PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Low-grade inflammation in type 2 diabetes: A cross-sectional study from a Danish diabetes out-patient clinic
AUTHORS	Okdahl, Tina; Wegeberg, Anne-Marie; Pociot, Flemming; Brock, Birgitte; Størling, Joachim; Brock, Christina

VERSION 1 – REVIEW

REVIEWER	Aso, Yoshimasa Dokkyo Medical University
REVIEW RETURNED	25-Mar-2022

GENERAL COMMENTS	The present study aimed to investigate low-grade inflammation in type 2 diabetes and explore associations to clinical aspects as well as micro- and macrovascular complications. The authors concluded that the level of low-grade inflammation in type 2 diabetes is associated with obesity, glycemic regulation, therapeutical management, sex, and complications. Comments 1. Please add prevalence of metformin use and anti-hypertensive drugs (ACEI, ARB, etc.) use to Table 1. 2. Please add data about eGFR or UACR to Table 1, because CKD is associated with chronic low-grade inflammation. 3. I am confusing about a significant difference in serum levels of IL-10 between short-term and long-term duration (0.3 vs. 0.3) in Table 2. Please explain this strange result. 4. How was an association of DPP-4 inhibitor use with serum eotaxin? In Table 3 C, serum eotaxin should be incorporated into these models. A previous data demonstrated that DPP-4 inhibitor teneligliptin significantly decreased serum eotaxin in patients with type 2 diabetes. 5. Did you look at effects of SGLT2 inhibitor on serum cytokines and chemokines? I think it is very interesting. Please add SGLT2 inhibitor as a predictor to Table 3. 6. The authors should describe its cross-sectional nature of the present study as a major limitation.

REVIEWER	Muilwijk, Mirthe Amsterdam UMC, Public Health
REVIEW RETURNED	24-Jun-2022

GENERAL COMMENTS	The authors describe a wide range of biomarkers reflecting levels of low-grade systemic inflammation amongst people with type 2 diabetes compared to healthy individuals. Although this might be of interest, the manuscript could benefit from better structuring, and clearly describing primary and secondary objectives. Many

	different comparisons were made, without any adjustments for multiple testing. The data handling and statistics section is too short. E.g. the methods lack a description of considered confounders and effect modifiers and how these were selected. Unclear why baseline characteristics of the study population are tested for statistical differences. It is strange that similar characteristics are reported with an SD in the Health group but with a median in the T2D group and the other way around (also in table 2). Figure 1 and table 2 are not adjusted for confounders at all. It would be good to see different models, or to explain why this is not needed. In paragraph 3.3 and several other occasions is spoken of prediction, while data is cross-sectional. In figure 2 remains unclear how biomarkers were selected. Be careful in how the discussion is written. Just report on associations and differences between groups, as data is cross-sectional, don't make bigger statements. How does your data support that CRP production is induced by the presence of TNF-a and IL-6? Please go through the discussion to avoid such statements. The authors suggest that free smoking cessation programs might be successful, something they did not study at all. Selection or reporting bias seems much more realistic here. I did not see a reporting checklist.

VERSION 1 – AUTHOR RESPONSE

Response to Reviewer 1

1. Please add prevalence of metformin use and anti-hypertensive drugs (ACEI, ARB, etc.) use to Table 1.

Our reply: We agree with the reviewer that the requested information is relevant and have thus updated Table 1 to include information on prevalence of metformin and antihypertensive drugs.

2. Please add data about eGFR or UACR to Table 1, because CKD is associated with chronic low-grade inflammation.

Our reply: We thank the reviewer for pointing out the fact that CKD is relevant in regard to low-grade inflammation. We have added eGFR to Table 1.

3. I am confusing about a significant difference in serum levels of IL-10 between short-term and long-term duration (0.3 vs. 0.3) in Table 2. Please explain this strange result.

Our reply: We understand the confusion with these results. The explanation is the rounding of small values. However, we have decided to change the statistical approach to include adjustment of confounders, and Table 2 have been updated accordingly.

