Blood was collected from the retro-orbital sinus (80 μL) and the tail vein (~5 μL) just prior to oral gavage which was followed by blood sampling at 30 min (tail blood only), 1, 2, or 4 h post dosing, depending on meal challenge of interest. Please refer to Figure 1 for an illustration of the study design and further details.

**FIGURE 1** Illustration of the study design. C57BL/6 mice were dosed with glucose, lipid, or mixed meal after a 16-h fast. Blood samples were collected prior to and after oral dosing. Mice were euthanized at 2 h (glucose and mixed meal dosing), or 4 h post dosing (mixed meal and lipid dosing) which was followed by harvesting of tissue from the liver and jejunum. Eighteen mice were included in the glucose dosing study (n = 9 in each group), 64 mice were included in the lipid dosing study (n = 8 in each group for olive oil vs. water, olive oil vs. lard oil vs. water, and finally olive oil vs. fish oil vs. water) and 31 mice in the mixed meal study (n = 7–8 in each group for mixed meal versus water either euthanized at 2 [n = 16] or 4 h post dosing [n = 15]). Twenty mice were included in the study assessing LEAP2 levels in the systemic circulation versus portal vein upon dosing with olive oil versus water (n = 10 in each group).
In order to assess a potential contribution of LEAP2 secreted from the small intestine to systemic LEAP2 levels after meal digestion, we compared LEAP2 levels from blood collected from the portal vein and systemic circulation in 12-week-old male C57BL/6 mice (n = 20). All mice were fasted for 16 h prior to oral gavage with 10 mL/kg olive oil or a dose-matched volume of water (10 per group). Blood samples (from the tail and retro-orbital sinus) were collected prior to oral gavage and at 4 h post dosing. Mice were anesthetized with isoflurane prior to collection of the blood samples, enabling the final 4-h sample to also be collected from the portal vein.

The effect of stimulating receptors responsive to fatty acid digestion, including G-protein-coupled receptors (GPCRs) GPR40 (FFAR1), GPR120 (FFAR4), and GPR119, on the regulation of LEAP2 was assessed in 24 12-week-old male C57BL/6 mice. All mice were fasted for 16 h prior to oral gavage of 30 mg/kg AM-5262, Compound A and Arena231453 (GPR40, GPR120, and GPR119 agonists, respectively, from Merck Millipore, Burlington, Massachusetts, USA [AM-5262 and Compound A] and Arena Pharmaceuticals, Inc., San Diego, California, USA [Arena231453]) or vehicle (10% Tween 20 in MilliQ, Sigma-Aldrich, 2860 Soeborg, Denmark). Blood samples were collected from the retro-orbital sinus prior to oral gavage (baseline sample), at 2 and 4 h post dosing.

The effect of diet on the regulation of LEAP2 was assessed in 48 male C57BL/6 mice fed chow (n = 24) or a high-fat diet (HFD with 58% kcal from fat/w/sucrose, D12331, Research Diets, Inc., New Brunswick, NJ 08901, USA, n = 24) for 9 weeks of age. Body lean and fat mass was determined using EchoMRI Body Composition Analyser (Echo Medical Systems, Houston, TX, USA) just prior to the experiments. At 13 weeks of age, mice were orally dosed with 10 mL/kg olive oil, fish oil, or a dose-matched volume of water after 2.5 h of food deprivation from 6:30 AM in order to assess the effect of diet (n = 8 per group). As in the previous lipid dosing studies, blood from the retro-orbital sinus was collected just prior to oral gavage which was followed by blood sampling at 2 and 4 h post dosing.

All mice were euthanized by cervical dislocation at the end of the experiments. Tissue biopsies were collected from the left lateral liver lobe and the jejunum (10–16 cm distally to the pyloric sphincter) and immediately frozen in liquid nitrogen. The biopsies were stored at −80°C until analysis.

2.2 | Blood samples

Blood samples were collected in EDTA tubes with 154–195 KIU/mL aprotinin (catalogue no. RK-APRO, Phoenix Pharmaceuticals, Inc., Burlingame, CA, USA) and centrifuged at 8000 RPM, 4°C for 5 min. Plasma was isolated from the EDTA tubes and stored at −80°C until plasma analysis of LEAP2, triglycerides, cholesterol, free fatty acids (FFA), and glycerol. Blood glucose from tail blood was measured using a glucose meter (Contour XTM +, Ascensia Diabetes Care, Basel, Switzerland). Blood glucose levels were assessed by the average from two repeated measurements. If the values deviated >1 mmol/L, a third measurement was made.

2.3 | Tissue and plasma biochemistry

Tissue samples were prepared by homogenizing the biopsies in a 0.15M sodium acetate buffer (pH = 4.9) containing 0.75% Triton X-100 (Sigma-Aldrich, Soeborg, Denmark). Tissue lyser II (Qiagen, Hilden Germany) and digital homogenizer (T25 digital ULTRA-TURRAX, IKA, Staufen, Germany) were used to homogenize the liver and jejunal tissue samples, respectively. The samples were subsequently placed on a heating block (95 ± 5°C) for 2 min before being placed on ice. The cooled homogenate from each liver sample was split into two aliquots. Amyloglucosidase (Sigma-Aldrich, Cas no. 9032-08-0) was added to one of the aliquots which was then placed on a heating block (50°C) for 2 h to facilitate the breakdown of glycogen to glucose. Both aliquots were subsequently centrifuged at 9000 g for 10 min and the supernatant was used to measure lipid and glycogen levels. For the jejunal samples, the cooled homogenate was similarly centrifuged at 9000 g for 10 min and the supernatant was used to measure lipid levels.

Hepatic, jejunal, and plasma levels of triglyceride, total cholesterol, FFA, glycerol, and glycogen (hepatic samples, only) were determined using the Cobas 6000 c5010 instrument (Roche Diagnostics GmbH, Mannheim, Germany) according to manufacturer’s instructions. Levels of glycogen were calculated by subtracting the free glucose from the total glucose concentration (measured in untreated and amyloglucosidase-treated samples, respectively), whereas liver and jejunal triglyceride levels were calculated by subtracting free glycerol from total glycerol levels (measured after lipase treatment facilitated by the Cobas instrument).

2.4 | Stimulation of intestinal organoids with fatty acids

To assess the effect of specific fatty acids on the regulation of LEAP2, mouse intestinal organoids were generated from crypt fragments of mouse jejunum (8–18 cm distally to the pyloric sphincter) from 12-week-old male C57BL/6 mice (n = 2 for establishment of two independent organoid cultures). The jejunum was opened longitudinally, villi were removed by manual scraping and the tissue was washed...
with cold phosphate-buffered saline (PBS; 8 g NaCl, 0.2 g KCl g, 1.15 g Na₂HPO₄ 2H₂O, and 0.2 g KH₂PO₄ per liter Milli-Q water). After washing, the crypts were liberated by trituration with a 5 mM EDTA solution in PBS, followed by washing with a 1% BSA solution in PBS. Fifty microliters of organoid suspension in Geltex (Gibco™, Thermo Fisher Scientific, Massachusetts, USA) was plated in each well in a 24-well plate and covered with 550 μL culture medium (IntestiCult™ Organoid Growth Medium, Stemcell Technologies, Vancouver, Canada) supplemented with 100 U/mL penicillin–streptomycin (Gibco™, Thermo Fisher Scientific). Organoid cultures were passaged, and the medium was changed every 5–6 and 2–3 days, respectively.

