Author's response to reviews

Title: The relationship between N-terminal prosomatostatin, all-cause and cardiovascular mortality in patients with type 2 diabetes mellitus (ZODIAC-35)

Authors:

Peter R. van Dijk (p.r.van.dijk@isala.nl)
Gijs W.D. Landman (g.w.d.landman@isala.nl)
Larissa van Essen (essenl@isala.nl)
Joachim Struck (struck@sphingotec.de)
Klaas H. Groenier (k.h.groenier@umcg.nl)
Henk J.G. Bilo (h.j.g.bilo@isala.nl)
Stephan J.L. Bakker (s.j.l.bakker@umcg.nl)
Nanne Kleefstra (n.kleefstra@isala.nl)

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Reply to Reviewers

Title:

The relationship between N-terminal prosomatostatin, all-cause and cardiovascular mortality in patients with type 2 diabetes mellitus (ZODIAC-35).

Authors:

Peter van Dijk, Gijs Landman, Larissa van Essen, Joachim Struck, Klaas Groenier, Henk Bilo, Stephan Bakker and Nanne Kleefstra

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Corresponding author:

P.R. van Dijk

Diabetes Centre, Isala

P.O. box 10400

8000 G.K. Zwolle, The Netherlands

Email: P.R.van.Dijk@isala.nl
1. It was a very good idea of the authors to look at the predictive value of N-terminal prosomatostatin (NT-proSST) in patients with type 2 diabetes. Although this is a negative study because NR-proSST was not independently associated with all-cause mortality and with cardiovascular mortality, this work is of importance in its field. The strength of this study is that this is the first description of the predictive capability of NT-proSST in patients with diabetes. However, there are some criticisms that should be addressed in a revised manuscript.

   **Reply:** We would like to thank the reviewer for the comments and suggestions. We feel that his comments have improved the manuscript. According to the reviewer’s comments we have made changes throughout the manuscript (marked in yellow), which will be discussed in a point-by-point rebuttal below.

2. The present patient sample should be better described in a revised version (not only referring to references 19-21). In PubMed, I can find articles on several ZODIAC “studies” (up to ZODIAC-41) including those evaluating several other BRAHMS biomarkers. At least for me, the study population of the present work is unclear. If I correctly understand, approx. 3400 diabetic individuals at baseline in 1998 and 4500 diabetic individuals at 2-year follow up in 2000 were described in reference 20, obviously being the primary description of the ZODIAC trial (Eur J Epidemiol 2003;18:793-800). However, in the present manuscript the authors describe 1143 individuals from ZODIAC-1. In reference 19 (Neth J Med 2005;63:103-10), a target population consisting of 2660 patients with type 2 diabetes is described within the ZODIAC trial. How do the respective numbers fit together? Why was an additional number of 546 patients “enrolled” in 2001? Reference 21 (Diabetologia 2009;52:789-97) is related to the UK Prospective Diabetes Study (UKPDS); this publication refers to 973 individuals with diabetes (but not 546 as stated). Is the present manuscript a study reporting on a combined sample consisting of individuals from the Netherlands and the UK? Which kind of study did the local medical ethics committee approve? In the title of the manuscript, the authors use the acronym ZODIAC-35; what does “35” mean?

   **Reply:** ZODIAC is an acronym for Zwolle Outpatient Diabetes project Integrating Care and started in 1998 as a prospective observational study for patients with T2DM [1]. The patients participating in the ZODIAC study are known with T2DM and exclusively treated in primary care. In the first year, 1,143 patients with T2DM were included in this prospective observational study.
In 2001, another 546 unique patients with T2DM entered the study: originally these 546 patients participated in the observational study by Lutgers et al. and was part of a PhD project regarding skin-auto fluorescence (Skin-AF) measurements and the prediction of cardiovascular complications in T2DM (as compared to risk prediction scores in this cohort generated using the UKPDS risk engine) [2]. Of 973 participants in the ‘Skin-AF’ study, 427 persons already participated in the ZODIAC study, so there were 546 unique/new patients. Thus, the combined (original ZODIAC + ‘Skin-AF’) ZODIAC cohort consisted of 1,689 patients. Of all these patients plasma samples were available and for the current study we determined NT-proSST from this material. Plasma concentrations of NT-proSST was measured in 1,327 of these patients and one patient was excluded because of extremely high NT-proSST concentrations (71,300 pmol/L).

