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Influence of Dietary Habits on Oxidative Stress Markers in Hashimoto's Thyroiditis

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# Thyroid

## INFLUENCE OF DIETARY HABITS ON OXIDATIVE STRESS MARKERS IN HASHIMOTO'S THYROIDITIS

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| Abstract:                           | <p>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.</p> <p>Materials and Methods. Two hundred subjects (173 females and 27 males; median age, 37 years) were enrolled. None were under any</p>                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                                               |

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|  | <p>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.</p> <p>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, <math>P = 0.0001</math>; fish, <math>P = 0.0001</math>; dairy products, <math>P = 0.004</math>) compared to controls, who reported higher intake frequencies of plant foods (legumes, <math>P = 0.001</math>; fruits and vegetables, <math>P = 0.030</math>; nuts, <math>P = 0.0005</math>). The number of subjects who preferentially consumed poultry instead of red/processed meat was lower in HT subjects than in controls (<math>P = 0.0141</math>). 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 (<math>P = 0.0001</math>) than controls, while the activity of glutathione peroxidase and thioredoxin reductase, as well as total plasma antioxidant activity, were lower (<math>P = 0.020</math>, <math>P = 0.023</math>, and <math>P = 0.002</math>, 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.</p> <p>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.</p> |
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1 **INFLUENCE OF DIETARY HABITS ON OXIDATIVE STRESS MARKERS IN**  
 2 **HASHIMOTO'S THYROIDITIS**

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37  
 38 **Running title:** Diet and oxidative stress in Hashimoto's thyroiditis

39  
 40 **Keywords:** Hashimoto's thyroiditis – Diet - Oxidative stress – Vegetarianism – Thyroid  
 41 autoimmunity – Antioxidants – Mediterranean diet.

42 **ABSTRACT**

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

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

53 **Results.** Among the 200 recruited subjects, 81 (71 females and 10 males) were diagnosed with  
54 euthyroid Hashimoto's thyroiditis (HT); the remaining 119 (102 females and 17 males) served as  
55 controls. In questionnaires, HT subjects reported higher intake frequencies of animal foods (meat,  $P$   
56 = 0.0001; fish,  $P$  = 0.0001; dairy products,  $P$  = 0.004) compared to controls, who reported higher  
57 intake frequencies of plant foods (legumes,  $P$  = 0.001; fruits and vegetables,  $P$  = 0.030; nuts,  $P$  =  
58 0.0005). The number of subjects who preferentially consumed poultry instead of red/processed meat  
59 was lower in HT subjects than in controls ( $P$  = 0.0141). In logistic regression analysis, meat  
60 consumption was associated with increased odds ratio of developing thyroid autoimmunity, whilst  
61 Mediterranean diet traits were protective. In HT subjects, serum advanced glycation end products  
62 (markers of oxidative stress) were significantly higher ( $P$  = 0.0001) than controls, while the activity  
63 of glutathione peroxidase and thioredoxin reductase, as well as total plasma antioxidant activity, were  
64 lower ( $P$  = 0.020,  $P$  = 0.023, and  $P$  = 0.002, respectively), indicating a condition of oxidative stress.  
65 Stepwise regression models demonstrated a significant dependence of oxidative stress parameters on  
66 consumption of animal foods, mainly meat.

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

## 70 INTRODUCTION

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

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

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

102

## 103 MATERIALS AND METHODS

### 104 Subjects

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

117 A total of 200 healthy subjects (173 female and 27 male; median age, 37 years), who agreed to thyroid  
118 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  $\geq 10$  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  $1450 \times g$  at  $4^\circ\text{C}$  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

143 pmol/L FT4, and 0–10 IU/mL TPOAb. For all assays, the intra- or inter-assay CV was <5% and  
144 <10%, respectively.

