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A Long-term Treatment with Silybin in Patients with Non-alcoholic Steatohepatitis Stimulates Catalase Activity in Human Endothelial Cells

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Abstract. *Aim: To compare levels of oxidative stress markers in patients' sera with non-alcoholic steatohepatitis (NASH) treated for 12 months (T_{12}) with silybin conjugated with phosphatidylcholine (Realsil®) (R) or placebo (P) and investigate oxidative stress responses in human endothelial cells conditioned with patients' sera. Patients and Methods: We recruited twenty-seven patients with histological NASH. We measured thiobarbituric acid reactive substances (TBARS), superoxide dismutase (SOD) and catalase (CAT) activities in human endothelial cells conditioned with patients' sera exposed or not to H_2O_2. Results: We found in decreased-TBARS patients' sera, at T_{12}, a decrease of alanine aminotransferase (p=0.038), transforming growth factor-beta (p=0.009) and procollagen I (p=0.001). By dividing patients into two groups, increased (P-I/R-I) and decreased TBARS (P-II/R-II) at T_{12} compared to T_0, we found an increased CAT activity in conditioned endothelial cells at T12 in both groups (p=0.05 and p=0.001, respectively). Conclusion: Realsil® may be effective against endothelial dysfunction by stimulating the cellular antioxidant defense.*

Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver disease worldwide (1). NAFLD is characterized by fat accumulation in the liver, leading to clinical conditions ranging from isolated steatosis to chronic inflammatory status represented by fibrosis, cirrhosis and hepatocellular carcinoma (2). In NAFLD patients, the amount of liver enzymes has been demonstrated to predict the incidence of cardiovascular diseases (CVDs), independently from the traditional risk factors, including C-reactive protein and metabolic syndrome (MS). Moreover, the extent of liver damage has been correlated with early carotid atherosclerosis, suggesting that the injury to both vessels and liver share inflammatory mediators (3). For these reasons, NAFLD has been recently proposed as an early marker of atherosclerosis and endothelial dysfunction and, consequently, as an independent cardiovascular risk factor (4).

In insulin-resistant subjects, the presence of fatty liver has been correlated with an impairment of the systemic oxidant/antioxidant balance (indicated as oxidative stress) and endothelial dysfunction, independently from the presence of MS, adiposity and high levels of adipokines (4). It is well-known that oxidative stress is associated with endothelial dysfunction and CVD (5, 6) and, in NAFLD patients, it triggers an inflammatory cascade and extracellular matrix deposition in the liver, favoring the development of non-alcoholic steatohepatitis (NASH). Even if the mechanisms linking NALFD to increased oxidative stress and endothelial dysfunction have not yet been fully clarified, impaired mitochondrial β-oxidation, high levels of oxidized low-density lipoprotein (LDL), dietary saturated fat and reduced antioxidant intake have been proposed as potential pathogenetic factors (7-10).

Administration of several natural polyphenols is now considered to be a valid therapeutic strategy due to the ability of these compounds in preserving endothelial function and contrasting CVD (11). Silybin, the major active constituent of silymarin, is a potent antioxidant agent (12). It attenuates the

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oxidative stress by inhibiting accumulation of oxidants, interferes with lipid peroxidation and increases expression and activity of the antioxidant enzymes (13). These effects have been reported during both in *in vivo* and *in vitro* experiments carried out in rat Kupffer cells, hepatocytes, HepG2 cells, isolated mitochondria from rat hepatocytes, as well as in rat models of ischemia-reperfusion. Recently, it has been demonstrated that silybin also exerts a protective effect on endothelial cells and contributes to the improvement of endothelial dysfunction in the animal model of diabetes (14). Silybin is also administered as hepato-protective drug in patients with different forms of acute and chronic liver diseases, including NASH (15). The therapeutic response, mainly demonstrated by the improvement of liver damage, is reached in approximately 50% of the treated subjects. Moreover, we recently reported that this response was significantly correlated with the baseline serum oxidative stress, as well as with the variations of the serum oxidative and lipidomic profiles (16). In particular, we demonstrated that sera from patients with NASH induced lipid droplet accumulation in the cytoplasm of HepG2 cells, whereas a conditioning of the cells with sera of patients treated with silybin led to a reduction of intracellular fat accumulation. Of note, this effect was significantly correlated with baseline oxidative status and lipidomic variations induced by silybin. We identified two types of NASH patients differentiated by the presence of high or low systemic lipid peroxidation. Interestingly, these two groups of patients showed different therapeutic responses to silybin, evaluated by the following parameters: circulating oxidative stress, lipidomic profile and liver histology. In the present study, we evaluated whether sera isolated by NASH patients, treated with silybin conjugated with phosphatidylcholine (Realsil®) (R) for 12 months, modified the redox state and oxidative stress response of human endothelial cells (ECs) exposed, or not, to oxidative stress induction.

Patients and Methods

This interventional non-pharmacological prospective study is in compliance with ethical guidelines of the Declaration of Helsinki (1975) and has been approved by the Institutional Review Board at our Center (number 0018123/2015). All patients gave informed written consent.

