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Fasting levels of appetite regulating hormones predict caloric intake at breakfast in a group of Chilean adolescents

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ABSTRACT

***Background**: Appetite regulation is integral to food intake and is modulated by complex interactions between internal and external stimuli. Hormonal mechanisms which stimulate or inhibit intake have been characterized, but the physiologic effects of serum levels of such hormones in short-term appetite regulation have received little attention. **Aim**: To evaluate whether fasting levels of orexigenic/anorexigenic hormones were associated with energy intake at breakfast, served soon after drawing a fasting blood sample, in a group of adolescents. **Material and Methods**: Anthropometry, body composition and fasting blood levels of leptin, insulin, ghrelin, and orexin-A were measured in 655 Chilean adolescents aged 16.8 ± 0.3 years (52% males). Energy intake was measured at a semi-standardized breakfast. Associations between hormone levels and energy intake were studied using multivariate linear models. **Results**: Thirty nine percent of participants were overweight/ obese. After an overnight fast, median values for leptin, insulin, ghrelin and orexin-A were 7.3 ng/mL, 6.7 IU/dL, 200.8 pg/mL, and 16.1 pg/mL, respectively. Participants ate on average 637 ± 239 calories at breakfast. In multivariable models, insulin levels were inversely and independently associated with caloric intake at breakfast ($\beta = -18.65$; $p < 0.05$), whereas leptin, ghrelin and orexin-A levels were positively and independently associated with intake: $\beta = 5.56$, $\beta = 0.34$ and $\beta = 8.40$, respectively, $p < 0.05$. **Conclusions:** Fasting leptin, ghrelin and orexin-A were positively associated with energy intake during breakfast provided soon after the blood draw. Insulin was negatively associated with energy intake. Modifiable factors influencing levels of appetite regulating hormones could be a potential target for influencing food intake.*

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***Key words:** Appetite Regulation; Ghrelin; Insulin; Leptin; Orexins.*

Los niveles en ayunas de hormonas reguladoras del apetito predicen la ingesta energética en el desayuno en adolescentes

***Antecedentes:** La regulación del apetito es parte integral de la ingesta alimentaria y es modulada por complejas interacciones entre estímulos internos y externos. Se han caracterizado los mecanismos hormonales que estimulan*

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o inhiben la ingesta, pero los efectos fisiológicos de los niveles séricos de tales hormonas en la regulación del apetito a corto plazo han recibido poca atención. ***Objetivo****: Evaluar si los niveles en ayunas de hormonas orexigénicas/anorexigénicas se asocian con la ingesta energética en el desayuno, entregado inmediatamente después de una muestra de sangre en ayunas, en un grupo de adolescentes.* ***Material y Método:*** *Se efectuaron mediciones antropométricas, composición corporal y medición de niveles en ayunas de leptina, insulina, grelina y orexina-A en 655 adolescentes de 16,8 ± 0,26 años. La ingesta energética se midió en un desayuno semiestandarizado. Se estudiaron las asociaciones entre los niveles hormonales y la ingesta energética mediante modelos lineales multivariados.* ***Resultados:*** *Los valores de leptina, insulina, grelina y orexina-A fueron 7,3 ng/mL, 6,7 UI/dL, 200,8 pg/mL y 16,1 pg/mL respectivamente. Los participantes comieron un promedio de 637 ± 239 calorías en el desayuno. Los niveles de insulina se asociaron inversa e independientemente con la ingesta del desayuno (β = -18,65; $p < 0,05$), mientras que los niveles de leptina, grelina y orexina-A se asociaron positiva e independientemente con la ingesta: β = 5,65; β = 0,34; β = 8,40, ($p < 0,05$).* ***Conclusiones:*** *La leptina, grelina y orexina-A en ayunas se asociaron positivamente con la ingesta de energía durante el desayuno proporcionado poco después de la muestra de sangre. La insulina se asoció negativamente con la ingesta de energía. Los factores modificables que influyen en las hormonas reguladoras del apetito podrían ser un objetivo potencial para influir en la ingesta de alimentos.*

Palabras clave: *Insulina; Ghrelina; Leptina; Orexinas; Regulación del Apetito.*

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Appetite and satiety regulation are modulated by homeostatic pathways (i.e. gastro-enteropancreatic circuit, signals from adipose tissue, among others) and reward systems involving communication between hormones and the nervous system including the hypothalamus, cortical limbic system and the brain stem. In addition, environmental and social factors are involved. Thus, food intake is a complex response integrating both internal and external cues[1-3]. The role played by orexigenic and anorexigenic hormones in appetite regulation is increasingly appreciated[4-6] brain, and adipose tissue (AT).

