Leptin and amylin act in an additive manner to activate overlapping signaling pathways in peripheral tissues; *in vitro* and *ex vivo* studies in humans

**Short running title:** Leptin and amylin signaling

Hyun-Seuk Moon¹ PhD, John P. Chamberland¹ BS, Kalliope N. Diakopoulos¹ BS, Christina G. Fiorenza¹ BS, Florencia Ziemke¹ MD, Benjamin Schneider² MD, Christos S. Mantzoros¹,³ MD

¹Division of Endocrinology, Diabetes, and Metabolism, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA.
²Division of Minimally Invasive Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts 02215, USA.
³Section of Endocrinology, Boston VA Healthcare System, Harvard Medical School, Boston, Massachusetts 02130

**Correspondence should be addressed to:**
Christos S. Mantzoros
Email: cmantzor@bidmc.harvard.edu

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Objective - Amylin interacts with leptin to alter metabolism. We evaluated, for the first time, amylin- and/or leptin-activated signaling pathways in human peripheral tissues (hPT).

Research design and methods - Leptin and amylin signaling studies were performed in vitro in human primary adipocytes (hPA) and human peripheral blood mononuclear cells (hPBMC), and ex vivo in human adipose (hAT) tissue from male vs. female, obese vs. lean, and subcutaneous (SC) vs. omental (OM) subjects.

Results - The long form of leptin receptor (ObRb) was expressed in human tissues and cells studied in ex vivo and in vitro, respectively. Leptin and amylin alone and in combination activate STAT3, AMPK, Akt and ERK signaling pathways in hAT ex vivo, and hPA and hPBMC in vitro; all phosphorylation events were saturable at leptin and amylin concentrations of ~50 ng/ml and ~20 ng/ml respectively. The effects of leptin and amylin on STAT3 phosphorylation in hPA and hPBMC in vitro were totally abolished under endoplasmic reticulum stress and/or in the presence of a STAT3 inhibitor. Results similar to the above in vitro studies were observed in hAT studied ex vivo.

Conclusions - Leptin and amylin activate overlapping intracellular signaling pathways in humans and have additive, but not synergistic, effects in signaling pathways studied in hPT in vitro and ex vivo.

Leptin is an adipocyte-secreted hormone that plays a major role in energy homeostasis and weight balance (1). Leptin activates not only central nervous system networks that suppress appetite (1) but also acts in the periphery to alter immune function and metabolism (2). Leptin administration to leptin deficient (ob/ob) mice has been shown to reduce food intake and body mass and improve insulin resistance even before body weight is reduced (3). Moreover, leptin improves insulin resistance in leptin deficient lipoatrophic mice and humans with the metabolic syndrome and insulin resistance (3). Amylin is a 37-amino acid peptide hormone that is co-secreted with insulin from pancreatic β-cells (4). The physiologic effects of amylin receptor agonism include decreased food intake (4) and reduction of postprandial glucagon release in a glucose-dependent manner (5). Moreover, it has been proposed that treatment with a combination of amylin and leptin may be more effective than leptin or amylin alone for obesity treatment in both animals (2) and humans (5).

Comprehensive pharmacological studies demonstrated that concurrent amylin and leptin infusion synergistically reduce body weight and adiposity in diet-induced obesity (DIO) rats (6). It has also been demonstrated that acute amylin infusion amplifies central leptin signaling, and with sustained treatment, amylin and leptin elicit synergistic weight and fat loss (6). Amylin-treated rats demonstrate a trend for a greater number of p-STAT3-positive cells within the arcuate nucleus (ARC) than vehicle-treated controls, an effect similar to leptin’s, whereas rats treated with both amylin and leptin have significantly more p-STAT3-positive cells than vehicle-, amylin-, or leptin-treated rats (7). Furthermore, this study also demonstrated that leptin receptor mRNA was significantly increased by amylin/leptin co-infusion both centrally and peripherally i.e. in white adipose tissue (WAT) and liver (7). Moreover, it has been shown that peripheral leptin injection in
Leptin and amylin signaling in rodents increases STAT3 and MAPK phosphorylation in liver and AT (8). No previous study has evaluated leptin and amylin signaling in human peripheral tissues (hPT) nor studied how amylin interacts with leptin to alter signaling in hPT.

