PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	Towards biomarkers for outcomes after pancreatic ductal adenocarcinoma and ischemic stroke, with focus on (co-)morbidity and aging / cellular senescence (SASKit): protocol for a prospective cohort study
AUTHORS	Henze, Larissa; Walter, Uwe; Murua Escobar, Hugo; Junghanß, Christian; Jaster, Robert; Köhling, Rüdiger; Lange, Falko; Salehzadeh-Yazdi, Ali; Wolkenhauer, Olaf; Hamed, Mohamed; Barrantes, Israel; Palmer, Daniel; Möller, Steffen; Kowald, Axel; Heussen, Nicole; Fuellen, Georg

VERSION 1 – REVIEW

REVIEWER	Hideo Baba Kumamoto University, Japan
REVIEW RETURNED	17-Jun-2020

GENERAL COMMENTS	The authors report a very interesting protocol that links PDAC and IS from the perspective of aging and leads to the discovery of biomarkers for each disease or the discovery of factors that may lead to elucidation of the disease mechanism. The relationship between cancer and arterial thrombosis as Torso syndrome has been said for a long time, but the mechanism is still unknown, and this research may lead to its elucidation, which is very interesting. I am sorry but the article is difficult to accept BMJ Open by the concerns bellow. Major concerns 1. The author also states in a limitation, but I think the sample size of 50 patients per arm is too small. Although there is a description that venous thromboembolism is the most common event occurring in up to 34% of patients with metastatic PDAC, I think that it is difficult to show the relationship between them because there are not many cases of comorbidity of PDAC and IS. I think the author recruit at least 100 cases per arm. 2. Basic diseases that cause IS include cardiogenic cerebral infarction caused by atrial fibrillation. Do these cases also enter the cohort? 3. I think that cirrhosis is a factor involved in coagulation. I think that liver function should be an exclusion criteria. 4. In the introduction, I think that cancer and venous thrombosis are mainly considered. I think it is better to add consideration to cancer and arterial thrombosis. 5. Please explain in more detail how you do and what it means for optional skin biopsy and skin microbiome analyzes. 6. Grip strength is used as an indicator of sarcopenia, but I think that it may have an effect if there is paralysis in IS, so I think it is better to

	use other indicators as well.

REVIEWER	Mingquan Li Medical School of Nanjing University, China
REVIEW RETURNED	06-Jul-2020

GENERAL COMMENTS	This is a protocol for a cohort study about the prognostic value of serum biomarkers for long-term outcome in pancreatic cancer and ischemic stroke patients. Due to severe clinical outcocmes, early detection of (bio)markers is crucial for these disease, especially genomic and proteomics, which is less reported in current literature. Power analysis instead of sample size in this protocol. However, sample size calculation is a key step for overall study, so I recommend to estimate sample size based on previous study results. The manuscript is clearly written and straightforward.

REVIEWER	W.H.Mess Dept. of Clinical Neurophysiology; MUMC+ Maastricht, the Netherlands
REVIEW RETURNED	23-Jul-2020

GENERAL COMMENTS	This manuscript focusses on an interesting and innovative concept combining several medical disciplines as well as advanced data analysis. The writing is excellent and clear. An extremely thorough data analysis is incorporated, including a machine learning approach. As the authors themselves mention the amount of included subjects is rather low which is determined by external factors not related to the goals or contents of this study. However, if in the future the biomarkers under study are going to be used in patient care there should be a definite predictive value in 50 patients. If a relationship between a risk factor and the outcome is only found in a (much) larger patient group it is highly unlikely that identifying and eventually modifying these risk factors will be relevant for the single patient presenting. So, taken together there are no comments from my part that would need any modification. The only remark that I would like to make is that the level of detail when describing the data analysis is much less in the abstract than in the manuscript itself, but assuming that there is a word count limit I agree with the choice the authors made (i.e. emphasising other aspects of the study). Concerning the data analysis and statisttics: The very advanced level of data analysis is not my field of expertise. As far as I can see it is sound but somebody with more knowledge in this field should have a look at this part (this is why I checked "n/a" for the statistics part)

REVIEWER	Markus Scholz Universität Leipzig, Germany
REVIEW RETURNED	30-Aug-2020

GENERAL COMMENTS	In this paper, the authors present a protocol for a prospective cohort study of two disease entities (stroke, pancreatic carcinoma) with the aim to find similarities and differences of cellular senescence. I have the following questions and comments: 1. The considered disease entities are very different with respect to disease pathology, dynamics and prognosis. Please explain, why exactly these entities were chosen.