Sera. Serum samples of twenty-seven out of 30 patients of a previous study with histological diagnosis of NASH (16) were used. Eleven serum samples from patients treated with placebo (P) and 16 from patients treated with R for 12 months were collected at 0 (T_0) and after 12 months (T_{12}) and stored at –80˚C for later use.

Serum markers. Aspartate aminotransferase (AST), alanine amino-transferase (ALT), gamma-glutamyl transpeptidase (GGT), blood glucose and insulin were evaluated using enzyme-linked immuno-sorbent assay (ELISA) commercial kits (R&D Systems, Minneapolis, MN, USA). Insulin-resistance was calculated by the homeostatic model assessment of insulin resistance (HOMA-IR test) (17).

Table I. *Clinical and serological characteristics of enrolled patients (mean±standard deviation).*

Number	27
Age (years)	42±11
Gender (Male/Female)	14/13
BMI (kg/m^2)	29.38±4.56
Obesity	5
Diabetes mellitus	3
Hypercholesterolemia	3
Hypertriglyceridemia	2
ALT (IU/l)	59.65±30.18
AST (IU/l)	32.24±9.85
GGT (IU/l)	39.75±25.61
Glucose (mg/dl)	85.13±38.21
Insulin (μU/ml)	21.72±14.64
HOMA-IR	4.05±2.07

BMI, Body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyltranspeptidase; HOMA-IR, the homeostatic model assessment of insulin resistance.

Cytokines and markers of liver fibrosis were assessed using ELISA kits: interleukin (IL)-10, transforming growth factor (TGF)-β, tumor necrosis factor (TNF)-α and matrix metalloproteinases (MMP)-2 (R&D Systems); hyaluronic acid (Echelon Biosciences Inc., Saltlake City, OK, USA); procollagen I and amino-terminal propeptide of type III procollagen (PIIINP) (Takara Bio Inc., Otsu, Shiga, Japan); tissue inhibitor of metalloproteinases (TIMP)-I (Invitrogen, Carlsbad, CA, USA); TIMP-II (Chemicon International, Millipore, Billerica, MA, USA).

Markers of oxidative stress. We evaluated the following markers of oxidative stress: (i) Thiobarbituric acid reactive substances (TBARS) (18).The assay was performed with 10 μl of serum. The chromogen TBARS was quantified using a spectrophotometer at a wavelength of 532 nm with 1,1,3,3-tetramethoxyprophane as standard. The amount of TBARS was expressed as nmol/μg of protein. Presented data are the mean (M)±standard deviation (SD) resulting from three independent experiments.

(ii) Superoxide dismutase (SOD) and catalase (CAT) activities were measured in both sera and cell lysates (Cayman Chemical, Ann Arbor, MI, USA) (19). For both assays, samples were previously diluted with buffer (1:10 for sera; 1:2 for cell lysates). One unit of SOD activity was defined as the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. SOD assay measured all three types of SOD (Cu/Zn, Mn and FeSOD). One unit of CAT activity was defined as the amount of enzyme that led to the formation of 1.0 nmol of formaldehyde per min at 25˚C. The values are reported as U/μg of protein. The results derived from three independent experiments.

Cell culture and treatments. Human umbilical vein endothelial cells (HUVEC) (Clonetics, Walkersville, MD, USA) were used for *in vitro* experiments. ECs were cultured in EGM-2 Bullet Kit (Clonetics), containing vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor (EGF), hydrocortisone, heparin, gentamicin

Table II. *Biochemical parameters of patients at T0 and T12 of placebo (P) or Realsil® (R) administration.*

	P (10 patients)			R (17 patients)		
	T_0	T_{12}	*p*-Value	T_0	T_{12}	*p*-Value
BMI (kg/m^2)	29.41±4.19	29.50±4.47	0.343	29.32±5.00	29.22±5.06	0.796
ALT (IU/l)	56.25±32.26	40.38±13.61	0.110	66.21±32.91	45.86±30.23	0.038
AST (IU/l)	33.38±11.86	26.63±5.93	0.095	31.93±8.54	26.79±10.51	0.090
GGT (IU/l)	36.83±20.83	39.17±14.57	0.795	45.69±29.94	39.21±20.77	0.271
Glucose (mg/dl)	95.13±48.23	104.29±23.03	0.548	81.73±39.89	85.27±29.88	0.184
Insulin (μU/ml)	22.41±13.73	9.53±6.39	0.134	21.82±15.87	16.83±7.22	0.501
HOMA-IR	4.25±2.27	6.48±7.43	0.961	3.94±1.93	3.61±1.82	0.433
IL-10 (pg/ml)	37.15±23.95	31.22±19.16	0.194	32.76±19.87	28.15±12.16	0.324
MMP-2 (ng/ml)	155.64±27.58	184.18±62.44	0.178	152.73±58.08	167.44±83.78	0.544
TGF-β (ng/ml)	20.83±13.41	20.75±13.23	0.987	20.82±9.50	14.74±9.24	0.009
TNF-α (pg/ml)	19.70±11.82	23.39±9.55	0.400	24.66±14.48	28.51±13.09	0.373
TIMPI (ng/ml)	304.36±126.40	296.18±78.31	0.805	316.67±75.73	316.69±90.62	0.997
TIMPII (ng/ml)	47.73±22.94	67.36±51.48	0.196	65.13±45.15	54.06±34.47	0.493
Hyaluronic acid (ng/ml)	277.36±218.71	291.55±246.99	0.722	234.27±120.39	244.56±147.46	0.684
PIIINP (ng/ml)	108.28±47.01	109.54±53.87	0.665	128.04±44.57	116.61±33.76	0.736
Procollagen I (ng/ml)	308.45±60.08	293.18±80.11	0.538	308.20±88.61	244.44±58.40	0.001