4. How was an association of DPP-4 inhibitor use with serum eotaxin? In Table 3 C, serum eotaxin should be incorporated into these models. A previous data demonstrated that DPP-4 inhibitor teneligliptin significantly decreased serum eotaxin in patients with type 2 diabetes.

Our reply: For simplicity of Table 3, we have chosen only to show analytes with p-values below 0.05 in the various models. Thus, in our models, eotaxin was not significantly associated with DPP-4 inhibitor therapy (Unadjusted model: p=0.124; Model 1: p=0.161; Model 2: p=0.149). We have edited the Table 3 legend to emphasize the rationale of the shown analytes.

5. Did you look at effects of SGLT2 inhibitor on serum cytokines and chemokines? I think it is very interesting. Please add SGLT2 inhibitor as a predictor to Table 3.

Our reply: This is an intriguing suggestion. We have made the calculations and added use of SGLT2 inhibitors as a predictor in Table 3E. We have also added the following to the results and discussion section, respectively:

"Lastly, SGLT2 inhibitor therapy was associated with lower levels of MDC."

"In our cohort, SGLT2 inhibitor therapy was associated with a decrease in MDC, known to facilitate and amplify type II immune response (36). The anti-diabetic effects of SGLT2 inhibitors rely on the inhibition of renal reabsorption of glucose, but anti-inflammatory effects have also been reported including attenuation of IL-6 production (37) and modulation of macrophage polarization (38). The prospect of utilizing the anti-inflammatory potential of SGLT2 inhibitors in various pathologies is currently receiving much attention (39). "

6. The authors should describe its cross-sectional nature of the present study as a major limitation.

Our reply: We thank the reviewer for this comment and we completely agree. The following has been added to section 4.4:

"A major limitation of this study is the cross-sectional study design, which hinders any assumptions of the predictive potential of low-grade inflammation and clinical characteristics of type 2 diabetes."

Likewise, we have added a bullet to "Strengths and limitations of this study":

"*The cross-sectional design is a limitation of the study and hinders any assumptions of causality"*

<u>Response to Reviewer 2</u>

1. … the manuscript could benefit from better structuring, and clearly describing primary and secondary objectives.

Our reply: After critically reviewing the manuscript again we agree with the reviewer. We have revised the last section of the introduction to be clearer:

"The aim of this study was to investigate the level of low-grade systemic inflammation in a cohort of individuals with type 2 diabetes with varying disease duration. We hypothesized that individuals with type 2 diabetes exhibited higher levels of pro-inflammatory biomarkers than healthy controls, and accordingly, the primary endpoint was differences in circulating inflammatory biomarkers in healthy and people with type 2 diabetes. Furthermore, we hypothesized that levels of pro-inflammatory biomarkers in type 2 diabetes were associated with disease duration, obesity, glycemic control, therapeutical management, and presence of diabetes-related micro- and macrovascular complications. The secondary endpoints were thus to investigate associations between inflammatory biomarkers and clinical characteristics of type 2 diabetes."

Moreover, a renaming/restructuring of subsections in the results and discussion sections have been made (all highlighted in yellow in the main document).

2. Many different comparisons were made, without any adjustments for multiple testing.

Our reply: We agree with the reviewer that multiplicity can be a problem when predictors are identified, but we do, however, believe that the Bonferroni correction in this dataset is a too conservative way of dealing with that. We have focused on the scientifically sensible pro-inflammatory markers (which contributes to the pathogenesis) and not on every possible serum marker. Furthermore, we have expanded the tables to include 95% confidence intervals on the odd's ratio. Thus, we have added the following to the limitations:

"On this dataset, we tested for association between low grade inflammation in type2 diabetes, and we selected á priori the anti-inflammatory markers, as they are part of the underlying pathogenesis. According to the study design, each of the serum markers were tested individually, and based on our unadjusted and adjusted models we suggest an association to the specific marker IL-10. As the manufacturer of the multiplex assay had defined division of serum markers into cytokines (n=4), chemokines (n=6), pro-inflammatory cytokines (n=5), vascular injury (n=1), we believe that Bonferroni's correction is too conservative."