We performed ex vivo and in vitro signaling studies to clarify the role of leptin and amylin in activating signaling pathways in hPT as well as their potential interactions. We first investigated in vitro leptin and amylin signaling in human primary adipocytes (hPA) and human peripheral blood mononuclear cells (hPBMC), known cell targets of leptin action. We then performed ex vivo leptin and amylin signaling studies in human adipose tissue (hAT) from subcutaneous (SC) vs. omental (OM), male vs. female, and obese vs. lean subjects.

**RESEARCH DESIGN AND METHODS**

**Subject collection** - We used human adipose tissue (hAT) from subjects undergoing laparoscopic adjustable gastric banding, hernioplasty, liposuction or abdominoplasty. The study protocol was approved by the Institutional Review Board at the Beth Israel Deaconess Medical Center (BIDMC) and subjects gave written informed consent to participate. All subjects were otherwise healthy, had no evidence of immunologic or endocrine disease based on physical examination and routine blood tests, and had no history of recent infection. The subjects’ age, vitals and BMI were recorded. We collected tissue samples from obese (6 males and 6 females; age, 31-54 year-old; BMI, 42-44 kg/m2) and lean (3 males and 4 females; age, 22-32 year-old; BMI, 21-23 kg/m2) subjects.

**Materials** - All primary and secondary antibodies were purchased from Santa Cruz Biotech (San Francisco, CA). Leptin human recombinants were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). Amylin human recombinants were purchased from Phoenix Pharmaceuticals Inc. (Burlingame, CA). FBS and FCS were provided by Gibco Life Technologies (New York, USA). BSA, α-MEM, RPMI, NaHCO₃, Hepes, biotin, pantothenate, human transferrin, gentamycin, insulin, cortisol, T₃, DTT, TUN and ciglitazone were purchased from Sigma-Aldrich (St. Louis, MO).

**Real-time polymerase chain reaction (RT-PCR)** - The long form of leptin receptor (ObRb) expression was detected with RT-PCR. RNA was extracted with Trizol® (Invitrogen, Carlsbad, CA) and first-strand cDNA synthesis was performed using Superscript III® (Invitrogen, Carlsbad, CA), according to the manufacturer’s protocol. For RT-PCR, 100ng of cDNA per 25µl reaction were amplified using TaqMan Gene Expression system (Applied Biosystems, Foster City, CA), specific primers and FAM tagged probes set (Applied Biosystems, Foster City, CA), and the standard real-time 7500 protocol (Applied Biosystems, Foster City, CA), with an initial polymerase activation step at 95°C for 10 min and 40 cycles. This included a 15 sec melting step at 95°C and a 1 min annealing-elongation step at 60°C. The analysis of relative gene expression was based on ΔCt values obtained from RT-PCR (9).

