Author's response to reviews

Title: Serum homocysteine levels are decreased in levothyroxine-treated women with autoimmune thyroiditis.

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Author's response to reviews: see over
Dear Editor,

Please find below our responses to Reviewer's comments. We hope that you will find them satisfactory. We do appreciate very much your input in making this paper better. If any further explanations would be required, we will be more than happy to answer any questions. The study was approved by the Poznan University of Medical Sciences Ethical Committee.

Maciej Owecki & co-authors

Reviewer: Diarmuid Smith
We thank the Reviewer for all comments.

Reviewer's report:
The article was well written and is of interest in its field
Major Compulsory Reviews
1. Include Standard Deviation data for the homocysteine levels as well as median and interquartile ranges, in the control group there appears to a number of outliers with very high homocysteine levels, please comment.

We provided means and Standard Deviations (SD) for Hcy in Table 1.

\[
\text{HCY (µmol) Mean} \pm \text{SD:}
\]
- Non treated Hashimoto: 10.33 µmol ± 3.36 µmol
- Treated Hashimoto: 9.84 µmol ± 4.24 µmol
- Controls: 12.97 µmol ± 6.71 µmol

\[
\text{HCY (µmol) Median (IQR):}
\]
- Non treated Hashimoto: 11 µmol (4.2 µmol)
- Treated Hashimoto: 10.8 µmol (6.9 µmol)
- Controls: 13.35 µmol (6.34 µmol)

In general we express results as medians and IQR because data did not follow normal distribution.

There was only one case in control group with high HCY level 44.6 µmol and we included it to further analyses. The rest of values varied from 4 µmol to 19.2 µmol. Since data were compared by non-parametric test (Kruskal-Wallis), outlying measurement did not influence the results of comparison between groups. Non-parametric test operates with ranks not original values.

2. In the discussion I would like the authors to discuss in more detail why they feel they did not see a difference in homocysteine levels between treated and non-treated TPO antibody patients and why there was no difference between non-treated hypothyroid patients and the control group. This would suggest that there is no association between thyroid autoimmunity and atherosclerosis. Yet the authors do not highlight this point in the discussion and the reader is left confused by the conclusion of the author. Therefore I would suggest the 3rd paragraph in the discussion and conclusion section is rewritten and made clearer.

We added the third paragraph in the discussion section speculating on the possible reason of the Hcy reduction during LT4 treatment:

Serum FT4 concentration is considered as an independent determinant of Hcy level [29]. As was mentioned above, Hcy level is generally decreased in hyperthyroid patients in contrast to hypothyroid subjects, who have higher Hcy concentration. Moreover, restoration of thyroid
function leads to normalization of Hcy concentration. In general, our study and control groups were euthyroid, but this state was achieved by LT4 replacement therapy in a group of treated HT women. This group had lower Hcy levels than normal controls, despite similar FT4 levels. A possible explanation of this finding is the fact that, in spite of similar hormone concentrations, these patients had different sources of thyroxine: it was endogenous in one group, and exogenous in the other. In our opinion, there is a causative relationship between LT4 replacement therapy and decreased Hcy levels. Patients who are on LT4 therapy have daily changes of FT4 serum concentration that result from pharmacokinetic properties of this medication. The maximum FT4 concentration occurs approximately two hours after the drug ingestion [30]. Moreover, there is a transient increase of FT4 serum level after ingestion of LT4 for 5 hours [31]. Since FT4 directly influences Hcy concentration, during this time Hcy metabolism is similar to hyperthyroid state and it may lead to decreased Hcy levels in treated HT patients in contrast to healthy controls, in whom FT4 output is adjusted to real needs and the rate of physiological elimination.


31 Ain KB, Pucino F, Shiver TM, Banks SM: Thyroid hormone levels affected by time of blood sampling in thyroxine-treated patients. Thyroid 1993 3: 81-85.

We added the following comment to conclusion section:
It seems that non-treated HT in euthyroidism is not associated with Hcy increase, as compared with overt hypothyroidism. This may be just another argument against the concepts about the role of “euthyroid HT” in the development of atherosclerosis, which is of quite importance considering the high prevalence of high TPOAbs titers in Europe. Second, that Hcy was lower in the treated group may point to the beneficial role of LT4 treatment in general.