On the day of experiment, mature organoids from each well were transferred to 1.5 mL Eppendorf tubes. Six-hundred milliliter DMEM/F12 (Dulbecco’s Modified Eagle Medium: Nutrient Mixture F-12, Gibco™, Thermo Fisher Scientific) supplemented with 100 U/mL penicillin–streptomycin (Gibco™, Thermo Fisher Scientific) was plated in each well in a 24-well plate and covered with 550 μL culture medium (IntestiCult™ Organoid Growth Medium, Stemcell Technologies, Vancouver, Canada). Tissue samples were used to disrupt and homogenize prior to centrifugation at 5000 g, whereas 250 or 500 ng RNA was used from the organoids, followed by 1:25 or 1:50 dilution, respectively.

Primers for qPCR were purchased from TAG Copenhagen (2000 Frederiksberg, Denmark) and diluted in MilliQ water according to manufacturer’s instructions. qPCR was performed using PrecisionPLUS qPCR Master Mix with SYBRgreen (Primerdesign, Southampton, UK). Five microliters cDNA mixed with 5.5 μL SYBRgreen, 0.1 μL forward primer, 0.1 μL reverse primer, and 0.8 MilliQ water was added in each well. Samples were exposed to preincubation at 95°C for 2 min, 45 cycles (95°C for 15 sec and 60°C for 1 min) for amplification which was followed by generation of melt curves (95°C for 20 min and 60°C for 1 min) and cooling (40°C for 30 sec). The fluorescence signal was measured on the LightCycler® 480 (Roche Applied Science, Penzberg, Germany) from which the quantification cycle (C_q) values were extracted from the associated software. Specific amplification of each gene was confirmed by the presence of a single melting peak, and the overall qPCR efficiency for all genes was in the range of 95%–105%. No-template controls were checked for either missing C_q values or values >35. Gene expression was expressed as the average C_q value from triplicates. Expression levels were normalized to constitutively expressed Ywhaz gene and the relative mRNA expression was calculated by the 2^-ΔΔCT method in Microsoft Excel (Microsoft Corporation, Washington, USA). Invariant expression of Ywhaz across the study groups was confirmed by statistical comparisons across the groups as described in Section 2.7.

For an overview of genes for qPCR and the associated primers, please refer to Table 1.

2.5 Quantitative reverse transcription PCR

RNA from tissue and organoids was isolated using the RNasy Lipid Tissue Mini Kit and RNasy Plus Micro kit according to manufacturer’s instructions (Qiagen, Hilden, Germany). TissueLyser II and 5 mm stainless steel beads were used to disrupt and homogenize tissue samples, and DNase digestion was facilitated by the RNase-Free DNase set (Qiagen, Hilden, Germany). RNA from tissue and organoids was collected in 40 or 14 μL RNA-free water where 1 μL was used to determine RNA concentration and purity (Nanodrop™ 2000 Spectrophotometer, Thermo Fisher Scientific, Massachusetts, USA; mean A260/280 ~ 2 for cells and tissue).

cDNA was subsequently synthesized using Superscript III Reverse Transcriptase kit (Invitrogen, California, USA). Ten microliters RNA in RNA-free water was mixed with 4 μL FS buffer, 1 μL DTT (0.1 M), and 1 μL random primers, generating a total reaction volume of 16 μL that was exposed to 70°C for 3 min for cDNA synthesis (C1000 Touch Thermal Cycler, Bio-Rad Laboratories, California, USA). One microliter of each dNTPs, RNAse out and superscript II, in addition to 2 μL MilliQ water was subsequently added for cDNA amplification of the 21 μL reaction (25°C for 5 min, 50°C for 60 min, 70°C for 15 min prior to by cooling at 4°C). For animal tissue, 1 μg RNA was used for the cDNA synthesis, followed by 1:100 dilution of cDNA in MilliQ water prior to qPCR analysis, whereas 250 or 500 ng RNA was used from the organoids, followed by 1:25 or 1:50 dilution, respectively.

Primers for qPCR were purchased from TAG Copenhagen (2000 Frederiksberg, Denmark) and diluted in MilliQ water according to manufacturer’s instructions. qPCR was performed using PrecisionPLUS qPCR Master Mix with SYBRgreen (Primerdesign, Southampton, UK). Five microliters cDNA mixed with 5.5 μL SYBRgreen, 0.1 μL forward primer, 0.1 μL reverse primer, and 0.8 MilliQ water was added in each well. Samples were exposed to preincubation at 95°C for 2 min, 45 cycles (95°C for 15 sec and 60°C for 1 min) for amplification which was followed by generation of melt curves (95°C for 20 min and 60°C for 1 min) and cooling (40°C for 30 sec). The fluorescence signal was measured on the LightCycler® 480 (Roche Applied Science, Penzberg, Germany) from which the quantification cycle (C_q) values were extracted from the associated software. Specific amplification of each gene was confirmed by the presence of a single melting peak, and the overall qPCR efficiency for all genes was in the range of 95%–105%. No-template controls were checked for either missing C_q values or values >35. Gene expression was expressed as the average C_q value from triplicates. Expression levels were normalized to constitutively expressed Ywhaz gene and the relative mRNA expression was calculated by the 2^-ΔΔCT method in Microsoft Excel (Microsoft Corporation, Washington, USA). Invariant expression of Ywhaz across the study groups was confirmed by statistical comparisons across the groups (as described in Section 2.7). For an overview of genes for qPCR and the associated primers, please refer to Table 1.

2.6 ELISA

Jejunal tissue segments were homogenized in 600 μL lysis buffer (Invitrogen™ Cell Lysis Buffer and Thermo Scientific™ AEBSF Protease Inhibitor, both from Thermo Fisher Scientific, Massachusetts, USA; Protease Inhibitor Cocktail powder from Sigma-Aldrich, 2860 Soeborg, Denmark). TissueLyser II and 5 mm stainless steel beads (Qiagen, Hilden, Germany) were used to disrupted and homogenize tissue samples prior to centrifugation at 5000g
for 15 min at 4°C. The supernatant was collected, and protein concentration was measured on samples diluted 1:25 (Thermo Scientific™ Pierce™ BCA Protein Assay Kit from Thermo Fisher Scientific; SpectraMax 190 Microplate Reader, Molecular Devices, San Jose, USA). Samples were frozen at −80°C before analysis. LEAP2 in plasma and intestinal samples were analyzed with an ELISA kit for human and mouse LEAP2 detection (Cat. No. EK-075-40, Phoenix Pharmaceuticals Inc, Burligame, USA). Plasma and tissue samples were diluted 1:50 and 1:20, respectively, before being analyzed in accordance with the manufacturer's instructions.