Hormones including leptin, insulin and ghrelin have been extensively investigated in dietary intervention and experimental research (e.g., pharmacologic effect)[7-9]. In addition, observational studies have described the association between appetite regulating hormone levels and food intake[10-12] between habitual high-fat (HF. To date, most studies have shown negative associations between serum anorexigenic hormones (e.g. leptin and insulin) and energy intake, and positive associations between serum levels of orexigenic hormone (e.g. ghrelin in the acylated form) and energy intake[13-16]. However, some studies have found no association or associations in the opposite direction[1721] it is possible that this system might be impaired by the sustained intake of highly palatable foods. Short-term feeding studies suggest that the appetite-stimulating hormone ghrelin is suppressed less effectively by dietary fat intake, and diets high in sucrose decrease levels of the adipose hormone leptin. We hypothesized that higher habitual intake of dietary fat and carbohydrate (CHO. Published studies used diverse methodology and did not always consider important covariates that might influence the association between hormone levels and intake[22-24].

In addition to peripherally synthetized appetite hormones, several factors produced in central nervous system (CNS) are important in appetite regulation, but cannot be measured in blood. Orexin-A is an exception as an orexigenic factor released by orexin neurons in the lateral hypothalamus, which is measurable in blood[25-27] discove-

red through positional cloning 15 years ago, is an adipocyte-secreted hormone with pleiotropic effects in the physiology and pathophysiology of energy homeostasis, endocrinology, and metabolism. Studies in vitro and in animal models highlight the potential for leptin to regulate a number of physiological functions. Available evidence from human studies indicates that leptin has a mainly permissive role, with leptin administration being effective in states of leptin deficiency, less effective in states of leptin adequacy, and largely ineffective in states of leptin excess. Results from interventional studies in humans demonstrate that leptin administration in subjects with congenital complete leptin deficiency or subjects with partial leptin deficiency (subjects with lipoatrophy, congenital or related to HIV infection, and women with hypothalamic amenorrhea. Orexin-A functions, including appetite modulation, are pivotal at the level of the CNS. However, questions remain about the role of Orexin-A in peripheral tissues and whether serum levels reflect brain levels; some reports have shown the existence of orexin type 1 receptors, and the ability to synthetize the peptide by extra CNS organs[28,29].

Given the limited data available regarding the physiologic role of different orexigenic and anorexigenic hormone levels on almost immediate food intake, our hypothesis was that fasting levels of the orexigenic hormones, ghrelin and orexin-A, are directly associated with the energy intake of breakfast, while the associations with leptin and insulin are inverse. Thus, the aim of the current study was to assess the independent association of fasting levels of these hormones on caloric intake during a semi-standardized breakfast offered shortly after a fasting blood draw in a sample of adolescents.

Methods

Participants belonging to longitudinal cohort, originally studies as part of an infancy iron deficiency anemia preventive trial were considered for this analysis. Participants were originally recruited at 4 months old, from low to middle income communities in Santiago, Chile[30]. The preventive trial took place when the infants were 6 to 12 months old; 1657 infants completed the trial. Infants found to be anemic (n = 73) did not enter the trial. They entered a parallel neuromaturation study along with the next non-anemic control (n = 62)[31]. All participants were comprehensively assessed for developmental functioning at 12 months and invited to participate in follow up studies at 5 years, 10 years, and at multiple time points during adolescence. Sample sizes varied at each wave of testing with the smallest recruitment at 5 years (n = 888) due to a decrease in funding. This group did not differ from the original cohort (sex, birth weight, 1-year anthropometry, age at first bottle, maternal education, or maternal depression). At 16-17 years, those assessed at 5 years were invited to participate in a study related to obesity and cardiovascular risk, and 679 were assessed from 2009-2012[32]. All participants had completed puberty. Age at menarche was 12,4[1,4] in 95% females. The study was approved by the Ethics Committees at Institute of Nutrition and Food Technology (INTA), University of Chile, the University of California, San Diego, and the University of Michigan. Parents gave informed consent and participants signed informed assent. For this study, only participants with complete data for energy intake serum hormones in adolescence and with z-score for body mass index (BMI) >-2 were included (n = 655).

