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ANALYSIS OF LEPTIN GENE EXPRESSION IN SEVERLY OBESE PATIENTS

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ABSTRACT: Obesity is a major health issue in much of the human population. Obesity is a disorder of energy balance, excess energy is stored as fat whenever energy intake exceeds energy expenditure. Leptin is a hormone that regulates body weight by decreasing food intake and increasing energy expenditure. Leptin is expressed in the adipocytes: both its expression and its secretion are highly correlated with body fat and adipocyte size. Circulating leptin concentrations are closely related to adipose tissue. Adipose tissue total RNA from 20 patients with severe obesity was reverse transcribed into complementary DNA, which was used as template for quantitative real-time PCR amplification. The aim of this study was to determine some preliminary information on the level of leptin gene expression in severe obese pacients. It has been found that leptin gene was overexpressed in 19 patients out of 20.

Keywords: leptin, obesity, Real-Time PCR, humans, GAPDH

INTRODUCTION:

Obesity is a significant health problem, carrying an important number of social and economic issues. Obesity is frequently associated with cardiovascular disease (high blood pressure coronary atherosclerotic disease), metabolic (type 2 diabetes mellitus, dyslipidemia), pulmonary (sleep apnea, respiratory failure) or digestive (fatty liver disease) complications. All this compose the clinical picture of a high risk patient. Traditional therapeutic options are associating diet with physical exercise, changes in eating behavior and pharmaceutical treatment. Bariatric surgery is also an efficient method of delivering an important and sustained weight loss, frequently followed by the resolution of associated co-morbidities. The early recognition of those obese patients harboring an increased risk of failure to traditional therapies is very important; minimizing the period while the patient is at risk would contribute to lowering the incidence of side effects, optimizing the cost-efficiency parameters and it would also induce an improvement in the quality of life of these patients.

Leptin, the product of the *ob* gene, is a recently discovered single-chain proteohormone with a molecular mass of 16 kDa that is thought to play a key role in the regulation of body weight (Friedman &Halaas, 1998). Its amino acid sequence exhibits no major homologies to other proteins (Zhag *et al.*, 1994). This product of the *ob* gene (obesity mice) is named leptin from the Greek word "leptos", meaning thin. Leptin acts on the central nervous system, in particular the hypothalamus, suppressing food intake and stimulating energy expenditure (Webber, 2003).

Other tissues in which leptin was observed are: mammary gland, tests, ovary, placenta, stomach, pituitary gland. This hormone is mainly produced by differentiated adipocytes (Zhang *et al.*, 1994). The main factors that regulate serum leptin concentrations are short-time caloric intake and the amount of energy stored in adipocytes. Among other factors there are: fat mass/fat distribution, sex, hormones and cytokines. According to various studies the leptin concentrations are directly correlated with the amount of body-fat (Chan *et al.*, 2006; Chan *et al.*, 2005; Kelesidis *et al.*, 2006, Lee *et al.*, 2006; Bluher and Mantzoros, 2007). The LEP gene encodes for leptin. It has been localized in humans on the 7alpha31.3 chromosome and consists of three exons separated by two introns (Isse et al., 1995).

It has been shown that the visceral quantity of fat is a strong predictor for metabolic adverse consequences of obesity such as diabetes, hyperlipidemia and cardiovascular disease (Montague and O'Rahilly, 2000). An increase of age in children has been correlated to an increase in leptin gene expression in subcutaneous adipose tissue (Lindgren *et al.*, 1997). The same pattern was observed as well in rats: the leptin mRNA expression also increases with age, following characteristic ontogenetic patterns in different adipose tissue depots (Oliver *et al.*, 2001).

Our purpose was to evaluate leptin gene expression from visceral fat tissue from severly obese pacients.

MATERIALS AND METHODS

Sampling and RNA extraction

Visceral adipose tissue samples were collected from 20 patients suffering from severe obesity and 5 normal weight patients. The samples were immediately and completely submerged in RNA preserving solution (RNAlater - Qiagen GmbH Germany) and then stored at -80° C, for further analysis of leptin gene expression (mRNA). The starting material for extraction was of 100 mg of tissue and the RNA isolation was performed using guanidine-thiocyanate acid phenol TRI Reagent (Sigma-Aldrich) according to the manufacturer's instructions. The RNA concentration was quantified using a spectrophotometer Biospec - Nano (Shimadzu Biotech, Kyoto, Japan)

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Revers transcription and Real Time PCR

One microgram RNA was reverse transcribed in a volume of 20 ml for 5 min at 25ºC, 30 min at 42ºC and 5 min at 85ºC. The kit used was the iScript synthesis kit (Bio-Rad, Hercules, CA, USA)

The set of primers used in the amplification reaction were selected using Primer 3 and are shown in Table 1.

