

Could high levels of tissue polypeptide specific antigen, a marker of apoptosis detected in nonalcoholic steatohepatitis, improve after weight loss?

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Abstract. *Background:* Tissue Polypeptide Specific antigen has recently been proposed as diagnostic marker of apoptosis in NonAlcoholic SteatoHepatitis.

The aim of this study was to validate in patients suffering from NonAlcoholic SteatoHepatitis the clinical utility of this marker after different programs of weight reduction.

Methods: Overweight/obese patients with visceral adiposity and liver histology compatible were assigned to a Calorically-Restricted diet ($n = 22$), a Calorically-Restricted diet plus EXercise ($n = 19$) or No Healthy Life Style (control group, $n = 21$) for six months. The presence of Body-Weight loss was assessed by a Body Mass Index decrease of at least three points. Serum ALanine aminoTransferase, HOmeostasis Model Assessment method value and Tissue Polypeptide Specific antigen concentrations were determined at time 0, after 3 and 6 months in both the Intervention groups and in the controls' one.

Results: In NonAlcoholic SteatoHepatitis patients who obtained Body-Weight reduction, a significant decrease of the serum Tissue Polypeptide Specific antigen values was showed with a clear linear trend across time, $P = 0.0001$.

Decrement of Tissue Polypeptide Specific antigen concentrations best differentiated the Body-Weight loss from the body-weight maintenance in respect to Tissue Polypeptide Specific antigen and HOmeostasis Model Assessment method values.

Conclusion: This study support the clinical utility of serum Tissue Polypeptide Specific antigen levels in the follow-up of overweight/obese patients with NonAlcoholic SteatoHepatitis on weight reduction programs.

Keywords: TPS, NASH, Body-Weight loss

Abbreviations: MS, Metabolic Syndrome; CV, Cardio Vascular; NAFLD, NonAlcoholic Fatty Liver Disease; IR, Insulin Resistance; NASH, NonAlcoholic SteatoHepatitis; TPS, Tissue Polypeptide Specific antigen; B-W, Body-Weight, BMI, Body Mass Index; WC, Waist Circumference; HDL, High-Density Lipoprotein; NHLS, No Healthy Life Style; CR, Calorically-Restricted; EX, EXercise; ALanine aminoTransferase (ALT); NV, Normal Values; HOMA, HOmeostasis Model Assessment method; TNF- α , Tumor Necrosis Factor-alpha; CI, Confidence Intervals.

1. Introduction

Obesity, often associated with Metabolic Syndrome (MS), promotes the development of atherosclerosis, increases Cardio Vascular (CV) morbidity and mortality from near all causes [1]. NonAlcoholic Fatty Liv-

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er Disease (NAFLD) is frequently associated to Insulin Resistance (IR) and MS. NAFLD includes fatty liver, NonAlcoholic SteatoHepatitis (NASH), cirrhosis and it can progress to the end stage liver disease [2]. The prevalence of NAFLD in obese adults is estimated from 40 to > 90% [3,4]. Some studies have suggested that individuals with obesity or overweight and with central distribution of fatty are more likely to develop NAFLD [5]. There is strong evidence that Body-Weight (B-W) reduction in overweight and obese individuals improves risk factors for diabetes and CV diseases. Some Interventions control the progression of NAFLD, decrease IR indexes and improve liver enzymes and morphology [6,7]. Liver biopsy [8], is the gold standard for diagnosing NASH, but approval for this invasive tool (mainly after a short-term follow-up period) is difficult to obtain from patients. In fact, Authors started proposing alternative measures of NASH presence [9,10].

Accordingly, we have recently reported the diagnostic accuracy of the serum Tissue Polypeptide Specific antigen (TPS) determinations [11]. TPS, a serological mirror of the cytokeratin 18, is widely used as a marker for various cancers. It can be abundantly released into the extracellular space during the intermediate stage of epithelial cell apoptosis [12]. At later stages of the apoptotic process, when the integrity of the cytoplasmic membrane becomes compromised, keratin aggregates are shed from the cell [13]. As a matter of fact, hepatocyte apoptosis is significantly increased in patients with NASH [14]. In this context, serum TPS levels could be of some interest, likely representing a clue of cellular damage.

