

Favorable effects of myo-inositol, selenomethionine or their combination on the hydrogen peroxide-induced oxidative stress of peripheral mononuclear cells from patients with Hashimoto's thyroiditis: preliminary *in vitro* studies

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Abstract. – OBJECTIVE: The aim of this study was to assess whether blood mononuclear cells (PBMC) from Hashimoto's thyroiditis (HT) and control women, were protected from *in vitro* H₂O₂-induced oxidative stress after addition of antioxidants.

PATIENTS AND METHODS: PBMC, from 8 HT women and 3 healthy women (controls), were cultured in the presence of 200 μM H₂O₂ alone, with subsequent addition of myo-inositol (Myo) (0.25, 0.5, 1.0 μM), selenomethionine (SelMet) (0.25, 0.5, 1.0 μM), or their combination (0.25+0.25, 0.5+0.5, 1.0+1.0 μM). PBMC proliferation, vitality, genotoxicity (Comet score) and secretion in the medium of the chemokines CXCL10 [IP10], CCL2 e CXCL9 [MIG] were the indices measured.

RESULTS: PBMC proliferation was decreased by H₂O₂ alone, and it decreased further and dose-dependently in either group (greatest decrease with Myo+SelMet in HT). H₂O₂ alone decreased vitality by 5% in controls and 10% in the HT group, but vitality was rescued by the three additions. The addition of H₂O₂ alone increased the Comet score at +505% above baseline in controls and +707% in HT women. In either group, each addition dose-dependently contrasted genotoxicity. Concentrations of chemokines in the medium were increased by H₂O₂ alone, and in HT women more than in controls. Each addition dose-dependently decreased these concentrations in either group, and often below baseline levels, with Myo+SelMet being the most potent addition (up to approximately -80% of baseline).

CONCLUSIONS: The tested antioxidants exert beneficial effects on PBMC exposed *in vitro* to H₂O₂-induced oxidative stress in both control and HT women. Particularly, the association Myo+SelMet is the most effective. After the demonstration of a favorable *in vitro* outcomes in a large cohort of HT patients, we could predict favorable *in vivo* outcomes given by the same supplement. Thus, one can select HT patients with a high chance of benefit from supplementation.

Key Words

Myo-inositol, Selenomethionine, Hashimoto's thyroiditis, Oxidative stress, Chemokines.

Introduction

Primary hypothyroidism is a common disorder, with a prevalence of approximately 5% and incidence of approximately 250/100,000 per year in the adult population, but both prevalence and incidence keep raising^{1,2}. The subclinical form of primary hypothyroidism (also known as initial or mild hypothyroidism) is much more frequent than the overt form. The leading cause of primary hypothyroidism is Hashimoto's thyroiditis (HT), the annual frequency of which has increased markedly starting from the early/mid 90's³⁻⁵.

Enhanced oxidative stress was documented in autoimmune thyroid disease (AITD)⁶⁻⁹. Over-

production and/or decreased disposal of hydrogen peroxide (H_2O_2), a reactive oxygen species, is involved in the pathogenesis of the inflammation of AITD and AITD-associated apoptosis of thyrocytes^{10,11}. Indeed, H_2O_2 participates in the regulation of multiple inflammatory pathways¹², and culturing cells in the presence of H_2O_2 is a practical way to induce oxidative stress in several experimental settings. Illustratively, such cultured human or animal cells are as diverse as thyrocytes¹¹, cardiomyocytes¹³, neurons¹⁴, glia cells^{15,16}, gingival fibroblasts¹⁷, pancreatic beta cells^{18,19}, myoblasts²⁰, retinal pigment epithelium²¹ stem cells²², peripheral blood mononuclear cells (PBMC)^{9,23,24}, and even embryos²⁵.

Among other aspects, including environmental factors that trigger intrathyroid oxidative stress²⁶, AITD is characterized by intrathyroid lymphocytic infiltration and overproduction of cytokines by lymphocytes and thyrocytes, including the small chemotactic cytokines (chemokines), the secretion of which is often induced by pro-inflammatory cytokines themselves²⁷.

CCL2 is the prototype Th2 chemokine, that is produced by lymphocytes and other cells^{28,29}. The interferon gamma ($IFN-\gamma$)-inducible protein 10 (IP-10, also called CXCL10), and monokine induced by $IFN-\gamma$ (MIG/CXCL9) were first recognized as a chemokine $IFN-\gamma$ -induced³⁰⁻³². CXCL10 and CXCL9 bind to chemokine (C-X-C motif) receptor 3 (CXCR3), contributing to the pathogenesis of various organ-specific autoimmune diseases (i.e. Graves' disease [GD] and ophthalmopathy, type 1 diabetes mellitus), or systemic autoimmune diseases, (i.e. mixed cryoglobulinemia, systemic lupus erythematosus, Sjogren syndrome or systemic sclerosis)^{33,34,35,36,37}. The secretion of CXCL9 and CXCL10 by CD4+, CD8+, and natural killer (NK) depends on $IFN-\gamma$. Stimulated by $IFN-\gamma$, CXCL10 is secreted by thyrocytes or other cell types^{35,36}. Hence, high CXCL9, CXCL10 levels in peripheral fluids are a marker of a T helper (Th)1 orientated immune response³⁷. Serum levels of both CXCL9 and CXCL10 in patients with HT are significantly greater than in patients with non-autoimmune nodular goiter or healthy controls³⁸. In HT patients, serum CXCL9 levels were significantly higher in those with a hypoechoic pattern, and in those with hypothyroidism. In the same HT patients, CXCL10 was significantly higher in those with a hypoechoic pattern ($p = 0.008$), and in those with hypothyroidism³⁸. CXCL9 and CXCL10 serum levels were significantly related to each other ($r = 0.719$, $p < 0.001$) in HT patients³⁸.

A nutraceutical approach for the medical management of AITD with selenium³⁹⁻⁴², selenium plus myo-inositol (Myo)⁴³ or L-carnitine^{44,45} has appeared in the literature. A common denominator among these substances is antioxidant activity^{39,46-48}. In studies on HT patients supplemented with selenium (most often at 200 $\mu\text{g}/\text{d}$) for 3 to 12 months, the outcome has been the decline in thyroperoxidase autoantibodies (TPOAb)³⁹. Supplementation with selenomethionine (SelMet) performed better than supplementation with sodium selenite. As described below under Discussion, beneficial effects on indices of thyroid autoimmunity and thyroid function were obtained in TPOAb positive women treated with SelMet alone or SelMet plus Myo^{41,43}.

