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Thyroid

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Abstract:	Objective. There is growing awareness that nutritional habits may influence risk of several inflammatory and immune-mediated disorders, including autoimmune diseases, through various mechanisms. The aim of the present study was to investigate dietary habits and their relationship with redox homeostasis in the setting of thyroid autoimmunity. Materials and Methods. Two hundred subjects (173 females and 27 males; median age, 37 years) were enrolled. None were under any

	pharmacological treatment. Exclusion criteria were any infectious/inflammatory/autoimmune comorbidity, kidney failure, diabetes, and cancer. In each subject, serum TSH, free thyroxine, anti-thyroid antibodies, and circulating oxidative stress markers were measured. A questionnaire on dietary habits, evaluating the intake frequencies of food groups and adherence to the Mediterranean diet, was submitted to each participant. Results. Among the 200 recruited subjects, 81 (71 females and 10 males) were diagnosed with euthyroid Hashimoto's thyroiditis (HT); the remaining 119 (102 females and 17 males) served as controls. In questionnaires, HT subjects reported higher intake frequencies of animal foods (meat, P = 0.0001; fish, P = 0.0001; dairy products, P = 0.004) compared to controls, who reported higher intake frequencies of plant foods (legumes, P = 0.001; fruits and vegetables, P = 0.030; nuts, P = 0.0005). The number of subjects who preferentially consumed poultry instead of red/processed meat was lower in HT subjects than in controls (P = 0.0141). In logistic regression analysis, meat consumption was associated with increased odds ratio of developing thyroid autoimmunity, whilst Mediterranean diet traits were protective. In HT subjects, serum advanced glycation end products (markers of oxidative stress) were significantly higher (P = 0.0001) than controls, while the activity of glutathione peroxidase and thioredoxin reductase, as well as total plasma antioxidant activity, were lower (P = 0.020, P = 0.023, and P = 0.002, respectively), indicating a condition of oxidative stress. Stepwise regression models demonstrated a significant dependence of oxidative stress parameters on consumption of animal foods, mainly meat. Conclusions. The present study suggests a protective effect of low intake of animal foods towards thyroid autoimmunity and a positive influence of such nutritional patterns on redox balance and potentially on oxidative stress-related disorders.

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ABSTRACT

Objective. There is growing awareness that nutritional habits may influence risk of several inflammatory and immune-mediated disorders, including autoimmune diseases, through various mechanisms. The aim of the present study was to investigate dietary habits and their relationship with redox homeostasis in the setting of thyroid autoimmunity.

Materials and Methods. Two hundred subjects (173 females and 27 males; median age, 37 years) were enrolled. None were under any pharmacological treatment. Exclusion criteria were any infectious/inflammatory/autoimmune comorbidity, kidney failure, diabetes, and cancer. In each subject, serum TSH, free thyroxine, anti-thyroid antibodies, and circulating oxidative stress markers were measured. A questionnaire on dietary habits, evaluating the intake frequencies of food groups and adherence to the Mediterranean diet, was submitted to each participant.

Results. Among the 200 recruited subjects, 81 (71 females and 10 males) were diagnosed with euthyroid Hashimoto's thyroiditis (HT); the remaining 119 (102 females and 17 males) served as controls. In questionnaires, HT subjects reported higher intake frequencies of animal foods (meat, $P = 0.0001$; fish, $P = 0.0001$; dairy products, $P = 0.004$) compared to controls, who reported higher intake frequencies of plant foods (legumes, $P = 0.001$; fruits and vegetables, $P = 0.030$; nuts, $P = 0.0005$). The number of subjects who preferentially consumed poultry instead of red/processed meat was lower in HT subjects than in controls ($P = 0.0141$). In logistic regression analysis, meat consumption was associated with increased odds ratio of developing thyroid autoimmunity, whilst Mediterranean diet traits were protective. In HT subjects, serum advanced glycation end products (markers of oxidative stress) were significantly higher ($P = 0.0001$) than controls, while the activity of glutathione peroxidase and thioredoxin reductase, as well as total plasma antioxidant activity, were lower ($P = 0.020$, $P = 0.023$, and $P = 0.002$, respectively), indicating a condition of oxidative stress. Stepwise regression models demonstrated a significant dependence of oxidative stress parameters on consumption of animal foods, mainly meat.

Conclusions. The present study suggests a protective effect of low intake of animal foods towards thyroid autoimmunity and a positive influence of such nutritional patterns on redox balance and potentially on oxidative stress-related disorders.

INTRODUCTION

Hashimoto's thyroiditis (HT) is the most common autoimmune endocrine disease and the main cause of hypothyroidism in iodine-sufficient areas (1). Incidence has increased significantly over the last few decades (2, 3), paralleling the steady rise in frequency of other autoimmune disorders (ADs) mostly in Western countries compared to the East and Global South (4, 5). This rapid increase of ADs in developed countries and its clear relationship with socioeconomic status points to a strong influence of changing environmental factors in driving such geoepidemiologic trends as opposed to constancy of genetic basis (5,6).

Among the many environmental triggers of autoimmunity, growing interest has been focused on a Western lifestyle since several significant changes have occurred over the past decades in more industrialized and richer societies. They include modified infectious habitat and personal hygiene, increased pollution exposure, psychological stress overload, sedentary lifestyle, and changes in dietary habits (5-12). In particular, in Westernized countries, a diet rich in calories, fats, and proteins, high in salt and refined sugars, and low in fibers is often preferred to dietary regimens rich in fruits and vegetables, along with more frequent consumption of processed and fast foods. This dietary regimen, the so-called Western-type diet, might influence risk of ADs either directly by increasing inflammation and altering immune ($CD4^+$ effector and regulatory T cells) balance and intestinal microbiota composition or indirectly through increasing fat mass and obesity (10-14). Another possible mechanism is enhanced oxidative stress, which is an imbalance between reactive oxygen species (ROS) production and removal by antioxidant mechanisms (15, 16). A correlation between increased oxidative stress and the Western-type diet has been demonstrated since consumption of large amounts of fats and refined sugar in the long run results in intestinal dysbiosis and inflammation with ROS overproduction, while low intake of fruits and vegetables causes lack of exogenous antioxidants (16, 17).