On the other hand, B-W reduction leads to a health improvement as previously reported and could be used to test the clinical utility of an index parameter when dealing with obesity. So, we aimed at evaluating the usefulness of monitoring the TPS concentrations associated to the caloric restriction-induced B-W loss or caloric restriction- and exercise-induced B-W loss, however gained, comparing them to a classic parameter sensible to this type of Intervention, i.e., the ALanine aminoTransferase (ALT) levels [15], and to an other, expression of IR, i.e., HHomeostasis Model Assessment method (HOMA).

2. Patients and methods

This pilot cross-sectional study, implanted to validate the diagnostic accuracy of the index test (TPS)

before and after Intervention, was conducted in a tertiary, Medical School Hospital; the inclusion criteria were overweight/obese patients with visceral adiposity and histology compatible with NASH presence; the exclusion criteria were defined by the co-presence of chronic liver diseases (hepatitis C, hepatitis B, autoimmune diseases, genetic haemochromatosis and possibly hepatotoxic drugs), cancer, renal dysfunction, and use of drugs impacting on lipid or glucose metabolism including aspirin.

2.1. Diagnosis of NASH

Histological evidence followed recent suggestion [8]; negative or occasional (≤ 20 g day⁻¹) history of alcohol consumption was always present.

2.2. Anthropometrics

Body Mass Index (BMI) and Waist Circumference (WC) were evaluated according to the World Health Organization criteria (1997). Weight was measured in light clothing, without shoes, in kilograms and height was measured in centimetres, using a scale-integrated stadiometer. BMI was calculated as body weight (kg)/height² (m²) and patients were categorized as normal weight (< 25.0 kg m⁻²), overweight (≥ 25.0 and ≤ 29.9 kg m⁻²) and obese (≥ 30.0 kg m⁻²). The obesity was classified in grade I (30.0–34.9 kg m⁻²), grade II (35.0–39.9 kg m⁻²) and grade III (≥ 40.0 kg m⁻²).

Standing WC was measured at the midway between the lowest rib and the iliac crest with a flexible tape. WC was classified according to the risk of metabolic complications: low (men < 94 cm, women < 80 cm); slightly increased (men 94–101 cm and women 80–87 cm) and increased (men ≥ 102 cm and women ≥ 88 cm).

MS was defined according to the revised Adults Treatment Panel III (2001) [16], and three or more criteria were considered: plasma glucose concentration of at least 100 mg dL⁻¹, WC ≥ 102 cm in men and ≥ 88 cm in women, serum High-Density Lipoprotein (HDL) cholesterol concentration < 40 mg dL⁻¹ in men and < 50 mg dL⁻¹ in women, blood pressure of at least 130/85 mm Hg, and serum triglyceride concentration of at least 150 mg dL⁻¹.

2.3. Research design

Among 146 consecutive overweight/obese patients with abdominal fatness, NAFLD was diagnosed by hyper-echogenicity at Ultra Sound (US) or ALT increase and no other liver disease in 124 subjects. Ninety-seven underwent liver biopsy. A part of them (66 patients) had a histological diagnosis of NASH and was broken into three well-balanced groups. The participants were openly assigned to a Calorically-Restricted diet (CR, $n = 22$, 10 Females, F), a CR diet plus EXercise designed to produce an additional 20% energy deficit (CR*EX, $n = 19$, 10 F, three withdrawals), or No Healthy Life Style (NHLS, control group, $n = 21$, 10 F, one withdrawal) for six months. Initially, weight loss was induced by a two-week, very-low-energy diet, i.e., 600–800 kcal/day. Then, for the following 22-week-period, the subjects in the two treated groups were asked to reduce their energy intake by 1,000–1,200 kcal/day.

Being walking safe and accessible for our patients, 30 to 45 minutes of this physical activity of moderate intensity, performed 3 to 5 days a week, was strongly encouraged as EX.

We analyzed data from all participants that completed the research; therefore, this was not an intention-to-treat analysis.

US features of liver hyper-echogenicity were quantified according to the following scale: 0, absent; 1, mild; 2, moderate; 3, severe [17].

As laboratory data were measured serum ALT (Normal Values, NV, ≤ 40 U/L), insulin and TPS levels (by a chemiluminescent enzyme immunoassay, IMMULITE, Diagnostic Products Corporation, Los Angeles, CA, USA; our cut off obtained in young lean subjects was 80 ng/mL) together with lipid profile and plasma glucose. IR was considered when HOMA value was ≥ 2.5 , lightly different from that of a previous study [18].