Based on all this knowledge, the aim of the pilot study reported here was to check whether experiments on PBMC from euthyroid HT patients that were exposed, *in vitro*, to 200 μM H_2O_2 would have provided evidence for protection conferred by Myo, SelMet or their association (Myo+SelMet). The main interest was for a reduction of CXCL10 (IP10), CCL2 e CXCL9 (MIG).

Patients and Methods

To avoid confounding variables and appreciate the effect of thyroid autoimmunity *per se*, upon informed written consent we enrolled a group of 8 non-smoking, euthyroid HT women with no personal history of autoimmune diseases other than HT, and under no drug known to alter thyroid function tests and/or to trigger thyroid autoimmunity. However, they had thyroiditis demonstrable by positivity for serum thyroid autoantibodies (TPOAb and thyroglobulin antibodies [TgAb]) and sonographic signs of inflammation. We anticipated that these criteria would have allowed enrollment of a limited number of women.

For the same aim of avoiding confounding variables, to form the control group we aimed at enrolling 3 healthy, non-smoking age-matched euthyroid women with no personal history of any autoimmune disorder, and under no drug known to alter thyroid function tests and/or to trigger thyroid autoimmunity. Before enrollment and upon informed written consent, thyroid sonography demonstrated normal thyroid volume and absence of both nodules and sonographic signs of thyroiditis. Again, these criteria allowed enrollment of a limited number of women.

No Ethic Committee approval was required, as the study involved neither *in vivo* administration of compounds nor invasive procedures.

General Outline of the Experiments

Experiments were aimed at stressing peripheral blood mononuclear cells (PBMC) with H_2O_2 and then assess whether, in the presence of H_2O_2 , the addition of equimolar concentrations of Myo alone, SelMet alone or a combination of Myo+SelMet would have protected PBMC from the effects given by H_2O_2 . Myo-inositol (Myo) was obtained by Lo.Li Pharma S.r.l (Rome, Italy) and L-selenomethionine was purchased from Sigma-Aldrich Chemie (Darmstadt, Germany). Both Myo and SelMet were dissolved in sterile phosphate buffered saline (PBS) prior to use.

We used a fixed concentration (200 μ M) of H_2O_2 and three concentrations of each of the above compounds (0.25, 0.5 and 1.0 μ M) based on clear-cut results of dose-dependent inhibition of H_2O_2 -induced genotoxicity on initial experiments. A number of cell culture models have been set up using 200 μ M H_2O_2 ^{14,18-20,22,24,25} or concentrations of H_2O_2 that include 200 μ M^{11,15,21}. Worthy of note, because the molecular weights of SelMet and selenium are 196.1 and 79.0, respectively, 0.25, 0.5 and 1.0 μ M SelMet correspond to 0.1, 0.2 and 0.4 μ M elementary selenium.

The indices (outcomes) evaluated were PBMC proliferation, viability, genotoxicity, secretion into the medium of three chemokines.

Cells and Medium

Blood was collected by venipuncture using tubes containing K-EDTA. Blood samples were processed for PBMC isolation by density gradient centrifugation with Lympholyte[®] cell separation media (Cedarlane, Burlington, Ontario, Canada). Lymphocytes were cultured in RPMI-1640 medium (Euroclone S.p.A., Pero (Milan), Italy), supplemented with 10% fetal calf serum (Euroclone), penicillin (5 μ g/mL), streptomycin (10 μ g/mL) and 1% MEM amino acid solution (Sigma-Aldrich, St. Louis, MO, USA). PBMC were cultured at 37°C with 5% CO_2 in 96 wells microtiter plates for five days. However, a 30 μ L aliquot of the freshly collected blood was used immediately for the genotoxicity experiments using a protocol described elsewhere⁴⁹.

Cell Proliferation and Viability

PBMC, isolated from 8 HT and 3 control women, were plated in 96-well plates at a seeding concentration of 100,000 per well in the absence (baseline) or presence of 200 μ M H_2O_2 . In designated wells, further to 200 μ M H_2O_2 , we added specified concentrations of Myo alone (0.25, 0.5 or 1.0 μ M), SelMet alone (0.25, 0.5 or 1.0 μ M), or

Myo plus SelMet (0.25+0.25, 0.5+0.5 or 1.0+1.0 μ M). PBMC were cultured in the above medium and after 5 days, cells were harvested and assessed for proliferation and viability. Cell culture supernatants were collected for chemokine assays (see below). This scheme of culture and additions was applied for the other experiments described below, except for the Comet assay in which an aliquot of blood sample was used separately.

After harvesting from the wells, PBMC were diluted in PBS, and counted in a Neubauer counting chamber using a 0.5% trypan blue staining solution. As known, nonviable cells internalize the solution and appear blue, whereas viable cells with intact cell membranes, do not internalize the dye. Trypan blue positive and negative cells were counted using a hemocytometer in an optic microscope to estimate the % change in the number of viable cells.

Trypan blue dye exclusion assay was used to assess actual viable cell number, but it lacks sensitivity compared to other cell viability tests. For this reason, PBMC viability was also evaluated by a resazurin-based assay (CellTiter-Blue; Promega, Madison, WI, USA) which uses the indicator dye resazurin to measure the metabolic capacity of cells. Nonviable cells rapidly lose the ability to reduce resazurin and do not generate the fluorescent signal of resorufin (λ_{ex} = 560 nm, λ_{em} = 590 nm), which was measured with a plate reader (SynergyTM HT; Biotek, Winooski, VT, USA). These assays were performed in triplicate.

Data were normalized as percentage of control. Thus, PBMC incubated in the absence of H_2O_2 and any of Myo, SelMet or Myo+SelMet were considered as 100%.

Genotoxicity (Comet Assay)

As known, the Comet assay is a simple and sensitive genotoxicity test for the detection of deoxyribonucleic acid (DNA) damage in eukaryotic cells.

As said above, a 30 μ L aliquot of blood was incubated with 30 μ L Myo, SelMet or their combination at 37 °C for 1h. As stated above, the final concentrations of Myo or SelMet were 0.25, 0.5 and 1.0 μ M; the final concentrations of the combination were 0.25 μ M Myo plus 0.25 μ M SelMet, 0.5 μ M Myo plus 0.5 μ M SelMet, and 1.0 μ M Myo plus 1.0 μ M SelMet. Cell suspensions were then processed for the alkaline version of the Comet assay as described elsewhere⁴⁹, including ensuring protection from light to avoid further DNA damage. Experiments were repeated twice, with each condition (baseline, H_2O_2 , and each of the three concentrations of the three additions) being tested in triplicate.