Several studies have evaluated the possible association between nutrition and autoimmunity in different settings of patients and consistently suggest dietary traits as risk factors for rheumatoid arthritis (RA), multiple sclerosis, psoriasis, and celiac and inflammatory bowel diseases (13, 17-23). In the field of thyroid diseases, however, very few studies have evaluated the role of different dietary patterns, mainly in relation to thyroid dysfunction rather than autoimmunity *per se* (24, 25), and none have investigated the possible relationship with oxidative stress. The present study investigated nutritional habits in euthyroid HT subjects compared to healthy controls and their relationship with changes in redox balance.

MATERIALS AND METHODS

Subjects

All subjects were recruited randomly from voluntary participants (>18 years-old) in thyroid disorders awareness campaigns that were run at the Endocrinology Unit of the University Hospital "Policlinico G. Martino" of Messina, Italy, during International Thyroid Awareness week, World Thyroid Day, and in the preceding weeks to stimulate population awareness. To obtain as homogeneous a study population as possible with regard to geographic location, ethnic group, and lifestyle/diet, inclusion criteria at recruitment were Caucasian subjects stably living in the city of Messina, those with stable dietary habits in the last 5 years, and no history of pharmacological treatment, antioxidant agent, and/or vitamin supplements in the preceding 6 months. Exclusion criteria were obesity [body mass index (BMI) > 30 kg/m^2]; diabetes mellitus; kidney failure; history of neoplastic disease; existence of any comorbid cardiovascular, autoimmune, infectious, or inflammatory disease; current or past smoking history; and current or former alcohol abuse. Patients who had already been diagnosed with thyroid disorders or who had already been treated for thyroid dysfunction were also excluded.

A total of 200 healthy subjects (173 female and 27 male; median age, 37 years), who agreed to thyroid function and autoantibodies tests and provide a blood sample, took part in the study and were

119 administered a validated Italian questionnaire aimed at collecting data regarding lifestyle and dietary
120 habits (26). Each subject underwent a careful history, physical examination, and thyroid
121 ultrasonography. A food frequency questionnaire was used to evaluate the intake frequencies of food
122 groups (meat, fish, cereals, fruits and vegetables, and dairy products). With this step, the 14 items
123 included in PREDIMED, a validated questionnaire on Mediterranean diet adherence, were also
124 obtained (27); thus, adherence to the Mediterranean diet in the present cohort was assessed. Briefly,
125 the PREDIMED score was calculated as follows: for each item, a score of 1 or 0 was assigned; a
126 score of 0–5 meant low adherence, 6–9 represented average adherence, and ≥10 equated to high
127 adherence (27). Concerning the iodine nutritional status, all subjects were from the same area of mild
128 iodine deficiency (28). The design of the present study did not include individual urinary iodine
129 measurements. However, all participants were asked if they used iodized salt to evaluate any
130 difference in the iodine nutritional habits of the study population. All subjects were informed of the
131 study aims according to the Helsinki Declaration and provided written informed consent. The study
132 was approved by the local Ethics Committee.

133 Blood collection and biochemical analysis

134 Venous peripheral blood samples were collected after overnight fasting. Blood samples were
135 centrifuged at 1450 × *g* at 4 °C for 10 min, and each sample was divided into aliquots. Processing
136 and scoring of samples were performed blind and concurrently. At the end of the study, information
137 regarding thyroid status and data from the questionnaire were linked to a code number and became
138 available for statistical analysis.

139 Main metabolic parameters (fasting glucose, insulin, and lipids) and thyroid function indices were
140 immediately measured. Serum thyroid stimulating hormone (TSH), free thyroxine (FT4), and anti-
141 thyroperoxidase (TPOAb) antibodies were measured by electrochemiluminescence immunoassay
142 (Roche Diagnostics, Mannheim, Germany). Normal values were 0.27–4.5 mIU/L TSH, 9.0–22.0

pmol/L FT4, and 0–10 IU/mL TPOAb. For all assays, the intra- or inter-assay CV was <5% and <10%, respectively.

Aliquots for other assays were stored at −20 °C. Two markers of oxidative stress, advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs), were measured in serum samples as previously reported (29). Activity of antioxidant enzymes superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), thioredoxin reductase (TRxR), and total plasma antioxidant activity (TEAA) were measured in plasma samples as described elsewhere [see Supplementary Materials] (30, 31). Overall, determination of the study parameters occurred within 2 months from sera collection.

Statistical analysis

Numerical data are expressed as medians and ranges (minimum and maximum), and categorical variables were expressed as number and percentage. A nonparametric approach was used since most numerical variables were not normally distributed, as verified by the Kolmogorov-Smirnov test. To assess the existence of significant differences between HT subjects and controls, the Mann Whitney test (for numerical parameters) and Chi square, Fisher exact, or Likelihood ratio tests were applied as appropriate (for categorical variables). Spearman correlation was applied to evaluate interdependence between the studied oxidative stress markers, both in all subjects and in each group (HT subjects and controls, separately). Multivariable linear regression models (with stepwise procedure) were estimated to assess the possible dependence of each oxidative stress parameter (AGEs, AOPPs, SOD, GPx, GR, TRxR, and TEEA) on some potential explicative covariates, including age, sex, BMI, biochemical parameters [homeostatic model assessment (HOMA), high-density lipoprotein (HDL)-cholesterol, triglycerides, thyroid function indices, and anti-thyroid antibodies], and dietary habits (food group intake frequencies, and adherence to Mediterranean diet evaluated by PREDIMED score). Finally, a multivariable logistic regression model (with stepwise procedure) was estimated to identify significant predictive factors of AbTPO positivity; covariates were age, sex (female 0, male

1), BMI, PREDIMED score, and intake frequency of main food groups (fish, meat, dairy products, eggs, cereals, fruits and vegetables). Statistical analyses were performed using SPSS 22.0 for Windows. A $P < 0.05$ was considered statistically significant.

RESULTS

Demographic, clinical, and biochemical features of the study population are summarized in **Table 1**. Eighty-one subjects (71 females and 10 males; median age, 40 years; age range, 18–66) were diagnosed with euthyroid HT by currently accepted laboratory and ultrasonographic criteria [serum anti-thyroid antibody positivity and/or heterogeneous echostructure with diffuse or patchy hypoechogenicity at ultrasound] (1). The remaining 119 subjects (102 females and 17 males; median age, 37 years; age range, 18–65) had no evidence of thyroid disease (normal thyroid function, absence of serum thyroid autoantibodies, and no ultrasound alterations) and served as controls. The two groups of age- and sex-matched HT subjects and healthy controls did not differ significantly regarding main anthropometric and metabolic parameters, with the exception of HDL-cholesterol (**Table 1**). All subjects were euthyroid, naïve to L-T4 therapy, and not taking any drugs affecting thyroid function at the time of sampling nor during the previous 6 months. However, HT subjects had higher TSH and lower FT4 values, though within normal ranges, compared to controls ($P = 0.006$ and $P = 0.0001$, respectively).