Table 1.
The conditions used for RealTime PCR Reactions

LEPTIN	**Forward**	TGCCTTCCAGAAACGTGATCC
	Reverse	CTCTGTGGAGTAGCCTGAAGC
GAPDH	**Forward**	TTCATTGACCTCAACTACAT
	Reverse	GTGGCAGTGATGGCATGGAC

The protocol for the Real Time PCR reactions is described in Table 2. In order to obtain the reaction mix for Real Time PCR reaction the components described in Table 3 were used. The reaction was performed on a thermocycler iCycler iQ (Bio-Rad) using excitation filter set/transmitter specific for the SYBR Green (λex 490 nm/λem 530 nm). The fluorescence intensity reflecting the amount of actually double-stranded formed PCR-product was read in real – time at the end of each elongation step.

Table 2.
The conditions used for Real-Time PCR Reaction

Program	Number of cycles	Temperature	Time
Pre-incubation	1	95 ºC	3,30 min
		95 ºC	30 sec
Amplification	45	57 ºC	30 sec
		72 ºC	45 sec
	1	95 ºC	1 min
Melting curve	1	55 ºC	1 min
	85	raise from 55 ºC by 0.5 ºC	10 sec for the first cycle
Cooling	1	20 ºC	HOLD

Table 3. Components of Real-Time PCR reaction.

Components	Volumes (µl)
IQMix	12.5
Forward primer	0.4
Reverse primer	0.4
Deionized water	6.7
cDNA (100ng/ul)	5
Total Volume	25

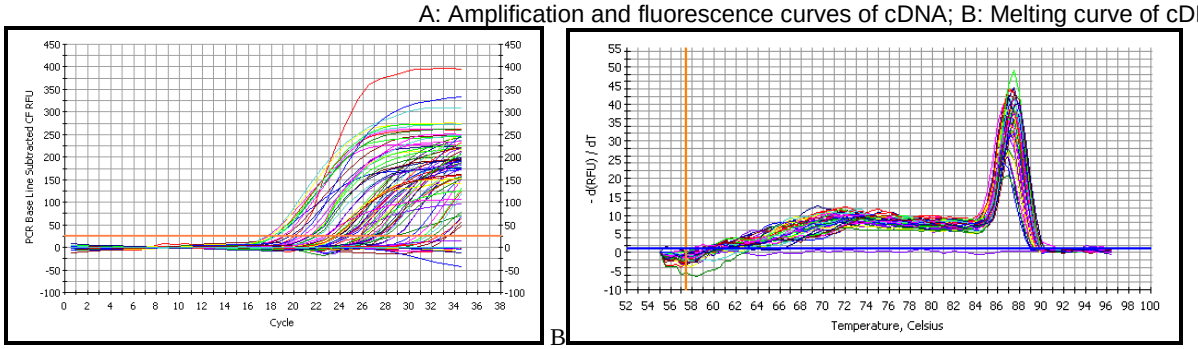
The relative quantification levels for the gene expression were calculated using the $2^{-\Delta\Delta CT}$ method (CT = crossing points, cycle number where the fluorescence crossed the threshold): ΔCT = CT (target gene) – CT (reference gene); ΔΔCT = ΔCT patients - ΔCT normal controls. Using this method, the comparative level of expression is: $2^{-\Delta\Delta CT}$ (Livak & Schmittgen, 2000).

RESULTS AND DISCUSSIONS:

The quantity of RNA ranged between 20.41-944.7 ng/µl with an average of 120.15 ng/µl and median of 82.295 ng/µl, concentrated in 20 µl elution solutions. The obtained A260/280 ratio (absorption ratio at 260/280nm) varied between 1.78 and 2.08.