	2. When I understand it right, there are longitudinal comparisons within the disease groups and with their respective controls. But, I could not find an approach to perform comparisons between the two disease groups. Could you please elaborate on that issue in more detail? 3. I have concerns regarding the selection of control groups. The authors propose some kind of matched pair design by selecting controls from the same household. However, this could result in unbalanced distribution of risk factors between cases and controls (e.g. sex / age). Moreover, this pairing needs to be accounted for in all analyses, i.e. cases and controls cannot be considered as independent. 4. I find the envisaged follow-up scheme ambitious in view that especially PDAC patients are moribund. I would expect several drop-outs here. How do you cope with this issue? 5. Regarding endpoints: b) and c) could occur more than once so that multiple endpoints are possible. Is this intended or how do you deal with this issue? 6. The manuscript could be better arranged in my opinion: a. Study design with blood sampling and follow-ups could be presented as a figure. b. The manuscript is very lengthy and difficult to read. In particular, the bioinformatics part is very detailed and I am confused regarding the different approaches proposed there and their relative merit / added value. c. Conversely, the design aspects (as mentioned above) are a bit underdeveloped.

VERSION 1 – AUTHOR RESPONSE

> Reviewers' Comments to Author:

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> Reviewer: 1

> Reviewer Name: Hideo Baba

> Institution and Country: Kumamoto University, Japan

> Please state any competing interests or state ‘None declared’: None declared

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> The authors report a very interesting protocol that links PDAC and IS from the perspective of aging and leads to the discovery of biomarkers for each disease or the discovery of factors that may lead to elucidation of the disease mechanism. The relationship between cancer and arterial thrombosis as Torso syndrome has been said for a long time, but the mechanism is still unknown, and this research may lead to its elucidation, which is very interesting. I am sorry but the article is difficult to accept BMJ Open by the concerns bellow.

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> Major concerns

> 1. The author also states in a limitation, but I think the sample size of 50 patients per arm is too small. Although there is a description that venous thromboembolism is the most common event occurring in up to 34% of patients with metastatic PDAC, I think that it is difficult to show the relationship between them because there are not many cases of comorbidity of PDAC and IS. I think the author recruit at least 100 cases per arm.

Indeed, we list the size of the study as one of its limitations. However, co-morbidity is only one aspect of our study; reviewer #4 in fact overlooked it, mostly, in favor of the disease-specific analyses that we also do, specifically in the "standard" (that is, not exploratory) analyses. Moreover, as described in the text, we are doing a biomarker discovery study; we do in-depth measurements at the expense of power. Our study was explicitly approved as a discovery study by the funding agency. We will of course report results with all necessary caveats.

> 2. Basic diseases that cause IS include cardiogenic cerebral infarction caused by atrial fibrillation. Do these cases also enter the cohort?

Yes, patients with cardiogenic IS caused by atrial fibrillation can also enter the cohort but only if they did not undergo any therapeutic anticoagulation for longer than 1 month within the past 2 years (as an exclusion criterion). This normally excludes patients and controls with previously known atrial fibrillation (since these usually have been receiving therapeutic anticoagulation), limiting the number of such cases to those with newly detected atrial fibrillation. The history of atrial fibrillation is recorded as a standard item as part of the baseline clinical data.

> 3. I think that cirrhosis is a factor involved in coagulation. I think that liver function should be an exclusion criteria.

Since the study had to be started in the meantime, we should not change the exclusion criteria, but the data analysis plan can be improved. Since we measure markers of liver (dys-)function or disease, such as INR, albumin, bilirubin, ALT, AST and AP, we suggest checking whether liver dysfunction or disease is a confounder. We thus added the following to the study protocol:

1) We amended the paragraph “Blood sampling will be done (...) INR (...) albumin, bilirubin (...)” by “Among the standard measurements, we also measure the liver parameters ALT, AST and AP as surrogate markers of liver disease”. 2) We also amended the section on “Predictors” by adding *liver dysfunction or disease* to the list of *further covariates:* “Further covariates are smoking, liver dysfunction or disease, the baseline NIHSS score in case of IS, as well as locally-advanced vs metastatic PDAC and modality of treatment in case of PDAC.”

> 4. In the introduction, I think that cancer and venous thrombosis are mainly considered. I think it is better to add consideration to cancer and arterial thrombosis.

In the second paragraph of the introduction, it reads: "Venous thromboembolism is the most common event occurring in up to 34% of patients with metastatic PDAC, but arterial ischemic events, like stroke, are also reported, see also Box 2." To further stress the importance also of arterial thrombosis in PDAC, we added two more 2 references (Poirée 2004, Schattner 2002), see text.

> 5. Please explain in more detail how you do and what it means for optional skin biopsy and skin microbiome analyzes.