Concerning oxidative stress parameters, AGEs were increased in HT subjects ($P = 0.0001$), while AOPP levels were similar between HT subjects and controls ($P = 0.162$). In the same HT subjects, GPx, TRxR, and TEAA were lower than in controls ($P = 0.020$, $P = 0.023$, and $P = 0.002$, respectively), indicating a condition of oxidative stress (**Table 2**). Correlation analysis assessing interdependence between oxidative stress markers demonstrated a significant inverse correlation between AGEs and TEAA in all participants ($P = 0.018$), as well as in HT subjects ($P = 0.013$), but not healthy controls ($P = 0.747$).

In questionnaires, HT subjects reported higher intake frequencies of animal foods (meat, $P = 0.0001$; fish, $P = 0.0001$; dairy products, $P = 0.004$) compared to controls, who, in turn, reported higher intake frequencies of plant foods, including legumes ($P = 0.001$) and fresh fruits and vegetables ($P = 0.030$) (**Figures 1 and 2**). The two groups mainly differed regarding consumption of meat, as HT subjects reported higher intake frequencies of animal meat in general, specifically red/processed meat, compared to controls. Indeed, the number of subjects who reported preferential consumption of white meat and poultry instead of red/processed meat was significantly lower in the HT group than in the control (29% versus 52%; $P = 0.014$). None of the subjects who did not eat meat at all or no more than twice a month were found to have thyroid autoantibodies. Moreover, consumption of other animal foods, like fish and dairy products, was higher in HT individuals than controls, but no difference in egg consumption was observed ($P = 0.081$). Finally, the number of subjects who reported consumption of ≥3 servings per week of nuts was significantly lower in HT subjects compared to controls (23% versus 55%; $P = 0.0005$).

HT and control groups did not differ concerning intake of cereals (88.5% versus 93%, $P = 0.315$) and whole-grain (51% versus 58%, $P = 0.563$). Furthermore, most subjects reported use of olive oil as main culinary fat (at least 4 tablespoons daily), without difference between HT and control subjects (94% versus 98%, $P = 0.433$). Similarly, most subjects in both groups did not use butter or cream at all, while less than one-third of subjects consumed butter, margarine, or cream <3 d/week, without differences between the groups ($P = 0.733$). Finally, HT subjects reported a higher frequency of consumption of fats and refined sugars from commercial sweets ($P = 0.010$) despite no differences in the consumption of sweetened or carbonated beverages (all <1 per day). Moreover, control and HT subjects did not differ concerning weekly consumption of wine or general alcohol intake ($P > 0.05$). Finally, there was no difference in the use of iodized salt between HT subjects and controls, but individual urinary iodine concentrations were not available.

Concerning lifestyle, most of the subjects reported doing physical activity no more than twice a week (70% of HT subjects versus 60% of controls), without significant differences between subjects with

or without HT (P = 0.176). Current or past smokers were excluded to avoid biases. The largest part of the cohort presented a medium-high grade of adherence to the Mediterranean diet according to PREDIMED scores. However, HT subjects displayed significantly lower scores than controls (P = 0.0001; **Figure 3**). The main determinants of this difference were the higher consumption of animal meat in general, red/processed meat in particular, the lower consumption of vegetables, fruits, and legumes, the higher consumption of commercial sweets or pastries, and the lower consumption of nuts in HT subjects compared to controls.

In the multivariable logistic regression model, adherence to the Mediterranean diet, as evaluated by PREDIMED score, was a significant predictive factor of TPOAb positivity. A higher score of adherence to the Mediterranean diet along with higher intake frequencies of fruits, vegetables, and cereals was protective against the risk for developing thyroid autoimmunity, while higher intake frequency of meat and dairy products were significantly associated with an increased risk of thyroid autoimmunity (**Table 3**). As expected, increasing age and female gender were also associated with an increased probability of developing thyroid autoantibodies in the regression model (**Table 3**).

Stepwise regression models demonstrated a significant dependence of oxidative stress parameters on age and consumption of animal foods; meat intake was associated with lower levels of the antioxidants GPx (P = 0.048), GR (P = 0.010), and TRxR (P = 0.007) but higher levels of the oxidants AGEs (P = 0.045) and AOPPs (P = 0.048). Similarly, dairy product intake was associated with low levels of both GR (P = 0.048) and TEAA (P = 0.020; **Table 4**). Eggs, which represent a source of animal proteins and saturated fats, were slightly but significantly associated with reduced levels of GPx (P = 0.010) despite consumption being similar between the two groups (P = 0.221; **Table 4**). Finally, TPOAb positivity was an independent predictor of increased AGEs and reduced GPx and GR activities in multivariate analysis (**Table 4**).

DISCUSSION

In the present pilot study, the nutritional habits of a cohort of euthyroid HT subjects compared to healthy subjects, and the relationship between intake of different foods and changes in redox balance was investigated. A main finding was that dietary habits significantly differed between subjects with and without HT. HT subjects reported a higher intake of animal products and a lower level of adherence to the Mediterranean diet than healthy controls, who reported higher intake of plant foods. Overall, the nutritional pattern of HT subjects according to the survey was characterized by increased consumption of animal proteins, higher intake of saturated fats and refined sugars, and lower intake of fibers and antioxidants compared with healthy subjects. In other words, nutritional patterns of HT subjects resembled the Western-type diet, while controls displayed a higher level of adherence to the Mediterranean diet. Such a significant difference supports the hypothesis of a possible predisposing role of nutritional patterns in autoimmunity.

