PEER REVIEW HISTORY

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ARTICLE DETAILS

TITLE (PROVISIONAL)	Study Protocol: The TRAnsplant BIOpsies (TRABIO) Study: A Prospective, Observational, Multicenter Cohort Study to Assess the Treatment of Kidney Graft Rejections
AUTHORS	von Samson-Himmelstjerna, Friedrich; Esser, Grit; Schulte, Kevin; Kolbrink, Benedikt; Krautter, Markus; Schwenger, Vedat; Weinmann-Menke, Julia; Matschkal, Julia; Schraml, Florian; Pahl, Anne; Braunisch, Matthias; Feldkamp, Thorsten; Kunzendorf, Ulrich; Renders, Lutz; Heemann, Uwe

VERSION 1 – REVIEW

REVIEWER	David Rush University of Manitoba
REVIEW RETURNED	09-Apr-2021

GENERAL COMMENTS	von Samson-Himmelstejrna et al propose an interesting and pragmatic 5 year study in renal transplant patients, in which the treatment of rejection episodes is standardized, and in whom the outcomes (primary: all cause mortality, graft survival; secondary: >30% drop in GFR, proteinuria, recurrent acute rejection), are determined yearly for 5 years. More than 800 patients are to be recruited (495 have been included already). The decision when to stop recruitment (after how many patients) is not stated. Methods and Analysis (pages 9-12) The study will include all adult consenting patients that require biopsy with deteriorating function in whom rejection is suspected. This practice may result in patients with rejection (not suspected) not being biopsied. This possibility should be taken into account/clarified.The degree of functional deterioration that triggers an indication biopsy is not stated. It appears that patients may have had one (or more) prior kidney transplants. This may affect the results and should be considered as a potential variable. It is stated that the treatment of rejection is standardized according to the Banff 2019 criteria, but that treatment decisions may be modified by the treating physician, which is contradictory, and which may obscure the main study objective ('to analyze rejection outcomes and their association with different treatment strategies"). At the time of enrollment (at biopsy), one of the data recorded is that of "previous biopsy results". Is this correct? Does this mean that a biopsy for delayed graft function, for example, is allowed prior to the enrollment? This should be clarified. Will biopsies be allowed during follow up, after the enrollment biopsy, if clinically indicated? How will the successful resolution of treated rejection episodes be determined, if at all. This should be clarified.

	The biopsies are to be treated as per the Banff 2019 criteria (published in 2020). A list of treatments for various forms of rejection is provided in Figure 2. A strong point of this study is the aggressive (by current standards) treatment of borderline rejection. Standard treatment, as is proposed for other pathologies, has not been successful, as pointed out in the introduction, so it is unlikely that outcomes will be improved overall for the majority of patients. Banff 2019, has a new proposed histopathological entity, that of "chronic active T cell mediated rejection" (the treatment of which is not agreed upon). The investigators have made no comment about this entity in the text (or how it will be treated, as are other entities described in Figure 2). Is there a standardized protocol for polyoma virus detection? This infection may occur post-treatment of rejection and should be systematically monitored. There is very little said about biopsies and antibody. For example, will biopsies would be read locally or centrally? Will C4d staining be done routinely? How will mixed rejections (cellular inflammation and antibody-mediated lesions present concomitantly) be treated? And for antibody, will thisl be tested for in all biopsied patients, to exclude these mixed forms of rejection? Is the methodology for biopsies and antibody testing also standardized?

REVIEWER	Juilan Singer The University of Sydney, Kidney Node Laboratory
REVIEW RETURNED	15-May-2021