**Ex vivo signaling study in hAT** - The ex vivo culture was established according to the method described by Kim et al. (8). Briefly, five to seven grams of fresh subcutaneous (SC) and/or omental (OM) hAT were placed into Krebs-Ringer-HEPES buffer (20mM, pH 7.4) with 2.5% BSA and 200nM adenosine at 37°C in the operating room and immediately taken to the laboratory for further analysis. In the laboratory, the tissue samples were minced into pieces of approximately 1-mm in diameter, and any non-adipose tissue and non-muscle tissue were removed by washing with fresh buffer. The samples were aliquoted and incubated at 37°C either with or without amylin and leptin.
**Human primary adipocytes (hPA) culture** - The hPA culture was established according to the method described by Ribet et al. (10). Briefly, SC and OM hAT samples were obtained from lean (35-41 years old, BMI 22-25 kg/m^2) and obese (34-48 years old, BMI 39-50 kg/m^2) males and females, respectively. The hAT was then digested with PBS/collagenase solution (3 mg collagenase per gram of tissue and 1 ml PBS per 1 mg collagenase) + 3.5% fatty acid free BSA and then filtered using filter-bottle unit (sterile funnel with double layered gauze), then the solution was centrifuged at 1,200 rpm for 10 minutes. The pellets were re-suspended in α-MEM supplemented with 15 mmol/L NaHCO₃, 15 mmol/L Hepes, 33 µmol/L biotin, 17 µmol/L pantothenate, 10 mg/ml human transferrin, 0.05 mg/ml gentamycin, and 10% FBS and then plated overnight. After confluence, α-MEM was removed and washed once or twice with HBSS. To induce adipocyte differentiation, the cells were exposed to differentiation medium containing 66 nmol/L insulin, 100 nmol/L cortisol, 0.2 nmol/L triiodothyronine (T₃) and 1 µg/ml ciglitazone. The medium was changed every 2 days and cells were kept in culture for 28 days.

**Human peripheral blood mononuclear cells (hPBMC) culture** - The hPBMC was isolated by density gradient sedimentation on Ficoll-Paque (Pharmacia, Uppsala, Sweden) as described previously (11). The cells were washed twice in PBS and re-suspended in medium appropriately for cell culture, RPMI 1640 supplemented with 25 mM Hepes, 2 mM L-glutamine, 100 µg/ml penicillin, 100 µg/ml streptomycin, and amphotericin B (2.5 µg/ml).

**Endoplasmic reticulum (ER) stress induction** - The induction of ER stress was established according to the method described by Hagiwara et al. (12). Briefly, to induce ER stress, the cells were pre-treated with tunicamycin (TUN, 3 µg/ml) and/or dithiothreitol (DTT, 1 mM) for 5 hr, and subsequently taken to leptin and/or amylin.

**Protein extraction** - For total cell extracts, collected cells were suspended in a lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 5 mM ethylene diamine tetra acetic acid (EDTA), 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 0.05% aprotinin, and 0.1% Igepal and then incubated for 30 min at 4°C. The suspension was centrifuged for 25 min at 14,240 g and the supernatant was saved as the total extract. Next, the pellet was re-suspended in a lysis buffer containing 50 mM Hepes-NaOH (pH 7.8), 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF and 10% (v/v) glycerol. The suspension was mixed for 30 min at 4°C and centrifuged for 15 min at 890 g; the supernatant was saved as the nuclear extract.

**Western Blotting** - For Western Blotting, proteins were loaded in each lane. After SDS-polyacrylamide gel electrophoresis, proteins were blotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, NH). The membranes were blocked for 1 hr in TBS containing 5% nonfat dry milk and 0.1% Tween 20. Incubation with primary antibodies was performed in TBS containing 5% nonfat dry milk for overnight and then incubated with horseradish peroxidase secondary antibodies for two hour. After incubation with antibodies, membranes were washed with TBS containing 0.1% Tween-20. Enhanced chemiluminescence was used for detection. Measurement of signal intensity on nitrocellulose membranes after Western Blotting with various antibodies was performed using Image J processing and analysis software.

**Statistical Analysis** - Data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Analyses were carried out using SPSS (version 11.5, SPSS).

**RESULTS**
1. Leptin receptor expression in hAT *ex vivo*, and in hPA and hPBMC *in vitro* - The long form of leptin receptor (ObRb) was expressed in human tissues and cells studied in *ex vivo* and *in vitro*, respectively (data not shown). We did not observe any increase in ObRb expression as a result of leptin, amylin, and/or leptin + amylin administration for 30 min when compared to control. Also, we did not observe any difference in ObRb expression in SC vs. OM hAT, male vs. female and obese vs. lean (data not shown) in the limited number of subject studied.