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

## 152 Statistical analysis

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

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

171

## 172 RESULTS

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

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

193 In questionnaires, HT subjects reported higher intake frequencies of animal foods (meat,  $P = 0.0001$ ;  
194 fish,  $P = 0.0001$ ; dairy products,  $P = 0.004$ ) compared to controls, who, in turn, reported higher  
195 intake frequencies of plant foods, including legumes ( $P = 0.001$ ) and fresh fruits and vegetables ( $P =$   
196  $0.030$ ) (**Figures 1 and 2**). The two groups mainly differed regarding consumption of meat, as HT  
197 subjects reported higher intake frequencies of animal meat in general, specifically red/processed  
198 meat, compared to controls. Indeed, the number of subjects who reported preferential consumption  
199 of white meat and poultry instead of red/processed meat was significantly lower in the HT group than  
200 in the control (29% versus 52%;  $P = 0.014$ ). None of the subjects who did not eat meat at all or no  
201 more than twice a month were found to have thyroid autoantibodies. Moreover, consumption of other  
202 animal foods, like fish and dairy products, was higher in HT individuals than controls, but no  
203 difference in egg consumption was observed ( $P = 0.081$ ). Finally, the number of subjects who  
204 reported consumption of  $\geq 3$  servings per week of nuts was significantly lower in HT subjects  
205 compared to controls (23% versus 55%;  $P = 0.0005$ ).

206 HT and control groups did not differ concerning intake of cereals (88.5% versus 93%,  $P = 0.315$ ) and  
207 whole-grain (51% versus 58%,  $P = 0.563$ ). Furthermore, most subjects reported use of olive oil as  
208 main culinary fat (at least 4 tablespoons daily), without difference between HT and control subjects  
209 (94% versus 98%,  $P = 0.433$ ). Similarly, most subjects in both groups did not use butter or cream at  
210 all, while less than one-third of subjects consumed butter, margarine, or cream  $< 3$  d/week, without  
211 differences between the groups ( $P = 0.733$ ). Finally, HT subjects reported a higher frequency of  
212 consumption of fats and refined sugars from commercial sweets ( $P = 0.010$ ) despite no differences in  
213 the consumption of sweetened or carbonated beverages (all  $< 1$  per day). Moreover, control and HT  
214 subjects did not differ concerning weekly consumption of wine or general alcohol intake ( $P > 0.05$ ).

215 Finally, there was no difference in the use of iodized salt between HT subjects and controls, but  
216 individual urinary iodine concentrations were not available.

217 Concerning lifestyle, most of the subjects reported doing physical activity no more than twice a week  
218 (70% of HT subjects versus 60% of controls), without significant differences between subjects with

219 or without HT ( $P = 0.176$ ). Current or past smokers were excluded to avoid biases. The largest part  
220 of the cohort presented a medium-high grade of adherence to the Mediterranean diet according to  
221 PREDIMED scores. However, HT subjects displayed significantly lower scores than controls ( $P =$   
222  $0.0001$ ; **Figure 3**). The main determinants of this difference were the higher consumption of animal  
223 meat in general, red/processed meat in particular, the lower consumption of vegetables, fruits, and  
224 legumes, the higher consumption of commercial sweets or pastries, and the lower consumption of  
225 nuts in HT subjects compared to controls.

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

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

242

243 DISCUSSION

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

255 The association between diet and risk of developing ADs was proposed as early as 50 years ago by  
256 Trowell, who observed that a number of ADs, including RA, type 1 diabetes, and HT, were extremely  
257 rare among isolated rural sub-Saharan populations following traditional near-vegan diets (32, 33). A  
258 similar low incidence of ADs was reported in Asian societies whose diets were almost vegan (33). In  
259 the last few decades, further evidence has accumulated on the influence of nutritional factors in the  
260 development of several ADs, including RA, celiac and inflammatory bowel diseases, type 1 diabetes,  
261 multiple sclerosis, and psoriasis (13, 17-23). To date, only two studies have assessed the dietary habits  
262 of subjects suffering from thyroid diseases. In 2013, Tonstad *et al.*, using data from the Adventist  
263 Health Study-2, evaluated the prevalence and incidence of hypothyroidism among a large cohort ( $n$   
264 = 65,981) of Seventh-day Adventist church members who exhibited a wide range of diets from vegan  
265 to omnivorous, with a high proportion of vegetarians (24). They found a lower, though not significant,  
266 prevalence and incidence of hypothyroidism among subjects following vegan diets compared to  
267 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  $\omega$ -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