GENERAL COMMENTS	Authors identify that despite improvement in short-term outcomes, driven largely by advances in immunosuppression, long-term patient and graft outcomes remain poor. The development of chronic allograft nephropathy through both immune and non-immune mediated injury and its treatment remains an area of high research priority. The study is a multi-centre, prospective, cohort study to capture the outcomes of standardised treatment for renal allograft rejection. aiming to recruit 800 participants. Recruitment commenced in 2016. The trial is feasible, and well designed to meet its aim. The author's treatment protocols are well-founded and consistent with usual clinical practice in most treatment centres. The treatment administered is determined by Banff criteria for rejection – modification to treatment may be made by the treating physician. Major concerns: 1. The enrolment of patients is unclear. How are potential participants identified and at which time point are they approached, pre or post-biopsy? If post-biopsy, this may introduce selection bias. 2. How are patients screened? What is the process for consent, or is there a waiver of consent? Are all patients who undergo an indication biopsy for graft deterioration screened, or just some? 3. At times in the paper the study is referred to as a “registry” – this is likely misleading, especially as it is unclear whether all patients who meet the eligibility criteria are enrolled. 4. Inclusion/exclusion criteria: are recipients of combined-organ transplant included? i.e. kidney/pancreas or kidney/liver? 5. Safety monitoring

	a. How are adverse events monitored and recorded? Are they only captured at 14 days and yearly follow-up? b. Exposure to augmented immunosuppression may increase the risk of adverse events, particularly, infection, cancer, and cardiovascular disease. These events should be recorded. Minor concerns: 1. Will CNI trough levels be recorded at follow-up, or just doses? 2. Will DSAs be monitored yearly, or just at initial diagnosis? 3. Second primary outcome – graft survival; is only defined as a return to dialysis. It should also include re-transplantation or death from graft failure. 4. Secondary outcomes; proteinuria; - should include more than one measurement i.e positive urinalysis for proteinuria on at least 2 occasions b. Secondary outcomes; recurrent of rejection - What definition will be used to delineate persistent or treatment-resistant rejection from a subsequent, second rejection episode? 5. Loss to follow-up – is there a protocol to manage loss to follow-up. Ie to ensure the patient is not deceased?

VERSION 1 – AUTHOR RESPONSE

1. Reviewer

David Rush, University of Manitoba, Canada

1.) The decision when to stop recruitment (after how many patients) is not stated.

The reviewer is correct in pointing this out and we have now specified that recruitment will be stopped, when 800 eligible patients have been enrolled and biopsied. A longer recruitment period is currently not planned. The first sentence in the sub-section 'study design' now reads as:

> (p. 7, l. 15 – 16) This study was designed as a prospective, observational, multicenter cohort study. Recruitment started in September 2016 and will last until 800 patients have been enrolled.

2.) The study will include all adult consenting patients that require biopsy with deteriorating function in whom rejection is suspected. This practice may result in patients with rejection (not suspected) not being biopsied. This possibility should be taken into account/clarified. The degree of functional deterioration that triggers an indication biopsy is not stated.

The reviewer correctly points out that this study is exclusively aiming at patients treated for rejections seen in indication biopsies. This study will not evaluate surveillance biopsies (biopsies taken without suspicion of graft rejection). As kidney transplant recipients are very closely monitored with regular appointments and routine blood and urine tests by their transplant center and their primary care physician, we are confident that we have a solid protocol in place to detect all cases of significant transplant deterioration. Any patient that is evaluated for acute kidney injury (KDIGO 2012) or in whom new-onset high proteinuria is detected without presenting with an alternate obvious cause for acute kidney injury will be suspected to have a rejection and consequently receive a kidney biopsy. We believe that through this pragmatic approach the number of missed rejections should be very low. We have specified this in the 'study purpose' section and 'study population and enrollment' sub-section:

(p. 6, l. 1 – 2) To analyze the association of different treatment strategies with clinical outcomes after rejection episodes diagnosed through indication biopsies

(p. 6, l. 8 – 16) All patients who undergo an indication biopsy for suspected kidney graft rejection due to deteriorating kidney function at the participating transplant centers will be screened for participation in the study. Graft rejection can either be suspected by the primary care physician with a subsequent referral for an indication biopsy at the transplant center or directly by the transplant center during a regularly scheduled appointment. Deterioration of graft function with graft rejection is suspected when acute kidney injury as defined by the 2012 KDIGO guidelines is present in the absence of another highly suggestive cause of kidney injury, e.g. hypovolemic shock or ureteric obstruction, or when new-onset high proteinuria (> 300 mg/dl in urine dipstick test) is detected.

Additionally, the following phrase has been added to the section 'strengths, significance, and limitations':

(p. 12, l. 13 – 15) Since we do not conduct surveillance protocol biopsies, we will not be able to detect subclinical rejections. However, we have a strong protocol in place to diagnose and register all episodes of significant graft deterioration.