We expanded the paragraph to that effect, which now reads as follows. “A separate ethics approval was granted for an optional skin biopsy; skin microbiome analyses are planned as well. More specifically, participants have the option to provide a skin biopsy of 5mm from an area that is not usually visible. We expect that about 30-50% of the participants will opt in. We keep the biopsy in culture for several days and divide it into several pieces. Using these, we measure biomarkers of cellular senescence (specifically, senescence-associated beta-galactosidase, which cannot easily be measured in blood) and we treat some pieces with compounds that may affect cellular senescence, such as quercetin or fisetin. Moreover, we plan to sample the microbiome of the forehead using a standard swab. This is a very simple procedure, motivated by the claim that a competitive epigenetic aging clock can be based on such a sample (Huang et al, DOI: 10.1128/ mSystems.00630-19)“.

> 6. Grip strength is used as an indicator of sarcopenia, but I think that it may have an effect if there is paralysis in IS, so I think it is better to use other indicators as well.

Grip strength is measured for both hands. For each study participant, the measure of the stronger hand is used for further analysis in order to exclude the effects of (unilateral) paralysis. Grip strength is used to define what is formally known as “probable sarcopenia”.

> Reviewer: 2

> Reviewer Name: Mingquan Li

> Institution and Country: Medical School of Nanjing University, China

> Please state any competing interests or state ‘None declared’: None declared

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> This is a protocol for a cohort study about the prognostic value of serum biomarkers for long-term outcome in pancreatic cancer and ischemic stroke patients.

> Due to severe clinical outcocmes, early detection of (bio)markers is crucial for these disease, especially genomic and proteomics, which is less reported in current literature.

> Power analysis instead of sample size in this protocol. However, sample size calculation is a key step for overall study, so I recommend to estimate sample size based on previous study results. The manuscript is clearly written and straightforward.

Yes, we agree that a formal sample size calculation would have been ideal. Given the limited resources provided in terms of funding and a limited ability to recruit PDAC patients within the given time-frame, we fixed the expected number of available patients and provided a power analysis to make clear which effects can be shown. We would like to point out that our study was designed as an exploratory study for the identification of biomarkers; it was the specific aim of the funding agency to sponsor biomarker discovery, not biomarker validation.

> Reviewer: 3

> Reviewer Name: W.H.Mess

> Institution and Country: Dept. of Clinical Neurophysiology; MUMC+ Maastricht, the Netherlands

> Please state any competing interests or state ‘None declared’: None declared

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> This manuscript focusses on an interesting and innovative concept combining several medical disciplines as well as advanced data analysis. The writing is excellent and clear. An extremely thorough data analysis is incorporated, including a machine learning approach.

> As the authors themselves mention the amount of included subjects is rather low which is determined by external factors not related to the goals or contents of this study. However, if in the future the biomarkers under study are going to be used in patient care there should be a definite predictive value in 50 patients. If a relationship between a risk factor and the outcome is only found in a (much) larger patient group it is highly unlikely that identifying and eventually modifying these risk factors will be relevant for the single patient presenting.

> So, taken together there are no comments from my part that would need any modification. The only remark that I would like to make is that the level of detail when describing the data analysis is much less in the abstract than in the manuscript itself, but assuming that there is a word count limit I agree with the choice the authors made (i.e. emphasising other aspects of the study).

> Concerning the data analysis and statisttics: The very advanced level of data analysis is not my field of expertise. As far as I can see it is sound but somebody with more knowledge in this field should have a look at this part (this is why I checked "n/a" for the statistics part)

Indeed, we are describing an observational trial aimed at biomarker discovery, and validation of findings must be done in followup studies; only then can we find out whether any biomarker is fit for clinical routine diagnostics.

> Reviewer: 4

> Reviewer Name: Markus Scholz

> Institution and Country: Universität Leipzig, Germany

> Please state any competing interests or state ‘None declared’: None

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> In this paper, the authors present a protocol for a prospective cohort study of two disease entities (stroke, pancreatic carcinoma) with the aim to find similarities and differences of cellular senescence.

> I have the following questions and comments:

> 1. The considered disease entities are very different with respect to disease pathology, dynamics and prognosis. Please explain, why exactly these entities were chosen.

Please let us refer to Box 2, “Cellular senescence and the comorbidity of cancer and vascular events.”, though we failed to refer properly to this box in the Introduction. Thus, we now write, “The background of the cancerous and vascular comorbidity is described in Box 2. Importantly, despite differences in disease pathology, dynamics and prognosis, there is a lot of evidence that cellular senescence is, in part, an important contributor to disease etiology, progression and consequences for both diseases.”