We agree with the reviewer that this manuscript would profit from more information on the ZODIAC study in general. Accordingly, we added this information in the Materials section:

“The Zwolle Outpatient Diabetes Project Integrating Available Care (ZODIAC) study was initiated in 1998, in the Zwolle region of the Netherlands. One of the initial study goals was studying the effects of task delegation from physicians to specialist nurses, details have been published previously [20, 21]. As a subcategory of the ZODIAC study the effects of several biomarkers, including NT-proSST, on risk prediction in T2DM were planned and blood was stored for this purpose. The ZODIAC study cohort consisted of Dutch T2DM patients treated exclusively in primary care. Patients were only excluded if they were already treated in secondary care for their diabetes, if they had a very short life expectancy (including patients with active cancer) or if they were considered to have insufficient cognitive abilities [20]. In the first year, 1,143 patients with T2DM were included, and in 2001, 546 patients with T2DM were enrolled, which resulted in a combined study population of 1,689 patients [22]. The ZODIAC study was approved by the local medical ethics committee (Isala, Zwolle), and all patients gave informed consent.”

The local ethics committee of Isala (Zwolle, The Netherlands) approved the ZODIAC study. We have also added the name of the ethical committee to the manuscript.”

“The ZODIAC study was approved by the local medical ethics committee (Isala, Zwolle), and all patients gave informed consent.”
3. Is this (i.e., the data of the present manuscript) a prospectively or retrospectively conducted study? Was it a post hoc decision to measure NT-proSST plasma concentrations in the present study sample? The nature of this study should be clarified in a revised manuscript.

Reply: The data presented in the manuscript were prospectively collected. In addition, prior to the start of the ZODIAC study in 1998 the decision was made to collect blood samples to research the predictive capabilities for biomarkers in the future. As such, one could argue that this study has a prospective design. One the other hand, we decided to analyse NT-proSST specifically later. Taken together, in accordance with studies with a similar design [3, 4], we feel that this is a prospective study design. Nevertheless, this matter is open for debate and therefore we think it is appropriate to give the reader more detailed information concerning the study design:

“The Zwolle Outpatient Diabetes Project Integrating Available Care (ZODIAC) study was initiated in 1998, in the Zwolle region of the Netherlands. One of the initial study goals was studying the effects of task delegation from physicians to specialist nurses, details have been published previously [20, 21]. As a subcategory of the ZODIAC study the effects of several biomarkers, including NT-proSST, on risk prediction in T2DM were planned and blood was stored for this purpose.”

“Baseline data were collected in 1998 and 2001, including a full medical history”

“NT-proSST was measured in non-fasting plasma samples collected at baseline and kept frozen at -80° Celsius until analysis in 2010.”

4. The setting of this study is not clearly described in the present manuscript. I suppose it was a primary care setting in both patient samples?

Reply: The reviewer is right in pointing us to this omission, this was indeed a study in a primary care setting. Accordingly, we added the information to the paper.

“The study cohort consisted of Dutch T2DM patients treated exclusively in primary care.”

5. It is unclear of whether the study sample is a consecutive patient series or not. Inclusion and exclusion criteria should be stated explicitly in the revised manuscript.

Reply: Indeed, the current study includes two series of consecutive patients. Accordingly, we have added the inclusion and exclusion criteria to the manuscript.
“The ZODIAC study cohort consisted of Dutch T2DM patients treated exclusively in primary care. Patients were only excluded if they were already treated in secondary care for their diabetes, if they had a very short life expectancy (including patients with active cancer) or if they were considered to have insufficient cognitive abilities.”

6. The study was approved by the local ethics committee. What was the a priori study hypothesis in the respective study protocol? The study hypothesis (H1 vs. H0) should be provided in a revised manuscript. The authors should provide their a priori sample size calculation according to the study hypothesis.