2.4. Outcome measures

Serum ALT, HOMA value, TPS concentrations were determined at time 0, 3 and 6 months of the Intervention in the CR*EX, CR and control groups. In the same periods were evaluated the BMI and WC of all participants. Although B-W loss was not the final end-point, its presence was assessed by a BMI decrease of at least three points, corresponding to a reduction of 7–10% of the initial B-W. The basal MS criteria, when abnormal, were re-tested after six months.

2.5. Statistics

To analyze frequencies the chi square test was evaluated. For paired observation the paired T test was adopted.

Relations between TPS or ALT levels and BMI, WC and IR were determined by using Pearson correlation expressed as coefficient of correlation (r) plus 95% CI. To eventually detect structure in the relationships among variables, that is to classify variables, Factor Analysis was applied. The Cattell's Scree plot, with relative eigenvalues, was performed to screen the real factors. Factors, marked by high loadings on some variables, unravelled a structure among them. The critical value was established following the formula $2 \times \text{Pearson's correlation coefficient for 5\% level of significance} / \text{square root of patients minus 2}$, i.e., 0.645.

The one-way ANalysis Of VAriance (ANOVA) with the Bonferroni post hoc comparison test was performed to examine differences among groups; when dealing with ordinals the Kruskal-Wallis test was adopted.

ANOVA with repeated measures was adopted to study the differences between three and six months data, using basal values as Covariates (ANCOVA).

To appreciate the simultaneous Effect Sizes (ES) of Intervention (CR, CR*EX and NHLS) and gender on final BMI and WC values, Multivariate ANalysis Of VAriance (MANOVA), a three per two groups (between) crossed design was used. They were expressed as Standardized (Std) discriminant function coefficient, larger values of ES meaning better outcomes.

The power of ALT, HOMA, TPS values in discriminating the eventual B-W loss was identified by the Area Under the Receiver Operator characteristic Curve (AUROC).

Variables potentially predicting the final BMI were analyzed by Multiple Linear Regression (Least Squares, Entner method). To predict the B-W loss the Logistic Regression (Entner method) was adopted, selecting as independent variables the treatment and the initial values of BMI and WC.

Statistical analysis was performed operating on Systat 12 (Richmond, CA, USA) and MedCalc Version 9.4. software packages.

The study was conducted according to guidelines of the International Conference on Harmonization for Good Clinical Practice, in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Table 1
Demographics and principal parameters of the 66 NASH patients at entry

	Intervention											
	CR				CR*EX				NHLS			
	Mean	95% CI	SD	25–75 P	Mean	95% CI	SD	25–75 P	Mean	95% CI	SD	25–75 P
AGE yrs ¹	57.7	55.4–60	5.3	55–61	59.3	57–61	4	57–61	58.7	56–61	6	55–66
ALT U/L ²	71.4	62.6–80	2	59–88	77.5	67–88	21	67–91		63–81	19	64–88
HOMA ³	3.4	2.9–4	1.3	2.5–4.2	3.3	2.7–4	1.4	2.3–4	3.5	2.8–4	1.3	2.2–4
TPS ng/mL ⁴	164.3	139–189	56.8	128–198	166	147–184	38.6	139–185	166	139–194	60	135–213
BMI ⁵	30.9	30–32	2.1	29–33	31.4	30.3–32.6	2.4	29–34	31	30–32	2	29–33
WC cm ⁶	104	102–106	4.6	100–106	106	102–110	8	98.7–110	106	103–110	7.6	99–114

¹p value = 0.63, ²p value = 0.59, ³p value = 0.94, ⁴p value = 0.99, ⁵p value = 0.76, ⁶p value = 0.47, all analyzed at ANOVA.

NASH, NonAlcoholic SteatoHepatitis; CI, Confidence Intervals; 25–75 P, 25–75 Percentile; SD, Standard Deviation; ALT, ALanine aminoTransferase; HOMA, HOmeostasis Model Assessment method; TPS, Tissue Polypeptide Specific antigen; BMI, Body Mass Index; WC, Waist Circumference; NHLS, No Healthy Life Style; CR, Calorically-Restricted; EX, Exercise.

Table 2

Frequency distribution of the 66 NASH patients with normal laboratory values at entry

	FN	TP	X ²	p value
TPS ng/mL	0	62		
ALT U/L	7	57	5.58	0.0018
HOMA	17	44	17.778	0.0001

NASH, NonAlcoholic SteatoHepatitis; TPS, Tissue Polypeptide Specific antigen; ALT, ALanine aminoTransferase; HOMA, HOmeostasis Model Assessment method; FN, number of patients with normal values (False Negative); TP, True Positive patients; X², chi square.