2. Leptin and amylin signaling: Dose- and time-response curves in hAT *ex vivo*, and in hPA and hPBMC *in vitro* from obese female subjects - *Ex vivo* and *in vitro* leptin and amylin administration significantly induced phosphorylation of STAT3 by ~2.8-fold higher than control at 30 min in hAT (Fig. 1A and 1B) *ex vivo*, and in hPA (Fig. 1C) and hPBMC (Fig. 1D) *in vitro*. Importantly, all leptin and amylin signaling pathways were activated in a dose-dependent manner but activations of all signaling pathways were saturable at a leptin concentration of ~50 ng/ml and amylin concentration of ~20 ng/ml. We did not observe any difference in STAT3 activation in SC vs. OM hAT, obese vs. lean and in male vs. female subjects (data not shown).

3. Activation of STAT3 signaling by administration of leptin and amylin alone or in combination in hAT *ex vivo* and in hPA and hPBMC *in vitro* from obese female subjects - Based on the above (Fig. 1), we chose a representative administration time (30 min) and leptin (50 ng/ml) and amylin (20 ng/ml) concentrations. Either leptin or amylin stimulated phosphorylation of STAT3 by ~2.8-fold in SC and OM hAT *ex vivo* (Supplemental Figure 1A available at [http://care.diabetesjournals.org](http://care.diabetesjournals.org)), by ~2.9-fold in hPA *in vitro* (Supplemental Figure 1B) and by ~2.8-fold in hPBMC (Supplemental Figure 1C) *in vitro*. Also, co-administration of leptin and amylin activated STAT3 signaling in hAT *ex vivo* by ~4.7-fold, in hPA *in vitro* by ~5.1-fold and in hPBMC *in vitro* by ~4.9-fold. Moreover, nuclear translocation of STAT3 was increased by leptin, and this effect was more enhanced by co-administration of amylin in hPA and hPBMC *in vitro* (data not shown). By contrast, increased STAT3 phosphorylation by leptin + amylin administration was totally abolished by pre-treatment with AG490, a STAT3 inhibitor, in hPA (Supplemental Figure 1D) and hPBMC (Supplemental Figure 1E) *in vitro*. Importantly, we observed an additive, but not synergistic, effect of amylin on leptin-treated tissues and/or cells. There was no difference in STAT3 activation in experiments comparing SC vs. OM hAT, obese vs. lean and male vs. female subjects (data not shown).

4. Activation of ERK signaling by administration of leptin and amylin alone or in combination in hAT *ex vivo* and in hPA and hPBMC *in vitro* from obese female subjects - ERK signaling was activated in leptin-treated (by ~2.3-fold) and amylin-treated (by ~2.2-fold) hAT *ex vivo*, and this activation was further increased in response to co-administration of amylin and leptin by ~3.4-fold (Supplemental Figure 2A). As expected, ERK signaling was activated in leptin- and amylin-treated hPA *in vitro* by ~2.9-fold and ~2.8-fold (Supplemental Figure 2B) and in hPBMC *in vitro* by ~2.8-fold and ~2.7-fold, respectively (Supplemental Figure 2C). Also, activation of ERK signaling in response to leptin and amylin co-administration in hPA *in vitro* was increased by ~5.1-fold and hPBMC *in vitro* by ~4.8-fold but leptin + amylin-activated ERK signaling was totally blocked by pre-treatment with an ERK inhibitor (Supplemental Figure 2B and 2C). We did not observe any difference in ERK activation when comparing cells or tissues from SC vs. OM hAT, obese vs. lean and male vs. female subjects (data not shown).
5. Activation of Akt and AMPK signaling by administration of leptin and amylin alone or in combination in hAT ex vivo and in hPA and hPBMC in vitro from obese female subjects - Leptin activated Akt and AMPK signaling in hAT ex vivo by ~1.9-fold and ~3.5-fold (Supplemental Figure 3A), in hPA in vitro by ~2.6-fold and ~2.8-fold (Supplemental Figure 3B) and in hPBMC in vitro by ~3.0-fold and ~3.2-fold (Supplemental Figure 3C). Amylin also activated Akt and AMPK signaling in hAT ex vivo by ~2.0-fold and ~3.6-fold (Supplemental Figure 3A), in hPA in vitro by ~2.6-fold and ~2.8-fold (Supplemental Figure 3B) and in hPBMC in vitro by ~3.0-fold and ~3.1-fold (Supplemental Figure 3C). Moreover, activation of Akt and AMPK signaling by leptin and amylin alone in hAT ex vivo as well as hPA and hPBMC in vitro was increased in response to leptin and amylin co-administration. By contrast, activation of Akt and AMPK signaling in leptin + amylin-stimulated cells was blocked by pre-treatment with each inhibitor. We did not observe any difference in Akt and/or AMPK activation in cells or tissues from SC vs. OM hAT, obese vs. lean and from male vs. female (data not shown).