T_0, Baseline; T_{12}, after 12 months; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, the homeostatic model assessment of insulin resistance; IL-10, interleukin-10; MMP-2, matrix metalloproteinase-2; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TIMPI/II, tissue inhibitor of metalloproteinase-I/II; PIIINP, amino terminal propeptide of type III procollagen.

sulfate amphotericin, ascorbic acid and 2% fetal bovine serum (FBS). The cells were subcultured by trypsinization, seeded on cell culture dishes and grown at an atmosphere of 5% CO_2 at 37˚C.

The cells were cultured in medium supplemented with patients' sera (10%) (P-ECs or R-ECs, corresponding to patients treated with P or R, respectively) or FBS (10%) as a control. Some cells were exposed to oxidative stress with hydrogen peroxide (H_2O_2), similarly to what has been described in previous studies (5, 20). Briefly, the cells were seeded and cultured for 48 h in medium supplemented as previously described. Later, the culture medium was aspirated and ECs were exposed to 500 μM H_2O_2; four hours after H_2O_2 exposure, growth medium was replaced with fresh medium containing FBS and cells were maintained in culture for another 48-h period.

All experiments were performed at a population doubling level (PDL) of 8 to 12.

Statistical analysis. Continuous variables are expressed as M±SD and compared with the use of Student's *t*-test (normally distributed) or as median±interquartile range value and compared with the use of Mann-Whitney *U*-test (not normally distributed). Normality of data distribution was evaluated using Kolmogorov-Smirnov test. All data were analyzed by SPSS version 15.0 (SPSS, Inc., Chicago, IL, USA). Statistical significance was accepted at $p<0.05$ in a two-tailed test. Alessandro Federico and Valeria Conti analyzed the data.

Results

Effects of placebo (P) and R on serum markers of oxidative stress. Clinical and serological characteristics of the enrolled patients are reported in Table I.

Table II reports biochemical parameters of the enrolled patients at baseline (T_0) and after 12 months (T_{12}) of P or R administration. After 12 months, no differences in P group were found; conversely, ALT ($p=0.038$), TGF-β ($p=0.009$) and procollagen I ($p=0.001$) values significantly decreased at T_{12} after R treatment. Figure 1 (panels A, B and C) summarizes the serum levels of TBARS, SOD and CAT activities found in the sera at T_0 and T_{12} of P or R administration. As shown, no significant changes were found.

As reported in a previous study (16), we identified patients showing very low or very high levels of TBARS at baseline when compared to those of healthy controls. Thus, we divided patients (both P and R) into two groups on the basis of the trend of TBARS serum levels between baseline and after 12 months of treatment with P or R, in order to evaluate any potential differences of antioxidant response between these two groups. Seven patients who showed a significant increase of TBARS values at T_{12} compared to T_0 ($p<0.01$) were included in the first group of patients treated with R (R-I), whereas 10 patients who showed a decrease of TBARS at T_{12} ($p<0.01$ *vs*. T_0 values) were included in the second group (R-II). Of note, in the P group, we did not find any statistically significant variation in the TBARS levels after 12 months (Figure 2, panel A).

Taking into consideration the first division in two groups in relation to TBARS trend, we analyzed two additional

markers of oxidative stress (SOD and CAT) activities at baseline and after 12 months of treatment with P or R.

No significant differences in SOD activity between T_0 and T_{12}, both in P and R groups, were found (Figure 2, panel B). Conversely, CAT activity exclusively decreased in the R-I group ($p<0.01$) after 12 months of R administration (Figure 2, panel C).

Biochemical variables of the study population, divided into two groups (I and II) for each considered treatment (P and R), are listed in Tables III and IV. In patients treated with P, both P-I and P-II, no differences were found before and after treatment (Table III). By contrast, a significant reduction of procollagen I after 12 months of R administration was found in both R-I ($p=0.018$) and R-II ($p=0.030$) groups. Interestingly, in R-II group, we also recorded a significant reduction of ALT ($p=0.041$) and TGF-β ($p=0.015$) after 12 months of R administration (Table IV).