At the end of the electrophoretic procedure⁴⁹, slides (three per each condition) containing processed PBMC were stained with 5 µg/ml propidium iodide and examined at 100X magnification with an E800 microscope (Nikon, Düsseldorf, Germany) equipped to detect the fluorescence of propidium iodide. One hundred cells were randomly selected in each slide and scored 0 (no damage) to 4 (very significant damage), based on comet-tail length. The final Comet score was calculated by the formula: (n cells scored 1) + (2 x n cells scored 1) + (3 x n cells scored 3) + (4 x n cells scored 4).

Chemokine Assay

CXCL10 (IP-10), CCL2 (MCP-1) and CXCL9 (MIG) levels in cell culture supernatant were assayed by a quantitative sandwich immunoassay kits (R&D Systems, Minneapolis, MN, USA), with a sensitivity range of 0.41-4.46 pg/ml, 0.57-10.0 pg/ml, and 1.37-11.31 pg/ml, respectively. The absorbance was measured at 450 nm, with correction wavelength set at 540 nm, using a plate reader (SynergyTM HT; Biotek, Winooski, VT, USA). Experiments were performed in triplicate. When the assay is performed in cell culture supernatants, the intra-assay coefficients of variations (CV) for CXCL10, CCL2, and CXCL9 are 3.0, 3.6, and 4.2%. The inter-assay CV are 6.9, 7.2, and 4.8%.

Statistical Analysis

Data are reported as mean ± SD and median. Except for vitality and Comet score (genotoxicity), the other variables had nongaussian distribution. Differences between continuous variables were analyzed by the two-sided Wilcoxon rank sum test, except vitality and Comet score (differences analyzed by ANOVA). A p -value <0.05 was considered statistically significant, while a p -value between 0.10 and 0.05 as a borderline significant.

Results

Characteristics of the Healthy Group and HT Group

Pertinent data are shown in Table I. Noteworthy in the HT group (8 women) is that, though serum hormones were within normal limits, FT4 was 9% lower, FT3 5% lower and TSH 39% greater than in the healthy group (3 women). This pattern was strongly suggestive of an initial decline in thyroid function.

Proliferation and Vitality

After 5 days of culture in the absence of H₂O₂ and any addition (baseline), the median number of PBMC per well was 92,000 for the healthy group and 92,500 for the HT group, a physiological but statistically significant decline in either group ($p = 0.008$ and $p = 0.005$, respectively). The corresponding mean ± SD numbers were 92,890 ± 11,973 and 90,600 ± 14,284, which were statistically similar ($p = 0.60$). In the presence of 200 µM H₂O₂ alone, there was a median decrease of 4.2% (-6.2 ± 5.2%, $p = 0.008$ vs baseline) in the healthy group and 7.8% (-10.4 ± 7.5%, $p = 0.005$ vs baseline) in the HT group (Figure 1). The difference between these two decreases in one group compared with the other was borderline significant ($p = 0.092$).

When the PBMC of healthy women were cultured in the presence of H₂O₂ plus any of the three compounds (Figure 1, left panel), there was a further decline in the number of PBMC, with an evident dose-dependency displayed by Myo. Due to the small standard deviation, the highest concentration of Myo alone was the sole addition that resulted at least borderline significantly lower compared to H₂O₂ alone ($p = 0.07$) and also statistically lower than baseline ($p = 0.04$) (Figure 1). The highest inhibition of proliferation was observed at the two highest concentrations of

Table I. Demographics of the study population*.

Index	Reference range	Hashimoto' thyroiditis (n=8)	Healthy controls (n=3)
Age, years	N/A	43.1 ± 11.3 [40.5] (31-61)	45.3 ± 8.7 [43] (38-55)
TSH, mU/L	0.27-4.2	1.96 ± 0.63 [1.93] (0.89-2.98)	1.41 ± 0.65 [1.30] (0.82-2.1)
FT3, pg/ml	2.0-4.2	2.88 ± 0.21 [2.90] (2.57-3.15)	3.03 ± 0.55 [3.20] (2.4-3.5)
FT4, pg/ml	8.5-17.1	12.1 ± 1.9 [11.0] (10.4-15.6)	13.3 ± 1.9 [13.6] (11.3-15)
TgAb, UI/ml	0-115	212 ± 98.9 [160] (144-347)	18.0 ± 10.4 [13] (11-30)
TPOAb, UI/ml	0-34	199 ± 236.4 [84] (42-619)	6.7 ± 3.4 [6.1] (3.6-10.4)

* Data are given as mean ± SD, [median] and (range).

the Myo+SelMet combination (median: -29 and -29%; mean \pm SD: -21.5 ± 20.5 and $-25.8 \pm 21.4\%$). Of note, the combination showed the highest inter-individual variability, while Myo showed the lowest one.

In the HT group, the decrease in viable PBMC number was somewhat less evident than in the healthy group (Figure 1, right panel). This was particularly true for SelMet, which only at the highest concentration was barely more cytotoxic than H_2O_2 alone. It was confirmed that the combination showed the highest inter-individual variability, while Myo showed the lowest one. Only upon removing an outlier patient, at last one concentration of Myo+SelMet was borderline significantly lower compared to both H_2O_2 alone and baseline ($p = 0.10$ to $p = 0.016$).

In the presence of H_2O_2 , vitality as measured by the resazurin test was $94.2 \pm 11.2\%$ of baseline

(corresponding to a mean decrease of 5.8%) in the healthy group, and 93.2 ± 9.5 (corresponding to a mean decrease of 6.8%) in the HT group (data not shown). Because of a fewer number of women in the healthy group, only the decrease in the HT was significant ($p = 0.01$). With additions, vitality improved with a mean increase of approximately 5% to 15% over baseline, and with no superiority of the Myo+SelMet combination over either Myo alone or SelMet alone. For the same reason as above, changes over H_2O_2 alone were statistically more significant in the HT group than in the healthy group (up to $p < 0.0001$ vs. up to $p = 0.004$) (data not shown).