6. Inhibition of STAT3 signaling by ER stress in hPA and hPBMC in vitro from obese female subjects - Stimulation of the cells with leptin + amylin led to a marked and significant increase in phosphorylation of STAT3 in hPA by ~5.0-fold (Supplemental Figure 4A) and in hPBMC by ~4.8-fold (Supplemental Figure 4B) but, when challenged with ER stress inducers, TUN and/or DTT, the leptin + amylin-activated STAT3 phosphorylation was totally abolished, suggesting that amylin could not overcome ER stress-induced inhibition of leptin-activated STAT3 signaling. We did not observe any difference in ER stress-blocked STAT3 activation in hPA and hPBMC from obese vs. lean and male vs. female subjects (data not shown).

CONCLUSIONS
Leptin, the prototype adipocyte-secreted adipocytokine, regulates food intake and body weight acting primarily in the hypothalamus (1). It may also act in the periphery to modulate immune and metabolic functions (2). Peripheral leptin administration has been demonstrated to phosphorylate STAT3 and MAPK in rodent AT and liver (8). Similar effects have not yet been explored in humans. Amylin, a hormone secreted from the pancreas, functions to decrease food intake, decrease gastric emptying and glucagon secretion (4). Although amylin has been proposed to amplify leptin signaling in the hypothalamus, thereby synergistically effecting weight and fat loss in animal models (6), the molecular mechanisms underlying these effects remain to be fully clarified. We have previously demonstrated that altering circulating leptin levels in humans does not alter circulating amylin levels (13). Whether amylin’s action can increase leptin signaling in human tissues remains to be fully elucidated. More specifically, there have been no leptin and amylin signaling studies evaluating hPT, including hAT and hPBMC. Hence, we performed ex vivo and in vitro signaling studies to investigate the role of amylin and leptin in activating signaling pathways in hPT as well as their potential interaction.

STAT3 mediates the expression of a variety of genes in response to cell stimuli, and thus plays a key role in many cellular processes such as cell growth and apoptosis (8). In fact, disrupting the ability of the leptin receptor to activate the STAT3 pathway in mice leads to severe obesity and several other neuroendocrine abnormalities (14). The STAT3 pathway is the first identified signaling mechanism found to be associated with the leptin receptor (8). Leptin induces
phosphorylation of STAT3 in keratinocytes, endometrial cancer cells, hypothalamic cells, murine adipocytes and mouse AT (3, 8, 15-16). Amylin, similar to leptin, has been shown to increase the number of p-STAT3 cells in the ARC of rats (7) and this effect was significantly greater with co-administration of leptin and amylin when compared to individual treatments (7). Based on these results, we checked for the first time whether leptin signaling interacts with amylin in humans and whether co-treatment of amylin and leptin could further increase leptin-stimulated STAT3 signaling in hPT. We observed that leptin and amylin increased STAT3 signaling in hAT \textit{ex vivo} and hPA and hPBMC \textit{in vitro}, and the effect of these two hormones was similar in magnitude and peaked around the same time. Our data in peripheral tissues is consistent with rodent studies previously demonstrating that amylin-treated rats tend to have a greater number of p-STAT3-positive cells than vehicle controls in hypothalami (7). Although it is important to study leptin’s and amylin’s effects in human hypothalamic or other central nervous system cells, our data suggest that amylin-increased STAT3 signaling in leptin-stimulated hAT and cells may have important clinical implications.