Effects of sera on ECs. We used HUVEC in cultures conditioned with medium supplemented with NASH patients' sera (10%) in order to evaluate if the sera were able to determine an antioxidant response in ECs with or without an oxidative H_2O_2 stimulus. We performed this experiment in order to create a microenvironment similar to the oxidative state present in serum of patients. As shown in Figure 3 (panel A), no difference in SOD activity between T_0 and T_{12} in cell cultures supplemented with patients' sera treated with P, both PI and PII (PI/II-ECs), was found. Treatment with H_2O_2 did not induce any change in SOD activity between T_0 and T_{12} in both PI-ECs and PII-ECs. In cells cultured in medium supplemented with patients' sera (10%), treated with R (RI/II-ECs), we recorded a drastic reduction in SOD activity at T_{12} ($p=0.001$) after induction of oxidative stress in RI-ECs, whereas no difference was found in RII-ECs.

No difference in CAT activity between T_0 and T_{12}, in both PI-ECs and PII-ECs, was found; moreover, treatment with H_2O_2 did not induce any change between T_0 and T_{12}. Conversely, we recorded a significant increase in CAT activity, with and without induction of oxidative stress, between T_0 and T_{12} in both RI-ECs and RII-ECs ($p=0.05$ and $p=0.001$; Figure 3, panel B).

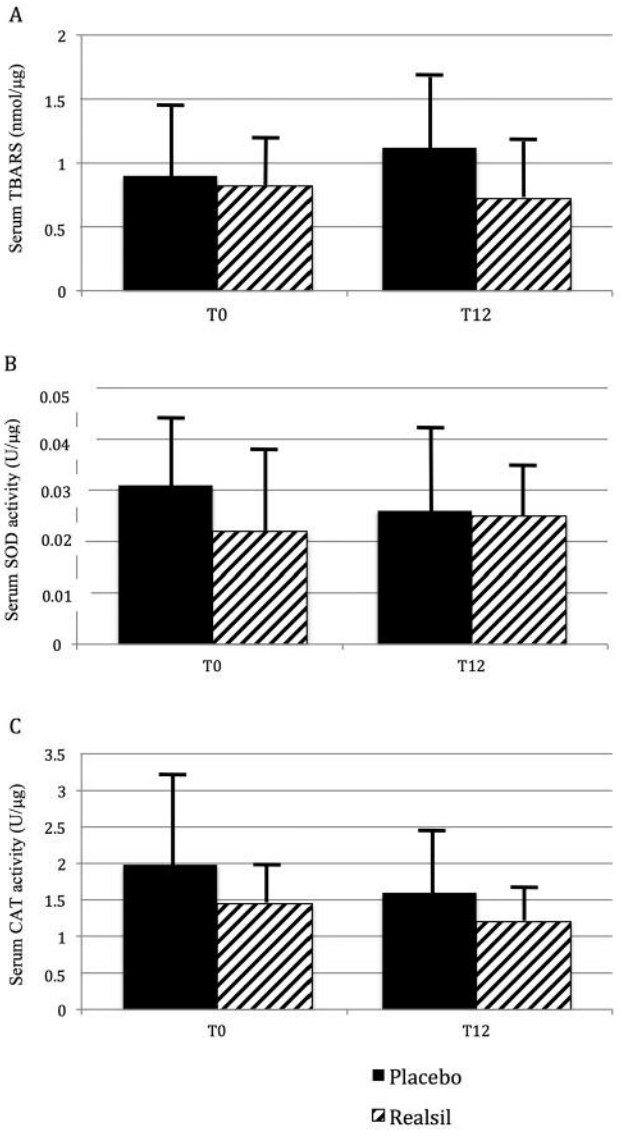

Figure 1. *Serum levels of thiobarbituric acid reactive substances (TBARS) (mmol/μg), superoxide dismutase (SOD) (U/μg) and catalase (CAT) (U/μg) activities at baseline (T_0) and after 12 months (T_{12}) of placebo or Realsil® administration.*

Discussion

NAFLD is associated with MS, which is characterized by the coexistence of atherogenic dyslipidemia, elevated blood pressure, elevated glucose/insulin resistance, prothrombotic state and a low-grade inflammation (21, 22).

These conditions are cardiovascular risk factors and patients affected by this syndrome have an elevated probability to develop a cardiovascular pathology that, in turn, represents one of the main causes of death in this population (23).

In recent years, the role of NAFLD in inducing CVD has been investigated; moreover, it may be considered as an independent risk factor for cardiovascular pathologies (2, 24).

Among the several processes involved in the mechanism linking NAFLD and CVD, oxidative stress and endothelial dysfunction (in turn, strongly inter-correlated) appear to play an important pathogenic role (25). Recent studies have showed that NAFLD is associated to endothelial dysfunction, that is characterized by oxidative stress accumulation and considered as a surrogate marker of CVD (26).