Anthropometry and Body Composition

Trained physicians assessed participant height and weight in the Frankfurt position, without shoes, wearing underwear. Weights and heights were measured twice, with Precision Hispana scales (SECA) and a stadiometer (Holtain) to 0.1 kg and 0.1 cm, respectively. Using the average of the two measurements, BMI was computed as weight [kg]/ height [m]^2; BMI z-score was determined for sex and age using World Health Organization standards (AnthroPlus software). The participants were classified as underweight/ normal weight (z-score ≥ -2 to < 1), overweight (z-score ≥ 1 to < 2) and obese (z-score ≥ 2). Lean body mass [kg] and fat-free mass [kg] were quantified via the absorptiometry of dual energy X-rays (DEXA, Lunar Prodigy®) according to standard protocol in the same machine, calibrated every two days. Fat mass index (FMI: fat mass [kg]/ height [m]^2) and fat-free mass index (FFMI: fat-free mass [kg]/ height [m]^2) were computed, FMI was considered high if ≥ 6.6 kg/m^2 for males and ≥ 9.5 kg/m^2 for females[33].

Measurement of Leptin, Insulin, Ghrelin, and Orexin-A

Blood samples were obtained after an overnight fast (time elapsed since the evening meal was not recorded) and processed within two hours. Serum aliquots were kept at -80ºC until analysis. The enzyme-linked immunosorbent assay (ELISA) was used to determine serum leptin levels (DRG International, Inc., New Jersey, NJ, USA) and the radioimmunoassay (RIA) technique was used for a quantitative measurement of insulin (DCP Diagnostic Products Corporation LA, USA), ghrelin (Phoenix Pharmaceuticals, INC. Burlingame CA, USA.) and orexin-A (Phoenix Pharmaceuticals, INC. Burlingame CA, USA.).

Breakfast intake

Following anthropometry and the blood draw, participants were offered a breakfast tray including juice, fruit in syrup, sandwiches (ham and cheese or jam and butter), flavored milk (chocolate or strawberry) and tea or coffee (with or without sugar). Each participant ate breakfast, without other participants, in the presence of a researcher who invited them to eat as much as they wanted; they have no time limit for eating this meal (registered) and the process was considered ad-libitum. Participants confirmed verbally that they had eaten until satisfied. They were unaware that their breakfast intake was being recorded. After the meal was complete and the participant had left the room, a nutritionist calculated the intake of calories and macronutrients based on the nutritional information on the labels of each product and the difference between the original weight of each food and what was left after consumption [34].

Other covariates

Start time of the evaluation, which ranged from 08:07 to 11:50 AM, was transformed to hours elapsed since midnight the night prior and used as a covariate in multivariate models. Other covariates included nighttime sleep duration of the previous night (self-reported as bedtime and waking time), and whether the participant usually ate breakfast (self-reported as yes/no).

Statistical analysis

Variable distribution was evaluated through visual analysis of histograms and the Shapiro Wilk test. Descriptive statistics included mean and standard deviation for normally distributed variables, or median and interquartile range or frequency for non-normally distributed variables. Descriptive statistics by sex were compared using appropriate parametric and nonparametric analyses (Student t-test, Mann-Whitney *U* test, or chi-square test). The potential association between fasting appetite-related hormone level and energy intake at breakfast was conducted using separate linear regression models. Potential confounders of the relationship between hormone levels and intake were considered. Final adjusted models included all relevant co-variates (i.e., > 10% change in β coefficient) for at least one of the associations between hormones and energy intake. Nighttime sleep duration and usual consumption of breakfast were not statistically significant in the models studied and removed for parsimony. Interactions between target hormones and sex or FMI categories were also tested in every model and removed if non-significant (p-value > 0.1); when appropriate, final models were stratified by sex and higher FMI. A model including fasting levels of the 4 studied hormones was also performed, adjusted for relevant variables; interactions were not studied. Similar models using log-transformed hormone levels as predictors were performed. All final models met the assumptions for linear regression.

