Peer Review File

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Reviewer A

The current study by Camacho-Ordonez and colleagues measured tear levels of cytokines in 16 subjects with PD 16 age- and gender-matched controls. Associations between cytokines, motor 53 features, and dry eye symptoms were made for persons with PD. Here, TNF-α tear levels were notably higher in PD subjects than in controls. Furthermore, no differences were found in cytokine concentrations between PD patients with DED and those without DED. IL-8 was associated with the HY stage, TBUT, DEQ-5, and a better MoCA score. A higher BR correlated moderately with a lower HY stage, and DED patients have lower BR in PD. Overall, this is an important line of research, but please address the following points:

Comment 1. Please consider adding diagrams of the experiment's setup, specifically for the Tear Film Break-Up Time (TBUT) and fluorescein staining and for the tear collection.

Reply 1. We thank the reviewer for this comment. We have added the diagrams for the Tear Film Break-Up Time (TBUT), fluorescein staining, and for tear collection.

Changes in the text. We included the diagrams of the experiment, see Figure 1-3.

Comment 2. Statistical analysis: Did the researcher control for multiple comparisons when running the Spearman's rank correlation? If not, please do so.

Reply 2: We appreciate the commentary, the Bonferroni-adjusted confidence intervals of Spearman's rank correlation coefficients were employed for the analyses to account for multiple comparisons. This approach ensures that the probability of falsely detecting a significant correlation by chance is appropriately controlled, considering the increased likelihood of Type I errors due to conducting multiple tests. By applying the Bonferroni correction, each individual confidence interval was adjusted to maintain an overall desired level of confidence, preserving the validity of the statistical inferences made from the results.

Reviewer B

Camacho-Ordonez et al. measured pro-inflammatory cytokine levels in tears of patients with Parkinson's disease. The study cohort consisted of 16 patients with PD and 16 controls. The BD Human Inflammatory Cytokine Cytometric Bead Array was used to measure IL-1b, IL-6, IL-8,

IL-10, IL-12p70, and TNF levels. IL-1b and TNF levels were increased in tear samples of patients with PD compared to controls. IL-8 levels were increased in PD patients with dry eye disease.

The study is of interest, but enthusiasm is dampened due to the small cohort size and missing information on the demographics of the control groups.

Comment 1: Demographic data including age, sex, and possible medications that cause eye problems are not provided for the control cohort. Was the control cohort age- and sex-matched to the PD group?

Reply 1. We appreciate this observation and we have added a statement in the methods. The control cohort was age- and sex-matched to the PD group, see page 7, line 137.

Changes in the text. See page 9, line 184: ***"****The control group was excluded if the systemic or ocular medication caused possible eye problems".*

Comment 2: Table 3 needs to be visualized using a bar graph showing the individual data points for both groups.

Reply 2. We thank you for this observation, and we have changed the table to a bar graph of the cytokines comparison for both groups.

Changes in the text. Figure 4 included the bar graphs of the cytokines.

Comment 3: The authors need to write why IL-8 and IL-10 were measured.

Reply. 3: We thank the reviewer for the comment and we have expanded the discussion

Changes in the text: See page 14, line 311: "*It is reported that these cytokines are linked to microglial activation as a consequence of excessive localized brain inflammation and neurotoxicity in neurodegenerative diseases (41).*

IL-8 is a chemokine produced by macrophages. Its receptor, the CXCR2 has been detected in dystrophic neurites, suggesting that IL-8 mediates glial interactions with neurons and thereby contributes to neuronal damage (42). Previous studies have shown inconsistent results of serum IL-8 levels in PD. Williams-Gray et al. (43) and Fu J et. al (44) reported no statistical difference, but Gupta et al. (45) showed that IL-8 was significantly decreased in the serum in PD compared to controls (patients = 222.70 ± 200.91 pg/ml, controls = 1584.44 ± 504.44 pg/ml; p <0.001). Also, serum IL-8 level was positively associated with disease duration, depression, and UPDRS III in PD patients (45,46).

IL-10 is an anti-inflammatory cytokine modulator of glial activation in neurodegenerative diseases. High levels have been reported in PD compared with healthy controls (47). However, in another study, it was described that PD patients with more severe clinical characteristics and a non-tremor type showed reduced serum IL-10 levels (48)."