ERK, a member of the mitogen-activated protein kinase (MAPK) family, is an additional pathway downstream of the leptin receptor (16). Leptin has been shown to activate ERK1/2 in a time- and dose-dependent manner in several cultured mouse cells \textit{in vitro} as well as rodent tissues \textit{in vivo} (17). The ERK pathway has been reported to mediate leptin’s effects on human PBMC \textit{in vitro} as well as rat kidney and rat AT (8, 17). Based on these previous studies, we further investigated whether amylin could activate ERK signaling in hPT, since no relevant data in humans has been published to date. We observed that amylin increases ERK activation alone and in leptin-stimulated hAT \textit{ex vivo} as well as hPA and hPBMC \textit{in vitro}. Since ERK is a significant downstream target for leptin’s physiological effects (15-16), we suggest that amylin may also have a beneficial effect on human physio- and pathophysiology.

AMPK is an evolutionarily conserved serine/threonine protein kinase central to the regulation of energy balance at both the cellular and whole-body level (17). In its classical role as an intracellular metabolic stress-sensing kinase, AMPK switches on fatty acid oxidation and glucose uptake, while switching off hepatic gluconeogenesis, and exerts a broader role in metabolism by controlling appetite (17). In fact, AMPK is involved in cellular energy homeostasis by AMPK phosphorylation and inhibition of acetyl-coA carboxylase (ACC), and thus stimulates fatty acid oxidation (17-18). However, whether leptin can activate AMPK and/or whether amylin can activate AMPK to induce fatty acid oxidation in hPT has not yet been demonstrated. We checked leptin and amylin signaling in hPT \textit{in vitro} and \textit{ex vivo}, and observed that leptin and amylin increase AMPK activation in all cells. Moreover, we observed that leptin and amylin administration directly increase AMPK activation in hAT \textit{ex vivo}. Since AMPK is associated with fatty acid oxidation, which plays a key role in the pathophysiology of insulin resistance (17), leptin- and amylin-activated AMPK signaling in hAT and other peripheral tissues offers further support to the notion that the use of these hormones, alone or in combination, could be a viable strategy in treating obesity and type II diabetes.

The Akt signaling pathway is responsible for cellular processes like cell growth, proliferation and glucose metabolism (19). It has been shown that obesity induces increased levels of leptin, insulin, insulin-like growth factor, TNF-α and IL-6, and reduces adiponectin levels (19). These altered factors change Akt signaling activity, which, in turn,
Leptin and amylin signaling regulates downstream targets leading to increased cell survival and cell growth, and promoted cell cycle (19). Based on these previous results, we further investigated whether leptin and/or amylin could activate Akt signaling in hPT \textit{in vitro} and \textit{ex vivo}. We observed that leptin and amylin alone and in combination increase Akt phosphorylation in hAT \textit{ex vivo} as well as hPA and hPBMC \textit{in vitro} suggesting that leptin and amylin, alone or in combination, could have beneficial effects on cell survival.