The association between diet and risk of developing ADs was proposed as early as 50 years ago by Trowell, who observed that a number of ADs, including RA, type 1 diabetes, and HT, were extremely rare among isolated rural sub-Saharan populations following traditional near-vegan diets (32, 33). A similar low incidence of ADs was reported in Asian societies whose diets were almost vegan (33). In the last few decades, further evidence has accumulated on the influence of nutritional factors in the development of several ADs, including RA, celiac and inflammatory bowel diseases, type 1 diabetes, multiple sclerosis, and psoriasis (13, 17-23). To date, only two studies have assessed the dietary habits of subjects suffering from thyroid diseases. In 2013, Tonstad *et al.*, using data from the Adventist Health Study-2, evaluated the prevalence and incidence of hypothyroidism among a large cohort (n = 65,981) of Seventh-day Adventist church members who exhibited a wide range of diets from vegan to omnivorous, with a high proportion of vegetarians (24). They found a lower, though not significant, prevalence and incidence of hypothyroidism among subjects following vegan diets compared to omnivorous diets, even after adjusting for BMI and demographic variables (24). Among the same

268 population from the AHS-2 study, a strongly reduced risk of hyperthyroidism was also reported in
269 those consuming a vegan diet when compared to omnivores, while lacto-ovo and pesco vegetarian
270 diets were associated with intermediate protection (25). These two studies provided congruent,
271 though not always statistically significant, data in favor of a protective role of diets excluding meat
272 against both hypo- and hyperthyroidism, commonly autoimmune in etiology (24, 25).
273 The present study further points to meat in omnivorous diets as a main nutritional factor associated
274 with increased risk of thyroid autoimmunity. Also, intake of animal proteins and saturated fats from
275 dairy products seems to be relevant to the development of HT, while plant foods, containing high
276 amounts of antioxidants and fibers and no fats, may be protective. Compared to healthy controls, HT
277 subjects reported the highest intake of saturated and trans fats from animal products as well as
278 commercial sweets and pastries. In the survey, HT subjects reported significantly higher intake of
279 fish than controls. These results partially contradict previous reports on the protective role of seafood
280 and fish oil supplementation against ADs, including HT (21, 23, 32, 34). Perhaps this different
281 influence could be related to the variable content of polyunsaturated fatty acids, like ω-3
282 polyunsaturated acids, in the fish species consumed (oily fishes or other species). Since we assessed
283 the frequencies but not the quality of consumed seafood in our survey, we can only infer that protein
284 content may account for the association between fish consumption and thyroid autoimmunity.
285 Finally, HT subjects in the present cohort displayed significantly lower adherence to the
286 Mediterranean diet compared to healthy controls, and the PREDIMED score was an independent
287 predictor of the presence of thyroid autoantibodies, suggesting the Mediterranean diet is associated
288 with reduced risk for thyroid autoimmunity. The Mediterranean diet is a nutritional model inspired
289 by traditional dietary regimens of populations living in the Mediterranean basin and is characterized
290 by high intake of vegetables, legumes, fresh fruits, nuts, whole grains, and olive oil; frequent and
291 moderate consumption of red wine; moderate intake of seafood, dairy products, poultry, and eggs;
292 and low consumption of red meat and processed meat products (35). This dietary pattern is rich in
293 fibers, natural antioxidants and vitamins, and consequently, had anti-inflammatory and antioxidant

294 effects which are beneficial to health status (33, 34). Much evidence exists in favor of the protection
295 imparted by the Mediterranean diet against diseases associated with chronic inflammation, including
296 diabetes, obesity, cardiovascular diseases, cancer, and cognitive disorders (36). The current study
297 provides the first evidence of a protective role of the Mediterranean diet also against thyroid ADs. It
298 is conceivable that adoption of this dietary pattern could also be protective against ADs, counteracting
299 the deleterious effects of oxidative stress and exerting anti-inflammatory and immunomodulatory
300 actions, most likely by affecting cytokine production and gut microbiota composition. Indeed, gut
301 dysbiosis may represent another possible pathogenetic mechanism linking diet to autoimmunity (10,
302 37).
303 Notably, despite the excess caloric intake of their nutritional habits (high-fat and high-sugar), HT
304 subjects did not differ from controls regarding body weight and BMI. This suggests that the
305 pathogenetic link between diet and thyroid autoimmunity cannot be represented by being overweight
306 or obesity, whose pro-inflammatory effects are well known, at least in this current cohort. Indeed,
307 BMI was not a significant predictor of thyroid autoantibody positivity nor alterations in oxidative
308 stress parameters in regression models.
309 Another important finding of the present study was the significant influence of nutritional pattern on
310 oxidative stress parameters. Oxidative stress, defined as an imbalance between free radical production
311 and antioxidant defense mechanisms, has been implicated in the pathogenesis of several inflammatory
312 and immune-mediated disorders, including thyroid ADs, and the role of antioxidants is intensely
313 debated (29, 38-42). Excess ROS production due to environmental agents could induce modification
314 of tissue proteins or may dysregulate the immune system, influencing the onset of an AD. Moreover,
315 excess ROS increases the pro-inflammatory state and leads to tissue damage, further contributing to
316 the progression of ADs (43). In the current cohort of euthyroid HT subjects, measured oxidants were
317 increased and antioxidants decreased, confirming redox dysregulation in HT subjects compared to
318 controls. Enhanced oxidative stress seems to be related to chronic autoimmune inflammation rather
319 than variations of thyroid hormone levels despite the fact that a slightly intracellular decrease of

thyroid hormones cannot be ruled out in such subjects. The dietary habits of the subjects seemed to influence the redox balance independent from thyroid autoimmunity and function. Indeed, in all subjects, the intake of animal foods, mostly meat, significantly increased levels of oxidants and significantly lowered levels of antioxidants.

A major strength of the present study was the collection of nutritional data in a group which was homogeneous for ethnicity, stable residence, stable dietary habits, and normal anthropometric and metabolic parameters. Another key strength was all recruited subjects had well-characterized thyroid profiles, with thyroid autoantibodies and hormones as well as oxidative stress parameters measured to investigate possible pathogenetic links between nutrition and thyroid autoimmunity. Major limitations of the present study were the relatively small number of recruited subjects and rather high prevalence of HT in the study group. Despite an involuntary selection bias that cannot be excluded, this finding of a high prevalence of HT subjects was in line with previous studies which reported a higher frequency of HT in the Messina area than in neighboring areas. Moreover, a more relevant increase in HT incidence has been reported in this area in recent decades (44, 45). Consequently, this study group cannot be considered representative of the general population or other populations Moreover, the observational design of the study, which reports descriptive data, does not allow establishment of any causal relationship between imbalanced redox ratios and HT as well as diet.