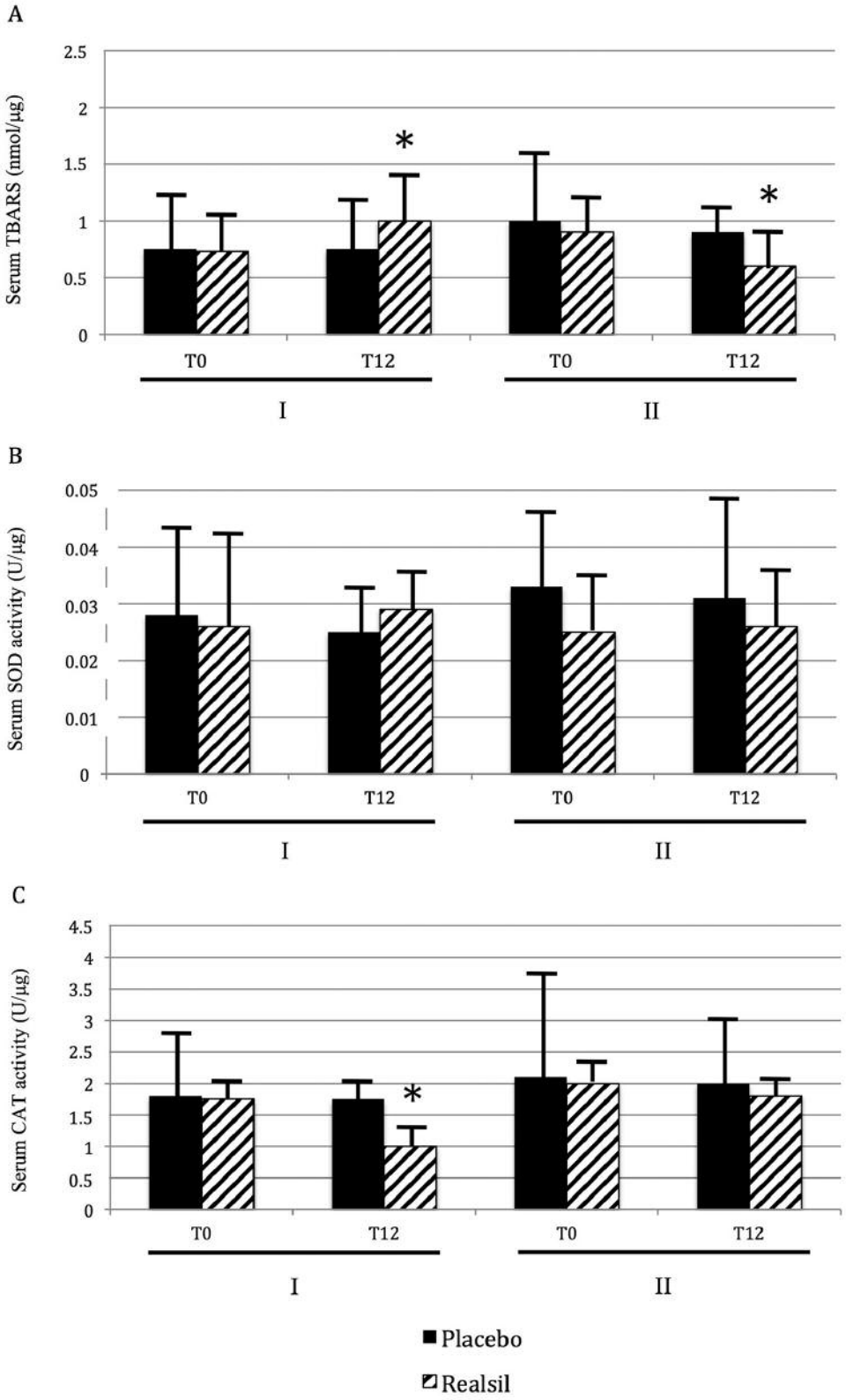

Figure 2. *Serum levels of thiobarbituric acid reactive substances (TBARS) (mmol/µg), superoxide dismutase (SOD) (U/µg) and catalase (CAT) (U/µg) activities at baseline (T_0) and after 12 months (T_{12}) of placebo or Realsil® administration in stratified patients into two groups on the basis of the trend of TBARS serum levels. A: *p<0.01vs. T_0; C: *p<0.01 vs. T_0.*

Table III. *Biochemical variables of patients stratified into two groups (I and II) for placebo (P) treatment.*