We observed that amylin activates Jak2 signaling pathways in hAT \textit{ex vivo} and hPA and hPBMC \textit{in vitro} (data not shown). Since OBRb has the capacity to activate Jak2 (20), we suggest that amylin-mediated intracellular signaling pathways \textit{ex vivo} and \textit{in vitro} might be activated not only through cGMP (21) but also potentially through Jak2 signaling. This is the first report not only on amylin interacting with Jak2 signaling in human peripheral tissues, but also on amylin signaling in human peripheral tissues. It has been suggested that amylin may act through up-regulation of the leptin receptor and that this induces STAT3 activation in hypothalami in rodent \textit{in vivo} (7). We did not observe any difference in ObRb expression in human peripheral tissues treated with leptin, amylin and leptin + amylin but the treatment time was only 30 min. Longer term exposure to amylin and leptin as well as a receptor knock-down experiment would also be useful in determining whether amylin could act through ObRb in human peripheral tissues. Since it is impossible to perform the receptor knock-down studies in humans, alternative methods such as ObRb knock-down \textit{in vivo} rodent or mouse studies may have to be performed. In any case, the lack of ObRb up-regulation in our studies excludes this potential mechanism and suggests that most probably amylin may activate signaling pathways downstream of Jak2. This original observation has to be replicated by other studies in the future.

It is generally accepted that obese vs. lean subjects have differences in leptin responsiveness (3). We also expected that there would be differences between obese vs. lean group in terms of leptin responsiveness. Despite minor differences in the timing of signaling activation, we did not observe any differences in several leptin-activated signaling pathways from obese vs. lean subjects in the tissues studied. There are a number of potential explanations for this observation. First, although there are differences between obese vs. lean subjects in response to leptin accumulation in the medium of cultured OM and SC AT in the early differentiation stages \textit{in vitro} (22), leptin and leptin mRNA levels are not different in obese vs. lean subjects after full differentiation (23). The effect of leptin on lipolysis is not different between cultured obese and lean human adipocytes \textit{in vitro} (24). These results demonstrate that there are major quantitative changes in leptin responsiveness between obese and lean during differentiating stages, but not differentiated stages, of hAT \textit{in vitro}. We have used differentiated adipocytes herein. Signaling interactions of leptin and amylin in various differentiation stages remain to be studied. Second, although we studied the signaling pathways considered primary targets of leptin, on the basis of current evidence deriving mainly from rodent studies, we have not looked at all signaling pathways potentially mediating leptin’s action in the periphery. Moreover, there might be other, totally unknown, pathways activated by leptin since there have been no prior studies on leptin signaling in human tissues. Much more needs to be learned in the future. Third, it is also possible that signaling pathways mediating leptin actions in peripheral tissues (and resistance thereof) could be totally different from those in hypothalami and/or other central nervous system areas (CNS) (25). Since it has been proposed that centrally administered leptin affects insulin sensitivity
Leptin and amylin signaling and metabolism in peripheral tissues, it is possible that differences in CNS signaling between lean and obese are much more important than leptin activated signaling pathways in the periphery. Fourth, leptin and amylin act not only in adipose tissue but also in several other metabolically active peripheral tissues, including but not limited to liver and reproductive organs (26). Since leptin signaling could differ between lean and obese in these tissues and since this could have direct clinical implications in metabolism, more studies, focusing on tissues other than the ones studied herein, need to be performed. Finally, it is possible that signaling pathways in humans may be different from those in animals. This may be analogous to the different effect of leptin in regulating neuroendocrine response to starvation we have previously shown in mice (27) vs. humans (28).

ER stress has recently been shown to play a role in the development of leptin resistance in the hypothalamus of rodents (13) and it has been suggested that ER capacity is directly related to leptin sensitivity (13, 29). This resulted in suggestions that ER stress reversal could be utilized as a strategy to sensitize obese mice, and by extension humans, to leptin. These previous studies have shown that the reduction in ER function creates ER stress, blocks leptin action, and generates leptin resistance in mice, suggesting that ER stress inhibits at an upstream step of STAT3 phosphorylation and provides a potential mechanism in which increased ER stress antagonizes STAT3-mediated leptin signaling (29). However, whether activation of ER stress interferes with leptin and/or amylin signaling in hPT has not yet been demonstrated. Here, we report for the first time that TUN and also DTT pre-treatment of human cells completely blocks leptin- and amylin-stimulated STAT3 activation in hPA and hPBMC in vitro. Our data demonstrate that amylin does not function as a leptin sensitizer to bypass ER stress-induced leptin resistance and suggests that increased ER stress in obese people may induce leptin and also amylin resistance. Since in vivo leptin and amylin actions may differ in comparison to in vitro, studies of in vivo leptin and amylin signaling in humans are needed to prove or disprove this hypothesis. It is currently impossible to perform human in vivo ER stress studies, however, and thus specific methods for ER stress induction in humans need to be developed.