> 2. When I understand it right, there are longitudinal comparisons within the disease groups and with their respective controls. But, I could not find an approach to perform comparisons between the two disease groups. Could you please elaborate on that issue in more detail?

Indeed, a lot of the work is done separately, per disease entity. We are however also deeply interested in joint analyses, and the following are planned:

1) For each disease, we check the "comorbid" outcome that the other disease occurs.

2) In the second-to-last paragraph of the Data Analysis Plan, we specifically write

"PDAC and IS data will be analyzed together in integrative exploratory analyses. In that case, the occurence of specific endpoints will be evaluated according to the group membership (PDAC or IS). This means that in addition to the biomarker signature, a group variable, indicating PDAC or IS patients, will be included in the analysis, to assess the difference in the progression of the respective endpoints between PDAC and IS patients."

> 3. I have concerns regarding the selection of control groups. The authors propose some kind of matched pair design by selecting controls from the same household. However, this could result in unbalanced distribution of risk factors between cases and controls (e.g. sex / age). Moreover, this pairing needs to be accounted for in all analyses, i.e. cases and controls cannot be considered as independent.

Thank you for raising this important point. We agree that the matching structure should be considered in a direct comparative analysis of diseased and controls. However, as our primary aim is the description of the changes within the PDAC or IS patient group, data of controls are considered when dealing with omics data only, to define contrasts. These contrasts, however, are based on the gene/protein expression *averaged* over all controls. To add clarity, we amended the bioinformatics section as follows.

“For genomic features as per (2), the feature measurements for an individual patient or control will then be the average linkscores of the 5 selected subnetworks, contrasting each patient with average control data, and each control with average patient data.”

> 4. I find the envisaged follow-up scheme ambitious in view that especially PDAC patients are moribund. I would expect several drop-outs here. How do you cope with this issue?

Indeed, and for this reason we explicitly set the main followup to 3 months for PDAC and 12 months for stroke. Late follow-ups will not be frequent in case of PDAC, unless there are therapeutic breakthroughs.

> 5. Regarding endpoints: b) and c) could occur more than once so that multiple endpoints are possible. Is this intended or how do you deal with this issue?

You are right that our endpoint could occur more than once and a multistate model would be an option for the analysis. We decided to use the first occurrence of each endpoint within the study period for the analysis. To make our intention more clear, we put *emphasis* on the word “first” at the beginning of the respective paragraph, as follows. “In both subtrials, the primary endpoint is a composite measure of “disease deterioration” defined as the *first* occurrence within a follow-up interval of at least one of the following, (...)”

> 6. The manuscript could be better arranged in my opinion:

> a. Study design with blood sampling and follow-ups could be presented as a figure.

Indeed, we agree that it is useful to have a figure regarding the study design; we therefore suggest to add the following figure.