Gene amplification was done in triplicate and was successful for all samples, except one. The mean of Ct values obtained and calculation for the 20 good samples are presented in Table 4. The amplification curves for both leptin and GAPDH genes are shown in Figure 1 A. The melting curve analysis showed no cross contamination or unspecific amplification (Figure 1 B).

Figure 1.
A: Amplification and fluorescence curves of cDNA; B: Melting curve of cDNA



A B

The aim of this study was to determine some preliminary information on the level of expression of leptin in severe obese pacients. Body weight is regulated by a complex system, including both peripheral and central factors.

Many genes are involved in the regulation of leptin secretion. Among these, three genes were most studied

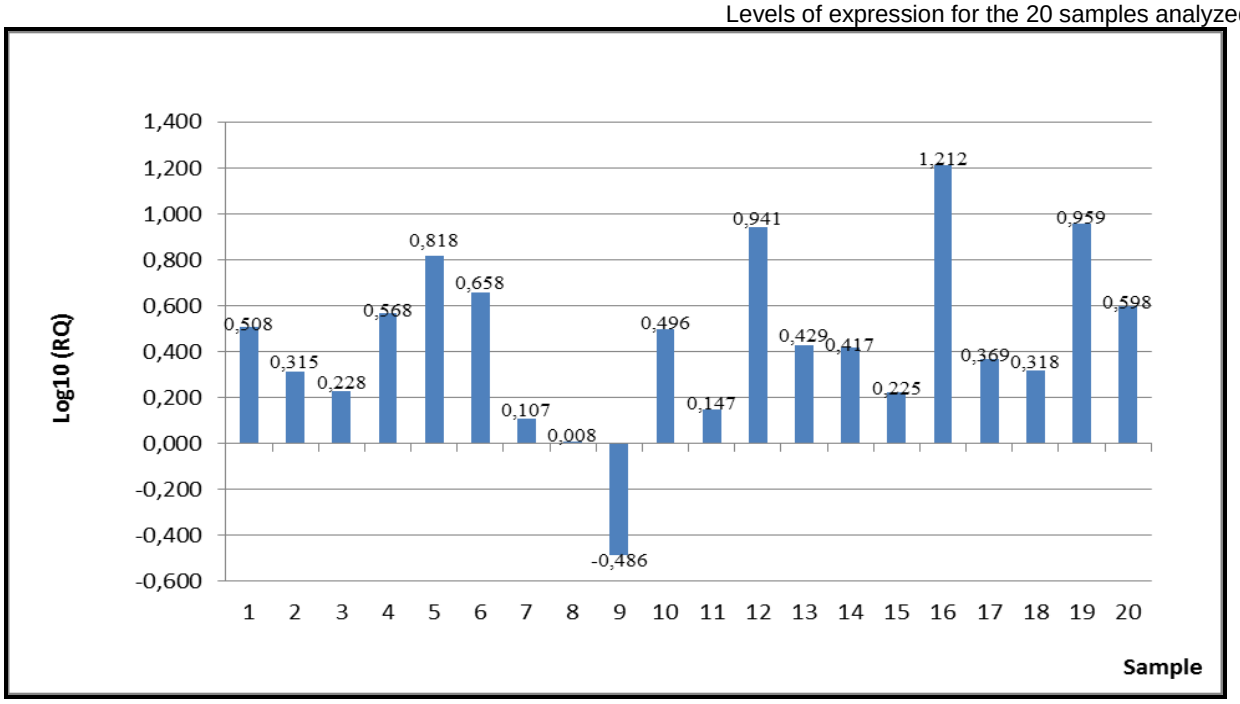
in association with obesity: leptin gene (LEP, Ob), the leptin receptor gene (LEPR) and the peroxisome proliferator-activated receptor-gamma gene (PPARG), (Paracchini *et al.*, 2005). But leptin protein is the product of leptin gene (LEP, Ob). Due to the fact that the coding region for leptin protein is located in exons 2 and 3 (Ahima and Filer, 2000), the analysis of mRNA expression was made using a set of primers that amplify a 163bp fragment belonging to exon 3 in the corresponding mRNA LEP sequence.

The expression of the reference genes should not be influenced by experimental conditions. In this experiment the GAPDH was used as reference gene to normalize the data. For 19 samples out of 20 the leptin gene was overexpressed. The relative quantification values for the 19 overexpressed samples varied between 0,008 (in case of patient 24) to 1,212 (in case of patient 41). The levels of expression for all samples are shown in Figure 2.