Nuclear Damage (Genotoxicity)

The addition of $200 \mu M H_2O_2$ was sufficiently dangerous for the nuclear integrity (Figure 2). Such genotoxicity at this concentration of H_2O_2

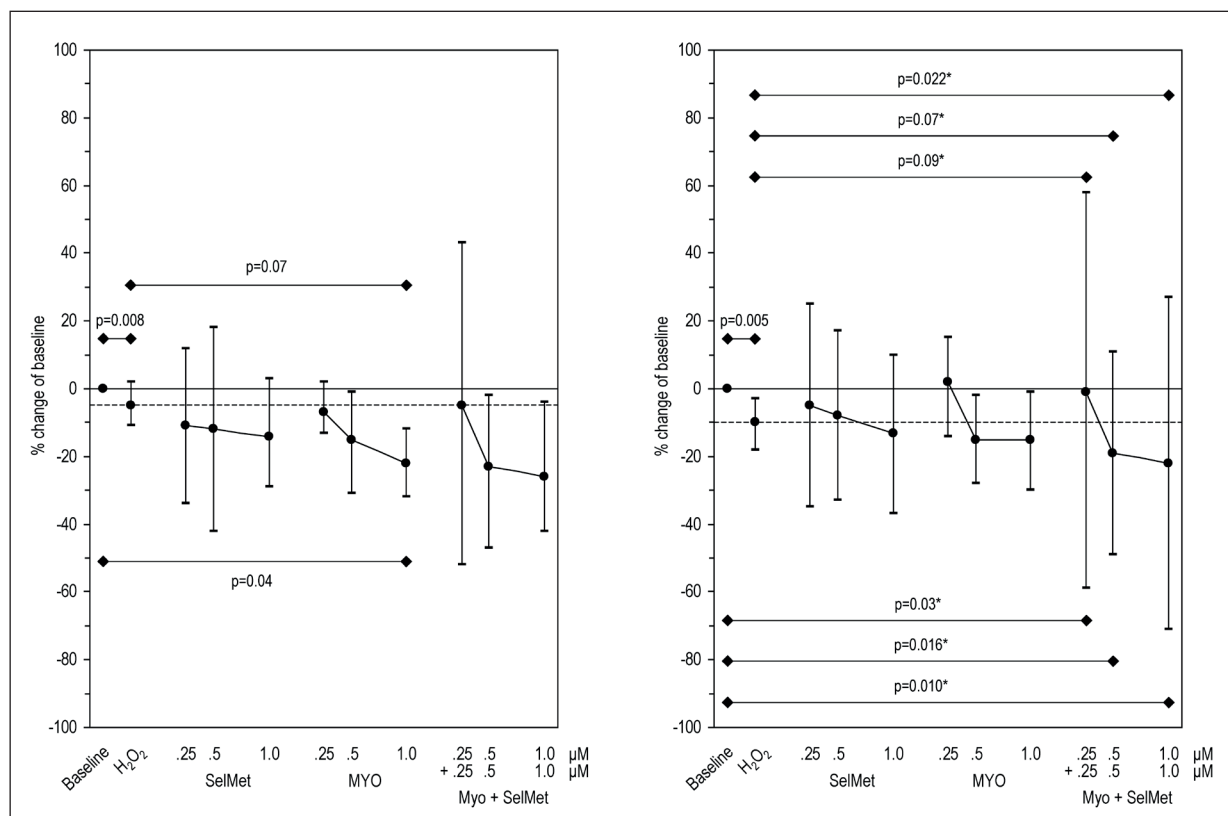


Figure 1. Proliferation of peripheral blood monocytes (PBMC) from three healthy women (*left panel*) or eight women with Hashimoto's thyroiditis (*right panel*) at baseline (no addition whatsoever), in the presence of $200 \mu M H_2O_2$ alone or $200 \mu M H_2O_2$ plus one at a time of these three additions: L-selenomethionine (SelMet), myo-inositol (Myo) or combination of Myo+ SelMet. The additions were tested at these equimolar final concentrations: 0.25, 0.5 or 1.0 μM ; for Myo+SelMet concentrations were 0.25+0.25 μM , 0.5+0.5 μM or 1.0+1.0 μM . Data are percent change in the number of cells per well after 5 days of culture with respect to baseline, baseline being zero. Data are illustrated as mean \pm SD (circles). Not to overload the graphs, only statistically significant ($p < 0.05$ minimum) and borderline significant differences (p -values between 0.05 and 0.10) are given. p -values written below the zero line refer to the comparison with baseline, while p -values above the zero line refer to the comparison with $200 \mu M H_2O_2$ alone.