Reviewer C

This is an interesting and nicely described paper. In this paper the author explained that PD patients have higher proinflammatory cytokines for example, for example, TNFa, IL-1b in tears in comparison with age-matched healthy controls. PD patients were subdivided into DED (Dry Eye Disease) and without DED. But there were no differences in cytokine differences between PD with DED and without DED. TNFa cytokine production was significantly higher in PD patients compared to healthy controls. Moreover, the author described that IL-8 in tears may be involved in the severity and dry eye in PD.

However, I have some remarks about the texts-The author mentioned TNFa cytokine secretion was higher in PD patients an observation that was also reported by Comoglu SS et al. (ref 24). Matilda Roda et al (2020), (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7246678/pdf/ijms-21-03111.pdf) showed that proinflammatory cytokine production was higher in DED patients compare to non- DED patients.

Comment 1: Could you please describe, what is the major accomplishment of this paper that makes it a unique article compared to the existing published paper?

Reply 1. Comoglu et al. study was interesting. The purpose of the study was to determine TNF-α levels in tear samples obtained from patients with PD and to analyze the relationship between TNF-α values and PD characteristics. We were inspired by his study and we added the quantification of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α in tears, but also, we investigated dry eye disease which is linked to an increase of cytokines in tears as shown in Matilda Roda et al. metanalysis. However, we reported no differences in cytokines concentrations between PD patients with DED compared to those without DED, but we showed significant differences among patients with DED in PD regarding their BR and HY stages.

Comment 2: (page 10, sentence 219) 50% (n=8) of PD subjects and 12.5% (n=2) controls had DED symptoms. Do you have any data on the proinflammatory cytokine profile with control DED groups? Is it possible to make any prediction or hypothesis or comments that proinflammatory cytokine in PD patients with DED has different pattern then DED patients without PD.

Reply 2. We thank for this comment and we have described in the text the pro-inflammatory cytokine profile with control DED. The different pattern of pro-inflammatory profile between

PD with DED and DED without PD could be explained by an incomplete blinking and a decrease BR in PD patients due to a deficiency in dopamine. These changes could lead to tear film hyperosmolality, accelerated tear evaporation, and finally corneal damage causing DED.

Changes in the text: See Page 12, line 261: ***"****The pro-inflammatory cytokine profile of HC with DED was: IL-1 (0.11±0.16 pg/mL), IL-6 (2.10±2.14 pg/mL), IL-8 (92.79±27.45pg/mL), IL-10 (0 pg/mL), IL-12 (0.41±0.57pg/mL), and TNF (0.1±0.01pg/mL)"*

See Page 16, line 350: *In PD incomplete blinking and a decrease in BR can be observed due to a deficiency in dopamine. These changes could lead to tear film hyperosmolality, accelerated tear evaporation, and finally corneal damage causing DED (52). In our study, a higher BR correlated moderately with a lower HY stage (r=-0.645, p=0.007), and patients with DED have lower blink rates (12.14±2.54 vs 9.0±2.06, p=0.031). In contrast, Fitzpatrick et al. could not find an association between BR and disease severity (53)*

Minor comments:

Comment 3: Reference number would be 24 instead of 23 (page 6, sentence 119).

Reply 1: We thank you for this observation, but the reference number 24 is about the MDS-UPDRS scale, and the sentence is describing the Çomoğlu S et al. study about TNF-α levels in tears in PD (reference number 23).

Reviewer D

Abstract

Comment 1: Results part need to as well introduce all results like iL-1β mentioned in the conclusion.

Reply 1. We appreciate this comment and we deleted these results from the conclusion because the levels of interleukin-1β were not statistically significant (1.75±4.36 vs 0.01±0.05, p=0.068).

Changes in the text: We deleted the sentence in the conclusion.

Comment 2: Next conclusion need to open shortly and tell why it is concluded like this "This study showed significant differences among patients with dry eye in PD regarding their BR and HY stages"

Reply 2: We thank the reviewer for the comment and we changed the sentence

Changes in the text: See Page 4, line 75: *"In addition, our findings suggest that as HY stage increases, indicating a more advanced stage, BR decreases, indicating greater motor impairment. Conversely, the presence of DED is associated with higher levels of bradykinesia in PD patients, suggesting a potential relationship between DED and motor impairment severity".*

Comment 3: Mention shortly why IL-8 and IL-1β are selected to measure. Why in next paragraph mentioned IL-4, IL6, IL-12 and interferon gamma are not measured?