Reply: We did not completely understand the reviewer on this point. We could provide the power calculations and hypothesis testing for both the original ZODIAC and Skin-AF studies, although we doubt whether this would add to the current manuscript. For the current study we believe there is no clear advantage for reporting a formal power calculation, over the 95% confidence intervals and we followed the stand-point by, amongst others Vickers and Altman, on this topic [5–7]. In addition there is no information available regarding what would be a sensible Hazard Ratio for NT-proSST to expect and to base a power calculation on.

7. Is there a trial number at ClinicalTrials.gov.

Reply: We did not register this study at ClinicalTrials.gov. We are not aware of any consensus on whether registration of observational studies is mandatory. The original ZODIAC study was performed before the era of registering clinical trials at Clinicaltrials.gov but it was registered it at our local medical ethics committee, which also approved the study.

8. What were exact diagnostic criteria for type 2 diabetes? Did the study participants suffer any comorbidity besides cardiovascular disease?

Reply: We agree with the reviewer that this information was missing from the manuscript. The diagnostic criteria for T2DM in both the original ZODIAC and the ‘Skin-AF’ cohort were the same and were based on the diagnostic criteria used in the primary care diabetes treatment guideline of the Dutch college of general practitioners of 1989 and 1999 (based on the 1985 World Health Organisation (WHO) and 1997 American Diabetes Association (ADA) criteria, respectively)[8, 9]. The validity of the diagnosis type 2 diabetes was checked in the individual patient files by looking up the glucose measurements the diagnosis was based upon, and comparing these measurements with the criteria for diabetes in the national guideline for each patient.
We have data on microvascular diseases at baseline but we do not have data on comorbidity besides cardiovascular disease and microvascular complications. To accommodate the comment of the reviewer, we added the following sentences to the manuscript and we have added the lack of data on non-cardiovascular comorbidity as a limitation of the study to the discussion section of the revised version.

“The diagnosis of diabetes was based on the diagnostic criteria used in the primary care diabetes treatment guideline of the Dutch college of general practitioners of 1989 and 1999 (based on the 1985 World Health Organisation (WHO) and 1997 American Diabetes Association (ADA) criteria, respectively). The validity of the diagnosis type 2 diabetes was checked in the individual patient files by looking up the glucose measurements the diagnosis was based upon, and comparing these measurements with the criteria for diabetes in the national guideline for each patient [10].”

“Unfortunately, data on relevant comorbidity besides cardiovascular diseases are unknown.”

9. The authors state that endpoints were all-cause mortality and with cardiovascular mortality, and endpoint ascertainment was made in 2009. Why did the authors chose to obtain mortality data in 2009? This is 5 years ago. What was the exact time point of outcome ascertainment?

Reply: Vital status or date and cause of death was retrieved in (January to March) 2009 using hospital records or by contacting the general practitioner.

The year 2009 was chosen based on the assumption that after a follow-up period of 10 years (for the original ZODIAC cohort) a relevant proportion of the population would be deceased, enabling us to do the first analyses. Furthermore, there were more pragmatic reasons: in those years we had the funding available for the biomarker determinations and a physician to retrieve the status of the T2DM patients in the ZODIAC cohort.

In order to clarify this matter, we added a more detailed description to the methods section:

“In 2009, vital status and cause of death were retrieved from records maintained by the hospital and general practitioners.”

10. The authors state that the causes of death were determined according to International Code of Diseases Version 9 (ICD9). Why didn’t they use the ICD10? How did they define cardiovascular mortality - ICD9/ICD10 codes 390-459/I00-I99?
Reply: The ICD-9 codes were used by the original ZODIAC investigators in 1998, we adapted the use of ICD-9 for reasons of uniformity. For cardiovascular mortality we indeed used the ICD-9 codes 390 – 459. To accommodate the comment of the reviewer, we have added this to the revised version of the manuscript:

“Cardiovascular death was defined as death in which the principal cause of death was cardiovascular in nature, using ICD-9 codes 390-459.”