Minor points
1. Did the authors have the baseline TSH levels before treatment in the women who were treated with thyroxine. Did these women have subclinical or overt hypothyroidism? Please include the data.
Unfortunately, we are not able to answer this question. Since in the majority of our patients hypothyroidism had been diagnosed many years before they were recruited into the study, we do not have data concerning the initial TSH levels.

2. Page 6 line 164 remove the word “for”
Thank you, we corrected this language mistake.

Reviewer: Lucy Ann Behan
Reviewer's report:
Major:
Methods and Results sections:
What is the benefit of the ROC curve in this situation? Are the authors proposing that Hcy levels can be used to distinguish between Hashimoto subjects and controls? I do not think this statistic adds anything to this paper.

ROC analysis is used to check if we can distinguish controls from HT. It discriminates HT patients from controls and it gives a proposed cut-off value of parameter to differentiate these groups. This analysis as well as Kruskal-Wallis test shows that Hcy level significantly differs between treated HT and controls. Roc analysis give the cut-off value for Hcy. It seems that treated HT tend to have lower value of Hcy than controls and this level for treated HT is less than 13.2 µmol. Sensitivity and specificity as well as AUC shows the accuracy of this discrimination. Of course AUC=0.68 can be interpreted as poor to fair accuracy. Therefore we agree with Reviewer’s suggestion and we decided to remove ROC analyses from manuscript.

Results and Discussion sections:
The treated-hypothyroid(HT) TPO positive group had higher FT4 compared to the non-treated group, but the same as controls, while homocysteine levels were lower in the treated-HT group compared to controls but not compared to the untreated-HT counterparts. There was no difference in Hcy levels in the non-treated group compared to controls. The significance of these findings remains unclear by the end of the manuscript. The authors do not postulate a reason why the Hcy is reduced in the treated versus controls but not versus the other autoimmune positive group, which suggests that the treatment may be more likely to be the factor rather than the autoimmunity. Could the authors please discuss this further? The medians of Hcy levels are very similar, it is suprising that the differences were significant.

We added the third paragraph in the discussion section speculating on the possible reason of the Hcy reduction during LT4 treatment:

Serum FT4 concentration is considered as an independent determinant of Hcy level [29]. As was mentioned above, Hcy level is generally decreased in hyperthyroid patients in contrast to hypothyroid subjects, who have higher Hcy concentration. Moreover, restoration of thyroid function leads to normalization of Hcy concentration. In general, our study and control groups were euthyroid, but this state was achieved by LT4 replacement therapy in a group of treated HT women. This group had lower Hcy levels than normal controls, despite similar FT4 levels. A possible explanation of this finding is the fact that, in spite of similar hormone concentrations, these patients had different sources of thyroxine: it was endogenous in one group, and exogenous in the other. In our opinion, there is a causative relationship between LT4 replacement therapy and decreased Hcy levels. Patients who are on LT4 therapy have daily changes of FT4 serum concentration that result from pharmacokinetic properties of this medication. The maximum FT4 concentration occurs approximately two hours after the drug ingestion [30]. Moreover, there is a transient increase of FT4 serum level after ingestion of LT4 for 5 hours [31]. Since FT4 directly influences Hcy concentration, during this time Hcy metabolism is similar to hyperthyroid state and it may lead to decreased Hcy levels in treated HT patients in contrast to healthy controls, in whom FT4 output is adjusted to real needs and the rate of physiological elimination.
We added following comment to conclusion section:

It seems that non-treated HT in euthyroidism is not associated with Hcy increase, as compared with overt hypothyroidism. This may be just another argument against the concepts about the role of “euthyroid HT” in the development of atherosclerosis, which is of quite importance considering the high prevalence of high TPOAbs titers in Europe. Second, that Hcy was lower in the treated group may point to the beneficial role of LT4 treatment in general.

If Hcy is a marker of atherosclerosis/cardiovascular risk then this premenopausal group of women is a low risk group at the outset. Do the authors think this has an impact on their findings?