Results

Participants (52.3% males) were 16.8±0.2 years old, weighted 65.6 ± 14.0 (kg) and were 1.65±0.1 height (m), respectively; 14.5% were obese, 24.9% were overweight, 60.6% were normal weight or underweight with, 6% having BMI z-score <-1 SD. Descriptive data for BMI z-score, body composition, intake and fasting hormone levels are shown in Table 1. Compared to males, females had higher FMI, lower caloric intake, and a greater percentage of calories from fats. Females also had higher leptin, insulin and ghrelin levels than males (Table 1).

Leptin and insulin were inversely associated with caloric intake at breakfast in unadjusted models, while ghrelin and orexin-A were positively associated with intake. All hormones were also significantly associated with energy intake at breakfast after accounting for relevant covariates

Table 1. Adiposity, intake at breakfast, and fasting hormone levels overall and by sex

	Total (N = 655)	Males (N = 341)	Females (N = 314)	P value[a]
Adiposity				
BMI z-score	0.70 ± 1.1	0.63 ± 1.1	0.76 ± 1.1	0.13
Fat mass index [kg/m][b]	7.3 ± 3.8	5.6 ± 3.2	9.1 ± 3.5	≤ 0.01
Free fat mass index [kg/m^2][b]	15.7 ± 2.1	17.2 ± 1.5	14.1 ± 1.5	≤ 0.01
Elevated fat mass index [kg/m^2][b,c,d]	225 (34.4)	105 (30.8)	120 (38.2)	0.04[e]
Breakfast intake				
Energy [kcal]	637 ± 239	689 ± 265	581 ± 193	< 0.01
Proteins [% of energy]	12.7 ± 2.8	12.5 ± 2.7	12.9 ± 3	0.05
Carbohydrates [% of energy]	56.8 ± 10.3	57.8 ± 10.1	55.7 ± 10.4	0.01
Fat [% of energy]	31.4 ± 7.7	30.6 ± 7.3	32.3± 7.9	< 0.01
Hormone concentrations[f]				
Leptin [ng/mL]	7.3 (1.2-19)	1.7 (1.0-6.8)	15.8 (8.8-25.7)	< 0.01[g]
Insulin [UI/dL]	6.7 (4.7-9.8)	6 (4.3-9.6)	7 (5.1-10)	0.01[g]
Ghrelin [pg/mL]	200.8 (143.3-291.2)	189.5 (136.7-282.4)	210 (148.6±307)	0.02[g]
Orexin-A [pg/mL][h]	16.1 (13.7-18.5)	16.5 ± 4	16.4 ± 4.2	0.83[g]

Values are mean ± standard deviation unless otherwise noted. [a]p-value assessing the difference between males and females, t-test unless otherwise noted. [b]n for body composition variables (total =647, males=339, females=308). [c]n (percentages)[a]. [d]Elevated fat mass index: male ≥ 6.6 kg/m^2 and female ≥ 9.5 kg/m^2 (33). [e]chi square. [f]Hormone concentrations described as median (Q1-Q3). [g]Mann Whitney. [h]n for orexin-A levels (total=652, males=339, females=313).

(sex, FMI, FFMI, start time of the evaluation). The association between leptin and energy intake became positive, after adjusting for these covariates (Table 2). Given that the adjusted model using leptin as a predictor showed significant interaction with sex, the association was analyzed separately for males and females. As shown in Table 3, leptin levels were significantly and positively associated with energy intake at breakfast among males (β: 3.35, 95IC: 0.19; 6.52) whereas no significant association was found among females. In the case of insulin, a significant interaction existed with FMI and thus the analyses were stratified by FMI category (i.e., normal vs elevated). Insulin levels were inversely associated with breakfast intake in both groups, but differed in magnitude, showing a