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<th>Gene name</th>
<th>Gene symbol</th>
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<th>Accession number</th>
<th>E value</th>
<th>Amplicon size (bp)</th>
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**TABLE 1** Gene name, gene symbol, primer sequences, GenBank accession numbers (RefSeq select database), E value and amplicon size for primers used.
2.7 | Data handling and statistical analyses

Differences in means across groups were tested by Welch’s t-test or one-way ANOVA (GraphPad Prism version 9.0.0 for Windows, GraphPad Software, San Diego, California USA, www.graphpad.com). For nonparametric data, Mann–Whitney or Kruskal–Wallis test were used to test differences in means between two or more groups, respectively. Tukey’s or Dunn’s test were used for post hoc analysis of parametric and nonparametric data, respectively. Two-way ANOVA was used to assess the effect of sampling site (portal vs. systemic) and treatment (olive oil vs. water) on LEAP2 levels. Inspection of Q–Q plots in Graphpad Prism was used to assess Gaussian distribution of collected data and residuals. The relationship between numeric variables was assessed by Pearson’s correlation analysis where the coefficient of determination ($R^2$) is reported. Glucose and triglyceride exposure in plasma were assessed by calculation of the area under the curve, AUC. Statistical significance was defined by a $p$-value less than .05, and data are shown as mean ± SE.

One outlier in jejunal Leap2 expression in the group dosed with water versus glucose was excluded (assessed by Grubb’s test in GraphPad Prism). When assessing LEAP2 protein levels in the jejunum, one outlier was omitted from the dataset when comparing olive versus water dosing, whereas four samples were omitted from the fish oil study (assessed by the ROUT method in GraphPad Prism). Three liver samples from the HFD mice, one from each dosing group, were excluded from the biochemical analyses of due to inadequate homogenization. Four of 144 Plasma samples in the HFD versus chow study could not be analyzed due to lack of sufficient plasma. One plasma sample from the glucose versus water study was excluded due to hemolysis of the sample. Finally, organoid wells with Leap2 $C_q$ values above 30 (from one of the technical replicates) were excluded from the analysis.

3 | RESULTS

3.1 | Effect of acute meal challenges on plasma and liver biochemistry

To assess how LEAP2 is regulated by the various meal challenges, the LEAP2 response associated with administration of glucose, lipid (olive, lard, and fish oil), and mixed meal was assessed in lean mice (Figure 1).

The mean body weight did not differ between mice dosed with glucose, mixed meal, lipids, or a dose-matched volume of water (data not shown).

Administration of glucose and mixed meal (8.2 and 12 kcal/kg from glucose or carbohydrates, respectively) significantly increased the blood glucose levels (all samples collected after dosing) and plasma glucose exposure ($AUC_{0-120min}$ and $AUC_{0-240min}$) compared to water ($p<.01$, Figure 2A,C). Conversely, olive oil versus water dosing resulted in a small decrease in blood glucose levels (120 and 240 min, $p<.05$, Figure 2B), in addition to increasing plasma triglyceride levels (120 and 240 min, $p<.01$, Figure 2D).

Hepatic glycogen levels increased after dosing with both glucose and mixed meal versus water ($p<.001$, Figure 2F,H). Except for 2 h after mixed meal versus water dosing, hepatic triglyceride levels were not affected by the meal challenges ($p<.05$, Figure 2I–K).

For a detailed overview of plasma/tissue biochemical profiles associated with the meal challenges that are not included in the figures, please refer to Tables 2 and 3 and Table S1 in the Supporting Information.

3.2 | LEAP2 may be regulated by local energy stores in the liver and jejunum, and the small intestine represents an important source of circulating LEAP2 upon lipid dosing

When assessing LEAP2 regulation in the liver in response to the meal challenges (glucose, olive oil, and mixed meal), only mixed meal versus water increased Leap2 expression in the liver 2 h post dosing ($p<.001$, Figure 3A–C). Liver glycogen levels and plasma glucose exposure showed a significant correlation with liver LEAP2 expression (mixed meal dosing 2 h: $R^2=0.86$ and $R^2=0.84$, $p<.001$; glucose dosing: $R^2=0.4$ and $p<.01$, $R^2=0.28$ and $p<.05$; Figure S1A–D).

In the jejunum, administration of glucose, mixed meal (2 h post dosing), and olive oil all increased the Leap2 expression compared to water ($p<.05$, Figure 3D–F). A tendency toward higher plasma LEAP2 levels was observed 1 h post dosing with glucose versus water ($p=.06$, Figure 3G), whereas levels were comparable at 2 h post dosing (data not shown). A significant positive correlation was found between plasma triglyceride exposure ($AUC_{0-240min}$) and jejunal Leap2 expression in the olive oil versus water dosing study ($R^2=0.69$, $p<.001$; Figure S1E).

When assessed for the olive oil study, LEAP2 levels also increased in jejunal samples from mice dosed with olive oil versus water both in terms of absolute LEAP2 levels ($p<.001$, Figure 3H) and relative to the total tissue protein concentration ($p<.001$, data not shown).

To assess the intestinal contribution to the systemic LEAP2 levels upon lipid digestion, plasma levels from the portal vein...
FIGURE 2  Metabolic profiles in mice orally dosed with glucose, olive oil, or mixed meal. Blood glucose profiles in C57BL/6 mice orally dosed with 2 g/kg glucose (A), 10 mL/kg olive oil (B), or 10 mL/kg mixed meal (C) versus a dose-matched volume of water. Plasma triglyceride levels in C57BL/6 mice orally dosed with 10 mL/kg olive oil (D) or 10 mL/kg mixed meal (E) versus a dose-matched volume of water. Hepatic glycogen (F–H) and triglyceride levels (I–K) in C57BL/6 mice orally dosed with 2 g/kg glucose (F and I), 10 mL/kg olive oil (G and J) or 10 mL/kg mixed meal (H and K) versus a dose-matched volume of water. N = 7–9 per group. *p < .05, **p < .01, and ***p < .001 by Welch’s t test.
TABLE 2  Plasma and liver biochemistry in C57BL/6 mice orally dosed with 2g/kg glucose, 10mL/kg olive oil, 10mL/kg mixed meal versus a dose-matched volume of water (N = 7–9 per group).