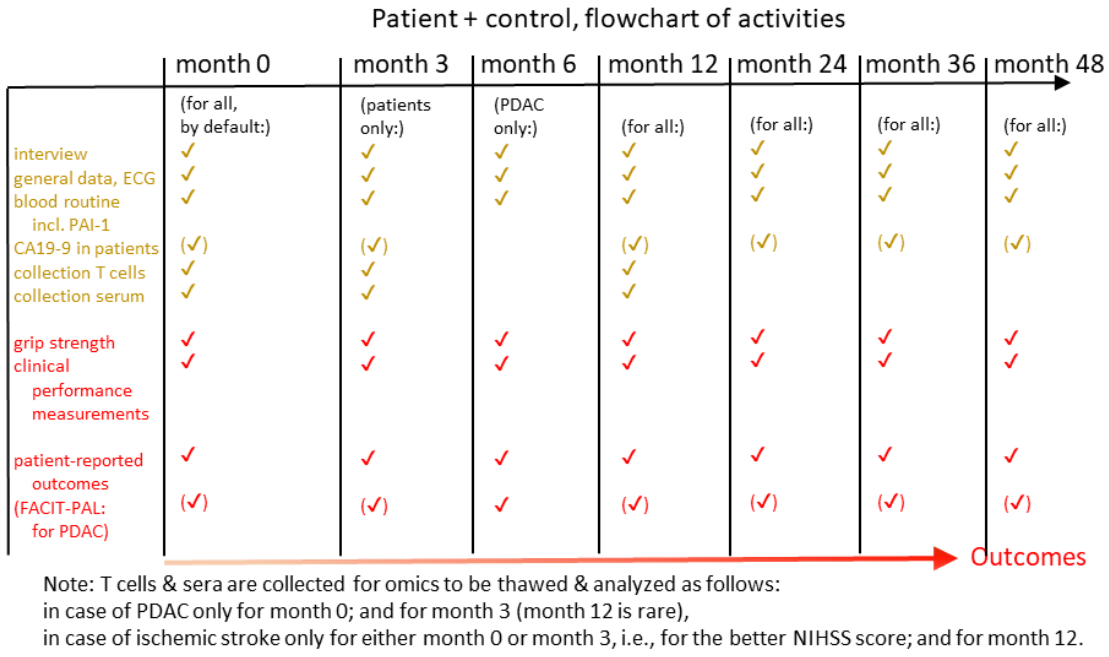

Figure 1: Study design of the SASKit study (human cohort; mouse studies designed to mirror the human study in part will be presented elsewhere). Predictor and outcome measurements along the time axis are described.

> b. The manuscript is very lengthy and difficult to read. In particular, the bioinformatics part is very detailed and I am confused regarding the different approaches proposed there and their relative merit / added value.

We hope that a second figure (see below) is helpful to better understand the data analysis plan. We strongly believe that the details presented are useful as a frame-of-reference. Essentially, these details document that we clearly specify the hypotheses and the data analyses upfront, and not post-hoc. Further, to tackle the over-optimism in the reporting of the biomarker candidates due to unreported multiple testing; based on the detailed data analysis plan one can clearly distinguish three types of biomarker candidates: (1) candidates found following the *standard* data analysis plan; (2) candidates found by any further *exploratory* analyses described in the data analysis plan; (3) candidates found by methods not mentioned in the data analysis plan (post-hoc). Only the first kind of candidates can be reported with high confidence. The third kind of candidates need to be treated with a lot of caution unless there is strong corroborative evidence, e.g., based on validation in a different data set.

To summarize the data analysis plan, we suggest adding the following figure.

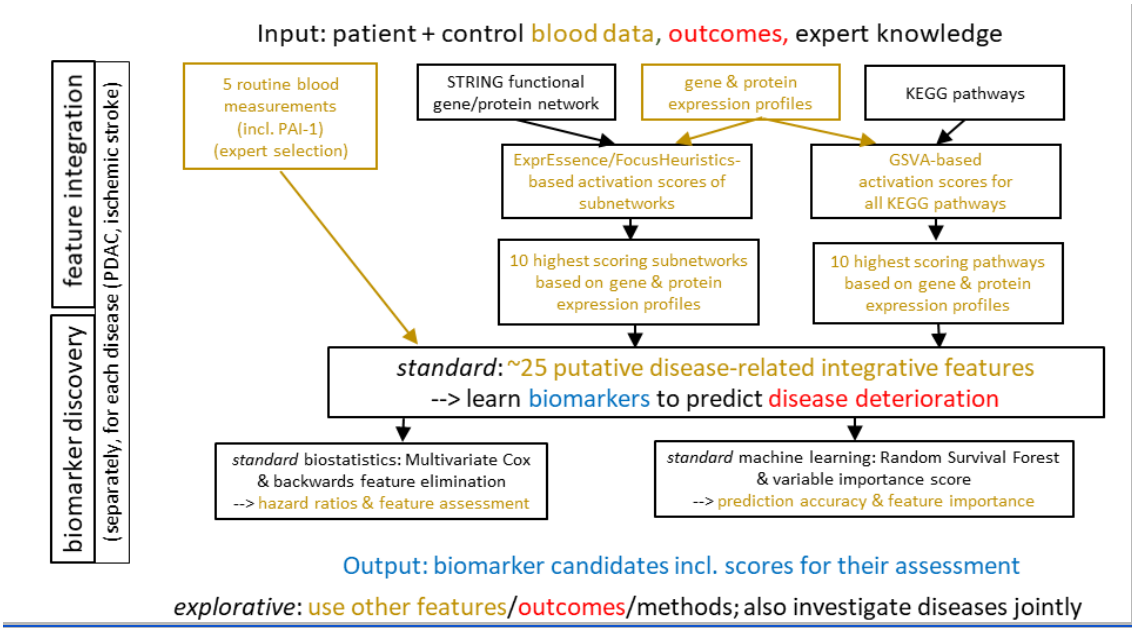

Figure 2: Data analysis plan of the SASKit study (human cohort). Input, methods and output of the standard (but not the explorative) analyses based on biostatistics and machine learning are described in detail.

> c. Conversely, the design aspects (as mentioned above) are a bit underdeveloped.

We hope that Figure 1 (see above) makes the design a lot clearer.

VERSION 2 – REVIEW

REVIEWER	Markus Scholz University of Leipzig Germany
REVIEW RETURNED	12-Oct-2020

GENERAL COMMENTS	No further comments.