In conclusion, pending confirmation with a large samples series and other populations, the present study suggests that low intake of animal foods has a potentially protective effect on thyroid autoimmunity as a result of the positive influence of this dietary habit on redox balance and consequent oxidative stress-related disorders. Reducing the intake of animal proteins and fats and increasing that of plant foods may represent a useful lifestyle strategy for reducing the risk for autoimmune thyroid disorders. In particular, a predominantly plant-based Mediterranean diet may represent a healthy food model in the setting of ADs.

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Table 1. Demographic, clinical and biochemical characteristics of the study population*.

	HT PATIENTS (n=81)	CONTROLS (n = 119)	*P*
Sex			
Male	10	17	
Female	71	102	
Age years, median (range)	40 (18-66)	37 (18-65)	0.615
Body weight (kg)	66 (41-73)	63.2 (42-76)	0.955
BMI (kg/m²)[#]	24 (19-30)	23 (19.4-30)	0.875
WHR[¶]	0.83 (0.7-1.0)	0.84 (0.7-1.0)	0.376
Fasting glucose (mg/dL)	86 (68-100)	84 (69-100)	0.199
Basal fasting insulin (μIU/L)	6.5 (1.08-10.7)	6.8 (1.4-10.3)	0.224
HOMA index[§]	1.35 (0.10-2.60)	1.4 (0.2-2.5)	0.746
Total cholesterol (mg/dL)	177.5 (125-233)	173 (130-228)	0.197
LDL cholesterol (mg/dL)	101 (59-140)	106 (60-138)	0.645
HDL cholesterol (mg/dL)	67 (46-119)	60 (39-115)	**0.030**
Triglycerides (mg/dL)	62.5 (39-144)	70 (32-150)	0.080
TSH (mIU/L)	2.1 (0.8-4.3)	1.8 (0.6-4.0)	**0.006**
FT4 (pml/L)	10.7 (9.0-16.2)	11.6 (9.54-16.3)	**0.0001**
TPOAb (IU/L)	286.8 (40-3890)	Absent	-

[*] Data are expressed as median and range, in parenthesis. Level of significance $P < 0.05$. In bolt statistically significant *P* values. Normal values are specified under Materials and Methods.

[#]The body mass index (BMI) was calculated by dividing the body weight (kg) with the square of height in meters. [¶]WHR, waist hip ratio, calculated by the formula waist circumference (cm)/hip circumference (cm). [§]Insulin resistance was estimated by the homeostatic model assessment index (HOMA). LDL-cholesterol, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol. TSH, thyroid stimulating hormone; FT4, free thyroxine; TPOAb, anti-thyroperoxidase antibodies.

Table 2. Circulating levels of oxidative stress parameters in subjects with Hashimoto's thyroiditis compared with healthy controls.

	OXIDATIVE STRESS MARKERS*						
	AGEs *(AU/g prot)^*	**AOPP** *(µmol eqClT/L)§*	**SOD** *(U/mL)*	**GPx** *(U/mL)*	**TRxR** *(U/mL)*	**GR** *(U/mL)*	**TEAA** *(mM TE)¶*
HT *(n=81)*	154.68 (38.04-363.98)	1.05 (0.73-2.46)	5.18 (3.66-6.25)	0.64 (0.31-0.86)	1.58 (0.59-3.60)	66.92 (27.59-107.35)	1.59 (0.001-1.83)
Controls *(n= 119)*	101.78 (30.52-325.33)	0.95 (0.63-2.02)	4.79 (2.87-8.48)	0.65 (0.31-0.97)	2.08 (0.69-5.13)	69.75 (23.91-127.96)	1.80 (1.28-1.97)
P	**0.0001**	0.162	0.121	**0.020**	**0.023**	0.282	**0.002**

* Data are expressed as median and range, in parenthesis. Comparison was made by the Mann-Whitney test. *P* values typed in bold are significant ($P \leq 0.05$).
HT: Hashimoto's thyroiditis; AGEs, advanced glycationeEnd products; AOPPS, advanced oxidation protein products (AOPPs); SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; TRxR, thioredoxin reductase; TEAA, total plasma antioxidant activity
^AU/g prot: arbitrary units (AU) per gram of protein.
§ µmol eq ClT/L, chloramine T units
¶ mM TE, millimole of Trolox equivalents.

Table 3. Multivariate logistic regression model with stepwise procedure.

	THYROID AUTOANTIBODIES POSITIVITY		
PREDICTORS	**Odds Ratio**	***95% CI***	***P***
Sex	0.859	*0.075 - 1.160*	**0.006**
Age	1.053	*1.016 - 1.092*	**0.005**
BMI	0.842	*0.759 - 0.958*	0.081
PREDIMED score	0.192	*0.074 – 0.500*	**0.001**
Meat	2.748	*1.721 - 4.387*	**0.0001**
Fish	1.219	*0.608 - 2.444*	0.577
Eggs	1.563	*0.845 - 2.891*	0.447
Dairy products	1.462	*1.042 - 2.050*	**0.028**
Fruit and Vegetables	0.322	*0.138 - 0.749*	**0.007**
Cereals	0.351	*0.137 - 0.900*	**0.029**
Legumes	0.446	*0.194 - 1.025*	0.057
Olive oil	0.455	*0.759 - 7.732*	0.060

*Level of significance $P < 0.05$. In bolt statistically significant *P* values.

CI: confidence interval; BMI, body mass index; PREDIMED score was calculated as specified under Materials and Methods to assess adherence to the Mediterranean diet.

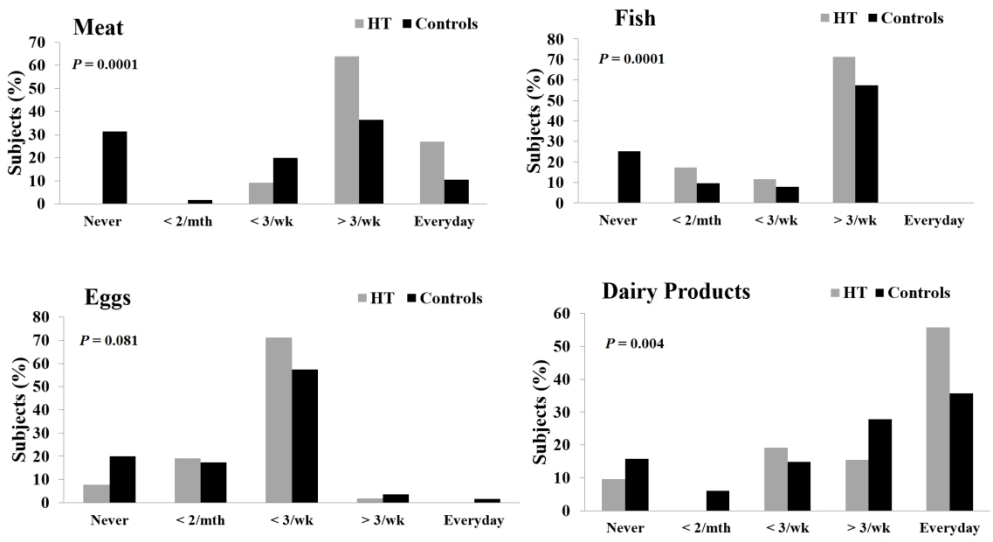
Table 4. Multivariate linear regression models with stepwise procedure.