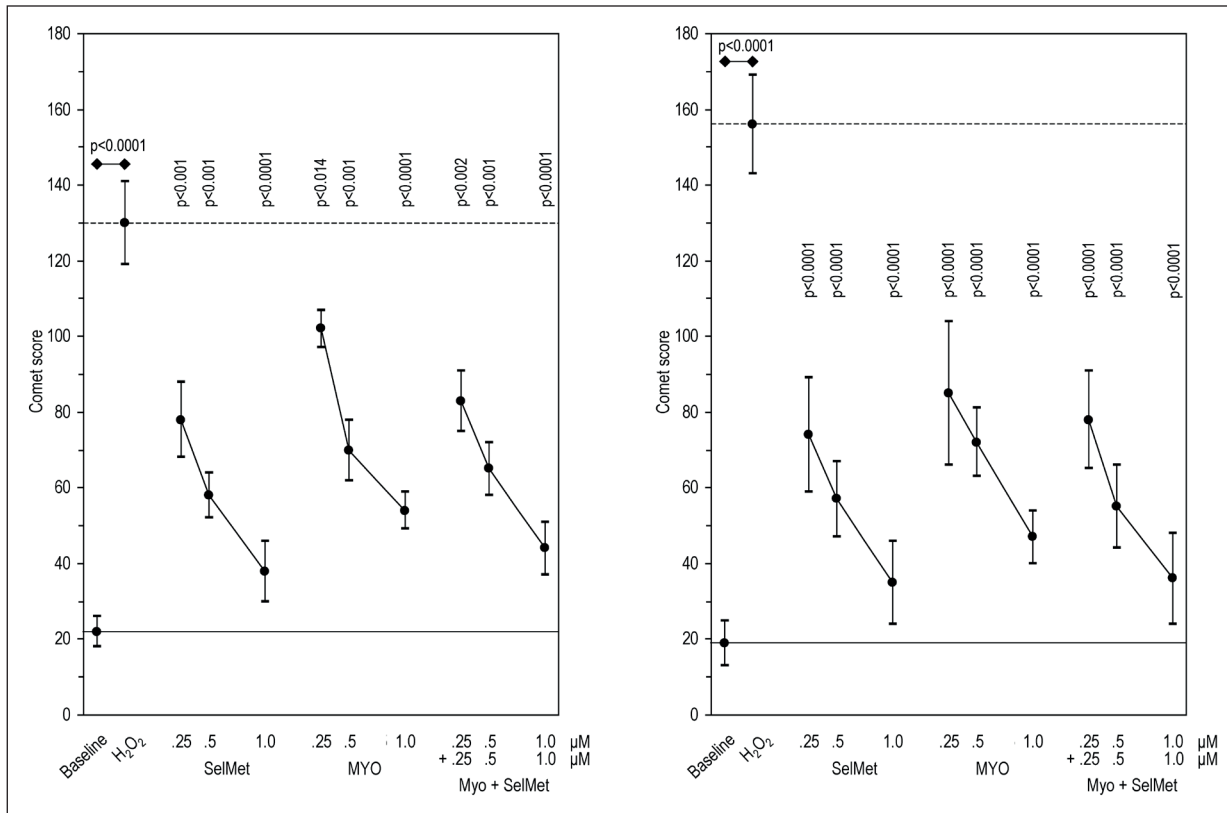


Figure 2. Genotoxicity (as indicated by Comet core plotted on the vertical axis) of peripheral blood monocytes (PBM) from three healthy women (*left panel*) or eight women with Hashimoto's thyroiditis (*right panel*) at baseline (no addition whatsoever), in the presence of 200 µM H₂O₂ alone or 200 µM H₂O₂ plus one at a time of these three additions: L-selenomethionine (SelMet), myo-inositol (Myo) or combination of Myo+ SelMet. The additions were tested at these equimolar final concentrations: 0.25, 0.5 or 1.0 µM; for Myo+SelMet concentrations were 0.25+0.25 µM, 0.5+0.5 µM or 1.0+1.0 µM. Note the similar basal genotoxicity in the two groups of women, and the greater genotoxicity induced by 200 µM H₂O₂ alone in the thyroiditis group. Also note the evident dose-dependent protection conferred by all three additions in either group (the Myo alone addition being the least potent). By extrapolation of the curves in either group, a return to basal genotoxicity is expected at approximately 1.25 µM SelMet alone, 1.25 µM SelMet+1.25 µM Myo, or 1.4 Myo alone.

was expected based on literature (see General outlines of the experiments, in Patients and Methods). The relatively narrow range between 0.25 and 1.0 µM dose of the tested compounds was sufficient to appreciate that these compounds were exerting a dose-dependent protection from H₂O₂ (Figure 2). Thus, we used this range of concentrations also for the experiments on chemokines (see below).

The Comet score at baseline was similar in the healthy subjects and HT patients (21.5 and 19.4, respectively). Addition of 200 µM H₂O₂ increased the Comet score (and therefore genotoxicity) to 130 (+505%) in the healthy group (Figure 2, left panel) and to a greater extent (156.1, or +707%) in the HT group (Figure 2, right panel), with a *p*-value < 0.0001 in either group. Also statistically different compared to H₂O₂ alone were all concentrations of all three additions in either group. In the healthy women, SelMet was the most po-

tent antagonist of H₂O₂, with a Comet score that increased to 263%, 170% and 81.4% above baseline at 0.25, 0.5 and 1.0 µM. The corresponding changes for Myo alone were +370%, +226% and +151%, while those for the Myo+SelMet combination (0.25+0.25 µM, 0.5+0.5 µM, and 1.0+1.0 µM) were +286%, +202% and +105%. In the HT group, it was confirmed that Myo alone was the least effective (+341%, +274% and +143%), whereas SelMet alone (+281%, +201%, and +81%) and Myo+SelMet (+300%, +185%, and +85%) were practically equipotent.

Cell Medium Concentrations of Chemokines in Healthy Women

The response of chemokines to the three additions (each addition in the presence of the fixed concentration of 200 µM H₂O₂) differed among chemokines, both in the healthy group (Figure 3,