3. The data handling and statistics section is too short. E.g. the methods lack a description of considered confounders and effect modifiers and how these were selected.

Our reply: We have elaborated the data handling and statistics section and agree with the reviewer that this was needed in order to clarify the applied statistical methods. The section now reads:

"Distribution of raw and log-transformed data was evaluated by Shapiro-Wilk test of normality. Pairwise comparisons among groups were achieved by independent samples t-test or Mann-Whitney U based on data distribution. Differences in inflammatory biomarkers between healthy and type 2 diabetes were investigated firstly by pairwise comparisons and secondly by a logistic regression model including age and BMI as confounders, as these factors were different between groups and known to influence systemic low-grade inflammation. For the volcano plot, the fold difference was calculated as the log2-ratio between two group means. Differences in inflammatory biomarkers between people with short- and long-term disease duration were likewise investigated by a logistic regression model including age and BMI as confounders. Multiple linear regression analyses were performed to investigate the association between clinical parameters and inflammatory biomarkers. The independent variables included obesity (BMI<30 versus BMI>30), blood glucose level (HbA1c<55 versus HbA1c>55), DPP-4 inhibitor therapy, GLP-1 receptor agonist therapy, and sex. Additionally, two models were applied in which associations were adjusted for the remaining clinical variables, and total plasma cholesterol or statin therapy, all of which may have an impact on the systemic inflammatory status. Differences in inflammatory biomarkers between people with 0, 1, 2 og ≥3 comorbidities were investigated by a Bonferroni-corrected ANOVA and subsequently the Dunn's Test. An α-level of 0.05 was applied for all analyses. The STATA software (StataCorp LLC, version 15.1) was applied for all statistical analyses."

4. Unclear why baseline characteristics of the study population are tested for statistical differences.

Our reply: A statistical test between baseline characteristics were done in order to assess aspects in which the two cohorts were different. We have applied this information in the selection of confounders in the statistical analysis of differences in analytes between healthy and type 2 diabetes (see item 6 below).

5. It is strange that similar characteristics are reported with an SD in the Health group but with a median in the T2D group and the other way around (also in table 2).

Our reply: We agree that this way of reporting data is confusion and we have thus standardized the way of reporting data in Table 1. Tabel 2 has been revised to reflect a different statistical approach (see item 6).

6. Figure 1 and table 2 are not adjusted for confounders at all. It would be good to see different models, or to explain why this is not needed.

Our reply: We are grateful for this comment, and we have adjusted our analyses accordingly to include adjustments for age and BMI as these are different in the healthy and type 2 diabetes cohort and known to influence low-grade inflammation (See the revised figure 1). Likewise, we have revised the analysis in Table 2 to include an unadjusted and adjusted model (See the revised Table 2)

7. In paragraph 3.3 and several other occasions is spoken of prediction, while data is cross-sectional.

Our reply: We thank the reviewer for pointing this out, and we agree that our data does not support any statements of prediction. We have therefore changed "prediction" with "association" throughout the manuscript (highlighted in yellow).

8. In figure 2 remains unclear how biomarkers were selected.

Our reply: All biomarkers were included in the statistical analysis, but only those with a p-value below 0.05 were shown in figure 2. We have specified this in the text:

"only analytes with p-values below 0.05 shown"

9. Be careful in how the discussion is written. Just report on associations and differences between groups, as data is cross-sectional, don't make bigger statements. How does your data support that CRP production is induced by the presence of TNF-a and IL-6? Please go through the discussion to avoid such statements.

Our reply: We agree with the reviewer that our data does not support such statements. We have revised the discussion (all changes highlighted in yellow)

10. The authors suggest that free smoking cessation programs might be successful, something they did not study at all. Selection or reporting bias seems much more realistic here.