Obviously, the young and otherwise healthy women that were enrolled into this study had low risk of atherosclerosis. Anyway, this risk may be present in some individuals even at younger age. It must be emphasized also that only such a group like this allowed us to investigate the influence of Hcy without other confounding and overlapping factors that would exist in any other groups with a higher risk of atherosclerosis (statins, hypertension, lack of oestrogens etc). Therefore, this premenopausal group helped us to eliminate other possible factors that might have the influence on Hcy level. In this setting, we had a study model formed strictly to analyze the parameter examined. In our opinion, the recruitment criteria are the strength of our study.

Discussion:
Paragraph 3 lines 170-173 – the authors mentions studies referenced 20-22 have been performed and that the conclusions of those studies are not comparable, however they have not stated what these studies examined or what their conclusions were – could the authors please elaborate slightly on these studies?

Thank you for these comments. We discussed cited articles:
Tamer et al. found that euthyroid HT (n=84) patients had higher LDL level as compared with controls (n=150) (p=0.0042) [20]. Moreover, TPOAbs level was negatively correlated with HDL (p=0.031; r=-0.137) and positively with TAG (p=0.013; r=0.158) and waist circumference (p=0.048; r=0.128). Ciccone et al. established that overweight or obese women with HT have increased IMT as compared with controls [22]. They suggested that the autoimmunity in HT patients is an independent factor that might accelerate atherosclerosis. However, they also found that HT patients had higher TSH levels and lower FT3.
The authors reference a study by Topaloglu et al which examines Hcy in context of thyroid autoimmunity in euthyroid premenopausal females with Hashimoto thyroiditis recently and state that CIMT was the only value that was higher in the study group. Please provide more detail regarding this study, was CIMT higher in the patients v controls or in the <2.5TSH v >2.5TSH?

We answered this question in manuscript:
CIMT was the only one evaluated parameter that was significantly higher in the study group regardless of TSH. CIMT was positively correlated with antithyroglobulin antibodies (p=0.014; r=0.328).

Minor:
Typographical error in Subjects and methods line 94 and 95 “tabel”
Typographical error in Table 1, column controls, row BMI – missing a closing bracket “)”

Many thanks for these comments, we corrected all typos.

Reviewer: Tommy Kyaw-Tun
Reviewer’s report:
This interesting study attempts to address whether TPO status is related to Hcy level in the presence of euthyroidism. Overall the study may be re-considered however quite a number of major and minor issues need to be addressed. Overall the main difficulty is that whilst a hypothesis is present, it is unclear from the results and discussion whether their hypothesis was affirmed or revoked, mainly because it is unclear whether comparisons for Hcy levels were done between TPO +ve untreated euthyroid (pre-clinical phase) patients had higher Hcy cf controls.

We thank the Reviewer for detailed revision.

(A) Major Compulsory Revisions

ABSTRACT
1. Ln 30-32: why is Hcy of interest?
We added that comment to the abstract:
Since some authors suggest that chronic autoimmune thyroiditis per se may be considered as a novel risk factor of atherosclerosis independent of thyroid function, the analysis of classical cardiovascular risk factors in context of thyroid autoimmunity might be helpful in evaluation the possible causative relationship. Data concerning the impact of thyroid autoimmunity in euthyroid state on homocysteine (Hcy) level is lacking.

Ln 34: How was Hashimoto’s thyroiditis - can one safely assume that all patients with Hashimoto's thyroiditis had positive TPO Abs?
Thank you for raising this question. We provided the information that all Hashimoto patients had positive TPOAbs.
"All women with HT had positive TPOAbs."

Ln 35: Healthy controls - were they all negative for TPO status? (these have to be clarified and addressed in methods section of main body also.)
We added a sentence clearly stating that controls were negative for TPOAbs in Methods section of both abstract and main body:

"Forty healthy females negative for TPOAbs comparable for age and body mass index (BMI) participated in the study as controls (Table 1)."

Ln 42-44 - so what is the significance of there being no difference in Hcy between non-treated HT and control. and if non-treated HT was not higher - does it matter that Hcy is lower in the treated group?