Table 2. Association between different predictor hormones and caloric intake at breakfast

	Leptin [ng/mL]	Insulin [μU/dL]	Ghrelin [pg/mL]	Orexin-A [pg/mL]
Unadjusted Model				
Hormone Conc.	-2.15 (-3.48; -0.82)[a]	-9.49 (-12.74; -6.24)[a]	0.28 (0.16; 0.4)[a]	8.87 (4.4; 13.2)[a]
R^2	0.01	0.04	0.03	0.02
Adjusted Model				
Hormone Conc.	5.65 (0.02; 11.27)[a]	-18.65 (-27.50; -9.80)[a]	0.34 (0.21; 0.47)[a]	8.40 (4.04; 12.77)[a]
Male sex	-10.22 (-85.59. 65;15)	-45.81 (-112.23; 20.59)	-47.96 (-114.46; 18.53)	-38.78 (-106.32; 28.76)
FMI [kg/m^2]	-9.43 (-16.71; -2.14)[a]	-5.68 (-14.77; 3.40)	-5.72 (-12.01; 0.56)	-7.94 (-14.29; -1.60)[a]
FFMI [kg/m^2]	14.53 (0.89; 28.18)[a]	17.77 (3.88; 31.66)[a]	15.38 (2; 28.76)[a]	13.03 (-0.55; 26.62)
Start time [h]	20.56 (-3.48; 44.6)	14.04 (-9.31; 37.39)	17.87 (-5.52; 41.27)	18.27 (-5.43; 41.97)
Hormone level x sex	-2.91 (-6.13; 0.33)	--	--	--
Hormone level x FMI	--	0.70 (-0.06; 1.46)	--	--
R^2	0.07	0.11	0.1	0.08

Values represent β coefficient (95% confidence interval). All models were built with the caloric intake at breakfast as dependent variable and only 1 hormone level as predictor. [a]Indicates p-value <0.05; -- indicates not included in the model. Abbreviations: Concentration (Conc.); Fat Free Mass Index (FFMI); Fat Mass Index (FMI).

Table 3. Association between leptin and insulin and caloric intake at breakfast in stratified models

	Leptin [ng/mL]		Insulin [μU/dL]	
	Males	Females	Normal FMI	Elevated FMI
Hormone Conc.	3.35 (0.19; 6.52)[a]	-1.67 (-3.78; 0.42)	-17.67 (-24.16; -11.18)[a]	-7.18 (-11.95; -2.40)[a]
Start time [h]	31.93 (-4.01; 67.87)[a]	5.97 (-24.67; 36.61)	22.30 (-5.54; 50.15)	-5.63 (-47.71; 36.43)
FFMI [kg/m^2]	20.17 (0.42; 39.93)[a]	2.69 (-15.77; 21.17)	36.32 (18.66; 53.99)[a]	1.46 (-17.37; 20.31)
FMI [kg/m^2]	-15.46 (-25.62; -5.31)[a]	1.89 (-8.50; 12.29)	NA	NA
Male sex	NA	NA	34.65 (-37.87; 107.17)	-127.22 (-208.84; -45.60)[a]
R^2	0.03	0.01	0.13	0.1

Values represent β coefficient (95% confidence interval). All models were built with the caloric intake at breakfast as dependent variable and only 1 hormone level as predictor. [a]Indicates p-value < 0.05; NA indicates non-available (given the stratification of the data). Abbreviations: Concentration (Conc.); Fat Free Mass Index (FFMI); Fat Mass Index (FMI).

greater decrease in breakfast intake for every unit of insulin increase among the normal FMI participants, compared with participants with elevated FMI (β: -17.67 to -7.18, respectively).

Discussion

Our results show that fasting levels of appetite-regulating hormones were independently associated with subsequent caloric intake at breakfast in a sample of healthy adolescents. The orexigenic hormones, ghrelin and orexin-A, were independently and positively associated with energy intake, whereas the anorexigenic hormone insulin had a negative association, which varied by adiposity category (i.e., greater association within participants with normal adiposity). In multivariate and sex-stratified models, leptin levels were positively associated with energy intake among males.