Reply 3: We appreciated this comment and we have expanded the discussion.

Changes in the text: See Page 14, line 311: *"It is reported that these cytokines are linked to microglial activation as a consequence of excessive localized brain inflammation and neurotoxicity in neurodegenerative diseases (41).*

IL-1β is a pro-inflammatory cytokine implicated as the main effector of the functional consequences of neuroinflammation on neurodegeneration in PD models. Prolonged pro-inflammatory IL-1β expression in the substantia nigra (SN) results in significant and permanent dopaminergic neuronal death in the SN (42).

IL-8 is a chemokine produced by macrophages. Its receptor, the CXCR2 has been detected in dystrophic neurites, suggesting that IL-8 mediates glial interactions with neurons and thereby contributes to neuronal damage (42). Previous studies have shown inconsistent results of serum IL-8 levels in PD. Williams-Gray et al. (43) and Fu J et. al (44) reported no statistical difference, but Gupta et al. (45) showed that IL-8 was significantly decreased in the serum in PD compared to controls (patients = 222.70 ± 200.91 pg/ml, controls = 1584.44 ± 504.44 pg/ml; p <0.001). Also, serum IL-8 level was positively associated with disease duration, depression, and UPDRS III in PD patients (45,46)."

* Indeed IL-4 and interferon-gamma were not measured although are increased in the serum of PD patients, and related to neuroinflammation in PD

Reply: We appreciate this observation, and we added a statement on limitations

Changes in the text: see Page 17, line 374: "Although we did not measure IL-4 and IFN-γ, it has been reported they are increased in serum of PD patients and linked to neuroinflammation.

Background

Comment 4: Explain at the end of the paragrpah why this study hase been conducted. Now there is basicly reported based to the previous experiments same cytokine detected already. Shortly explain the novelty value of the present study.

Reply 4: We appreciate this suggestion and have added a statement in the text.

Changes in the text: See Page 6, line 127: *"To the best of our knowledge, this is the first study to investigate the quantification of the proinflammatory profile in tears and also the existence of DED in PD patients. DED is usually linked to an inflammatory profile and seen frequently in patients with PD"*

Comment 5: Confusing end of the background " We present the following article in accordance with the STROBE reporting checklist". May this need to be some other part of the manuscript? At the end of the manuscript?

Reply 5: We thank the reviewer for the comment and we change the statement at the end of the manuscript since this statement is required by the journal.

Materials

Comment 6: Include all detailed related to product/kit information.

Reply 6: We appreciated the observation and we have added the link to consult the kit information.

Changes in the text: See page 10, line 224: "*BD Cytometric Bead Array Human Inflammatory Cytokine Kit (BD Biosciences, USA) was used to assess the cytokine profile (allowing one to measure the concentration of IL-1β, IL-6, IL-8, IL-10, IL-12p70, and TNF-α) in tear samples. The tests were performed according to the manufacturer's protocols. (In this link you can find the information on the Cytokine kit: https://www.bdbiosciences.com/en-us/products/reagents/immunoassay-reagents/cba/cba-kits/human-inflammatory-cytokine-cytometric-bead-array-cba-i-kit.551811)*".

Results

Comment 7: Table 1 should explain little bit.

Reply 7: We divided the patients into four groups (PD with and without DED, HC with and without DED) and described their demographic characteristics.

Changes in the text: Table 1 was modified.

Comment 8: When introduce result add short background sentence/sentences at the beginning and somekind conclusion related to presented results.

Reply 8: We appreciate the reviewer comments. On this matter the journal's Guidelines to Authors state that "It is important to note that the results should be presented objectively and not overly interpreted (interpretation should be done in the Discussion)." The discussion is structured in the same order as the results were presented to increase the readability.

Comment 9: Before table 2 there is mentioned many results and then refered to the table. Table is confusing short. Please collected as well results mentioned in the text before table 2 and put somekind table those results that it is easier to go results through.

Reply 9: We deleted Table 2 and added the results to the text.