First, it seems that non treated HT in euthyroidism is not associated with Hcy increase, as compared with overt hypothyroidism. This may be just another argument against the concepts about the role of “euthyroid HT” in the development of atherosclerosis, which is of quite importance considering the high prevalence of high TPOAbs titers in Europe. (we added this comment to conclusion section). Second, that Hcy was lower in the treated group may point to the beneficial role of LT4 treatment in general. As we write in lines 230-233 : “levothyroxine treatment may further add to medical approach aimed at atherosclerosis risk reduction with regard to Hcy decrease in LT4-treated women with chronic autoimmune thyroiditis. This last concept, however, as drawn only from a cross-sectional setting, requires further investigation in observational studies.”

We added the third paragraph in the discussion section speculating on the possible reason of the Hcy reduction during LT4 treatment:

Serum FT4 concentration is considered as an independent determinant of Hcy level [29]. As was mentioned above, Hcy level is generally decreased in hyperthyroid patients in contrast to hypothyroid subjects, who have higher Hcy concentration. Moreover, restoration of thyroid function leads to normalization of Hcy concentration. In general, our study and control groups were euthyroid, but this state was achieved by LT4 replacement therapy in a group of treated HT women. This group had lower Hcy levels than normal controls, despite similar FT4 levels. A possible explanation of this finding is the fact that, in spite of similar hormone concentrations, these patients had different sources of thyroxine: it was endogenous in one group, and exogenous in the other. In our opinion, there is a causative relationship between LT4 replacement therapy and decreased Hcy levels. Patients who are on LT4 therapy have daily changes of FT4 serum concentration that result from pharmacokinetic properties of this medication. The maximum FT4 concentration occurs approximately two hours after the drug ingestion [30]. Moreover, there is a transient increase of FT4 serum level after ingestion of LT4 for 5 hours [31]. Since FT4 directly influences Hcy concentration, during this time Hcy metabolism is similar to hyperthyroid state and it may lead to decreased Hcy levels in treated HT patients in contrast to healthy controls, in whom FT4 output is adjusted to real needs and the rate of physiological elimination.


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Table 1
a, b - what are these comparisons? between which pairs of groups or are they different p values e.g. <0.05 and <0.01?

Table 1 present data for three analyzed groups as well as the p-value for Kruskal-Wallis test. Kruskal-Wallis test is for comparing more than two groups and gives only information whether all groups are equal or not (meaning equal distributions). If p<0.05, significant difference was found, the next step is to find homogenous groups (the sets of groups that do not differ) – this is post-hoc analysis, which was performed by Dunn’s tests. The letters a,b denote groups that do not differ. This is a standard way of presenting homogenous groups in all ANOVA analyses.

The comparison is made at p<0.05. As it is written in subjects and methods part: “All tests were performed two-tailed and were considered as significant at p<0.05.”

To be clearer, we changed Table's instruction “a,b – values followed by the same letters do not differ significantly” into: "a,b – values followed by the same letters do not differ significantly at p<0.05".

MAIN BODY
Ln 72-74: are there references re: GFR / liver metabolism and Hcy levels
Yes, there are. We provided following references:


Ln 91-40: TPO status for each group should be clearly stated. We provided these information as mentioned above.

Ln 122: Were any comparisons made between all the HT (treated and untreated with LT4) and controls? if not then the Mann-Whitney test does not need to be used - since all the results were comparisons between 3 groups?

Thank you for this valuable question. In fact, we did not use Mann-Whitney test. We removed this information from Methods section. Comparisons between 3 groups were performed with Kruskal-Wallis test with further analysis of Dunn’s tests. Additionally we added the information about Spearman’s correlation coefficient which was used to measure the strength of relationship of analyzed parameters.

Ln 140: was there a difference in Free T4 between non-treated HT and controls? No, there was no difference. This information is provided in Table 1. Median of FT4 level of non-treated HT is 14.7 pmol/l and median of FT4 level of controls is 15.52 pmol/L. Both values are marked by the same letter a - it means (as stated under the table) that they do not differ significantly.