11. How was follow up data obtained? Were the hospital records of all hospitals of the Netherlands and the UK studied? Were all practitioners in the Netherlands and the UK surveyed for endpoint ascertainment of each study participant? What about individuals who died outside the Netherlands and the UK?

Reply: Vital status and cause of death were retrieved from records maintained by the hospital and the general practitioners. In the ZODIAC study, all participants were Dutch and no UK patients were included (please see our response to comment 2 for more information). The few patients (n=3) that moved out of the catchment area of the ZODIAC study were censored at the last contact date.

We have added the following sentence to the manuscript:

“In 2009, vital status and cause of death were retrieved from records maintained by the hospital and general practitioners.”

12. Were outcome data available for all patients? No missing data? What about censored data in the Kaplan-Meier curves including number at risk tables?

Reply: We agree with the reviewer that there was a lack of information on this topic. For all patients with NT-proSST measurements (n= 1,326) outcome data were available. To give the reviewer some additional information about the survival analyses, missing data and numbers at risk we performed extra analyses.

Total number of events and number censored per quartile for all-cause mortality:

<table>
<thead>
<tr>
<th>Quartile</th>
<th>Total N</th>
<th>N of Events</th>
<th>N Censored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartile 1</td>
<td>332</td>
<td>73</td>
<td>260</td>
</tr>
<tr>
<td>Quartile 2</td>
<td>330</td>
<td>74</td>
<td>254</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>332</td>
<td>115</td>
<td>218</td>
</tr>
<tr>
<td>Quartile 4</td>
<td>332</td>
<td>151</td>
<td>181</td>
</tr>
</tbody>
</table>
Total number of events and number censored per quartile for cardiovascular mortality:

<table>
<thead>
<tr>
<th></th>
<th>Total N</th>
<th>N of Events</th>
<th>N Censored</th>
</tr>
</thead>
<tbody>
<tr>
<td>Quartile 1</td>
<td>332</td>
<td>30</td>
<td>303</td>
</tr>
<tr>
<td>Quartile 2</td>
<td>330</td>
<td>25</td>
<td>303</td>
</tr>
<tr>
<td>Quartile 3</td>
<td>332</td>
<td>43</td>
<td>290</td>
</tr>
<tr>
<td>Quartile 4</td>
<td>332</td>
<td>78</td>
<td>254</td>
</tr>
<tr>
<td>Total</td>
<td>1326</td>
<td>176</td>
<td>1150</td>
</tr>
</tbody>
</table>

To give the reader a more comprehensive view of the outcome data, including the progressively lower number of patients at risk during the follow-up period, we added the number of patients at risk at the specific time points (2, 4, 6, 8 and 10 years) in figure 1. We would like to refer to this figure for more details.

3. The authors state that NT-proSST plasma concentrations were measured with a chemiluminescence assay. Where were the measurements done? How were the plasma aliquots stored? How many freeze and thaw cycles were evident in the study samples? What was the time interval between freezing the samples and performing NT-proSST measurements? What about in vitro analyte stability? Were the samples assayed in duplicate? Where from did the authors derive the imprecision data and the stability data at 22°C? The limit of detection is claimed to be 4 pmol/L. What is the measurement range of this assay? This information is essential for interpreting the authors’ data.

Reply: We agree with the reviewer that this information is vital. The measurements were performed at B.R.A.H.M.S. The aliquots were stored at -80°C, underwent only one freeze and thaw cycle. Measurements were performed in 2010: the subsequent interval was 12 years for samples taken in 1998 and 9 years for samples taken in 2001. We evaluated the stability of the native analyte at 22°C and 37°C in EDTA-plasma from 10 different individuals. Samples were analysed in duplicate. At 22°C the analyte was stable (<10% loss of immunoreactivity) for 72 h and at 37°C for 24 h. In 5 EDTA-plasma samples, freezing and thawing 4 times had no influence on the measured concentration of proSST (mean values, 99.1% [range, 93.8%-104.3%] of the original values). The lowest concentration detectable with an interassay CV of just below 20% was 19 pmol/L.
We adjusted the relevant part of the materials section by adding this information:

“NT-proSST was measured in non-fasting plasma samples collected at baseline and kept frozen at -80° Celsius until analysis in 2010. NT-proSST was measured using an assay in the chemiluminescence/coated tube-format (B.R.A.H.M.S. GmbH, Hennisdorf/Berlin, Germany) [11]. For this study, the assay used had a detection limit of 4 pmol/L; the inter-laboratory coefficient of variation (CV) was 20% at 18 pmol/l, 10% at 50 pmol/l, and <6% for NT-proSST concentrations above 100 pmol/L (highest calibrator concentration used was 2500 pmol/L). The stability of the native analyte at 22 °C and 37 °C was tested in EDTA-plasma from 10 different individuals. At 22 °C the analyte was stable (< 10 % loss of immunoreactivity) for 72 h and at 37 °C for 24 h. Samples were analysed in duplicate. And although the samples were only thawed for analysis, prolonged frozen storage and repeated (4 times) freeze-thaw cycles had no effect on measured NT-proSST concentration: in 5 EDTA-plasma samples, freezing and thawing 4 times had no influence on the measured concentration of proSST (mean values, 99.1% [range, 93.8%-104.3%] of the original values).”

14. NT-proSST plasma concentrations were measured in 1327 patients only (as stated in the materials section, 1689 individuals were studied). Why?

Reply: Unfortunately there material was either not available or there was too little to determine the NT-proSST plasma concentrations in all individuals studied, therefore it was determined in 1327 (79%) of the study population.

We added extra information about individuals without NT-proSST measurements to the methods section:

“Baseline plasma NT-proSST values could be measured in 1,327 (79%) patients. One patient was excluded because of extremely a high value of NT-proSST (71,300 pmol/L). Because not all patients had NT-proSST values, we compared the baseline characteristics of subjects from whom samples were available to those without. Besides a slightly higher, but not appreciably clinically relevant difference in serum creatinine among patients without NT-proSST measurements (92 [IQR 82 - 104] µmol/L versus 93 [IQR 84 - 106] µmol/L, p=0.02) there were no significant baseline differences in patients with and without NT-proSST measurements. In a separate Cox regression analyses the association between the presence or absence of a NT-proSST measurement and CV and all-cause mortality in the combined cohort of 1,689 patients was tested. For all-cause, but not for cardiovascular, mortality there was an increased hazard ratio (HR) (1.33, 95% CI 1.08 – 1.63) for patients with missing NT-proSST measurements as
compared to patients with NT-proSST measurements. This outcome may suggest an underestimation of the observed relationship between plasma concentrations NT-proSST and all-cause mortality."

15. In the Zodiac study other analytes were measured (Table 1). How were the other clinical and biochemical measures obtained?

Reply: We initially did not add these data to manuscript, in the revised version we added detailed information on and biochemical measures and added the following sentences:

“Baseline data were collected in 1998 and 2001, including a full medical history, including a history of cardiovascular diseases (CVD) and tobacco consumption. Patients were considered to have a history of CVD if they had a history of angina pectoris, myocardial infarction, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, stroke or transient ischemic attack. Laboratory and physical assessment data were collected annually and included non-fasting lipid profile, glycated hemoglobin (HbA\textsubscript{1c}), serum creatinine (sCr), albumin-to-creatinine ratio (ACR) in a portion of urine, and blood pressure. Scr was measured by a kinetic colorimetric Jaffe method (Modular P Analyzer, Roche Almere, the Netherlands), ACR was measured using immunonephelometry (Behring Nephelometer; Mannheim, Germany), and blood pressure was measured twice with a Welch Allyn sphygmomanometer in the supine position after at least 5 minutes of rest.”

16. For several reasons, all continuous data should be expressed as median (interquartile range), and only non-parametric tests should be used. How did the authors address the multiple testing problems in this work?

Reply: In the revised manuscript all continuous data are expressed as median with interquartile range. We also used non-parametric tests. Please see table 1.

Concerning multiple testing: We did not correct for multiple testing based on the advice made in a recent paper which states “adjustments for multiple testing are required in confirmatory studies whenever results from multiple tests have to be combined in one final conclusion and decision”, which we think is not applicable to this situation [12].