Our reply: We agree that the low number of current smokers may reflect selection or reporting bias, and have revised the text:

"*The low number of current smokers may reflect selection or reporting bias or perhaps successful free smoking cessation programs, as 40% of our participants reported to be previous smokers. This is, however, highly speculative. Nonetheless, the degree of a persistent pro-inflammatory effect of nicotine following smoking cessation is debated (38), and could potentially be influencing the results in the current study."*

11. I did not see a reporting checklist.

Our reply: We apologize for the inconvenience. We have uploaded the STROBE checklist along with the revised manuscript.

VERSION 2 – REVIEW

REVIEWER	Aso, Yoshimasa Dokkyo Medical University
REVIEW RETURNED	20-Sep-2022

GENERAL COMMENTS	The present study investigated low-grade inflammation in type 2 diabetes and explore associations to clinical aspects as well as micro- and macrovascular complications. The authors concluded that the level of low-grade inflammation in type 2 diabetes is associated with obesity, glycemic regulation, therapeutical management, sex, and complications. Comments 1. The authors should describe the definition of cardiac autonomic neuropathy in detail. How was the cut-off value for each reflex test? 2. Please add the prevalence of diabetic retinopathy, nephropathy, neuropathy, or cardiac autonomic neuropathy, respectively, in T2D group to Table 1. 3. How many patients did have cardiovascular disease (CVD) among people with T2D? please add the prevalence of CVD in people withT2D to Table 1. 4. Did you look at any differences in inflammatory biomarkers between people with and without CVD? 5. In Table 1, Hba1c should be corrected to HbA1c. 6. Plasma eotaxin supposed be decreased after administration of DPP-4 inhibitors. Please comment this.

REVIEWER	Muilwijk, Mirthe Amsterdam UMC, Public Health
REVIEW RETURNED	11-Oct-2022

GENERAL COMMENTS	Thank you for incorporating my comments.

VERSION 2 – AUTHOR RESPONSE

Response to Reviewer 1

1. The authors should describe the definition of cardiac autonomic neuropathy in detail. How was the cut-off value for each reflex test?

Our reply: We have added references regarding measurements and definitions of cardiac autonomic neuropathy to manuscript

2. Please add the prevalence of diabetic retinopathy, nephropathy, neuropathy, or cardiac autonomic neuropathy, respectively, in T2D group to Table 1.

Our reply: We thank the reviewer for this suggestion and have added prevalence of diabetic comorbidities to Table 1.

3. How many patients did have cardiovascular disease (CVD) among people with T2D? please add the prevalence of CVD in people withT2D to Table 1. Did you look at any differences in inflammatory biomarkers between people with and without CVD?

Our reply: We have thorough recordings on the neuropathic stage and manifestations, and fortunately we are able to identify that 40% of our cohort had early or manifest CAN. Encouraged by the reviewer, we have conducted a statistical analysis (logistic regression) to identify whether the levels of inflammatory markers were heightened in the group presented with CAN. Based on our cohort, we could not show such differences and thus the following have been added to the text:

"Similarly, we investigated whether presence of CAN (early or manifest) influenced the levels of inflammatory factors, however, none of these reached significant levels (data not shown)."

5. In Table 1, Hba1c should be corrected to HbA1c.

Our reply: We thank the reviewer for pointing out this misspelling in Table 1

6. Plasma eotaxin supposed be decreased after administration of DPP-4 inhibitors. Please comment this.

Our reply: We have added a comment of the conflicting results regarding eotaxin levels and DPP-4 inhibitor therapy in our and a previous study. The paragraph now reads:

"Surprisingly, we found lower levels of three DPP-4 substrates (IL-8, IP-10, and MDC) in connection with DPP-4 inhibitor therapy. Though seemingly in contrast with the expected result, similar observations have previously been reported e.g. lower levels of eotaxin in type 2 diabetes during DPP-4 inhibitor therapy (31). In our study, however, eotaxin levels were unaffected by DPP-4 inhibitor therapy, underlining the need for further research in the immunomodulating effects of these compounds."

VERSION 3 – REVIEW

REVIEWER	Aso, Yoshimasa Dokkyo Medical University
REVIEW RETURNED	18-Nov-2022

GENERAL COMMENTS	No comments.