	OXIDANTS		ANTIOXIDANTS				
	AGEs	**AOPPs**	**SOD**	**GPx**	**TRxR**	**GR**	**TEAA**
	B (*SE*) *P*	***B (SE) P***	**B (*SE*) *P***	**B (*SE*) *P***	**B (*SE*) *P***	**B (*SE*) *P***	**B (*SE*) *P***
Anthropometric parameters							
Sex	32.198 (*23.886*) *P* 0.181	0.117 (*0.138*) *P* 0.365	-0.132 (*0.258*) *P* 0.609	-0.003 (*0.030*) *P* 0.065	0.292 (*0.242*) *P* 0.230	3.834 (*5.817*) *P* 0.506	0.016 (*0.050*) *P* 0.750
Age	1.290 (*0.551*) ***P* 0.021**	0.007 (*0.003*) ***P* 0.012**	0.017 (*0.007*) ***P* 0.015**	-0.002 (0.001) ***P* 0.015**	0.004 (*0.007*) *P* 0.619	-0.477 (*0.173*) ***P* 0.008**	0.000 (*0.001*) *P* 0.937
BMI	0.780 (*1.578*) *P* 0.622	0.022 (0.009) *P* 0.014	0.015 (*0.019*) *P* 0.100	0.001 (*0.002*) *P* 0.626	-0.001 (*0.018*) *P* 0.940	-0.324 (*0.487*) *P* 0.531	0.001 (*0.004*) *P* 0.767
Metabolic parameters and thyroid profile							
HOMA	13.386 (*9.227*) *P* 0.151	0.022 (*0.022*) *P* 0.665	0.018 (*0.102*) *P* 0.862	-0.007 (0.12) *P* 0.556	-0.114 (*0.095*) *P* 0.233	-4.305 (*2.282*) *P* **0.002**	-0.001 (*0.019*) *P* 0.972
HDL-C	-0.933 (*0.590*) *P* 0.117	-0.002 (*0.003*) *P 0.579*	-0.001 (*0.007*) *P* 0.924	-0.001 (*0.001*) *P* 0.385	0.006 (*0.007*) *P* 0.389	0.130 (*0.155*) *P* 0.401	-0.001 (*0.001*) *P* 0.663
TG	0.065 (*0.374*) *P* 0.863	0.000 (*0.002*) *P* 0.935	-0.002 (*0.003*) *P* 0.447	0.000 (*0.000*) *P* 0.365	0.002 (*0.003*) *P* 0.520	-0.022 (*0.074*) *P* 0.773	0.005 (*0.001*) *P* 0.962
TSH	0.554 (*8.068*) *P* 0.945	0.090 (*0.035*) ***P* 0.012**	-0.033 (*0.98*) *P* 0.737	-0.011 (*0.013*) *P* 0.361	0.089 (*0.093*) *P* 0.336	-3.947 (*2.390*) *P* 0.102	-0.014 (*0.019*) *P* 0.449
FT4	0.496 (*3.327*) *P* 0.882	-0.019 (*0.018*) *P* 0.298	-0.031 (*0.040*) *P* 0.446	-0.002 (0.005) *P* 0.655	-0.017 (*0.038*) *P* 0.665	-1.389 (*0.879*) *P* 0.117	-0.008 (*0.008*) *P* 0.281
TPO-Ab	40.994 (*13.689*) ***P* 0.004**	0.024 (*0.097*) *P* 0.804	0.059 (*0.209*) *P* 0.776	-0.032 (0.025) ***P* 0.020**	-0.326 (*0.162*) ***P* 0.046**	-1.223 (4.793) *P* 0.799	-0.031 (*0.099*) *P* 0.429
Lifestyle and nutritional parameters							
PA	8.451 (*8.716*) *P* 0.335	0.015 (*0.030*) *P* 0.614	-0.031 (*0.092*) *P* 0.735	0.009 (*0.011*) *P* 0.456	-0.128 (*0.088*) *P* 0.149	2.110 (*2.281*) *P* 0.357	0.000 (*0.018*) *P* 0.982
PREDIMED score	-20.105 (*13.689*) *P* 0.141	-0.030 (*0.073*) *P* 0.680	0.033 (*0.169*) *P* 0.843	0.010 (*0.021*) *P* 0.616	0.099 (*0.0158*) *P* 0.532	1.835 (*3.896*) *P* 0.639	0.007 (*0.032*) *P* 0.817
Meat	6.680 (*3.727*) ***P* 0.045**	0.015 (*0.018*) ***P* 0.048**	-0.075 (*0.075*) *P* 0.325	-0.015 (*0.018*) ***P* 0.047**	-0.0143 (*0.053*) ***P* 0.007**	-0.031 (*0.012*) ***P* 0.010**	-0.004 (*0.014*) *P* 0.778
Fish	10.664 (*7.101*) *P* 0.135	-0.015 (*0.072*) *P* 0.684	0.099 (*0.086*) ***P* 0.020**	-0.011 (*0.014*) *P* 0.411	-0.105 (*0.107*) *P* 0.238	-3.092 (*2.684*) *P* 0.252	-0.022 (*0.022*) *P* 0.325