Few observational studies have reported on the association of appetite-related hormone levels and subsequent energy intake during a meal consumed immediately following hormone-level measurement. Most previous studies have assessed the relationship between appetite-related hormones and usual food intake (i.e., assessments of food intake over several days). Similar to our study, Buss et al., found a positive association between ghrelin levels and energy intake in overweight adult women[35]. On the other hand, prior studies of insulin and food intake[36-39] physical activity thermogenesis, diet-induced thermogenesis, and energy intake, did not demonstrate the expected negative association. Important methodological differences between these studies and ours could explain the discrepancy. Two of the prior studies did not adjust for covariates[36,37] physical activity thermogenesis, diet-induced thermogenesis, and energy intake. Two others focused on special populations or circumstances. He et al.[38], studied adult Pima Indians who had severe obesity (34.2 ± 9.4 kg/m^2), and Mars et al.[39] most human studies have failed to observe such a relationship. We studied the acute effects of severe caloric restriction on the association between serum leptin concentrations and subjective appetite. Subjects: A total of 44 healthy adult men (aged: 43 ± 5 years; BMI: 27.3 ± 3.2 kg/m^2, studied this association among male participants under caloric restriction.

To our knowledge, there are no prior observational studies reporting a positive association between peripheral orexin-A levels and energy intake in humans. Recently, peripheric synthesis and functions of orexin-A have been characterized, rising the doubt whether the peripheral levels could be used as a surrogate marker of the CNS levels[28,40]. Nevertheless, patients with type 1 narcolepsy, characterized by orexin deficiency, had increased energy intake than healthy controls[41]. More human clinical studies are needed to clarify the role of orexin in energy intake. Also, it should be studying eating behavior.

Regarding the association between leptin levels and caloric intake, four similar studies found negative associations between leptin levels and energy

intake[14,15,19,20]. But, in two of these, the associations did not remain significant after considering sex and fat mass as covariates[19,20]. In our study, an association between leptin levels and energy intake was found among males, but surprisingly, it was a positive association. In one other study, an independent positive association was found only among men[42]. These results may suggest that peripheral leptin levels do not represent the hormone action at the CNS, probably as a consequence of a central leptin resistance[43,44]. An important sexual dimorphism was found in the leptin levels, which could be in turn influencing the fact that there was no association between leptin and caloric intake among females

Associations between fasting levels of appetite regulating-hormones and caloric intake at breakfast explained 15% of the variance, although other pathways or hormones of the gastrointestinal system are not studied either. Several factors not considered in our study could also be associated with the intake at breakfast, such as behavioural, emotional, hedonic and sociocultural influences. Different limitations can be noted. The assessments were performed at a nutrition research institute, which may have influenced the participants eating behaviour; however, information or comments to participants related to eating behaviour, weight status or other were avoided. Tanner stage was not assessed because at 16.8 years all females and the vast majority of males were expected to have completed pubertal development; incomplete development could have affected hormonal levels in a few male adolescents. In addition, the stage of the menstrual cycle female was not evaluated at the blood draw, and this can affect the insulin sensitivity. Another limitation is that evaluations were made at different times in the morning, with differences in the circadian cycles between participants. However, hours since last meal was adjusted for in the final model. Participants were not assessed for eating disorders, which could also influence levels of appetite-regulating hormones.

This study has also important strengths including the precise measurement of hormone levels, food intake and body composition at a nutrition research institute. Future studies are needed to assess the role of other factors related to appetite and appetite hormone levels including sleep duration and quality and physical activity. It would also be interesting to assess day-to-day variability of fasting hormone levels in free-living individuals.

In conclusion, fasting blood levels of leptin, ghrelin, insulin, orexin-A, in a group of Chilean adolescents, were significantly and independently associated with caloric intake during an observed breakfast at will. Taking into account body composition, sex and the time of onset of the evaluation, orexigenic hormones, ghrelin and orexin-A, were positively associated with energy intake. Insulin was negatively associated with energy intake, and the degree of the association varied by adiposity category. Despite what was expected, fasting levels of leptin were independently and positively associated with energy intake among males. This study adds incremental knowledge about the role of appetite hormones in food intake in free living conditions.

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