3. Results

Demographic characteristics with main laboratory data and anthropometric measures of the 66 NASH patients at entry are represented in Table 1. Frequencies of the basal normal laboratory values in the three groups of patients were expressed in Table 2. The mid-term and final biochemistry and anthropometric evaluation are evidenced in Table 3. US scores (steatosis) and the histological features (grading and staging) of the enrolled patients showed no significant differences at Kruskal-Wallis test, $P = 0.90, 0.98$ and 0.94 , respectively.

3.1. Body weigh and visceral adiposity

The degree of B-W loss and WC reduction after Intervention is represented in Figs 1 and 2. Females had different compliance in managing W-L, Fig. 2. Frequencies of patients with B-W loss were 1 (0.045%), 8 (36.4%) and 17 (80.9%) subject(s) in NHLS, CR and CR*EX groups, respectively; chi square for trend was 29.8, $P < 0.0001$. ES studied the magnitude of the difference between treatments on final BMI and WC, respectively. Briefly, the best outcome was registered in females regarding to the visceral adiposity in

Table 3

Main outcome values during and after the period of Intervention, independently from the type, in the 66 NASH patients

TIME of Intervention				
	Mean	95% CI	SD	25–75 P
3 months				
ALT U/L	63.5	59.7–67.3	14.9	53–74
HOMA	3.1	2.8–3.35	1.1	2–3.6
TPS ng/mL	134.7	121.6–148	52.1	98–156
BMI	30.5	30–31	1.85	29–32
WC cm	102.4	101–104	5.9	99–105
6 months				
ALT U/L	57.2	52.2–62.2	19.65	43–69
HOMA	2.9	2.5–3.1	1.1	2–3.2
TPS ng/mL	109.5	97.3–121.6	47.7	78–135
BMI	29.8	29.3–30	2.03	28–31
WC cm	101.1	99.5–102.8	6.49	98–104

NASH, NonAlcoholic SteatoHepatitis; CI, Confidence Intervals; 25–75 P, 25–75 Percentile; SD, Standard Deviation; ALT, ALanine aminoTransferase; HOMA, HOmeostasis Model Assessment method; TPS, Tissue Polypeptide Specific antigen; BMI, Body Mass Index; WC, Waist Circumference.

the CR*EX group. Therefore, we conclude that both gender and combined Intervention contribute to W-L, coefficients = -3.14 and -3.52 , respectively; Wilks' Lambda, $P < 0.0001$.

3.2. Index test

The overall behaviour of TPS across the Intervention time reached the lowest values in the CR*EX group at the sixth month and at the same period showing a further discriminant capability between the Intervention groups, Fig. 3a. Indeed, a different pattern was registered for the final ALT and HOMA values, only discriminating the Intervention groups from the non Intervention one, Fig. 3b and 3c.

Using Intervention as grouping factor, the univariate and multivariate repeated measures analysis corrected for basal values showed a progressive-in-time reduction