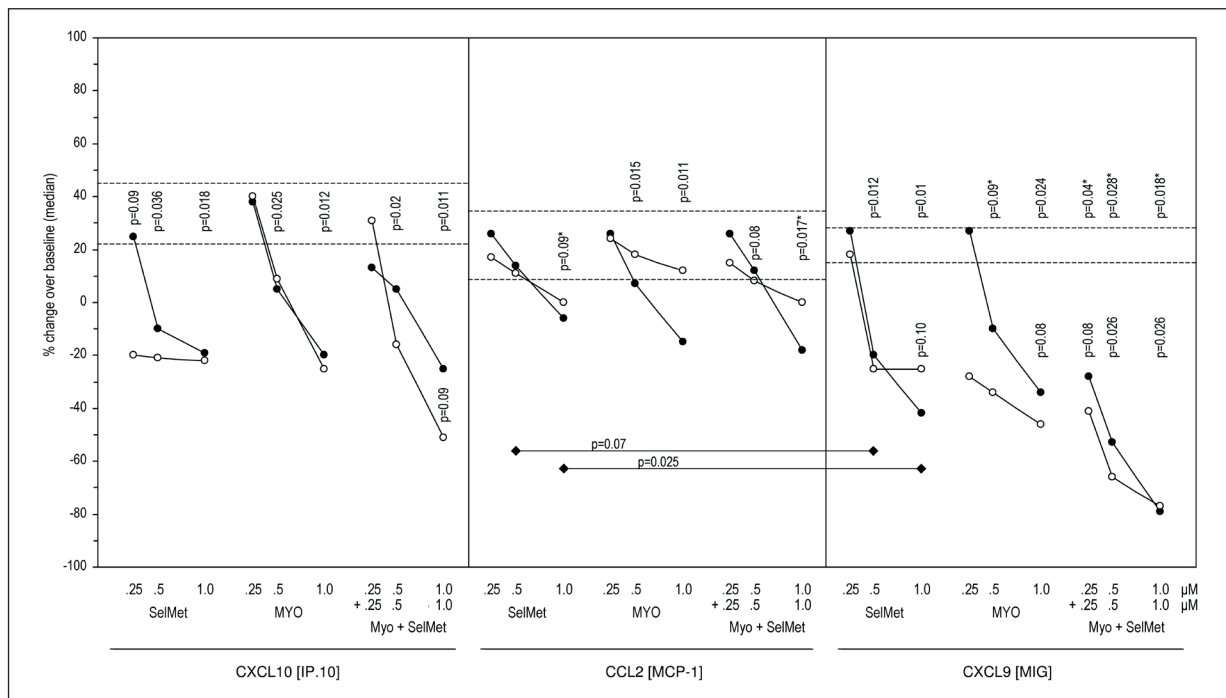


Figure 3. Concentrations of the three indicated chemokines in the medium of peripheral blood monocytes (PBMC) from three healthy women (*white circles*) or eight women with Hashimoto's thyroiditis (*black circles*) in the presence of 200 μM H_2O_2 , plus one at a time of these three additions: myo-inositol (Myo), L-selenomethionine (SelMet) or combination of Myo+ SelMet. The additions were tested at these equimolar final concentrations: 0.25, 0.5 or 1.0 μM ; for Myo+SelMet concentrations were 0.25+0.25 μM , 0.5+0.5 μM or 1.0+1.0 μM . Data (median) are percent change in the chemokine after 5 days of culture with respect to baseline, baseline (namely, no addition whatsoever) being zero. Median was preferred to mean \pm SD because of the large standard deviation, as it can be deduced from subsequent Figure. The horizontal dotted lines (two per each chemokine) refer to the change given by adding 200 μM H_2O_2 alone. The bottom dotted horizontal line pertains to the healthy group, while the top dotted horizontal line pertains to the Hashimoto's thyroiditis group. Not to overload the graphs, only statistically significant ($p < 0.05$ minimum) and borderline significant differences (p -values between 0.05 and 0.10) are given. p -values written vertically refer to the comparison with 200 μM H_2O_2 alone, and they are positioned above baseline (*zero line*) for the thyroiditis group, and below baseline for the healthy group. The P values written horizontally refer to significant or borderline significant differences between two chemokines given by the same addition at the same concentration. For instance, the decrease given by 1.0 μM SelMet alone on CXCL10 concentration was significantly greater ($p = 0.025$) than on CCL2, while it was statistically similar ($p > 0.10$) to that on CXCL9 which, in turn, was also statistically similar to that on CCL2. For p -values, the asterisk indicates that an at least borderline significant difference was obtained if an outlier women with Hashimoto's thyroiditis was removed from statistical analysis.

white circles) and in the HT group (Figure 3, black circles; see next heading). CXCL10 was the chemokine that was released with the greatest abundance in response to the addition of H_2O_2 alone (Figure 3, dotted horizontal lines). As can be deduced from inspection of Figure 4 (left panel), there was a large variability in individual responses to H_2O_2 alone and, especially, in individual response of chemokines. Overall, there was an evident dose-dependent fall for all chemokines, but only under SelMet or Myo+SelMet, the decline was more often not only below the stressed condition (200 μM H_2O_2 alone) but also below the baseline condition.

Figure 3 shows that CXCL10 decreased already maximally, and by approximately 20% of baseline, at the lowest SelMet concentration with no

further decline. In contrast, there was a wide dose-dependent inhibition with Myo, the maximum effect (-25% of the baseline concentration) being reached at 1.0 μM . The inhibition was greater in the presence of the combination, with a -16% of baseline at the intermediate dose and -51% of baseline at the highest dose.

The profile of CCL2 response was quite different from that of CXCL10 (see above) and CXCL9 (see below), with little differences among the three additions (Figure 3). There was a modest dose-dependency displayed by all three additions, with the SelMet curve and the Myo+SelMet curve almost overlapping. The greatest decline was at the highest concentration of either SelMet alone or Myo+SelMet.