	P-I (5 patients)			P-II (5 patients)		
	T_0	T_{12}	*p*-Value	T_0	T_{12}	*p*-Value
BMI (kg/m^2)	29.59±5.10	29.61±5.37	0.404	28.93±3.92	28.84±3.86	0.811
ALT (IU/l)	70.79±33.70	49.00±28.39	0.271	48.25±25.42	34.88±16.19	0.368
AST (IU/l)	34.21±9.85	29.00±10.00	0.251	29.38±8.96	22.75±5.12	0.245
GGT (IU/l)	44.46±30.93	40.50±20.93	0.871	39.50±18.25	32.43±15.71	0.751
Glucose (mg/dl)	76.00±40.22	94.75±15.28	0.777	125.00±49.67	117.00±28.58	0.503
Insulin (μU/ml)	21.85±16.58	13.11±8.18	0.136	22.45±11.39	15.18±6.76	0.212
HOMA-IR	3.63±1.73	3.35±1.91	0.356	4.88±2.40	7.06±7.04	0.110
IL-10 (pg/ml)	31.41±20.17	27.97±14.14	0.619	38.36±22.93	31.20±16.74	0.251
MMP-2 (ng/ml)	152.71±58.20	174.67±91.82	0.211	155.42±31.32	173.75±50.52	0.547
TGF-β (ng/ml)	23.35±12.75	17.40±11.64	0.606	17.88±8.28	16.92±11.16	0.621
TNF-α (pg/ml)	18.49±13.96	26.77±12.90	0.179	27.32±11.49	25.99±10.94	0.937
TIMPI (ng/ml)	340.36±113.82	315.00±83.00	0.628	277.75±65.57	300.00±90.10	0.719
TIMPII (ng/ml)	46.86±22.69	57.80±42.76	0.300	70.50±48.07	61.58±42.41	0.440
Hyaluronic acid (ng/ml)	179.07±89.93	174.20±101.40	0.839	338.17±196.41	375.58±220.80	0.799
PIIINP (ng/ml)	115.71±44.36	99.09±45.71	0.687	125.05±48.84	127.94±35.40	0.957
Procollagen I (ng/ml)	303.00±75.58	270.60±82.47	0.964	314.50±80.29	256.42±55.82	0.201

T_0, Baseline; T_{12}, after 12 months; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, the homeostatic model assessment of insulin resistance; IL-10, interleukin-10; MMP-2, matrix metalloproteinase-2; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TIMPI/II, tissue inhibitor of metalloproteinase-I/II; PIIINP, amino terminal propeptide of type III procollagen.

Table IV. *Biochemical variables of patients stratified into two groups (I and II) for Realsil® (R) treatment.*

	R-I (7 patients)			R-II (10 patients)		
	T_0	T_{12}	*p*-Value	T_0	T_{12}	*p*-Value
BMI (kg/m^2)	30.05±6.00	29.96±6.19	0.850	27.99±2.41	27.90±1.80	0.879
ALT (IU/l)	80.33±32.05	55.22±33.65	0.102	40.80±14.48	29.00±12.35	0.041
AST (IU/l)	33.56±7.30	29.56±11.81	0.258	29.00±10.68	21.80±5.63	0.266
GGT (IU/l)	48.25±36.03	40.67±24.15	0.501	41.60±19.58	36.60±14.91	0.282
Glucose (mg/dl)	79.17±29.54	82.43±37.48	0.311	84.80±53.53	90.25±10.69	0.385
Insulin (μU/ml)	25.68±19.90	16.32±8.45	0.485	16.03±4.59	18.35±1.20	0.877
HOMA-IR	3.23±0.89	3.60±2.15	0.404	5.00±2.72	3.66±0.62	0.815
IL-10 (pg/ml)	34.84±21.80	30.56±10.01	0.605	30.39±18.82	25.06±14.70	0.296
MMP-2 (ng/ml)	154.50±74.01	169.56±109.97	0.901	150.71±38.34	164.71±37.55	0.366
TGF-β (ng/ml)	24.85±9.99	17.00±10.58	0.090	16.21±6.90	11.83±6.82	0.015
TNF-α (pg/ml)	22.50±15.88	31.74±13.97	0.092	27.13±13.49	24.35±11.48	0.608
TIMPI (ng/ml)	340.38±82.87	316.11±74.40	0.357	289.57±61.36	317.43±114.67	0.385
TIMPII (ng/ml)	49.25±24.73	56.89±41.34	0.425	83.29±57.64	50.43±25.79	0.099
Hyaluronic acid (ng/ml)	169.00±79.22	155.67±51.47	0.349	308.86±119.68	358.86±154.06	0.505
PIIINP (ng/ml)	117.43±47.07	99.81±29.50	0.871	140.42±42.05	130.04±33.46	0.349
Procollagen I (ng/ml)	293.00±83.74	241.44±42.09	0.018	325.57±97.36	248.29±39.56	0.030

T_0, Baseline; T_{12}, after 12 months; BMI, body mass index; ALT, alanine aminotransferase; AST, aspartate aminotransferase; GGT, gamma-glutamyl transpeptidase; HOMA-IR, the homeostatic model assessment of insulin resistance; IL-10, interleukin-10; MMP-2, matrix metalloproteinase-2; TGF-β, transforming growth factor-beta; TNF-α, tumor necrosis factor-alpha; TIMPI/II, tissue inhibitor of metalloproteinase-I/II; PIIINP, amino terminal propeptide of type III procollagen.