3.) *It appears that patients may have had one (or more) prior kidney transplants. This may affect the results and should be considered as a potential variable.*

The reviewer is right with the assumption that patients may have had one or more prior kidney transplants. This will be considered as a variable and has now been included in the 'methods & analysis' section:

(p. 10, l. 6 – 9) Known confounders such as age, time since transplantation, cardiovascular disease, chronic hepatitis, cancer, and baseline kidney function as well as history of prior kidney transplantation will be included in the regression models.

4.) *It is stated that the treatment of rejection is standardized according to the Banff 2019 criteria, but that treatment decisions may be modified by the treating physician, which is contradictory, and which may obscure the main study objective ('to analyze rejection outcomes and their association with different treatment strategies").*

We agree that at first glance there appears to be a conflict between standardizing a therapeutic approach and simultaneously allowing individual modifications to that approach. However, this is not an interventional clinical trial in which the treatment of a patient can be dictated by the study protocol. As we are merely conducting an observational trial on the grounds of a predefined therapeutic standard, physicians may modify the treatment approach if they see a necessity to do so. Thus, there will be a cohort of patients that receives therapy according to the standardized therapeutic approach, and a cohort of patients in which treatment decisions deviate from that standard. In our analysis, we intend to use these discrepancies in order to examine whether the standardized therapeutic approach is associated with better outcomes. This has been re-phrased in the sub-section 'rejection treatment':

(p. 7, l. 9 – 12) Treating physicians are allowed to modify the proposed treatment strategy in the best interest of the individual patient. This will lead to a cohort of patients in which treatment has deviated from standard treatment. Treatment strategy and outcomes after

deviation from treatment standards will be closely monitored and will be a focal point of the analysis.

5.) *At the time of enrollment (at biopsy), one of the data recorded is that of "previous biopsy results". Is this correct? Does this mean that a biopsy for delayed graft function, for example, is allowed prior to the enrollment? This should be clarified.*

This is correct. Generally, patients who have had biopsies for delayed graft function can be enrolled. However, if this delayed graft function was caused by graft rejection, they will not be included in this study. 'Previous biopsy proven graft rejection' has now been added to the list of exclusion criteria (figure 1) and this has been clarified in the sub-section 'study population and enrollment':

> (p. 6, l. 19 – 21) Patients will be excluded, if they have previously had a biopsy proven kidney graft rejection. Patients with a previous biopsy for delayed graft function can be included, if the biopsy did not show signs of rejection.

6.) *Will biopsies be allowed during follow up, after the enrollment biopsy, if clinically indicated? How will the successful resolution of treated rejection episodes be determined, if at all. This should be clarified.*

Biopsies will be allowed during follow up, if they are clinically indicated. The results of those biopsies will be registered. A complete resolution of a treated rejection will be determined by a ≥ 90 % recovery of CKD-EPI-GFR back to baseline CKD-EPI-GFR and resolution of proteinuria. A partial response to treatment will be defined as a 50 – 89 % recovery of CKD-EPI-GFR back to baseline CKD-EPI-GFR. This has now been clarified in the 'data' and the 'outcomes' sub-sections, respectively, and reads as:

> (p. 8, l. 16 – 18) If a patient has to undergo a re-biopsy for graft deterioration of any cause, the findings of that biopsy will be recorded.

> (p. 9, l. 16 – 19) A complete resolution of a treated rejection episode is determined by presence of patient and graft survival, a ≥ 90 % recovery of CKD-EPI-GFR back to baseline CKD-EPI-GFR, and resolution of new-onset large proteinuria. A partial response is defined as a 50 – 89 % recovery of CKD-EPI-GFR back to baseline CKD-EPI-GFR.