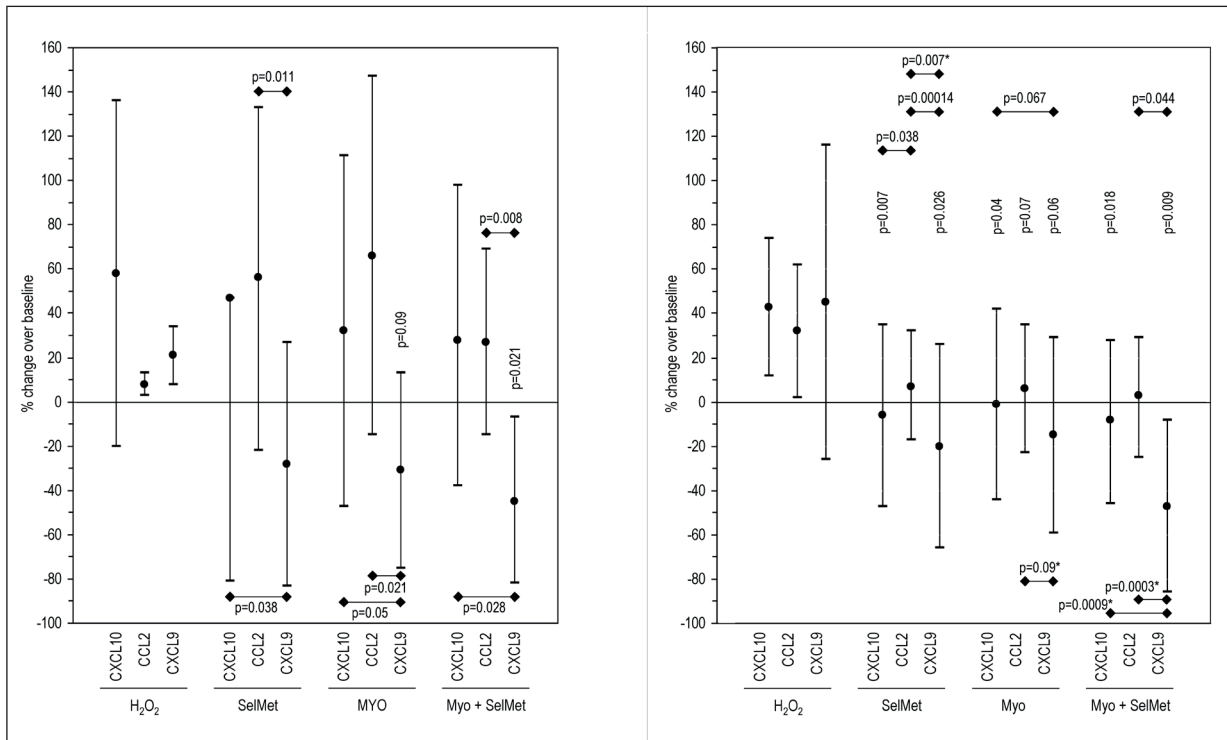


Figure 4. Concentrations of the three indicated chemokines in the medium of peripheral blood monocytes (PBMC) from three healthy women (*left panel*) or eight Hashimoto’s thyroiditis women (*right panel*) in the absence (*baseline, zero line*), in the presence of 200 μM H_2O_2 alone or 200 μM H_2O_2 plus one at a time of these three additions: L-selenomethionine (SelMet), myo-inositol (myo) or combination of Myo+SelMet. To maximize data, concentrations were pooled and considered as one. Data are percent change in the chemokine concentration after 5 days of culture with respect to baseline (baseline = zero). Data are illustrated as mean \pm SD (black circles). Not to overload the graphs, only statistically significant ($p < 0.05$ minimum) and borderline significant differences (p -values between 0.05 and 0.10) are given. The asterisk indicates that at least borderline significant difference was obtained if an outlier women with Hashimoto’s thyroiditis was removed from statistical analysis. p -values written vertically refer to the comparison with 200 μM H_2O_2 . The p -values written horizontally refer to significant or borderline significant difference between two chemokines given by the same addition.

The profile of CXCL9 resembled partially that of CXCL10 for SelMet (-25% of baseline at 0.5 and 1.0 μM). Myo gave a modest dose-dependent inhibition (-28 to -46% of baseline at 0.25 to 1.0 μM), while Myo+SelMet gave a wider and more potent inhibition (-41 to -77%, 0.25 to 1.0 μM).

Evident from Figure 3 is that only for CXCL9 there was an additive effect (greater inhibition at each dose) given by the Myo+SelMet combination compared to Myo alone and SelMet alone.

Cell Medium Concentrations of Chemokines in HT Patients

The inter-individual variability in chemokine responses was even greater than in the healthy group (Figure 4). This was particularly true for one HT patient (who will be referred to as “the outlier” throughout this paper). For instance, the CXCL10 response to 200 μM H_2O_2 alone was almost +2000% over baseline, and CXCL10 respon-

se to 0.5 μM Myo+0.5 μM SelMet (in the presence of 200 μM H_2O_2) was approximately +1000% over baseline, namely a 50% decline compared to H_2O_2 alone. As shown before for proliferation (Figure 2), omitting this outlier from statistical analysis allowed to reach at least borderline significant p values for comparisons concerning chemokines.

For CXCL10, only 0.5 and 1.0 μM SelMet gave an inhibition that approached the corresponding inhibition observed in the healthy subjects (Figure 3). With Myo, the curve was superimposable to that observed for the healthy subjects (Figure 3). With the Myo+SelMet combination, the curve was slightly shifted to the right, indicating a lesser degree of inhibition compared to the healthy subjects.

For CCL2, an inhibition was detected, and in the hierarchical order Myo+SelMet > Myo alone > SelMet alone (-31%, -15%, -9% of baseline). Only Myo showed a greater potency over the healthy women, at least at the two highest doses (0.5 and 1.0 μM).

Especially with Myo and the Myo+SelMet combination, the dose-dependency was wider compared to the healthy women. For CXCL9, a wider dose-dependency was evident with all three additions (Figure 3). Only SelMet showed possibly greater potency compared to the healthy group. With Myo alone and the Myo+SelMet combination, the corresponding curve was shifted to the right.

Confirming the pattern observed in the healthy group, in the HT group only for CXCL9, there was an additive effect (greater inhibition at each dose) given by the Myo+SelMet combination compared to Myo alone and SelMet alone (Figure 3).

Cell Medium Concentrations of Chemokines: Pooled Data in Either Group

To maximize data and permit statistics on a larger series of numbers, for each chemokine we pooled the concentrations given by the three doses of a given addition.

The results are shown in Figure 4. It is evident that, for none of the three chemokines, SelMet alone inhibits more potently in the HT group compared to the healthy group. At most, SelMet is equipotent (CCL2 and CXCL9). Myo alone inhibits relatively more potently in HT patients compared to healthy subjects only for CXCL10 and CCL2, though the decrease in either chemokine falls never below baseline. Myo alone exerts a stronger inhibition (*ie*, decline below baseline), and in healthy subjects more than in HT patients, only for CXCL9. The Myo+SelMet combination gives a relatively more potent inhibition, and in HT patients more than in healthy subjects, only for CXCL10.

Discussion

The therapeutic approach to AITD has also included biological agents^{50,51} but, as already mentioned in the Introduction, there is an increasing interest for a nutraceutical approach³⁹⁻⁴⁵ and, more recently, dietary approach⁵².

In one randomized, placebo-controlled study⁴¹, 77 TPOAb +ve women received 200 µg/d SelMet from the first trimester of pregnancy through 1 year postpartum, while 74 TPOAb +ve women received placebo. Starting at the third trimester, the SelMet group had significantly lower TPOAb levels and, during postpartum, significantly lower rates of postpartum thyroid dysfunction (including permanent hypothyroidism) and significant

amelioration of ultrasonographic signs of thyroiditis⁴¹. Of interest is also the randomized, 6-month duration study on 48 women with HT-associated subclinical hypothyroidism who received SelMet alone (83 µg/d) or SelMet (83 µg/d) plus Myo (600 mg/d)⁴³. Women were selected for having serum TSH between 4.01 and 9.99 mU/L, high levels (>350 U/ml) of TPOAb and/or TgAb and the typical thyroid hypoechogenicity at neck ultrasonography. At baseline, serum levels of TSH, TgAb, TPOAb and selenium were comparable in the two groups, and so were selenium levels at 6 months. However, rates of fall of both TgAb and TPOAb, and rates of thyroid hypoechogenicity improvement were greater in the Myo+SelMet group. TSH decreased by one-third in the Myo+SelMet group, which cannot be compared with the SelMet group, as it was not measured in this group.

In the first study⁴¹, serum selenium increased by approximately 40% compared to baseline (\approx 110 µg/L [1.4 µM] compared to 81 [1.0 µM]) in the supplemented women, while it remained unchanged in the nonsupplemented women (\approx 78 µg/L [1.0 µM]). In the second study⁴³, plasma selenium increased by 74% in the Myo+SelMet group (225.4 µg/L [2.85 µM]) compared to baseline (129.2 µg/L [1.6 µM]), while plasma Myo increased by 68% (37.3 µM compared to baseline levels of 22.2 µM). In the animal study summarized below⁵³, selenium-supplemented mice had serum levels of selenium 20% greater than unsupplemented mice (285 µg/L [3.6 µM] vs 237 µg/L [3.0 µM]). Thus, the 0.25 to 1.0 µM concentrations of SelMet (corresponding to 0.1 to 0.4 µM selenium) or Myo to which PBMC were exposed in our *in vitro* experiments are not supraphysiologic.