Ln 142: was there a difference in Hcy between non-treated HT and controls? and any differences between the treated and non-treated groups? Neither Figure 2 nor the table is clear about this. There is no difference between Hcy of non-treated HT and controls - in Table 1 the values of Hcy levels are followed by “b”. Similarly, Hcy levels of treated and non-treated HT are marked by “a” - it means that they do not differ significantly. The only difference is between treated HT and controls as they are marked by different letters (treated HT - a; controls - b). (Please see: notation of groups always starts from groups with the lowest value – this is a standard procedure).

Ln 149-153: are the authors proposing that Hcy levels be used to either diagnose HT (Fig 4) or whether they are adequately treated (Fig 3)? what is the reason for doing the ROC. ROC analysis is used to check if we can distinguish controls from HT. It discriminates HT patients from controls and it gives a proposed cut-off value of parameter to differentiate these groups. This analysis as well as Kruskal-Wallis test shows that Hcy level significantly differs between treated HT and controls. Roc analysis give the cut-off value for Hcy. It seems that treated HT tends to have lower value of Hcy than controls and this level for treated HT is less than 13.2 µmol. Sensitivity and specificity as well as AUC shows the accuracy of this discrimination. Of course AUC=0.68 can be interpreted as poor to fair accuracy. Therefore we agree with Reviewer’s suggestion and we decided to remove ROC analyses from manuscript.

(B) Minor Essential Revisions
ABSTRACT
Ln 40: TPOAbs titers were apparently higher (?) (also in line 140) do the authors mean to omit the word apparently?
Thank you. We omitted the word apparently.
Ln 122-134: Statistics - there are 4 paragraphs in this section. We put all in one paragraph.
Ln 172: state what is HOMA
HOMA means homeostasis model assessment- we added this information to the manuscript.

Ln 224: cost? funded(?) do the authors mean funded?
Yes, of course. Many thanks for detailed revision.

MAIN BODY
Ln 61: homocystynuria(?) do the authors mean homocysteinaemia?
No, we do not. We corrected typing error: homocystinuria (it is an autosomal recessive trait caused by cystathionine beta synthase deficiency).

Ln 64: Up to date(?) do the authors mean To date?
Yes, we do- we provided this change in manuscript.

Reviewer: Tríona O'Shea

Many thanks for all comments.

Reviewer's report:
Compulsory revisions: the patients in the untreated hypothyroidism group do not actually have evidence of subclinical hypothyroidism- they simply have elevated TPO Ab?
Yes, exactly. Non treated HT females had elevated TPOAbs and they were euthyroid without LT4 replacement therapy.
Can you comment on why patients with normal thyroid function had higher homocysteine levels than treated hypothyroid patients? Is there data on Hcy levels in patients prior to commencing levothyroxine therapy?

1. We added the third paragraph in the discussion section speculating on the possible reason of the Hcy reduction during LT4 treatment:

Serum FT4 concentration is considered as an independent determinant of Hcy level [29]. As was mentioned above, Hcy level is generally decreased in hyperthyroid patients in contrast to hypothyroid subjects, who have higher Hcy concentration. Moreover, restoration of thyroid function leads to normalization of Hcy concentration. In general, our study and control groups were euthyroid, but this state was achieved by LT4 replacement therapy in a group of treated HT women. This group had lower Hcy levels than normal controls, despite similar FT4 levels. A possible explanation of this finding is the fact that, in spite of similar hormone concentrations, these patients had different sources of thyroxine: it was endogenous in one group, and exogenous in the other. In our opinion, there is a causative relationship between LT4 replacement therapy and decreased Hcy levels. Patients who are on LT4 therapy have daily changes of FT4 serum concentration that result from pharmacokinetic properties of this medication. The maximum FT4 concentration occurs approximately two hours after the drug ingestion [30]. Moreover, there is a transient increase of FT4 serum level after ingestion of LT4 for 5 hours [31]. Since FT4 directly influences Hcy concentration, during this time Hcy metabolism is similar to hyperthyroid state and it may lead to decreased Hcy levels in treated HT patients in contrast to healthy controls, in whom FT4 output is adjusted to real needs and the rate of physiological elimination.

Unfortunately, we do not have data on Hcy levels in patients prior to commencing levothyroxine therapy.