Changes in the text: See Page 12, line 264: *"Statistical differences were found in TBUT, BR, and DEQ-5 when comparing PD subjects with PD. The mean of TBUT was lower in PD when compared to HC (8.18±4.03seconds vs 13.5±3.74 seconds, p=0.001). TBUT was less than 10 seconds in 62.5% (n=10) of PD subjects and 12.5% (n=2) of controls. The mean DEQ-5 score was higher in PD than in HC (5.68±4.39 vs 2.18±1.64, p=0.002). The mean BR was 10.37±2.68 blinks/minute in PD and 16.31±4.45 blinks/minute in HC (p=0.001)".*

Comment 10. Please mention as well other measured cytokines in the beginning of the manuscript.

Reply 10: We appreciate the comment and we add a statement in the introduction

Changes in the text: Page 6, line 122: *"The current study was designed to quantified the levels of cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) in tears in PD and HC, and to evaluate whether these inflammatory cytokines correlated with disease clinical characteristics and DED indicators."*

Comment 11: Different MDS-UPDRS scores need to explain little bit in some part of the manuscript example material and methods or supplementary data

Reply 11: We have extended the section on methods with an explanation of the components of the MDS-UPDRS scale

Changes in the text: Page 7, line 144 *"We recorded Movement Disorders Society-Unified Parkinson's disease rating scale (MDS-UPDRS) (24), this is a comprehensive assessment tool designed to evaluate various motor and non-motor aspects of Parkinson's disease. It consists of four parts that assess different domains of the disease. Part I: Non-Motor Experiences of Daily Living (NMEDL): This part assesses the impact of non-motor symptoms on daily activities and quality of life. It includes questions related to mood, cognition, hallucinations, apathy, sleep disturbances, and other non-motor aspects. The scale evaluates the frequency and severity of these symptoms and their impact on the patient's functioning. Part II: Motor Experiences of Daily Living (MEDL): This section focuses on motor symptoms and their impact on activities of daily living. It covers areas such as speech, salivation, swallowing, handwriting, cutting food, dressing, hygiene, falling, and freezing of gait. The scale rates the severity and frequency of these motor symptoms and their impact on the patient's ability to perform daily tasks. Part III: Motor Examination: Part III is a comprehensive assessment of motor symptoms using a standardized examination protocol. It evaluates various aspects of motor function, including tremor, rigidity, bradykinesia (slowness of movement), and postural instability. The examination assesses motor symptoms at rest, during posture maintenance, and during various movements. It provides a detailed evaluation of motor impairment and helps track disease progression. Part IV: Motor Complications: This part focuses on motor complications that may arise as a result of long-term treatment with dopaminergic medications. It assesses motor fluctuations (changes between "on" and "off" states) and dyskinesias (abnormal involuntary movements). The scale evaluates the frequency, severity, and impact of these complications on daily activities and quality of life. Each part of the MDS-UPDRS is scored independently, and the scores from all four parts are often combined to obtain an overall assessment of disease severity and progression. The scale provides a standardized and comprehensive evaluation of both motor and non-motor symptoms, helping clinicians and researchers assess the impact of Parkinson's disease and track changes over time."*

Discussion

Comment 12: Is this result showed in the manuscript?

Reply 12: We thank you for the comment, these results are shown in Table 1 and Table 2.

Comment 13: Last paragraph gives many reason to think is the manuscript too early for publication. As well usually methods and kits are not compared if not examide. Pleas, modify last paragraph or take it away and do somekind of conclusion paragraph based to the present results.

Reply 13. We appreciate this observation, but acknowledging study limitations is essential for maintaining scientific integrity, promoting accurate interpretation, guiding future research directions, upholding ethical principles, and fostering collaboration within the research community. It demonstrates a commitment to transparency, intellectual rigor, and responsible dissemination of knowledge, ultimately contributing to the progress of scientific inquiry.