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

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

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

344

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

|                                       | <b>HT PATIENTS<br/>(n=81)</b> | <b>CONTROLS<br/>(n = 119)</b> | <b><i>P</i></b> |
|---------------------------------------|-------------------------------|-------------------------------|-----------------|
| 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/m <sup>2</sup> ) <sup>#</sup> | 24 (19-30)                    | 23 (19.4-30)                  | 0.875           |
| WHR <sup>†</sup>                      | 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 <sup>§</sup>               | 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)                   | <b>0.030</b>    |
| 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)                 | <b>0.006</b>    |
| FT4 (pml/L)                           | 10.7 (9.0-16.2)               | 11.6 (9.54-16.3)              | <b>0.0001</b>   |
| TPOAb (IU/L)                          | 286.8 (40-3890)               | Absent                        | -               |

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

<sup>#</sup>The body mass index (BMI) was calculated by dividing the body weight (kg) with the square of height in meters. <sup>†</sup>WHR, waist hip ratio, calculated by the formula waist circumference (cm)/hip circumference (cm). <sup>§</sup>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.

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

\* 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 glycationEnd products; AOPPS, advanced oxidation protein products (AOPPs); SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; TRxR, thioredoxin reductase; TEAA, total plasma antioxidant activity

<sup>^</sup>AU/g prot: arbitrary units (AU) per gram of protein.

<sup>§</sup> $\mu$ mol eq CIT/L, chloramine T units

<sup>¶</sup>mM TE, millimole of Trolox equivalents.

**Table 3.** Multivariate logistic regression model with stepwise procedure.

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

\*Level of significance  $P < 0.05$ . In bold 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.

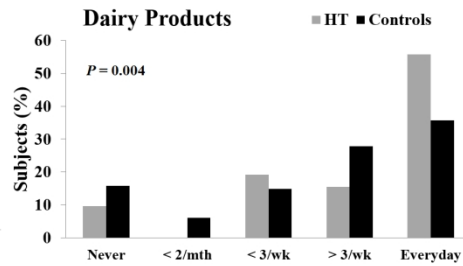
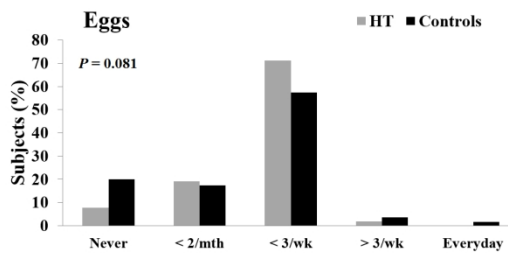
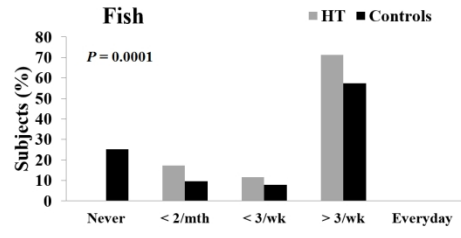
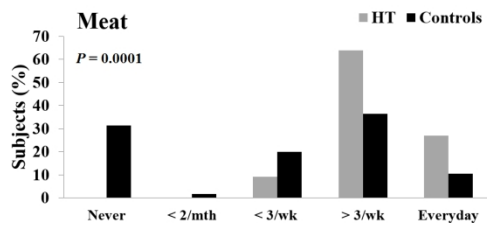
Table 4. Multivariate linear regression models with stepwise procedure.

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

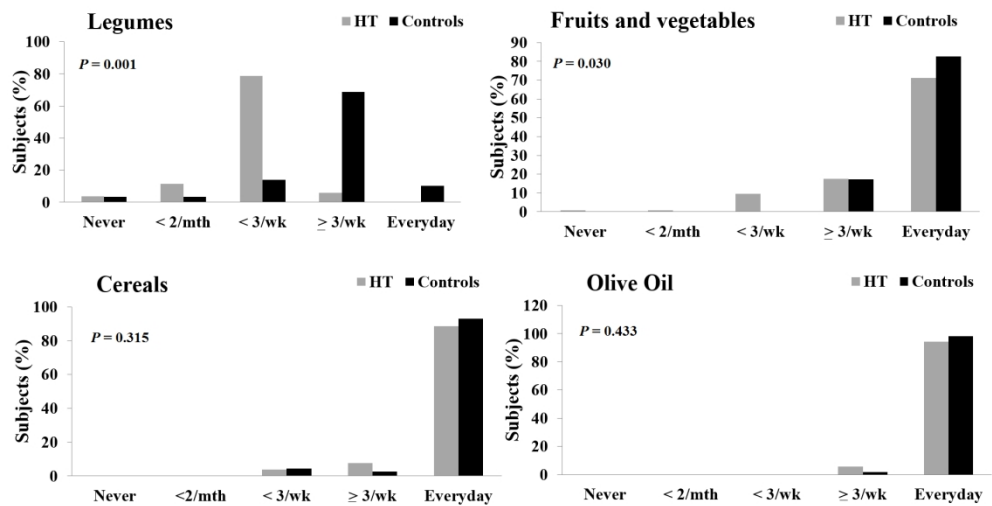
|                             |                                    |                                  |                                  |                                         |                                 |                                         |                                        |
|-----------------------------|-----------------------------------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