<table>
<thead>
<tr>
<th></th>
<th>Glucose vs. water</th>
<th>Olive oil vs. water</th>
<th>Mixed meal vs. water (2-h study)</th>
<th>Mixed meal vs. water (4-h study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver (μmol/g)</td>
<td>Plasma (mmol/L)</td>
<td>Liver (μmol/g)</td>
<td>Plasma (mmol/L)</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>11.40 ± 0.56</td>
<td>0.09 ± 0.06</td>
<td>11.82 ± 0.37</td>
<td>7.28 ± 0.6</td>
</tr>
<tr>
<td></td>
<td>vs. 12.86 ± 0.45</td>
<td>vs. 0.96 ± 0.07</td>
<td>vs. 11.47 ± 1.13</td>
<td>vs. 7.24 ± 0.56</td>
</tr>
<tr>
<td></td>
<td>2h: 1.3 ± 0.1</td>
<td>2h: 1.1 ± 0.07</td>
<td>2h: 1.39 ± 0.18</td>
<td>2h: 1.45 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>vs. 1.06 ± 0.13</td>
<td>vs. 1.39 ± 0.16</td>
<td>vs. 1.57 ± 0.14*</td>
<td>vs. 1.36 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>4h: 1.49 ± 0.11</td>
<td>4h: 1.49 ± 0.11</td>
<td>4h: 1.49 ± 0.11</td>
<td>4h: 1.49 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>vs. 1.24 ± 0.12</td>
<td>vs. 1.24 ± 0.12</td>
<td>vs. 1.24 ± 0.12</td>
<td>vs. 1.24 ± 0.12</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>7.9 ± 1.7</td>
<td>3.6 ± 0.11</td>
<td>8.56 ± 0.16</td>
<td>7.91 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>vs. 8.06 ± 0.16</td>
<td>vs. 3.29 ± 0.15</td>
<td>vs. 8.49 ± 0.17</td>
<td>vs. 7.99 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>2h: 3.65 ± 0.06</td>
<td>2h: 2.3 ± 0.08</td>
<td>2h: 2.9 ± 0.07</td>
<td>2h: 2.65 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>vs. 3.02 ± 0.23*</td>
<td>vs. 2.3 ± 0.15</td>
<td>vs. 2.2 ± 0.12</td>
<td>vs. 2.5 ± 0.13</td>
</tr>
<tr>
<td></td>
<td>4h: 3.59 ± 0.07</td>
<td>4h: 3.29 ± 0.07</td>
<td>4h: 3.29 ± 0.07</td>
<td>4h: 3.26 ± 0.08</td>
</tr>
<tr>
<td></td>
<td>vs. 3.19 ± 0.14*</td>
<td>vs. 2.2 ± 0.12</td>
<td>vs. 2.2 ± 0.12</td>
<td>vs. 2.47 ± 0.11</td>
</tr>
</tbody>
</table>

Note: Data are shown as mean ± SE.

*P < 0.05 vs. water by Welch’s t test.
TABLE 3  Plasma and tissue biochemistry in C57BL/6 mice orally dosed with 10 mL/kg olive oil, lard oil, fish oil, or a dose-matched volume of water (N=8 per group).

<table>
<thead>
<tr>
<th></th>
<th>Olive oil vs. lard oil vs. water</th>
<th>Olive oil vs. fish oil vs. water</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Liver (μmol/g)</td>
<td>Plasma (mmol/L)</td>
</tr>
<tr>
<td>Glycogen</td>
<td>0.33 ± 0.2</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>vs. 0.15 ± 0.06</td>
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<tr>
<td></td>
<td>vs. 0.7 ± 0.29</td>
<td></td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>8.71 ± 0.62</td>
<td>0h: 1.41 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>vs. 8.4 ± 0.18</td>
<td>vs. 1.2 ± 0.15</td>
</tr>
<tr>
<td></td>
<td>vs. 8.18 ± 0.41</td>
<td>vs. 1.3 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>2h: 1.67 ± 0.2</td>
<td>vs. 1.38 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>vs. 1.33 ± 0.11</td>
<td>vs. 1.33 ± 0.11</td>
</tr>
<tr>
<td></td>
<td>4h: 1.96 ± 0.07*</td>
<td>vs. 1.9 ± 0.16</td>
</tr>
<tr>
<td></td>
<td>vs. 1.51 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cholesterol</td>
<td>8.51 ± 0.23</td>
<td>0h: 2.66 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>vs. 8.95 ± 0.12</td>
<td>vs. 2.75 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>vs. 8.14 ± 0.34</td>
<td>vs. 2.66 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>2h: 2.77 ± 0.12</td>
<td>vs. 2.88 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>vs. 2.52 ± 0.05</td>
<td></td>
</tr>
<tr>
<td></td>
<td>4h: 2.85 ± 0.12</td>
<td>vs. 2.98 ± 0.09*</td>
</tr>
<tr>
<td></td>
<td>vs. 2.54 ± 0.06</td>
<td></td>
</tr>
</tbody>
</table>

Note: Data are shown as mean ± SE. *p < .05, **p < .01 vs. water by one-way ANOVA.

When assessing the association between jejunal lipids and LEAP2, a significant positive correlation was found between jejunal LeaP2 expression and levels of jejunal tri-glycerides ($R^2 = 0.69$ and 0.73 in the lard and fish oil study, respectively, $p<.001$, Figure S1G,I) and FFA (fish oil study: $R^2 = 0.60$, $p<.001$; lard oil study: $R^2 = 0.24$, $p<.05$; Figure S1H,J), whereas no correlation was found between jejunal cholesterol levels and LeaP2 expression.

3.3 Lipid dosing alters the expression of genes regulating lipid metabolism

Lipid dosing affected the regulation of a number of genes regulating lipid metabolism ($p<.05$, Figure 4Q,S). Similar to the described observation for LeaP2 expression (Figure 4I,K), administration of olive oil and lard oil versus water both increased the expression of SrebP1, whereas fish oil dosing failed to increase the expression of this gene (Figure 4Q,S). Furthermore, a positive correlation was found between LeaP2 and SrebP1 expression (fish oil vs. olive oil study: $R^2 = 0.76$; lard oil vs. olive oil study: $R^2 = 0.5$, $p<.001$, data not shown). Oil versus water administration also decreased the expression of peroxisome proliferator-activated receptor $\alpha$ (Ppara) and Liver X receptor (Lxr) and increased the expression of diacylglycerol O-acyltransferase 2 (Dgat2) ($p<.05$, Figure 4Q,S). Fish oil also decreased Lxr expression compared to water and olive oil, and olive oil did not decrease Lxr expression in the fish oil study. For an overview of the regulation of other genes associated with the administration of the different meal challenges, please refer to Table S2 in the Supporting Information.

3.4 Oleic acid increased LeaP2 expression in murine intestinal organoids

Stimulation of murine intestinal jejunal organoids (Figure 5A) was used to assess the LEAP2 regulation in response to specific fatty acids on a cellular level. Results showed that stimulation with oleic acid increased LeaP2 expression compared to vehicle and linoleic acid after 4h ($p<.05$, Figure 5B). No differences were observed in SrebP1 expression (data not shown).