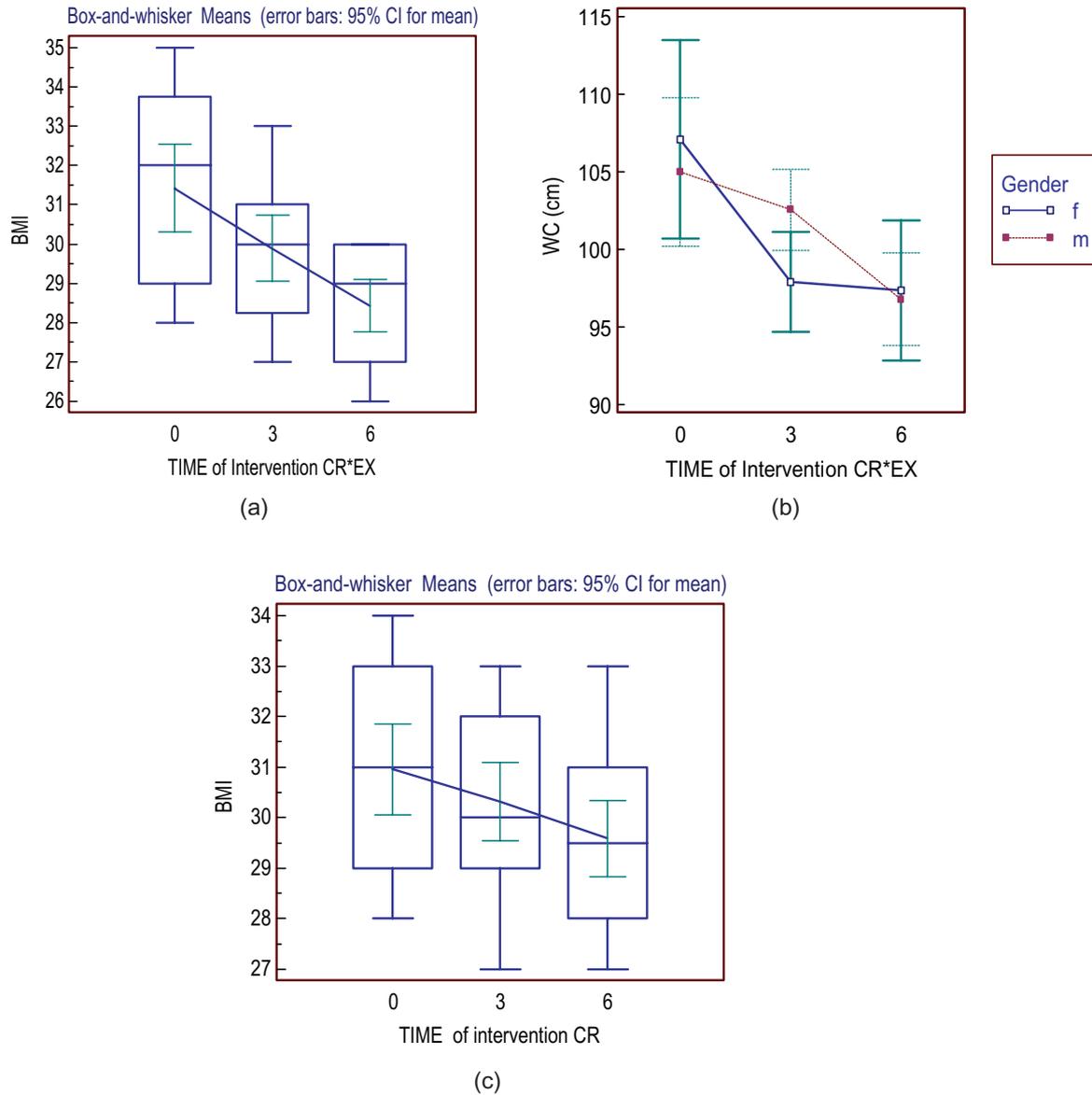


Fig. 1. Behaviour of the body weight loss and the pattern of the Waist Circumference reduction, corrected for gender, in NASH patients on Calorically Restricted diet plus EXercise. Behaviour of the body weight loss in patients on Calorically Restricted diet. NASH, NonAlcoholic SteatoHepatitis. (a) CI, Confidence Intervals; BMI, Body Mass Index; Time of Intervention 0, basal; 3, after 3 months; 6, after 6 months; CR*EX, Calorically Restricted diet plus EXercise; every group from the others, p value < 0.001 , ANOVA. (b) WC, Waist Circumference; f, female; m, male; CR*EX, Calorically Restricted diet plus EXercise; Time of Intervention 0, basal; 3, after 3 months; 6, after 6 months; every group from the others, p value = 0.004, for males and p value = 0.009 for females, ANOVA. (c) CI, Confidence Intervals; BMI, Body Mass Index; Time of Intervention 0, basal; 3, after 3 months; 6, after 6 months; CR, Calorically Restricted; ANOVA, p value = 0.061. (Colours are visible in the online version of the article at www.iospress.nl.)

for TPS and scarcely for ALT, but not for HOMA levels, $P = 0.0001$, 0.046 and 0.65, respectively. This is intuitive, being high HOMA values present only in 71% (44 out of 62) of patients at the study entry.

Among the selected independent variables, the prediction of B-W loss was assessed by treatment and ini-

tial BMI (OR = 1.92, 95% CI = 1.14 to 3.2).