7.) *Banff 2019, has a new proposed histopathological entity, that of "chronic active T cell mediated rejection" (the treatment of which is not agreed upon). The investigators have made no comment about this entity in the text (or how it will be treated, as are other entities described in Figure 2).*

The reviewer is certainly right with pointing out that this entity should be described in the manuscript. Our therapeutic approach to chronic active T cell mediated rejection has now been included in figure 2 (along with some further specifications and corrections) and it has been specifically addressed in the introduction. As the reviewer states, there currently is no widely recognized standard for the treatment of this Banff category. Generally, we do not increase immunosuppression after a diagnosis of chronic active T cell-mediated rejection. In some cases, where kidney function continues to deteriorate and no other cause can be identified, maintenance immunosuppression is increased (7.5 mg prednisolone instead of 5 mg for 6 months and/or tacrolimus trough level of 6-9 ng/l instead of 5-8 ng/l for 3 months).

(p. 4, l. 25 - p. 5, l. 2) Chronic active TCMR has recently been introduced to the Banff classification as a new entity of graft rejection, and there are signs that it could be a strong predictor of poor prognosis. (Nakagawa et al., Am J Transpl, 2021)

(p. 5, l. 17 – 19) While there is some preliminary data indicating that a sub-group of patients with chronic active TCMR might benefit from an immunosuppressive burst therapy, currently no clear consensus exists on how to approach this entity. (Kung et al, Kid Int, 2021)

8.) *Is there a standardized protocol for polyoma virus detection? This infection may occur post-treatment of rejection and should be systematically monitored*

We agree with the reviewer about the importance of polyoma virus monitoring. Polyoma virus infection is routinely tested for through serum-sample PCR and urine-sample PCR at each follow-up at the transplant center. This information has now been added to the 'data' sub-section:

(p. 8, l. 16) Presence of polyoma virus infection will be detected by PCR testing of serum and urine.

9.) *There is very little said about biopsies and antibody. For example, will biopsies be read locally or centrally? Will C4d staining be done routinely??*

Biopsies will be read locally and biopsy findings will be diagnosed according to the Banff classification. C4d staining will be performed routinely. A new sub-section titled 'biopsies' has been included in the manuscript:

(p. 8, l. 19 – p. 9, l. 6) Biopsies

Biopsies will be performed by experienced nephrologists under sterile conditions. The biopsy specimen will be stored in 4% formaldehyde and sent for histopathological analysis at the responsible nephropathology laboratory of the clinic performing the biopsy. Biopsies will be performed and read locally using a standardized protocol. All biopsies will be diagnosed and interpreted according to the Banff classification. The following Banff lesion scores and additional findings will be graded routinely:

number of glomeruli, number of sclerosed glomeruli, number of arteries, Banff i, Banff t, Banff v, Banff g, Banff ptc, Banff C4d, Banff ci, Banff ct, Banff cv, Banff cg, Banff mm, Banff ah, Banff ti, Banff i-IFTA, segmental arterial intima fibrosis without hyperelastosis, segmental arterial intima fibrosis with lymphatic infiltrates, thrombotic microangiopathy, glomerulonephritis, focal and segmental glomerulosclerosis, pyelonephritis, polyomavirusnephritis, post-transplant lymphoproliferative disease

10.) *How will mixed rejections (cellular inflammation and antibody-mediated lesions presenting concomitantly) be treated?*

When there is a diagnosis of acute antibody-mediated rejection and acute t-cell-mediated rejection concomitantly, patients will receive plasma exchange therapy for five days, a three-day steroid burst followed by steroid tapering, and treatment with anti-thymocyte globulins over a course of at least three days. Additionally, maintenance immunosuppression will be increased (7.5 mg prednisolone instead of 5 mg for 6 months, tacrolimus trough level of 6-9 ng/l instead of 5-8 ng/l for 3 months). This has now been added to figure 2.

11.) And for antibody, will this be tested for in all biopsied patients, to exclude these mixed forms of rejection?

DSAs will be tested for in all biopsied patients and the presence, type, and titer will be recorded. This information is included in figure 4 and in the 'data' sub-section of the manuscript and has been left unchanged.

12.) Is the methodology for biopsies and antibody testing also standardized?

Biopsies and antibodies will be obtained and read locally according to a standardized protocol. There will not be a centrally standardized methodology for biopsies or antibody testing. However, pathologists and laboratories of all participating centers have extensive experience in the field and we believe each center's methodology can be strongly relied upon. The Banff classification will be the basis for biopsy evaluation at all the sites. This has been included in the new sub-section 'biopsies' (see above, question #9).