Previous studies demonstrated that there is a strong positive correlation between leptin gene expression and protein levels in adipose tissue and circulating leptin levels (Maffei *et al.*, 1995; Frederich *et al.*, 1995; Considine *et al.*, 1996). Also, it has been noticed that in obese humans, not only serum leptin levels are elevated, but also adipose LEP mRNA (Hamilton *et al.*, 1995; Lonnqvist *et al.*, 1995).

Overexpression of LEP gene was found in subcutaneous and also in omental adipose tissue of patients with severe obesity (Lonnqvist *et al.*, 1995). Our study focused on visceral adipose tissue investigations and confirmed the previously obtained data. In the studied group of patients there is a strong correlation between leptin visceral adipose tissue expression and morbid obesity. Only one patient out of 20 manifested a down regulation of LEP gene.

Figure 2.
Levels of expression for the 20 samples analyzed



It has been shown that the leptin has dual regulation in human physiology. During weight maintenance, when the output and intake of energy output are equal, leptin concentrations reflect total body fat mass. However, in conditions of negative (weight-loss programs) and positive (weight-gain programs) energy balances, the changes in leptin concentrations function as a sensor of energy imbalance. Within 24 h of fasting, leptin concentrations decrease to about 30% of initial basal values. Massive overfeeding over a 12h period increases leptin concentrations by almost 50% of initial basal values (Meier and Gressner, 2004). Meal ingestion does not acutely regulate serum leptin concentrations. A few studies have shown a modest increase in leptin secretion at supraphysiologic insulin concentrations 4–6 h after insulin infusion (Radic *et al.*, 2003; Wong *et al.*, 2004). Similar to other hormones, leptin secretion shows circadian rhythm and oscillatory pattern. The nocturnal increase of leptin secretion is entrained by mealtime, probably as a result of the cumulative hyperinsulinemia that occurs over the entire day.

Some of the investigated obesity related genes were proven to have a more reduced expression in visceral fat than in subcutaneous compartment, which suggests that the latter may be more important for the circulating products (Frederiksen *et al.*, 2009).

So the direction of our future investigation is to analyze LEP gene expression in subcutaneous fat deposits and to evaluate the correlation of the expression results in the two types of adipose deposits with the circulating leptin levels and protein levels in adipose tissue.

Table 4.
Calculation of level of expression based on the mean of Ct obtained for the 20 samples.

Sample	GAPDH Obese patients	GAPDH Normal controls	Leptin Obese patients	Leptin Normal controls	RQ=2^(-ddCt)	Log10(RQ)
1	24,3	24,0	27,3	28,7	3,219	0,508
2	23,36	24,0	27	28,7	2,066	0,315
3	22,4	24,0	26,33	28,7	1,690	0,228
4	25,3	24,0	28,1	28,7	3,698	0,568
5	27,76	24,0	29,73	28,7	6,574	0,818
6	21,8	24,0	24,3	28,7	4,553	0,658
7	26,13	24,0	30,46	28,7	1,280	0,107
8	22,3	24,0	26,96	28,7	1,019	0,008
9	22,3	24,0	28,6	28,7	0,327	-0,486
10	21,56	24,0	24,6	28,7	3,131	0,496
11	22,46	24,0	26,66	28,7	1,401	0,147
12	27,5	24,0	29,06	28,7	8,734	0,941
13	20,8	24,0	24,06	28,7	2,688	0,429
14	21,7	24,0	25	28,7	2,615	0,417
15	26,86	24,0	30,8	28,7	1,678	0,225
16	30,8	24,0	31,46	28,7	16,298	1,212
17	21,3	24,0	24,76	28,7	2,340	0,369
18	22,9	24,0	26,53	28,7	2,080	0,318
19	25,63	24,0	27,13	28,7	9,105	0,959
20	26,13	24,0	28,830	28,700	3,963	0,598

CONCLUSIONS:

The obtained results show an overexpression of leptin gene in visceral tissue from severe obese patients. It has been shown that serum and plasma leptin levels are higher if both body fat and body mass index are higher (Schwarz *et al.*, 1996). Our study confers motivation for future work on development of new experiment in order to analyze the leptin concentrations from other tissues and see the involvement of this important hormone.

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