Recently, the protective role of silybin on endothelial cells has been demonstrated. Silybin has both antioxidant and anti-inflammatory properties (13, 27-31), as well as anti-fibrotic ones (32). However, even if the effectiveness of this natural compound against liver damage has been largely documented, its therapeutic potential in attenuating cardiovascular risk

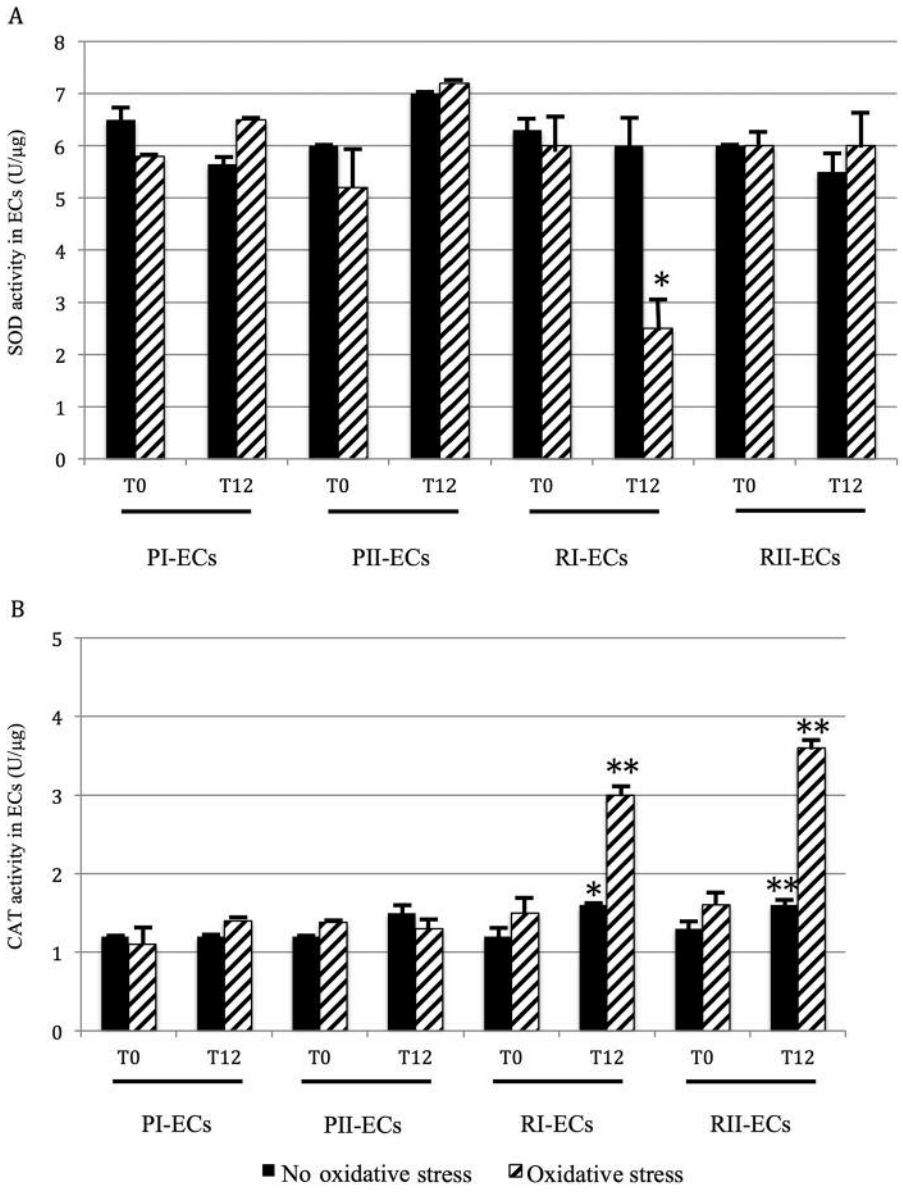

Figure 3. *Superoxide dismutase (SOD) (U/µg) and catalase (CAT) (U/µg) activities in cells cultured in medium supplemented with patients' sera at baseline (T_0) and after 12 months (T_{12}) of placebo (P) or Realsil® (R) administration, stratified into two groups on the basis of the trend of TBARS serum levels, with or without oxidative stress induction. A: *p=0.001 vs. T_0; B: *p=0.05 vs. T_0; **p=0.001 vs. T_0.*

factors, including endothelial dysfunction, has yet to be determined. For these reasons, we used R in order to try and modify the serum redox state of NASH patients and to evaluate if these modifications were able to induce an antioxidant response in ECs' cultures conditioned with serum of these patients.

In order to evaluate the redox state of NASH patients, we assessed the following markers of oxidative stress: TBARS, SOD and CAT activities before (T_0) and after (T_{12}) treatment with P or R.

As in a previous study (16), we identified an opposite trend in TBARS levels among the patients treated with R. Thus, we divided the patients into two groups on the basis of the incremental or decremental trend of TBARS levels, P/R-I and P/R-II, respectively. On the basis of this subdivision, we found, at the end of 12 months of R administration, a decrease of CAT activity in patients showing an increase of TBARS, whereas no change in patients showing a decrease of TBARS was found (R-I group).