Eggs	8.997 (*6.495*) *P* 0.168	0.003 (*0.034*) *P* 0.938	-0.064 (*0.107*) *P* 0.549	-0.031 (*0.012*) ***P* 0.010**	0.013 (*0.103*) *P* 0.900	-0.834 (*2.610*) *P* 0.750	-0.027 (*0.021*) *P* 0.193
Dairy products	6.003 (*4.006*) *P* 0.136	0.003 (*0.021*) *P* 0.904	0.017 (*0.063*) *P* 0.791	-0.001 (*0.008*) *P* 0.881	0.092 (*0.061*) *P* 0.132	-2.549 (*1.304*) ***P* 0.048**	-0.024(*0.011*) ***P* 0.027**
Fruit and Vegetables	-0.690 (*8.012*) *P* 0.931	-0.015 (*0.043*) *P* 0.729	0.065 *(0.122*) *P* 0.597	0.008 (*0.016*) *P* 0.613	0.018 (*0.128*) *P* 0.886	0.495 (*3.215*) *P* 0.010	0.012 (*0.026*) *P* 0.628
Cereals	-14.284 (*11.809*) *P* 0.228	-0.032 (*0.061*) *P* 0.600	0.263 (*0.207*) *P* 0.207	0.016 (*0.026*) *P* 0.056	0.162 (*0.102*) *P* 0.444	0.271 (*4.925*) *P* 0.9560	0.002 (*0.042*) *P* 0.960
Legumes	-4.490 (*7.746*) *P* 0.563	0.027 (*0.040*) *P* 0.508	0.114 (*0.124*) *P* 0.359	0.021 (*0.015*) *P* 0.167	0.094 (*.117*) *P* 0.423	3.247 (*2.858*) *P* 0.259	0.0167 (*0.024*) *P* 0.511
Olive oil	-12.984 (*11.764*) *P* 0.228	-0.049 (*0.061*) *P* 0.425	0.136 (*0.164*) *P* 0.406	0.000 (*0.022*) *P* 0.0987	0.074 (*.162*) *P* 0.649	0.700 (*4088*) *P* 0.864	0.005 (*0.034*) *P* 0.881

*Level of significance $P < 0.05$. SE: standard error. In bolt statistically significant *P* values.

BMI, body mass index;; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; HOMA, homeostatic model assessment index for insulin resistance. PA: physical activity. PREDIMED score was calculated as specified under Materials and Methods to assess adherence to the Mediterranean diet. AGEs, advanced glycation end products; AOPPS, advanced oxidation protein products (AOPPs); SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; TRxR, thioredoxin reductase; TEAA, total plasma antioxidant activity



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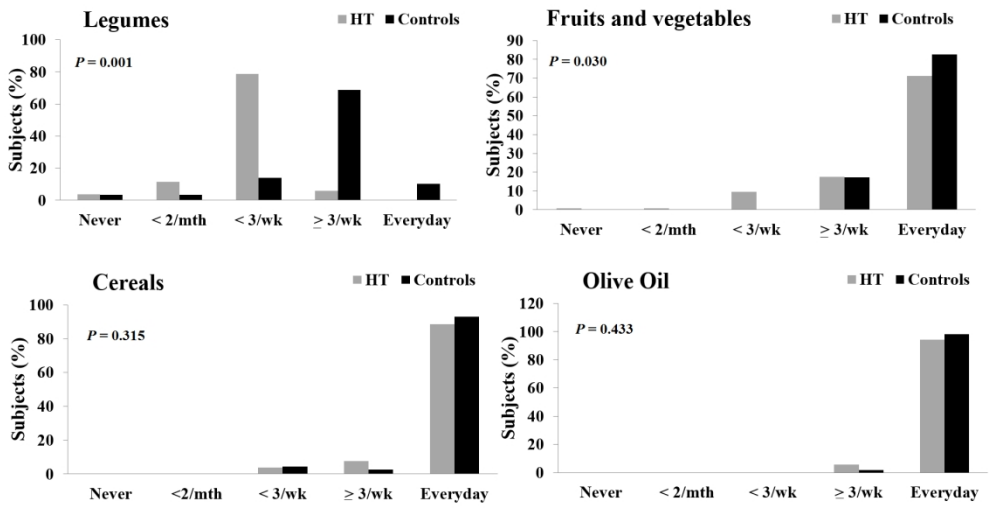

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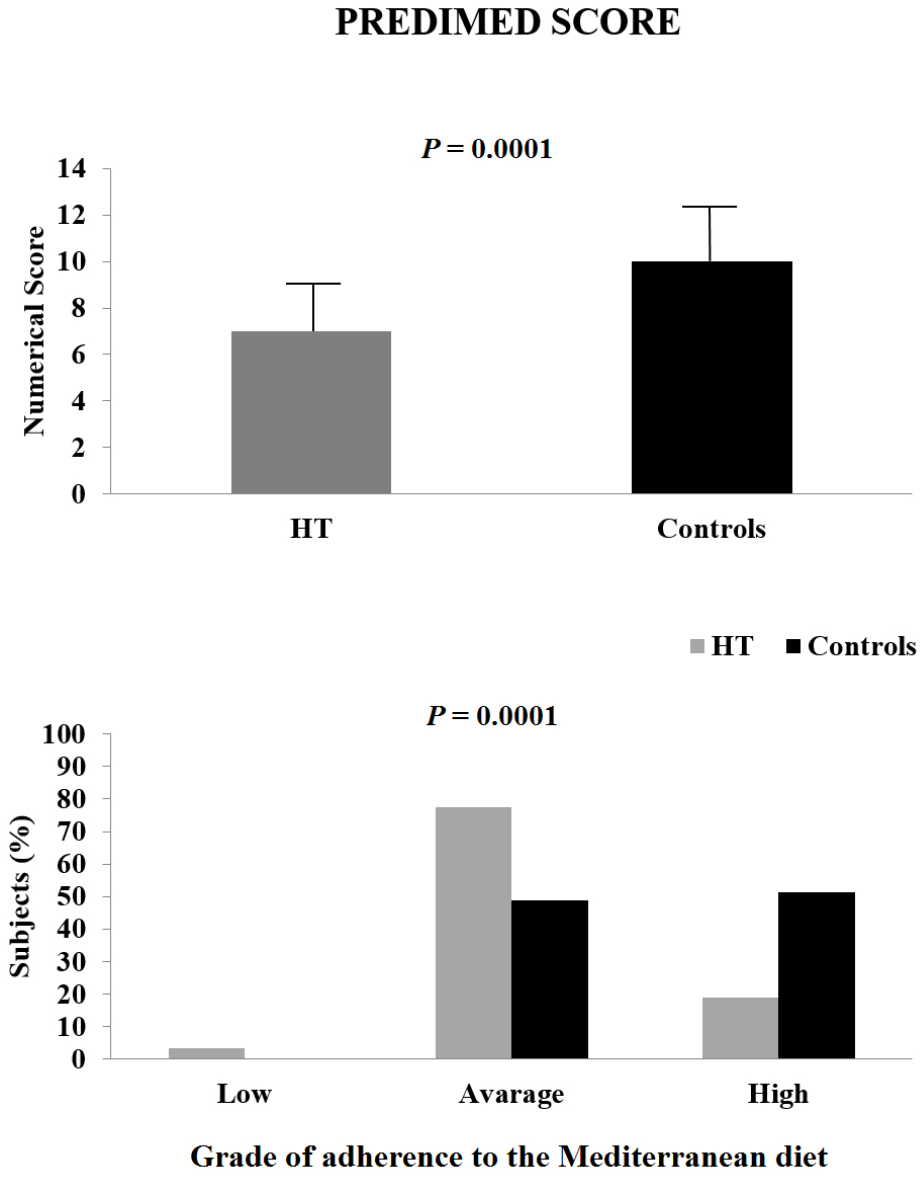