3.3. Reliability

Choosing as independent variables the initial and intermediate values of HOMA, ALT and TPS, the best

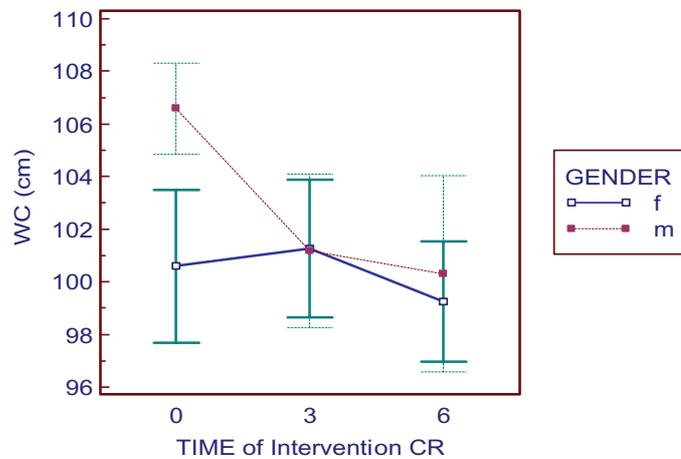


Fig. 2. Pattern of the Waist Circumference reduction in NASH patients after Calorically Restricted diet, corrected for gender. NASH, NonAlcoholic SteatoHepatitis; WC, Waist Circumference; f, female; m, male; CR, Calorically Restricted; Time of Intervention 0, basal; 3, after 3 months; 6, after 6 months. CI, Confidence Intervals; every group from the others, p value = 0.003, ANOVA, only for males. (Colours are visible in the online version of the article at www.iospress.nl.)

prediction of the final BMI in the entire population was made by the initial and mid-term values of HOMA.

Plotting the AUROCs of the ratios between final and initial values, a proportional decrement of TPS concentrations was showed; still, this parameter behaved better in differentiating the B-W loss from the B-W maintenance than ALT and HOMA, Fig. 4.

The pairwise comparison of ROC curves gave a significance only between the TPS and ALT ratios, $P = 0.01$.

3.4. Associations

In Intervention groups, there was an association between the initial values of TPS and ALT ($r = 0.29$, 95% CI = 0.043 to 0.50, $P = 0.0225$).

Similarly, a statistically evident correlation resulted by confronting the final values of the same variables ($r = 0.32$, 95% CI = 0.077 to 0.53, $P = 0.01$), explaining only a 10% of variance. HOMA and TPS final values showed a good correlation ($r = 0.43$, $P = 0.0004$), explaining the 18.5% of variance, differently from the initial values of the same parameters ($r = 0.12$, $P = 0.35$).

The association between final or initial data of HOMA and ALT was not significant ($r = -0.10$, $P = 0.41$ and $r = -0.12$, $P = 0.34$, respectively).

A valid association was found when correlating the final WC values to the final BMI ones ($r = 0.62$, 95% CI = 0.44 to 0.76, $P < 0.0001$). The final values of WC well correlated to the initial ones ($r = 0.55$, 95% CI = 0.35 to 0.70, $P < 0.0001$).

A sufficient association ($r = 0.32$, $P = 0.011$), explaining only the 10.2% of variance, was found between final ALT and TPS concentrations.

No hidden relationship was evidenced among HOMA, ALT and TPS values, casting doubts that the various components of NASH, i.e., IR, cellular damage and regenerative process, follow a similar expression, although possibly having common origin.

4. Discussion

Hypo-caloric diet and physical training are the mainstream to counteract overweight/obesity. Our findings confirm these strategies [19], even though evaluating modalities of B-W loss was not the main end-point. In fact, our study was implanted to verify the usefulness of an index parameter of apoptosis after Intervention.

The main finding was that in NASH patients with visceral adiposity, undergone B-W reduction programs, we found a near constant decrease of the serum TPS values. HOMA values and ALT concentrations resulted diminished but a lesser extend.

Besides, this research was characterized by an association among these three parameters. Probably, the necrotic process (ALT), the IR (HOMA) and the possibly apoptosis/necrosis-induced regenerative process (TPS) are linked even though they follow different pattern or time of expression in inducing/maintaining NASH. Interestingly, in overweight/obese subjects without NAFLD, the TPS levels were always below the upper limit of normality (data not shown); in the