2. Reviewer

Julian Singer, The University of Sidney, Royal Prince Alfred Hospital, Australia

Major Revision:

1.) The enrolment of patients is unclear. How are potential participants identified and at which time point are they approached, pre or post-biopsy? If post-biopsy, this may introduce selection bias.

We understand that the manuscript has not been precise enough in this regard and have now clarified this aspect in the sub-section 'study population and enrollment'. Potential participants are identified pre-biopsy when an episode of transplant rejection is suspected. This suspicion can be raised by their primary care physician/nephrologist, leading to a referral for an indication biopsy at the transplant center, or directly at the transplant center during a routine follow-up exam. All potential participants that receive a biopsy will be approached for enrollment before the biopsy, in order to exclude the risk of selection bias.

> (p. 6, l. 8 – 12) All patients who undergo an indication biopsy for suspected kidney graft rejection due to deteriorating kidney function at the participating transplant centers will be screened for participation in the study. Graft rejection can either be suspected by the primary care physician with a subsequent referral for an indication biopsy at the transplant center or directly by the transplant center during a regularly scheduled appointment.
>
> (p. 6, l. 21 – 23) Any kidney transplant patient that is scheduled to undergo a graft biopsy at a participating center will automatically be screened and approached for enrollment prior to biopsy.

2.) How are patients screened? What is the process for consent, or is there a waiver of consent? Are all patients who undergo an indication biopsy for graft deterioration screened, or just some?

All patients who undergo an indication biopsy for graft deterioration are screened and included, provided they give written consent.

(p. 6, l. 8 – 10) All patients who undergo an indication biopsy for suspected kidney graft rejection due to deteriorating kidney function at the participating transplant centers will be screened for participation in the study.

The process for consent includes a detailed discussion between the patient and a physician about the risks and benefits of study participation. The following sentence has now been moved from the 'ethical approval' section to the 'study population and enrollment' sub-section:

(p. 6, l. 23 – p. 7, l. 1) Before enrollment, each patient will be informed by a physician about the risks and benefits of the study, and of his/her rights. Only patients who provide written and informed consent will be enrolled.

3.) *At times in the paper the study is referred to as a "registry" – this is likely misleading, especially as it is unclear whether all patients who meet the eligibility criteria are enrolled.*

We agree with the reviewer that the current terminology can be misleading and have therefore unified it, uniformly replacing the term 'registry' with the term 'study'. All patients who meet the eligibility criteria will be enrolled, provided they give written consent.

4.) *Inclusion/exclusion criteria: are recipients of combined-organ transplant included? i.e. kidney/pancreas or kidney/liver?*

Recipients of combined-organ transplantation will be included. This has now been specified in the 'study population and enrollment' sub-section.

(p. 6, l. 17 – 20) Patients with a history of re-transplantation and patients that have received a combination-organ transplantation such as kidney/pancreas or kidney/liver will also be eligible. Patients will be excluded, if they have previously had a biopsy proven kidney graft rejection.

5.) *Safety monitoring*

a. *How are adverse events monitored and recorded? Are they only captured at 14 days and yearly follow-up?*
b. *Exposure to augmented immunosuppression may increase the risk of adverse events, particularly, infection, cancer, and cardiovascular disease. These events should be recorded.*

While we strongly agree with the reviewer about the importance of safety monitoring, this study is not designed to systemically register adverse events and we are primarily concerned with treatment efficacy of our therapeutic approach. It would have been enriching to record adverse events, yet unfortunately that was not possible in the scope of this project and funding. However, we believe that questions surrounding the safety profile of the substances used in this study have previously been thoroughly examined in phase 3 and/or phase 4 trials.

<u>Minor Revision:</u>

1.) *Will CNI trough levels be recorded at follow-up, or just doses?*

CNI trough levels will also be recorded. This has now been specified in the 'data' sub-section:

(p. 8, l. 6 – 7) […] induction and maintenance immunosuppressive treatment (drug classes and doses as well as calcineurin inhibitor and mTOR inhibitor trough levels) […]

2.) Will DSAs be monitored yearly, or just at initial diagnosis?

DSAs will only be monitored at initial diagnosis. No further specification has been made to the manuscript regarding this point and this aspect has been left unchanged in the 'data' sub-section and figure 4.