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FIGURES LEGEND.

Figure 1. Intake frequencies of animal foods in Hashimoto's thyroiditis (HT) subjects and healthy controls, as reported in food frequency questionnaires. HT subjects reported higher intake frequencies of animal products, including meat, fish and dairy products, compared to controls,

Figure 2. Intake frequencies of plant foods in Hashimoto's thyroiditis (HT) subjects and healthy controls, as reported in food frequency questionnaires. HT subjects reported lower intake frequencies of legumes, fruits and vegetables compared to controls. The two groups did not differ concerning olive oil and cereals consumption.

Figure 3. Adherence to the Mediterranean diet in our cohort, as evaluated by a validated 14-items questionnaire (PREDIMED score). The PREDIMED score was significantly lower in Hashimoto's thyroiditis (HT) subjects compared to healthy controls (top). Accordingly, HT subjects exhibited a significantly lower grade of adherence to Mediterranean diet than controls, calculated as follows: a score of 0–5 meant low adherence, 6–9 represented average adherence, and ≥10 equated to high adherence (bottom).

SUPPLEMENTARY MATERIALS

Assays for oxidants and antioxidants

Advanced glycation end products (AGEs) and advanced oxidation protein products (AOPPs), markers of oxidative stress, were measured in serum samples from each subject. Determination of AGEs was based on spectrofluorimetric detection as previously reported (29). Briefly, blood serum was diluted 1:50 with phosphate-buffered saline (PBS) (pH 7.4), and fluorescence intensity was recorded an excitation/emission of 350/440 nm by spectrofluorimeter (Shimadzu, Japan). The serum concentration of AGEs was normalized to the total protein amount determined by Bradford assay and expressed in arbitrary units (AU) per gram of protein. Determination of AOPPs was based on spectrophotometric detection as previously described (29). Blood serum (100 μL) or the same volume of chloramine-T (0–100 μmol/L) for calibration were diluted 1:5 with PBS (pH 7.4). Subsequently, 25 μL of 1.16 M KI and 50 μL of acetic acid were added to the diluted solutions and the absorbance measured immediately at 340 nm by spectrophotometer (Shimadzu, Japan). The concentration of AOPP is expressed as μmol equivalents of chloramine-T per liter.

The activity of antioxidant enzymes [superoxide dismutase (SOD), glutathione reductase (GR), glutathione peroxidase (GPx), and thioredoxin reductase (TRxR)], as well as the total plasma antioxidant activity, was measured on plasma samples from each subject. SOD activity was measured using an SOD assay kit (Sigma-Aldrich, Milan, Italy) according to the manufacturer's protocol. The kit allows for convenient SOD assessment using a highly water-soluble tetrazolium salt, WST-1, which produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction is linearly related to the xanthine oxidase activity and inhibited by SOD. Thus, the SOD activity can be determined colorimetrically at 450 nm using a microplate spectrophotometer (VICTOR3 V Multilabel Counter; PerkinElmer, Wellesley, MA, USA). Values obtained for each sample were compared to the concentration-response curve of standard SOD solutions and expressed as U/mL. One unit of enzyme activity is defined as the amount of enzyme

that inhibits the reduction of WST-1 by 50% in a coupled system with xanthine oxidase at pH 7.8 and 37 °C.

GR activity was assessed adapting a previously reported method (30). Briefly, 30 µL of plasma was added to 970 µL of reaction mix [100 mM phosphate buffer (pH 7.5) containing 1 mM EDTA, 2 mM NADPH, 3 mM 5,5-dithiobis(2-nitrobenzoic acid (DTNB), and 2 mM Glutathione Oxidized, Disodium salt (GSSG). The decrease in absorbance at 412 nm was monitored spectrophotometrically for 1 min at 25 °C. GR activity was expressed as mU/mL. One unit of enzyme activity is defined as the amount of enzyme that causes the reduction of 1.0 µmol of DTNB to 5'-thionitrobenzoic acid (TNB) per minute at 25 °C and pH 7.5. GPx activity was measured as previously described (31). Briefly, the reduction of GSSG coupled with the oxidation of NADPH, causing a decrease in absorbance at 340 nm, was spectrophotometrically monitored at 25 °C. GPx activity was expressed as U/mL. One unit of GPx activity was defined as the amount of enzyme that catalyzes the reduction of 1 µmol NADPH/min.

TRxR activity was assayed as previously reported with some adaptation (30). Plasma samples were mixed with reaction buffer containing 0.25 mM DTNB, 0.24 mM NADPH, 10 mM EDTA, and 100 mM phosphate buffer (pH 7.5). As different enzymes can reduce DTNB, a specific TRxR inhibitor was used to determine the reduction of DTNB due only to TRxR activity. The conversion of DTNB to TNB was measured spectrophotometrically at 412 nm at 10-s intervals over 1 min. TRxR activity is expressed as U/mL. One unit of TRxR causes an increase in absorbance at 412 nm of 1.0 /min/mL (when measured in a noncoupled assay containing DTNB alone) at pH 7.0 and 25 °C. Total plasma antioxidant activity was assessed as previously described (31). The antioxidant potential of the sample has been evaluated as its ability to reduce the radical cation of ABTS+ (ABTS•+ (2,20 -azino-bis(3-ethylbenzothiazoline-6- sulfonic acid) by decolorization and measured as quenching of absorbance at 740 nm. The values from each sample were compared to the concentration-response curve of a standard Trolox solution and expressed as mmol of Trolox equivalents.