Conclusion

Comment 14: Please check if you conclude that cytokines (e.g. IL-1β) are increased that statistics are really significant. If not there could not conclude as increased. Make conclusion more clear and concise

Reply 14. We thank you for this observation, and as you commented the IL-1β levels are not statistics significantly when compared with control, so we deleted the sentence from the manuscript " Higher tear interleukin-1β (1.75±4.36 vs 0.01±0.05, p=0.068)"

Changes in the text: We deleted the sentence from the manuscript in the conclusion: *"Higher tear interleukin-1β (1.75±4.36 vs 0.01±0.05, p=0.068)"*

Reviewer E

In the study entitled "Pro-inflammatory cytokines levels in tears and dry eye disease in Parkinson's disease" the authors analyze the tears of PD patients and healthy controls for their potential role as biomarkers of PD and/or DED. Prior to acceptance for publication, the following points need to be addressed:

Comment 1. In the methods is the abstract state which cytokines were analyzed and what technology was used to analyze those cytokines.

Reply 1: We thank the reviewer for the comment and we added a sentence in the methods of the abstract

Changes in text: See Page 3, line 60: "*pro-inflammatory cytokines (IL-1β, IL-6, IL-8, IL-10, IL-12p70 and TNF-α) were quantified in tears using the BD Cytometric Bead Array Human Inflammatory Cytokine Kit"*

Comment 2. Line 72, typo: It should read "in tears".

Reply 2: We appreciate the comment and we have changed the typo on line 75: “in tears”

Changes in the text: *see page* 3, line 73: “in tears”

Comment 3. The sentence “IL-8 in tears may be involved in the severity and dry eye in PD” is not clear, please rewrite.

Reply 3: We thank the observation and we have rewritten it.

Changes in the text: See Page 4, line 73: *“IL-8 in tears may be both involved in the severity of the disease and in the development of DED in PD”*

Comment 4. Please refer to “Dry Eye” as “Dry Eye Disease (DED)”

Reply 4: We value the comment and we have changed it to DED

Changes in the text: We changed “Dry Eye” to “Dry Eye Disease

Comment 5. Please add a table with the patient characteristics.

Reply 5: We recognize the commentary and we have modified Table 1 with patient and control demographic characteristics.

Changes in the text: see *Table 1*

Comment 6. “An increased staining panel labeled A to E according to the severity” is not a full sentence. Please correct.

Reply 6: We changed the sentence in the text

Changes in the text: see Page 9, line 202: *“An increased staining panel labeled A to E according to the severity is used (A, less severe, E, more severe)”*

Comment 7. Line 188, should it read “outer canthus”?

Reply 7: We have changed it

Changes in the text: see Page 10, line 222: *“outer canthus”*

Comment 8. In the methods state approximately how many microliters were collected per sample.

Reply 8: We appreciate the comment and we have added the information in the text.

Changes in the text: See Page 10, line 221: “we collected 100uL tear sample”

Comment 9. The first section in the methods that describes the patients should have a subtitle.

Reply 9: We have added a subtitle “*Subjects and controls”*

Changes in the text: See Page 6, line 134: “*Subjects and controls”*

Comment 10. The subtitle for Tear Collection Sample should also convey that the methods included also describe the quantification by flow cytometry.

Reply 10: We thank the observation and we have expanded the subtitle.

Changes in the text: See Page 10, line 221: "*Tear collection sample and flow cytometry analysis"*

Comment 11. The statistical methods do not describe the data being analyzed for whether the data was normally distributed or not.

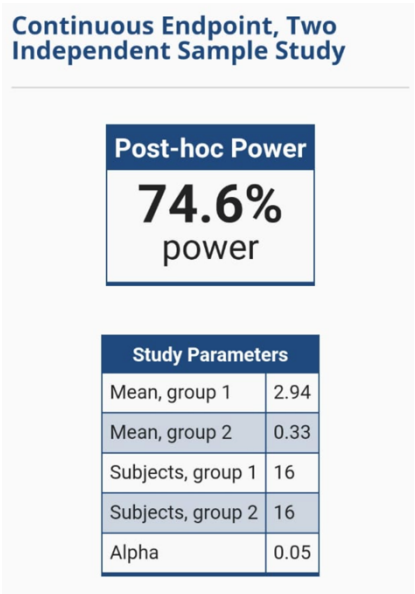
Reply 11: We recognize the commentary and we have added a statement in the methods.

Changes in the text: See Page 11, line 241: *"The Shapiro-Wilk test was used to examine if the continuous variables were normally distributed"*

Comment 12. Power analysis is needed to determine if the study was correctly powered to obtain a sound conclusion. Please provide the calculations.