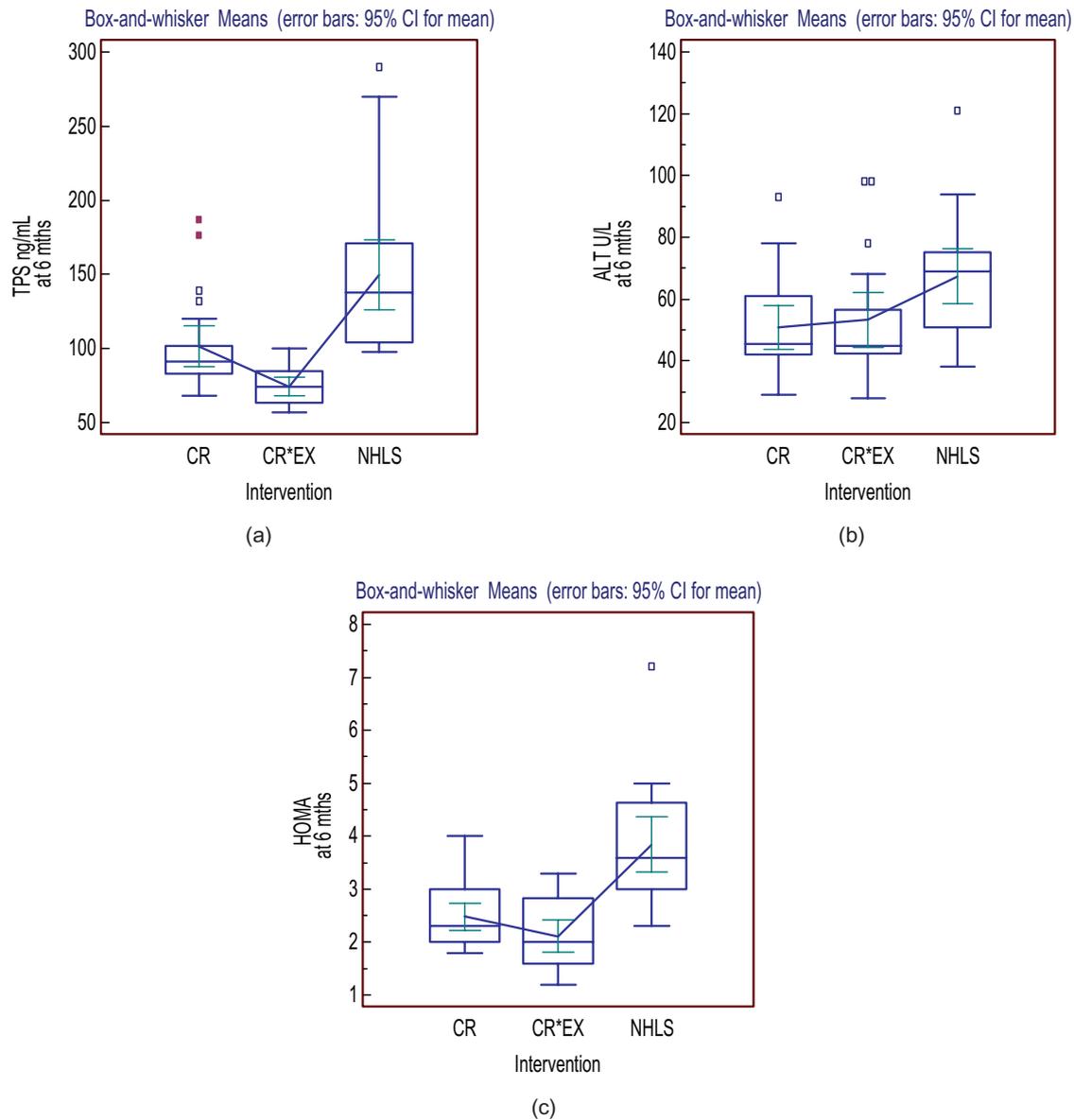


Fig. 3. Distribution of the Tissue Polypeptide Specific antigen, ALanine aminoTransferase concentrations and the HOmeostasis Model Assessment method values across groups at the sixth month of Intervention in NASH patients. NASH, NonAlcoholic SteatoHepatitis. a: CI, Confidence Intervals; mths, months; TPS, Tissue Polypeptide Specific antigen; NHLS, No Healthy Life Style; CR, Calorically-Restricted; EX, EXercise. Every group's TPS levels were significantly different from the others, p value < 0.001 , ANOVA. b: CI, Confidence Intervals; mths, months; ALT, ALanine aminoTransferase; NHLS, No Healthy Life Style; CR, Calorically-Restricted; EX, EXercise. The Intervention groups were only significantly different from the non Intervention one, p value < 0.011 , ANOVA. c: CI, Confidence Intervals; mths, months; HOMA, HOmeostasis Model Assessment method; NHLS, No Healthy Life Style; CR, Calorically-Restricted; EX, EXercise. The Intervention groups were only significantly different from the non Intervention one, p value < 0.001 , ANOVA. (Colours are visible in the online version of the article at www.iospress.nl.)

simple benign fatty liver, the TPS values were very few times and to little extent above the upper limit one [11], meanwhile no NASH patient showed TPS concentrations within the normal range.