3.5 Effect of GPCR agonists stimulation on LeaP2 expression in lean mice

Based on our results showing that LEAP2 is regulated by lipid digestion, we were interested in examining whether certain FFA receptors, that are responsive to lipid
digestion, could play a role in the regulation of LEAP2. The effect of stimulation by the GPCR agonists, AM-5262, Arena231453, and Compound A (targeting GPR40/FFAR1, GPR119, and GPR120/FFAR4, respectively), on Leap2 expression was assessed in C57BL/6 mice. Results show that the jejunal expression was not affected by oral administration of the agonists, whereas liver Leap2 expression decreased upon dosing with AM-5262 versus vehicle (p < .05, Figure 5C,D). As with Leap2, the liver Srebp1 expression was decreased in mice dosed with AM-5262 versus vehicle (p < .01, data not shown), whereas the expression of selected genes regulating lipid metabolism was unaffected by the treatments (Long-chain acyl-CoA dehydrogenase, Acadl; Carnitine palmitoyltransferase 1A, Cpt1a; Dgat2; Lxr).

### 3.6 High-fat versus chow diet increased both LEAP2 levels and the LEAP2 response associated with olive oil

To assess the effect of metabolic status and diet, the regulation of LEAP2 in response to olive versus fish oil dosing was investigated in mice fed an HFD versus chow.
Four weeks of HFD versus chow diet resulted in a small but significant increase in body weight, body fat, and body lean mass (p < .05, Figure 6A,B), where a higher fat:lean mass ratio was observed in mice fed HFD versus chow (p < .05, data not shown). Furthermore, higher blood glucose and LEAP2 levels were found in mice fed HFD versus chow after 2.5 h of food deprivation (p < .001, Figure 6C,D). Whereas the liver lipids were unaffected by diet intervention (Table 4), the HFD versus chow diet increased jejunal levels of triglycerides and FFA, and decreased levels of cholesterol (p < .05 when comparing chow and HFD mice dosed with water in Figure 7I-N).

Oral dosing with olive oil and fish oil increased plasma triglyceride and FFA levels compared to dosing with water (120 and 240 min, p < .05, Figure 6G,H and Table 4) where higher levels were observed in mice fed HFD versus chow.
Although lipid dosing versus water increased LEAP2 levels in mice fed chow, it did not give rise to significant differences across the dosing groups (Figure 6I). In mice fed an HFD, however, olive oil versus water dosing significantly increased LEAP2 levels (120 and 240 min, \( p < .01 \), Figure 6J). Fish oil did not increase LEAP2 levels in HFD mice where a significant increase in LEAP2 was only observed prior to dosing with fish oil versus water (\( p < .05 \), Figure 6J).

Furthermore, while the HFD versus chow diet increased the magnitude of the LEAP2 response associated with olive oil versus water dosing (\( \Delta \text{LEAP2}_{0-120\text{min}} \) and \( \Delta \text{LEAP2}_{0-240\text{min}} \), \( p < .01 \), Figure 6E,F) the opposite was observed with fish oil versus water dosing (\( \Delta \text{LEAP2}_{0-240\text{min}} \), \( p < .05 \), Figure 6F).

As in the previous studies, oral dosing with olive oil versus water decreased blood glucose levels (increased \( \Delta \text{Blood glucose}_{0-240\text{min}} \) in mice fed chow or decreased glucose exposure at 120 min in mice fed HFD upon dosing with olive oil, \( p < .05 \), Table S1).

Neither lipid versus water dosing (Figure 7A,C), nor diet intervention affected liver \( \text{Leap2} \) expression (data not shown). In mice fed HFD, liver \( \text{Srebp1} \) expression decreased in mice dosed with fish oil versus olive oil and water (\( p < .05 \), Figure 7D). Whereas no significant change in liver lipids was observed with lipid versus water dosing in mice fed chow, olive oil, and fish oil versus water dosing increased hepatic triglyceride and cholesterol levels in mice fed HFD (\( p < .05 \), Table 4). Furthermore, olive oil increased hepatic FFA levels compared to fish oil and water dosing (\( p < .05 \), Table 4). Liver glycogen levels were unaffected by both diet intervention and lipid administration (Table 4).

In the jejunum, olive oil dosing increased jejunal \( \text{Leap2} \) expression compared to water and fish oil, although this response was attenuated in mice fed HFD versus chow (150 vs. 223% increase compared to the control group, Figure 7E,G). Furthermore, while olive
FIGURE 6 Metabolic profiles and plasma levels of LEAP2 and triglycerides associated with dosing with olive oil and fish oil in mice fed chow or a high-fat diet (HFD). Body weight (A), body composition (B), blood glucose (C), and LEAP2 levels (D) in C57BL/6 mice fed chow or high-fat diet (HFD) for 4 weeks. N=24 per group. *p < .05, **p < .01, and ***p < .001 by Welch’s t test. (E and F) Change in plasma LEAP2 levels (ΔLEAP20-120min/0-240min) in C57BL/6 mice fed chow or HFD for 4 weeks associated with oral dosing with 10 mL/kg olive oil, fish oil, or a dose-matched volume of water. *p < .05, **p < .01, and ***p < .001 by Welch’s t test or Mann–Whitney test. Plasma triglyceride (G and H) and LEAP2 levels (I and J) in C57BL/6 mice fed chow (G and I) or HFD (H and J) for 4 weeks prior to oral dosing with 10 mL/kg olive oil, fish oil or a dose-matched volume of water. E–J: N=6–8 per group. *p < .05, **p < .01, and ***p < .001 by one-way ANOVA or Kruskal–Wallis test (G–J).
oil versus fish oil and water only increased Srebp1 expression in the jejunum in chow-fed mice, fish oil decreased its expression compared to olive oil in both diet groups ($p<.05$, Figure 7F,H). The alterations jejunal lipids associated with lipid dosing were comparable to those observed in the previous fish oil study (Figure 4). Hence, jejunal triglyceride levels increased with olive and fish oil versus water dosing ($p<.01$, Figure 7I,K). Furthermore, jejunal cholesterol levels increased upon dosing with fish oil versus olive oil and water ($p<.01$, Figure 7M,N). A decrease in jejunal cholesterol was however only observed with olive oil versus water in mice fed chow ($p<.05$, Figure 7M). Furthermore, whereas olive oil versus fish oil and water increased jejunal FFA levels in mice fed chow as in the previous studies (Figures 4O and 7J), no difference was observed between the dosing groups in mice fed an HFD (Figure 7L).

**4 | DISCUSSION**

This study assessed the dietary regulation of LEAP2, both in the liver and intestine under different metabolic conditions, by challenging mice with oral glucose, various lipids, or mixed meal. Here we show that LEAP2 can indeed be acutely regulated by meal ingestion in mice and that this regulation likely depends on local energy stores in the liver and small intestine. In the liver, this was reflected by increased Leap2 expression with mixed meal dosing, whereas in the jejunum, all meal challenges (glucose, mixed meal, olive, and lard oil) except fish oil increased Leap2 expression. In addition to increased LEAP2 levels in jejunal tissue, the increase in jejunal Leap2 expression was also associated with both increased portal and systemic LEAP2 levels following a lipid meal versus water, suggesting that the intestines represent a significant contributor to the systemic LEAP2 levels. We also show that LEAP2 regulation can be modified by diet since feeding mice with an HFD versus chow not only increased systemic LEAP2 levels but also the LEAP2 response (increment in plasma LEAP2) associated with olive oil.