We believe that our data are important from the physiological point of view. This is the very first attempt to map the intracellular signaling pathways downstream of leptin and amylin in human tissues. This first paper, if followed by other similar papers, will eventually allow full characterization of signaling pathways in all peripheral tissues in humans as well as the comparative evaluation of human vs. animal signaling pathways. This new knowledge in human physiology will consequently lead to the very first attempt to also study pathophysiology, and later therapeutics, in humans. Although we are initiating this effort with the current ground-breaking paper, much more needs to be done (additional tissues, other signaling pathways, other physiological conditions) in the future. Despite these limitations, our initial data in human tissues suggest the existence of 1) leptin tolerance (not resistance) in the studied tissues in human and 2) that unlike experiments in rodents showing synergistic effect of amylin on leptin-modulated food intake the effects of amylin and leptin in human peripheral tissues are additive and not synergistic.

In summary, despite minor differences in the timing of signaling activation in the different tissues and cells studied, we did not observe major differences in the magnitude of STAT3 activation in response to leptin and amylin administration in hAT ex vivo, and hPA and hPBMC in vitro. Also, we did not
observe any differences in STAT3, ERK, and AMPK phosphorylation when comparing male vs. female and obese vs. lean subjects. Importantly, leptin and amylin signaling pathways were saturable at a level of ~50 ng/ml (leptin) and ~20 ng/ml (amylin), suggesting that no additional signaling effect can be observed at higher doses than the above. Although leptin and amylin in combination increased phosphorylation of the pathways studied herein, we observed not a multiplicative, but rather an additive, effect of amylin on leptin-stimulated hPT \textit{ex vivo} and \textit{in vitro}. The present study adds to our recent clinical data demonstrating that leptin administration does not alter circulating amylin levels (13) and provides pre-clinical evidence that leptin and amylin administration activate overlapping intracellular signaling pathways in human peripheral tissues. These hormones have an additive effect in hPT further suggesting that leptin and amylin may play an additive role in regulating obesity and type-II diabetes in humans.

**Author contribution.** H.S.M. and C.S.M. wrote this manuscript. H.S.M., J.P.C., K.N.D., C.G.F., F.Z., B.S. and C.S.M. participated in the study design, performance and coordination. C.S.M. conceived and planned the study.

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FIGURE LEGEND
Figure 1. Leptin and amylin signaling: Dose- and time-response curves in hAT ex vivo and in hPA and hPBMC in vitro from obese female subjects - Ex vivo and in vitro leptin and amylin administration in hAT (A and B), hPA (C) and hPBMC (D) were performed as described in detail in section “RESEARCH DESIGN AND METHODS”. (A-D) The tissues and cells were incubated with leptin and/or amylin (50 ng/ml of leptin and 20 ng/ml of amylin for time-response study and 30 min incubation for dose-response study). All tissue lysates (whole protein extraction) were examined by Western blot with primary p-STAT3 and STAT3 antibodies. The secondary antibody used was horseradish peroxidase-conjugated anti-mouse and anti-goat antibodies. All figures showing quantitative analysis include data from at least three independent experiments. All density values for each protein band of interest are expressed as a fold increase. All data were analyzed using one-way ANOVA followed by post-hoc test for multiple comparisons. Values are means ± SD. Means with different letters are significantly different, p<0.05.