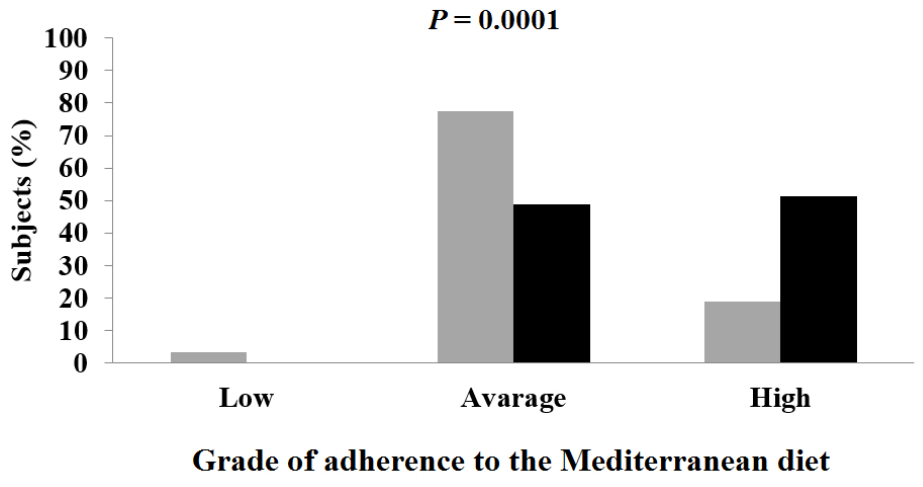
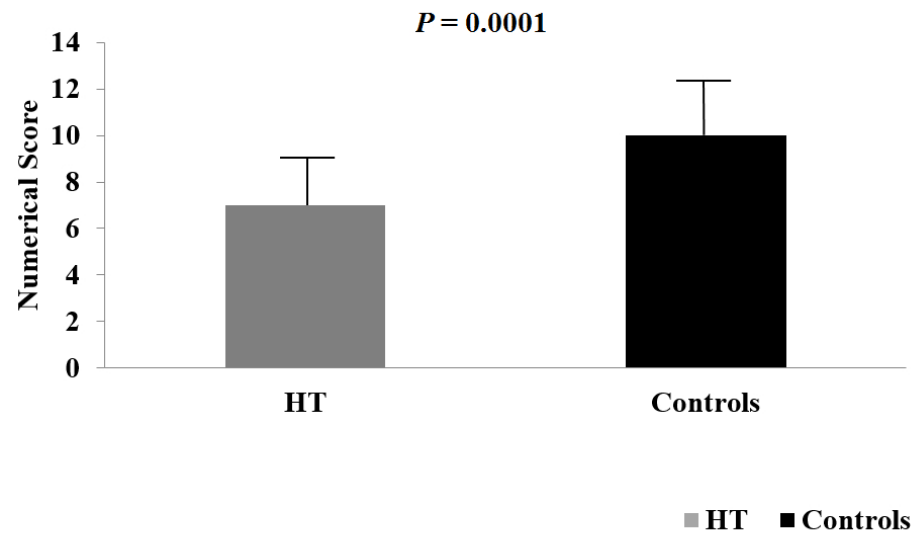


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529x288mm (96 x 96 DPI)

### PREDIMED SCORE



**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  $\geq 10$  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  $\mu$ L) or the same volume of chloramine-T (0–100  $\mu$ mol/L) for calibration were diluted 1:5 with PBS (pH 7.4). Subsequently, 25  $\mu$ L of 1.16 M KI and 50  $\mu$ L 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  $\mu$ mol 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  $\mu$ L of plasma was added to 970  $\mu$ L 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  $\mu$ mol 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  $\mu$ mol 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<sup>•+</sup> (ABTS<sup>•+</sup> (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.