In the liver, Leap2 expression correlated with both glucose exposure and hepatic glycogen levels, indicating that hepatic glycogen deposition can regulate LEAP2. Since glucose enters the liver through the portal vein...

<table>
<thead>
<tr>
<th>TABLE 4</th>
<th>Plasma and tissue biochemistry in C57BL/6 mice fed a high-fat diet (HFD) or chow orally dosed with 10 mL/kg olive oil, fish oil or a dose-matched volume of water ($N=8$ per group).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Olive oil vs. fish oil vs. water in mice fed chow</td>
<td>Olive oil vs. fish oil vs. water in mice fed HFD</td>
</tr>
<tr>
<td>Liver ($\mu$mol/g)</td>
<td>Plasma (mmol/L)</td>
</tr>
<tr>
<td>Glycogen</td>
<td>N/A</td>
</tr>
<tr>
<td>80.63 ± 15.76 vs. 96.96 ± 13.75 vs. 108.2 ± 28.85</td>
<td>vs. 60.94 ± 16.78 vs. 85.54 ± 20.34 vs. 126.1 ± 25.61</td>
</tr>
<tr>
<td>Triglycerides</td>
<td>Included in Figure 6</td>
</tr>
<tr>
<td>23.83 ± 1.35 vs. 24.4 ± 2.5 vs. 22.0 ± 2.8</td>
<td>36.13 ± 1.43* vs. 36.14 ± 2.88* vs. 26.09 ± 2.86</td>
</tr>
<tr>
<td>Free fatty acids</td>
<td>0h: 1.07 ± 0.09 vs. 1.35 ± 0.09 vs. 1.27 ± 0.08 2h: 2.18 ± 0.17* vs. 1.69 ± 0.22 vs. 1.33 ± 0.08 4h: 1.69 ± 0.2* vs. 1.42 ± 0.07 vs. 1.19 ± 0.07</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>5.55 ± 0.26 vs. 5.74 ± 0.34 vs. 5.59 ± 0.32</td>
</tr>
<tr>
<td>0h: 2.56 ± 0.09 vs. 2.37 ± 0.07 vs. 2.47 ± 0.05 2h: 2.76 ± 0.13 vs. 2.04 ± 0.26 vs. 2.41 ± 0.28 4h: 2.19 ± 0.15 vs. 2.12 ± 0.1 vs. 2.09 ± 0.07</td>
<td>0h: 3.73 ± 0.25 vs. 3.84 ± 0.18 vs. 3.52 ± 0.42 2h: 3.52 ± 0.18 vs. 3.67 ± 0.15 vs. 3.14 ± 0.37 4h: 3.29 ± 0.16 vs. 3.15 ± 0.19 vs. 3.06 ± 0.36</td>
</tr>
</tbody>
</table>

Note: Data are shown as mean ± SE. *$p<.05$, **$p<.001$ vs. water by one-way ANOVA.
upon digestion, dosing with glucose and mixed meal expectedly both increased liver glycogen levels. However, compared to lipid and glucose, mixed meal was the only dietary challenge that increased \textit{Leap2} expression more than twofold in the liver. This could be explained by a higher carbohydrate load with mixed meal dosing and thereby a higher accumulation of liver glycogen ($\Delta$Liver_glycogen where basal levels were lower in the mixed meal study) which also showed a stronger correlation with \textit{Leap2} in the mixed meal versus glucose dosing study ($R^2 = 0.86$ vs. 0.40). A higher caloric load and dietary protein present in the mixed meal versus glucose...
challenge could also have contributed to the increased 
Leap2 expression in the liver.

Our finding that lipid dosing did not affect hepatic 
Leap2 expression may not be surprising as most dietary 
long-chain fatty acids absorbed by the enterocytes of the 
small intestine are packaged into chylomicrons and trans- 
ported to the lymphatic system rather than the liver prior 
to entering the systemic circulation. Accordingly, as with 
the Leap2 expression, oil administration only gave rise to 
alterations in jejunal rather than hepatic lipid profiles, 
reflected by increased levels of jejunal triglycerides and FFA 
(olive and lard oil only). The positive correlation between 
Leap2 expression and jejunal triglyceride/FFA levels 
could suggest that in the small intestine, lipid deposition 
plays a role in regulating local Leap2 secretion. We did 
however also observe an increase in jejunal Leap2 levels 
upon dosing with glucose and mixed meal, suggesting that 
not only lipid uptake affects local Leap2 regulation in the 
small intestine. Compared to oil dosing, the increased 
Leap2 expression associated with glucose did not signifi- 
cantly increase plasma Leap2 levels (p = .06). This may 
be caused by insufficient study power as similar glucose 
dose and time of sample collection have been reported to 
increase Leap2 in larger study groups (n = 10–12 mice).7

The fact that Leap2 may show an acute regulation 
according to glycogen or lipid levels in the liver and in- 
testine, respectively, supports the notion that Leap2 is a 
peptide that is upregulated by a positive energy balance 
(i.e., an increase in plasma Leap2 or Leap2 expression 
levels reported to be associated with food intake/refeed- 
ing and obesity).7,10,15,20 Since Leap2 deletion has been 
reported to both increase food intake, body weight, and 
exaggerate hepatic fat accumulation in female mice,16 
the increase in Leap2 that occurs with a meal challenge 
may act to counteract further expansion of energy stores. 
It should however be mentioned that although the cor- 
relation between energy substrates and Leap2 expression 
suggests that Leap2 is regulated according to local energy 
availability, a causal link as well as the mechanism behind 
this regulation (e.g., the role of insulin-regulated glucose 
disposal), remains to be determined. Furthermore, while 
the mice included in our studies received equal dosing 
volumes, the meal challenges used in our studies were not 
isocaloric (approximately 80, 24, and 8.2 kcal/g for dos- 
ing with lipids, mixed meal, and glucose, respectively). A 
higher caloric load could therefore partly explain the large 
Leap2 upregulation associated with the lipid versus glu- 
cose and mixed meal challenges. On the contrary, ghrelin 
levels have been reported to be regulated according to 
content of macronutrients where the highest magnitude of 
ghrelin suppression was reported to occur with inges- 
tion of protein, followed by carbohydrate and lipid when 
consumed in isocaloric, isovolemic beverages.23

As with the increase in plasma Leap2, dosing with 
olive and lard oil both resulted in a small decrease in blood 
glucose levels. As Leap2 levels correlate with blood glu- 
cose levels and Leap2 administration has been reported to 
lower postprandial plasma glucose excursions in hu- 
mans,7,17 the decrease in blood glucose may partly be ex- 
plicated by the increased Leap2 levels associated with the 
oil challenges.