Reply 12: Post hoc power calculation involves estimating the probability of detecting an effect or difference in the sample based on the observed data. While post hoc power calculations can provide some insights, they have both advantages and disadvantages that should be carefully considered. Post hoc power calculations can provide valuable information for designing future studies. By examining the achieved power, researchers can assess the adequacy of their sample size and make adjustments for future investigations. Nevertheless, the calculations depend on the observed effect size, which may not be the true effect size. If the observed effect size is inflated or underestimated, the post hoc power estimate may provide a misleading interpretation of the study's actual power.

The Post-hoc Power was calculated as follows:



n1 = sample size for group #1
n2 = sample size for group #2
$\Delta = |\mu 2 - \mu 1|$ = absolute difference between two means
σ1, σ2 = variance of mean #1 and #2
α = probability of type I error (usually 0.05)
β = probability of type II error (usually 0.2)
z = critical Z value for a given α
Φ() = function converting a critical Z value to power

$$Power = \Phi\left\{ -Z_{1-\alpha/2} + \frac{\Delta}{\sqrt{\sigma_1^2/n_1 + \sigma_2^2/n_2}} \right\}$$

$$Power = \Phi\left\{ -(1.96) + \frac{2.61}{\sqrt{3.95^2/16 + 0.49^2/16}} \right\}$$

$$Power = \Phi\left\{ 0.663 \right\} = 0.746 = 74.6\%\ power$$

Comment 13. Line 214, break down the demographics into 4 groups: PD with DED, HC with DED, PD without DED and HC without DED.

Reply 13: We modified Table 1 and described the demographics of the 4 groups

Changes in the text: See Table 1

Comment 14. Line 215, what do the authors refer to as "side of onset"?

Reply 14: PD is an asymmetric disease and usually emerges with a unilateral side-of-onset,

Changes in the text: see Page 12, line 254: we have added *"side of the initial onset"*

Comment 15. Show the data on table 3 as box plots with whiskers. Same thing for the cytokines in table 4.

Reply 15: We have added a figure as box plots for both tables.

Changes in the text: See Figure 4 and figure 5

Comment 16. Show the AUC, the sensitivity, specificity, accuracy, PPV and NPV for the biomarkers proposed.

Reply 16: We thank for this comment, and we calculated the values for TNF-α: AUC 0.65, the sensitivity: 81%, specificity: 52%, accuracy: 71.72%, PPV:78.2%, and NPV: 56.29%

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) are statistical measures commonly used in diagnostic or screening tests. However, calculating these measures in studies not specifically designed for them can lead to several statistical drawbacks and

misinterpretations. If a study was not specifically powered to assess these measures, the sample size might not be appropriate, leading to imprecise or biased results. In addition, if the study was not designed to account for verification bias, the estimates may be flawed, affecting the accuracy and validity of the results.

Moreover, PPV and NPV are influenced by the prevalence of the condition or disease being tested. Being a case-control study the prevalence is fixed at 50% which deems inappropriate to calculate these values.

Comment 17. Line 257 should read "surrogate"

Reply 17: We have changed to "surrogate"

Changes in the text: See Page 14, line 300: *"surrogate"*

Comment 18. In the paragraph starting on line 264, state what tissue or tissue fluids were used to measure the levels of those cytokines in PD patients.

Reply: We have added the fluid that was used.

Changed in the text: See page 14, line 309: *"in serum, cerebrospinal fluid and tears"*

Comment 19. Please expand as to why the mechanisms behind the dry eye in PD seem to be related to BR and the severity of disease.

Reply 19: We appreciate this observation and we have expanded the discussion

Changes in the text: Page 16, line 350: "*In PD incomplete blinking and a decrease in BR can be observed due to a deficiency in dopamine. These changes could lead to tear film hyperosmolality, accelerated tear evaporation, and finally corneal damage causing DED. In our study, a higher BR correlated moderately with a lower HY stage (r=-0.645, p=0.007), and patients with DED have lower blink rates (12.14±2.54 vs 9.0±2.06, p=0.031). In contrast, Fitzpatrick et al. could not find an association between BR and disease severity".*

The study has a low sample size, so it is uncertain whether sound conclusion can be made from this study. No biomarker characteristics are presented for any analyte (area under the curve, sensitivity, specificity).