3.) *Second primary outcome – Graft survival - is only defined as a return to dialysis. It should also include re-transplantation or death from graft failure*

We agree with the reviewer's suggestion. This has now been specified and included in the 'outcomes' sub-section:

> (p. 9, l. 9 – 11) Dialysis-free graft survival at the time of follow-up is defined as complete independence from hemodialysis, peritoneal dialysis and absence of re-transplantation or death from graft failure.

4.) Secondary outcomes;

a. Proteinuria - should include more than one measurement, i.e positive urinalysis for proteinuria on at least 2 occasions

Patients will give a urine sample for measurement of spot proteinuria during their follow-up appointments. While we agree that measurement on two occasions (twice for each follow-up) might increase diagnostic accuracy, it would necessitate multiple appointments with the transplantation outpatient clinic and this would exceed the capacities of the outpatient clinics, as it would lead to up to 800 additional appointments a year. Hence, for organizational reasons we will only take one urine sample at each appointment.

b. Recurrence of rejection - What definition will be used to delineate persistent or treatment-resistant rejection from a subsequent, second rejection episode?

A persistent rejection will be diagnosed in the absence of resolution of graft dysfunction. If graft function has recovered at any point and subsequently deteriorates again, a second rejection episode will be suspected. This has been specified in the 'outcomes' sub-section:

> (p. 9, l. 14 – 16) If there is no resolution of graft dysfunction, this will be recorded as a persistent rejection. If graft function has recovered at any point and subsequently deteriorates again, a second rejection episode will be suspected.

5.) Loss to follow-up – is there a protocol to manage loss to follow-up. Ie to ensure the patient is not deceased?

The reviewer raises an important point. When a patient does not show up to their follow-up appointment and cannot be reached by phone or by mail, their primary care physician will be contacted in order to exclude the risk that the patient is deceased. In the rare occasion that the patient is not in contact with their primary care physician any longer, and the primary care physician does not know whether the patient is deceased, authorities will be contacted to gather this information. This has been added to the sub-section 'study design':

(p. 7, l. 22 – p. 8, l. 2) If a patient is lost to follow-up because he/she is not available for a scheduled follow-up appointment, the patient's primary care physician will be contacted to inquire about whether the patient is deceased. If the patient is alive, another appointment will be scheduled or, when this is not possible, the data required for follow-up will be directly obtained from the primary care physician.

VERSION 2 – REVIEW

REVIEWER	David Rush University of Manitoba
REVIEW RETURNED	27-Aug-2021

GENERAL COMMENTS	A pragmatic study., the results of which will be of interest. The "minor revision" comment refers to the fact that antibody testing is not standardized and that there is no mention of matching between donors and recipients that might be reflected in rejections (e.g. Class II HLA mismatching at the eplet level).

REVIEWER	Juilan Singer The University of Sydney, Kidney Node Laboratory
REVIEW RETURNED	22-Aug-2021

GENERAL COMMENTS	The authors addressed the concerns raised by the reviewers. The protocol is thorough and of research interest.

VERSION 2 – AUTHOR RESPONSE

1. Reviewer

David Rush, University of Manitoba, Canada

1.) The "minor revision" comment refers to the fact that antibody testing is not standardized and that there is no mention of matching between donors and recipients that might be reflected in rejections (e.g. Class II HLA mismatching at the eplet level).

While we agree with the reviewer that standardized antibody testing can be beneficial, because of the multicenter nature of this study and the currently well-established cooperation between the centers and their respective laboratories, centralized testing was not deemed to be sufficiently pragmatic for the study design and scope of funding. However, each of the laboratories involved is held to the highest standards of Good Laboratory Practice, producing test results with high reliability and reproducibility. Regarding information on matching between donors and recipients, it is true that we will not be able to examine the relationship between mismatching and rejections. Nevertheless, we believe this will not impede with the primary study purposes, which are to analyze the association of different treatment strategies with clinical outcomes after rejection episodes and to describe the prognostic and histopathological features of kidney graft rejections in Germany.

2. Reviewer

Julian Singer, The University of Sidney, Royal Prince Alfred Hospital, Australia

There were no additional requests for revision from the 2. reviewer.