SREBP1 is a transcription factor regulating lipid me- 
tabolism with a binding consensus sequence present 
at the LEAP2 promoter region,24,25 potentially indicating 
direct involvement in the Leap2 gene transcription. 
Hence, the attenuated plasma Leap2 levels and jejunal 
lipid deposition associated with fish oil, in addition to its 
ability to increase jejunal Leap2 expression levels, may 
partly be explained by a lack in SREBP1 upregulation in 
the jejunum. The regulation of Leap2 by SREBP1 could 
be mediated through the 5’ AMP-activated protein ki- 
nase (AMPK) since ghrelin has been reported to decrease 
Leap2 expression in the liver through AMPK phosphory- 
lation—an event that has also been reported to suppress 
the activity of SREBP1.14,26,27 However, the expression of 
ghrelin receptors in the liver is not consistently reported.28 
A definitive role of SREBP1 in Leap2 regulation in addi- 
tion to the potential regulation by other transcription 
factors (e.g., CDX4 which also binds to the Leap2 promo- 
tor region and has been suggested to regulate Leap2 in 
chicken20) remains to be investigated.

Since the main constituent (∼73%–75%) of olive oil is 
oleic acid,29 this fatty acid may partly be responsible for 
the Leap2 upregulation associated with olive oil dos- 
ing. Compared to olive oil and lard oil, fish oil contains 
a relatively high amount of omega-3 polyunsaturated 
fatty acids (n-3 PUFAs, ∼20%–31% in the fish oil used in 
our studies). These include docosahexaenoic acid (DHA) 
and eicosapentaenoic acid (EPA) that are reported to be 
associated with beneficial effects on plasma lipids and li- 
poproteins, blood pressure, and oxidative stress.30,31 The 
n-3 PUFAs have, for example, been reported to reduce 
plasma triglycerides levels by suppressing hepatic lipo- 
genesis and stimulating fatty acid oxidation through the 
regulation of transcription factors, including inhibition of 
SREBP1, carbohydrate regulatory element binding protein 
(ChREBP) and Max-Like Factor (MLX), and activation of 
peroxisome proliferator-activated receptor α (PPARα).32,33 
Hence, by limiting energy storage, the n-3 PUFAs present 
in fish oil may be responsible for the attenuated Srebp1/ 
Leap2 expression and jejunal lipid deposition in our study. 
To test this hypothesis and to assess the effect of oleic 
acid on Leap2 regulation, we stimulated organoids from 
the mouse jejunum with oleic acid (C 18:1 cis-9), linoleic 
acid (n-6 PUFA, 18:2 n-6), and docosahexaenoic/DHA 
(n-3 PUFA, 22:6 n-3). The results showed that oleic acid
inhibit both glucose production and food intake in rats, 34 central administration of oleic acid has been reported to inhibit both glucose production and food intake in rats. Interestingly, central administration of oleic acid has been reported to inhibit both glucose production and food intake in rats. 34

suggesting that the fatty acid may exert both peripheral (by increasing circulating LEAP2 levels) and central effects on the regulation of appetite and blood glucose levels.

As previously mentioned, n-3 PUFAs also induce fatty acid oxidation through the stimulation of PPARα. Although no difference in PPARα expression between mice dosed with olive versus fish oil argues against a role of PPARα in LEAP2 regulation, PPARα protein and activity levels are also needed to conclude on the role of the transcription factor.

The LEAP2 upregulation associated with oleic acid in the intestinal organoids was smaller than that observed for olive oil dosing in the in vivo studies. As the fatty acids likely enter the intestinal cells more rapidly in vitro versus in vivo, harvesting cells at an earlier time point might have enabled us to detect larger increases in LEAP2 expression and a difference in Srebp1 expression. Furthermore, although lard oil just like olive oil also contains a high concentration of oleic acid (~40%), future studies should assess the LEAP2 regulation in response to saturated fatty acids, such as palmitic and stearic acid, that represents a high fraction of the long-chain fatty acids present in lard oil (~47%).

Although the effect of lard oil and olive oil on Leap2 expression and jejunal lipids (triglycerides, FFA) was comparable, it should be mentioned that the levels associated with olive oil dosing were slightly lower in the lard oil study compared to the two other studies. Although the reason for this difference is not known (comparable plasma triglyceride levels were achieved in all studies), it could potentially have masked any difference between olive oil and lard oil with regard to effect on LEAP2 regulation.

The receptors GPR40, GPR120, and GPR119 are enriched in the enteroendocrine cells (EECs) and are involved in the release of gut hormones. 35 Whereas both GPR40 and GPR120 are responsive to long-chain fatty acids, both showing a preference for unsaturated fatty acids, the GPR119 is activated by 2-monoacylglycerols, produced by intestinal triglyceride digestion, and by oleoylethanolamides produced in the small intestine. 36

LEAP2 has been reported to be highly expressed in enterocytes along the luminal surface of the intestinal villi in mice. However, recent findings by Hagemann and colleagues showed that—in addition to a dense Leap2 expression in intestinal epithelial cells lining the crypts—a LEAP2 fragment, LEAP238–47, is expressed in human EECs from obese individuals. These findings could suggest that the EECs and associated receptors play a role in regulating intestinal LEAP2. In order to assess whether GPR40/119/120 could be responsible for the increase in LEAP2 observed with lipid dosing, we measured Leap2 expression in mice orally administered with agonists for GPR40, GPR120, and GPR119. The dose of 30 mg/kg was based on previous in vivo studies assessing the effect of GPR40 agonists on incretin secretion. 37 Our results do not suggest these GPCRs represent a significant contributor to the upregulation of LEAP2 with meal digestion, although we cannot rule out that earlier tissue sampling or use of a higher dose would have given us different results. However, administration of a GPR40 agonist, AM-5262 was associated with decreased liver Leap2 and Srebp1 expression. Interestingly, oral administration of a GPR40 agonist, SCO-267, has recently been reported to improve liver parameters through upregulation of lipid oxidation and inhibition of lipogenesis in a mouse model of NASH. 38 Furthermore, oral administration of the GPR40 agonist, GW9508, has been reported to inhibit lipogenesis through upregulation of AMPK, thereby attenuating lipid accumulation in mice fed a high-cholesterol diet and in HGeP2 cells. 39 The effect of GPR40 agonists on hepatic lipid storage could possibly explain the decrease in Leap2 expression in the liver if LEAP2—in addition being regulated according to metabolic status 7,10,19—is regulated according to local energy availability (hepatic glycogen, jejunal lipid) which our studies also suggest.