Each of the three fundamental AITD (GD, HT and postpartum thyroiditis) is widely heterogeneous^{3,26}. For instance, HT can present with transient thyrotoxicosis at one extreme or with overt thyroid failure at the other extreme, with euthyroidism and subclinical hypothyroidism in-between. The thyroid gland from HT patient can be large with or without nodules at one extreme or atrophic at the other extreme. Both thyroid autoantibodies (TgAb, TPOAb) can be enormously elevated at one extreme or both be undetectable at the other extreme. Thus, it is not unexpected that any given evaluated index will vary widely among individuals with the same AITD.

In the present study, we have shown that when PBMC are exposed to oxidative stress (as the one classically induced by H₂O₂), any of the anti-oxidants we tested (Myo alone, SelMet alone

or their combination, each in the range 0.25 to 1.0 μM) antagonizes H_2O_2 except for cell survival. In fact, all three antioxidant additions caused a further decrease in survival. If the same decline in lymphocyte population occurs in the thyroid, then this decline should be considered a beneficial response (that is, decreased mass of the lymphocytic infiltrate). In this context, it is pertinent to mention results obtained in the NOD.H-2h4 mice, which are used as an experimental model of autoimmune thyroiditis because they develop it when given iodide in drinking water⁵³. Compared with untreated mice, a decrease of both the lymphocyte infiltrates (-33%), thyroid weight (-26%) and serum concentrations of TgAb (-23%) were observed upon administration of selenium in the drinking water for 16 weeks⁵³. Finally, the percentages of CD4+CD25+Foxp3+ T cells were increased by one-third (3.24 ± 0.48 vs $2.48 \pm 0.40\%$, respectively, $p = 0.001$), and the expression of Foxp3 mRNA was doubled (1.50 ± 0.66 vs 0.77 ± 0.37 , $p = 0.011$) in the selenium-treated mice as compared with the untreated mice. These two last data are relevant because CD4+CD25+ regulatory T lymphocytes (Treg cells), the development of which is programmed by the transcription factor Foxp3, contribute to the prevention of autoimmunity⁵³. The same mechanisms as those reported in mice⁵³ are likely to have occurred in the women treated with SelMet⁴¹ or Myo+SelMet⁴³, in order to account for the decreased thyroid hypoechogenicity and thyroid Ab levels. Several studies reported that experimental mice develop multi-organ inflammation and autoimmune diseases, including thyroiditis, after injection of antiCD25 Ab or knock out of Foxp3⁵³. Thus, many autoimmune diseases could be prevented or suppressed by therapy that increases the number or function of Treg cells⁵³.

In one companion paper on 21 euthyroid HT women who received SelMet+ Myo (660 mg+83 μg) twice a day for 6 months⁵⁴, we report that TgAb, TPOAb, TSH, and CXCL10 decreased by 61%, 45%, 33% and 26%. Remarkably, this 26% reduction of CXCL10 agrees with the mean 31% fall over baseline (median = 27%) in the concentration of CXCL10 in the medium of HT PBMC cultured in the presence of 1.0 μM Myo+1.0 μM SelMet. Because of the minimal differences in PBMC viability between the healthy women and the HT women in the present *in vitro* study, the overtly appreciable differences in chemokine changes brought about by PBMC exposure to the antioxidants that we tested, and the time (5 days) elapsed to per-

mit measuring the chemokines in the cell medium, suggest that these compounds are likely acting at a gene-expression level by down-regulating the corresponding genes. However, there was no appreciable improvement in thyroid hypoechogenicity⁵⁴. This last finding can be explained by the marked interindividual variability among HT patients for various indices, as supported by the data reported here in the PBMC indices.

Conclusions

There are some obvious ramifications for future research that stem from the present study. First, we wish to confirm results on a larger cohort of HT and healthy controls, and measure simultaneously both the *in vitro* outcomes reported here on PBMC and the *in vivo* outcomes reported by Ferrari et al⁵⁴. Second, we can expand the outcomes to include TgAb and TPOAb levels in the medium of cultured PBMC. This is feasible, since literature exists on assay of these Ab from PBMC both under natural conditions and at stimulated/inhibited conditions. PBMC from patients with AITD (GD and HT) or healthy controls⁵⁵⁻⁵⁸ were studied. In one such study on HT patients⁵⁵, a direct correlation was found between serum titers of microsomal Ab (now termed TPOAb) and *in vitro* secretion of microsomal Ab by PBMC. Thus, at a practically translational level, assuming that PBMC would mirror thyroid-infiltrating lymphocytes, one can test *in vitro* the response of PBMC to SelMet, or Myo+SelMet in terms of proliferation, chemokine secretion and TgAb and TPOAb. Only upon evidence of significant inhibition of these indices *in vitro* on PBMC of a given patient, the physician would endorse patient-tailored supplementation with a reasonable expectation of decreasing thyroid lymphocytic infiltration, circulating thyroid Ab levels, intrathyroid and circulating chemokines, and circulating TSH as well. Third, as PBMC outcomes declined also in our healthy group, SelMet or Myo+SelMet supplementation could be given to consanguineous relatives of HT patients who have not developed HT yet. Also, this prophylaxis can be performed on a personalized basis. Fourth, there is literature⁵⁹⁻⁶⁴ on the association of HT or GD with differentiated thyroid cancer, particularly the papillary histotype, the increased risk for the associated malignancy being conferred by certain cytokines and, given the physiologic stimulus on thyrocyte proliferation, by serum TSH in the upper tertiles or quartiles of the normal range.

In summary, there is now experimental and clinical ground for the Myo+SelMet use in the clinical setting of AITD. Based on future studies stemming from the pilot study reported here, it is hoped that assessment of the *in vitro* responses to PBMC Myo+SelMet could predict clinically relevant outcomes on an individual basis. A long-term beneficial outcome might be protection against the oncogenic potential of the thyrocytes in the background of coexisting autoimmune thyroiditis.

Disclosure

S.B. has been an invited speaker for Lo.Li Pharma. Lo.Li Pharma provided us with pure myo-inositol, but had no role in the design, conduction of the experiments, their interpretation and writing of the manuscript. The others authors have no conflict of interests.

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