Actually, our study suffers from potential method-

ological limitations. Firstly, the sampling error of liver biopsy [20], that could have resulted in an erroneous initial enrolment of participants. But, this study was performed considering patients as control of themselves. Secondly, a successive biopsy was not performed for

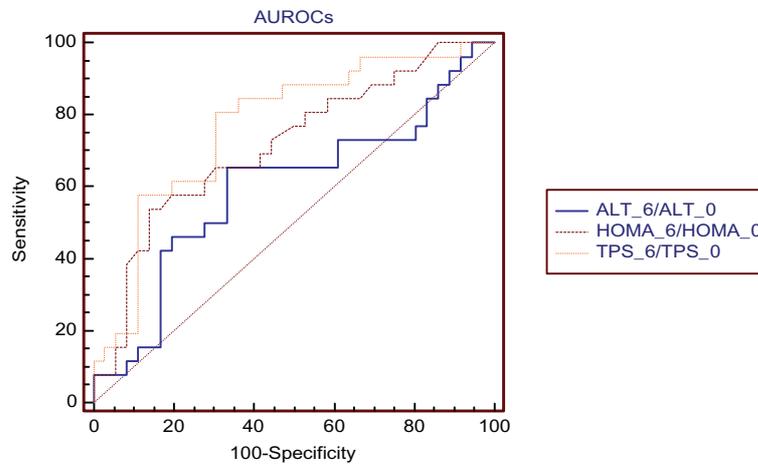


Fig. 4. Area Under the Receiver Operator characteristic Curves in NASH patients. NASH, NonAlcoholic SteatoHepatitis; AUROCs, Area Under the Receiver Operator characteristic Curves data with Confidence Intervals (CI) referred to the ratio between final (6) and initial (0) values of ALanine aminoTransferase, ALT; HHomeostasis Model Assessment method, HOMA and Tissue Polypeptide Specific antigen, TPS, i.e., 0.60, 95% CI = 0.46 to 0.72; 0.71, 95% CI = 0.57 to 0.82; 0.77, 95% CI = 0.65 to 0.87, respectively. (Colours are visible in the online version of the article at www.iospress.nl.)

ethical reason and for patients' refusal. Thirdly, one could cast doubts whether a six-month course is sufficient to obtain liver structural improvement [21]. As a matter of fact, a shorter period highlights a certain trend. Again, gamma-glutamyl transpeptidase levels could have been monitored, being this liver enzyme recently proposed as marker of MS [22]. Admittedly, this enzyme is mainly expressed in cholestatic forms of NAFLD [23]. Anyway, this laboratory parameter was not studied being rarely abnormal in our population. The lack of a randomization design may be regarded as a potential problem, although the fact that baseline characteristics were similar among groups, makes this point more of a theoretical issue than one that is playing an actual role in this study. The absence of a control group should be considered as a successive shortcoming. Finally, we might have studied the liver structural improvement evaluating the fibrosis status, using as marker the hyaluronic acid, although its accuracy is restricted to the more severe form [24], or other newly proposed single or combined markers of fibrosis [25]. It should be emphasized that the fibrosis regression is a very slow process and so difficult to appreciate. Indeed, part of the present data are supported by the results of previous studies [26–28].

But, what is the link between the B-W loss and improvement of metabolic features? Intriguing hypotheses highlight the role of the circulating Tumor Necrosis Factor- α (TNF- α) that is reduced after caloric restriction or exercise-induced B-W reduction [29]. In these conditions, a fall in TNF- α concentrations con-

tributes to the restoration of insulin sensitivity [30], as well as a modulated secretion of adiponectin does [31]. Still, a decrease in hyperinsulinemia stimulates lipoprotein lipase at a good extent. Thus, in adipocytes the triglycerides turnover changes and the Free fatty Acids (FFAs) disposal to liver lowers.

We hypothesize that the body-weight loss, independently from Intervention, improves the hepatic insulin-resistance before than the overt or incomplete peripheral one (HOMA). Thus, the decrease of TPS levels after body-weight loss could mirror the ongoing oxidative stress recovery that lessens the hepatic damage. It is necessary to wait for a longer period of time in order to highlight histological improvement, at least regarding the grading/staging scores.

Conclusion

The determination of serum TPS concentrations is a reliable tool in following up NASH patients on B-W reduction programs.

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