Moreover, RI and RII groups showed a decrease of procollagen I, a well-known marker of fibrosis, after 12 months of treatment. Finally, in R-II patients, who showed a decrease of TBARS at T_{12}, a reduction of ALT and TGF-β was also found. The reduction of TGF-β levels may contribute to limit fibrotic tissue deposition. Indeed, TGF-β represents one of the main cytokines able to sustain collagen synthesis and, therefore, fibrosis through transformation of hepatic stellate cells in myofibroblasts (33). On the contrary, in our study, tissue inhibitor of metalloproteinase (TIMP) I/II, matrix metalloproteinase (MMP)-2 and amino terminal propeptide of type III procollagen (PIIINP), other known serum parameters able to predict liver fibrosis, did not show statistically significant changes after R treatment. It is possible that this may be attributed to the limited sample size, as well as the limited time of observation (12 months).

In summary, in R-II patients' sera, we observed a decrease of lipid peroxidation after 12 months of treatment, concurrently with a decrease of ALT and two important pro-fibrotic factors: TGF-β and procollagen I.

As mentioned above, ALT has been recently shown to be associated with endothelial dysfunction and, therefore, proposed as a predictive factor for coronary events (3). Since the endothelial dysfunction is recognized as an early marker of NAFLD and taking into consideration the antioxidant property of silybin, we tested the hypothesis that R could be useful in contrasting endothelial dysfunction. Therefore, we adopted the *in vitro-in vivo* technique already used in our previous studies by which we demonstrated the capacity of sera isolated either from athletes (5, 20) or NASH patients treated with R (16) to stimulate the oxidative stress response in endothelial cells and HepG2 cells, respectively. Thus, we conditioned human endothelial cells with sera isolated from patients before and after P or R administration.

We performed this experiment in order to create a cellular microenvironment similar to the oxidative state present in the serum of patients. Obviously, this experiment does not reflect the intracellular redox state *in vivo*; however, our aim was to evaluate the response of ECs' cultures to the serum redox state modifications that treatment with P or R could cause.

Cells conditioned with sera from R-I patients showed an increase of CAT activity after R treatment (T_{12}), whereas cells conditioned with R-II sera showed a higher increase of CAT activity after R treatment. Importantly, this finding was observed both in the presence and absence of H_2O_2. No differences in the cells conditioned with sera isolated from patients treated with P were found.

We hypothesized that the increased levels of TBARS, reached in R-I patients sera at T_{12}, may have stimulated CAT activity in human ECs. The decrease of CAT serum activity and the increase of CAT activity in ECs conditioned with R-I patient's sera could be due to the possibility that CAT activity decreases since its function is partially substituted with the antioxidant effect of R. On the contrary, in ECs conditioned with RII patient's sera at T_{12}, increased CAT activity may be due to the direct effect of R, which could modify serum micromolecular composition, thus leading to the production of substances that increase CAT activity in ECs, through the interaction with specific receptors, independently of serum levels of TBARS at T_{12}. Particularly, the higher increase of cellular CAT activity, induced by R, in RII-ECs may be associated to a reduction of liver cytonecrosis indices. Further studies are needed in order to identify mediators responsible for the observed phenomenon.

The role of CAT in hepatic fibrosis has been recently investigated. Dong *et al.* demonstrated that overexpression of CAT leads to a decrease in the secretion of collagen type 1 in hepatic stellate cells (34). Moreover, Hernández-Ortega *et al.* showed that quercetin, a potent activator of SOD and CAT, improves hepatic fibrosis by reducing the expression of pro-fibrogenic molecules, including collagen 1α, connective tissue growth factor and TGF-β (35).

In this way, the trend of TBARS could be considered as a predictive marker of antioxidant response to R treatment; further studies, however, should be addressed in order to investigate whether the amount of TBARS in R-II patients could be reduced as a consequence of adaptive response in the endothelium of such patients.

In conclusion, the link between NAFLD and endothelial dysfunction, that is associated with cardiovascular pathologies, is a current research topic. The role played by oxidative stress in endothelial dysfunction is now recognized; however, further efforts should be addressed to clarify both its involvement in the onset and progression of NAFLD and the relationship between NAFLD and CVD.

The use of R, which has anti-inflammatory, antioxidant and anti-fibrotic properties, determines a slowdown of liver damage and could contribute, at least in part, to reduction of the incidence of CVD in patients with NAFLD, through the perturbation of redox state homeostasis.

Patients with higher levels of TBARS at T_0 demonstrated an improvement of fibrosis, pro-inflammatory and cytonecrosis biomarkers after 12 months of R administration. In this way, the trend of TBARS could be considered as a predictive marker of antioxidant response to R treatment. We hypothesized that these effects may be triggered by treatment with R, through a modulation of the response to oxidative stress both at the cellular and serum level. This may contribute to the improvement of the endothelial dysfunction in patients with MS, by intervening on the vicious circle that links MS and NASH to CVD.

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