To assess the effects of diet composition—playing a key role in the development of obesity—on LEAP2, we investigated the regulation of LEAP2 in response to dosing with olive and fish oil in mice fed an HFD or chow diet. As previously reported, 5,15 diet intervention increased plasma LEAP2 levels, reflected by higher levels in mice fed HFD versus chow. The fact that this increase is associated with increased levels of jejunal lipids (triglycerides and FFA) rather than liver lipids could suggest that the increase in LEAP2 levels is initially (~4 weeks) driven by metabolic changes in the small intestine rather than the liver when taking the correlation between Leap2 expression and levels of triglycerides/FFA into account. Comparable Leap2 expression levels in the liver from mice fed an HFD versus chow also argue against a key role for the liver in driving this initial increase in systemic LEAP2 levels. Rather, the accumulation of lipids in the small intestine may result in an upregulation of Leap2 expression, thereby increasing systemic LEAP2 levels, although the effect of diet on a number of reference genes (Ywhaz, Gapdh, Hprt, Rpl13a)
made it difficult to assess the effect of diet on jejunal Leap2 expression. Feeding mice HFD versus chow for 16 weeks has previously been reported to increase plasma LEAP2 levels with no increase in Leap2 expression, whereas longer diet interventions (23 weeks of HFD) have been reported to increase Leap2 expression in both the liver and small intestine. There is, therefore, a possibility that the increased LEAP2 levels associated with the HFD versus chow diet observed in our study is not caused by transcriptional upregulation but perhaps by alterations in LEAP2 degradation/blood peptidase activity or tissue uptake.

Despite increased jejunal Leap2 expression, oil versus water dosing did not significantly increase plasma LEAP2 levels in mice fed chow (p = .07) as observed in the previous oil dosing studies. This may be explained by the difference in the food restriction intervals (2.5 vs. 16 h with the latter favoring a high ghrelin/LEAP2 ratio with an increased chance to detect an increase in LEAP2). Plasma LEAP2 and jejunal Leap2 expression levels did however increase with olive oil versus water dosing in HFD mice. Although the Leap2 expression was attenuated and no increase in Srebp1c expression or FFA levels was observed with olive oil versus water dosing in this group, this may be caused by upregulation of Leap2/Srebp1c expression and increased lipid deposition induced by an HFD diet (as previously discussed), thereby potentially limiting a further increase.

Interestingly, in addition to increasing systemic LEAP2 levels, feeding mice with an HFD versus chow also increased the LEAP2 responsiveness to olive oil (ΔLEAP_{0-120min} and ΔLEAP_{0-240min}). The opposite was however observed with fish oil where an HFD versus chow diet decreased the LEAP2 response associated with fish oil versus water dosing (ΔLEAP_{0-240min}). The increased LEAP2 response associated with olive oil may be driven by the higher accumulation of lipids in the jejunum as well as in the liver. However, this does not explain why a decreased LEAP2 response was associated with fish oil versus water in mice fed HFD versus chow where triglyceride levels also increased in these tissues. This could suggest that, when it comes to LEAP2 regulation, the fatty acid composition overrules the amount of lipid incorporated to the lipid pool and lipid stores. It should however be mentioned that the fact that higher plasma LEAP2 levels were observed in the fish oil versus water group prior to dosing in HFD mice suggests that the fish oil data in this study should be interpreted with caution, as LEAP2 pharmacokinetics may differ between these two groups.

In humans, obesity and diabetes have been reported to be associated with an (perhaps compensatory) increase in LEAP2 levels. The regulation of LEAP2 in response to meal ingestion may also be altered in individuals with obesity and diabetes since changes in plasma LEAP2 associated with ingestion of a mixed meal of 600 kcal have been reported to positively correlate with BMI. However, the LEAP2 response seems to highly depend on meal size since no increase in postprandial LEAP2 levels has been reported in people with obesity (mean BMI 40.3 kg/m²) or healthy individuals with ingestion of a mixed meal of 300 kcal and ~241 kcal (1101 kJ), respectively. Although we also observed an increase in both LEAP2 levels and the meal-induced LEAP2 response in mice fed HFD versus chow, with the study groups deviating only ~9% in body weight, the individuals included in the clinical studies were all fasted prior to ingestion of a mixed meal (favoring a high ghrelin/LEAP2 ratio), thereby making it difficult to compare with our in vivo results where the primary purpose was to assess the effect diet/recent meal rather than the obesity status on LEAP2.

Mice fed HFD versus chow had much higher levels of plasma triglycerides upon lipid dosing. This is likely to cause the associated increase in hepatic lipids with lipid dosing, as the liver would be exposed to a higher flux of FFA from dietary chylomicrons. Here, the increased uptake of n-3 PUFAs from fish oil in mice fed HFD versus chow could be speculated to decrease liver Srebp1 expression. Since the mice were dosed according to body weight, a higher weight in the HFD group may have resulted in relatively higher exposure to plasma triglycerides which may also have contributed to the increased LEAP2 levels observed in this group. However, since the HFD mice only weighed slightly more compared to mice fed chow (9% increase vs. >2.3-fold increase in plasma triglycerides), this contribution is likely limited. The higher plasma triglyceride levels observed in HFD-fed mice may also result from decreased triglyceride uptake by peripheral tissues mediated by decreased insulin sensitivity, as higher blood glucose levels were observed in mice fed HFD versus chow after 2.5 h of food deprivation. Higher levels of jejunal triglycerides in mice fed HFD versus chow (by comparison of water-dosed groups) could also have contributed, as lipids stored in enterocytes from a previous meal can enter the systemic circulation upon ingestion of a new meal.

In conclusion, we found that LEAP2 is regulated by diet components in the small intestine and the liver of mice. In the liver, LEAP2 is upregulated in the setting of increased plasma glucose and liver glycogen. In the jejunum, lipid digestion and accumulation induce the largest LEAP2 upregulation, although this response highly depends on the fatty acid composition. Finally, a high-fat versus chow diet not only increases plasma LEAP2, but also the LEAP2 response associated with olive oil. Collectively, we show that LEAP2 is upregulated in response to a positive energy balance (as also reported in individuals with obesity and diabetes), most likely to counteract further expansion of energy stores. In
addition to exploring the mechanism behind the LEAP2 upregulation associated with meal digestion, further studies should assess how LEAP2 levels are regulated in response to different meal challenges/diets in both healthy individuals and in people living with obesity and diabetes. Furthermore, the therapeutic potential of LEAP2 in obesity and associated comorbidities remains to be explored.

**AUTHOR CONTRIBUTIONS**

All authors participated in the design and/or interpretation of the studies. In vivo studies, qPCR, ELISA, and the associated data analyses were performed by Anna Katrina Jógvansdóttir Gradel, Stephanie K. Holm, and Sarah Byberg. Organoid studies and tissue/plasma biochemistry were performed by Anna Katrina Jógvansdóttir Gradel and Jesper Damgaard. All authors were involved in drafting and revising the manuscript.

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**DISCLOSURES**

The authors declare no conflicts of interest.

**DATA AVAILABILITY STATEMENT**

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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**SUPPORTING INFORMATION**

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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