17. At least to my knowledge, the authors performed interventions in the ZODIAC trials. Does this hold true for the individuals enrolled into the present work as well? Were the results on the predictive value of NT-proSST independent of the intervention groups or did the have an impact on the results provided?
Reply: The initial cohort received an intervention as was part of the ZODIAC study. This interventions involved a more extensive (including a diabetes register, structured recall, facilitated generalist–specialist communication, audit and feedback, patient-specific reminders and it emphasised patients’ education) or limited form of shared care [1].

As the blood samples were taken at baseline of this study, i.e. before the intervention, and the nature of this intervention does not interfere with concentrations of NT-proSST (to the best of our knowledge) we don’t feel that this could have influenced our results. Nevertheless, we cannot exclude the possibility that it did have an influence. Therefore we feel we have to mention this as a limitation of this study.

“Furthermore, we cannot exclude the presence of difference in outcomes between patients who received task delegation care during the first 3 years of the ZODIAC study and patients who did not.”

18. The authors cite 25 references. Of them only 6 were published after 2000 and only one was published after 2010 (an abstract on the AACC 2013). The authors are recommended to update the literature and, thus, to include more recently published references.

Reply: We thank the reviewer for this suggestion. Unfortunately there is very little relevant recent literature on this topic. Most research original research on NT-proSST was performed before the year 2000. Nevertheless, we did another literature search, and added 9 references to the manuscript. We kindly refer to the reference list in the revised manuscript for an overview of these new references (marked in yellow).

19. The authors could use different measurements of performance (discrimination, calibration and reclassification by IDI and NRI) to test the potential incremental prognostic value of NT-proSST in the setting evaluated.

Reply: We thank the reviewer for this suggestion. We discussed this in advance and we had several reasons not to use the NRI or the IDI. Firstly, concerns have recently been raised for the use of these measures because they are not ‘proper scoring rules’, meaning that the prognostic performance can be manipulated [13–15]. Secondly, there are no generally accepted cut-off points for either NT-proSST or 10 year all-cause or cardiovascular mortality categories. And thirdly, the NRI and IDI were not developed in the context of censored data.
20. The authors use terms such as “serum levels” and “plasma values” throughout the entire manuscript. The authors should instead use the term “NT-proSST plasma concentrations” consistently.

Reply: We want to thank the reviewer for this comment and changed this throughout the manuscript.
The opinion about using N-terminal prosomatostatin as risk indicator deserves more discussion. Do you advice the reader not to use this measurement in that way or do you see (future) applications?

First of all, we want to thank the reviewer for his thoughts on the present study and think his effort has improved the manuscript.

At present, there is scarce literature on N-terminal prosomatostatin (NT-proSST) which makes it difficult to give an clear advice regarding the use of NT-proSST in daily practice. Based on this one study, NT-proSST should not be used as a marker for (cardiovascular) mortality among stable outpatients with T2DM.

We added a more detailed interpretation of our findings. In the last paragraph of the discussion paragraph we give the reader an unambiguous conclusion on the use of NT-proSST for risk prediction in T2DM and we also speculate on possible future research areas for NT-proSST.

“Adding plasma NT-proSST concentrations to a model with potential confounders and well-known cardiovascular risk factors for mortality did not improve the Harrel’s C statistic compared to the fully adjusted model without plasma NT-proSST concentrations, indicating a lack of benefit in risk prediction when adding NT-proSST to the model.”

“Nevertheless, the present study adds to the current literature by describing for the first time the predictive capabilities of NT-proSST in a large cohort of patients with T2DM with sufficient follow-up. Based on these results, NT-proSST appears to be no suitable biomarker for cardiovascular an all-cause mortality prediction in patients with T2DM. However, these results do not exclude a role for NT-proSST a potential marker for short term risk prediction and further research should focus on the use of NT-proSST as a biomarker in specific areas such as acute heart failure in non-DM subjects and several neuro-endocrine and gastro-intestinal processes [3, 19, 33, 34].”
References


11. Diagnostic use of prosomatostatin. 


