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DISSERTATION

Association of the Banff-categories of acute rejections and the Expression of interferon-stimulated genes with the clinical outcomes after kidney transplantation

Assoziierung der Banff-Rejektionskategorien und der Expression von Interferon-stimulierten Genen mit dem klinisch-Outcome nach Nierentransplantation

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List of abbreviations

AMR	Antibody-mediated rejection
AVR	Acute vascular rejection
ATG	Anti-thymocyte globulin
AUC	Area under the curve
Aza	Azathioprine
BMI	Body mass index
BPAR	Biopsy-proven acute rejection
CI	Confidence intervals
CIT	Cold ischemic time
CyA	Cyclosporine A
DCGS	Death-censored graft survival
DGF	Delayed graft function
DSA	Donor-specific antibodies
ELISA	Enzyme-linked immunosorbent assay
ESRD	End stage renal disease
GS	Graft survival
HLA	Human leukocyte antigen
HR	Hazard ratio
IFN	Interferon
IL	Interleukin
ISGs	Interferon-stimulated genes
LAT	Linker for activation of T-cells
MI	Microcirculation injury
MICA	Major histocompatibility complex classI-related chain A
MMF	Mycophenolate mofetil
PPH	Plasmapheresis
PRA	Panel reactive antibody
PRA-STAT	an ELISA test with soluble HLA class I molecules as targets

PS	Patient survival
PTLD	Post-transplant-lymphoproliferative disease
ROC	Receiver operating characteristics
Scr	Serum creatinine
SD	Standard deviation
SGF	Stable graft function
UTI	Urinary tract infection
Tac	Tacrolimus
TCMR	T-cell-mediated rejection
TI	Tubulo-interstitial inflammation
TMA	Thrombotic microangiopathy

Abstrakt (Deutsch)

Einleitung

Die Banff Klasssifikation, eine international akzeptierte Richtlinie für die Interpretation der Transplantatbiopsien, fokussiert mehr auf histologische Typen der Gewebeschäden als auf die Graduierung der Rejektion. Wir führten retrospektive Studien an unseren Patienten und deren Biopsiematerialien mit folgenden Fragestellungen durch: sind Schwere der akuten zellulären Rejektion (ACR) oder der akuten vaskulären Rejektion (AVR) assoziiert mit dem Transplantatüberleben. Außerdem messen wir die Expression von Interferon-stimulierten Genen (ISGs) als ein nicht-invasiver Weg zur Differenzierung von Antikörper-vermittelte Rejektion (AMR) von anderen Komplikationen nach Nierentransplantation (Tx).

Methoden

Entsprechend der revidierten Banff Kriterein 2009/2013 sind die Borderline Läsionen, die T-Zell vermittelte Rejektion (TCMR) Typ1 und Typ 2/3 definiert als akute zelluläre Rejektion geringen, mäßigen und schweren Grades. 270 Biopsien wurden ausgewählt nach der schwersten Form der ACR für jeden Patienten, 370 Patienten ohne Biopsie dienten als Kontrollgruppe.

Die drei gradige Arterienwandentzündung (Banff v1, v2, v3) wurde definiert als geringe, mäßige, starke AVR, 148 Patienten mit mindestens einer AVR Episode wurden ausgewertet und eingeteilt in 3 Typen: DSA-C4d- AVR, DSA+C4d- AVR und DSA+C4d+ AVR.

185 Patienten wurden einschließlich AMR (n=20), SGF (n=51), UTI (n=17), Borderline (BL) (n=22), TCMR I (n =19), TCMR II/III (n=26) und IFTA (n=30) rekrutiert. Gesamt-RNA wurde zum Zeitpunkt der Biopsie aus Vollblut von Patienten isoliert. Quantitative Echtzeit-PCR und ELISA wurden durchgeführt, um das Expressionsniveau von ISGs zu messen, die IFIT1, IFI44L, RSAD2, ETV7, IFIT3, IFI44 enthielten.

Ergebnisse

Bis zu 8 Jahren nach Transplantation beträgt die Todeszensierte Transplantatüberlebensrate (death-censored graft survival DCGS) in der

Kontrollgruppe, der Borderline Gruppe, der TCMR I und TCMR II/III Gruppe 97.6%, 93.3%, 79.6% und 73.6% (log rank Test, p<0.001), die Kontrolle Gruppe hatte eine signifikant höhere DCGS als die übrigen 3 ACR Gruppen (jeweils paarweiser Vergleich p<0,05). Die DCGS Rate der späten ACR war signifikant kleiner, verglichen mit der frühen ACR (63.6% vs. 87.4%, p<0.001).

Die 10 Jahres DCGS Rate DSA-C4d-, DSA+C4d- und DSA+C4d+ AVR Gruppen waren 39.5%, 16.7% und 14.3% (im Allgemeinen p=0.06, jeweils paarweiser Vergleich p>0.05). Die DCGS Raten der v1, v2 und v3- AVR waren 43.9%, 11.1% und 0.0% (im Allgemeinen p<0.001, jeweils paarweiser Vergleich mit v1-AVR p<0.01).

Die mRNA-Expressionsspiegel von IFIT1, IFI44L, RSAD2, ETV7, IFIT3, IFI44 waren im Blut von AMR-Patienten im Vergleich zur SGF-Gruppe signifikant erhöht (*P*<0,05).

Schlussfolgerungen

Alle Typen der ACR zeigten ein langes Transplantatüberleben mit Schädigungen. Die vaskuläre oder späte ACR sagt ein schlechteres Transplantatüberleben voraus; der Schweregrad der AVR ist enger mit dem Langzeittransplantatversagen assoziiert, als die AVR Typen. Die Messung der ETV7-mRNA-Expression könnte einen neuen und nicht-invasiven Ansatz darstellen, eine AMR von anderen transplantierten Komplikationen zu differenzieren.

Abstract (English)

Introduction

The Banff classification, an internationally accepted guideline to interpret the allograft biopsy by consensus definitions and arbitrary thresholds, focuses large extent on histological “types” rather than “grade” of rejection. We performed retrospective studies to observe whether the severity of the acute cellular rejections (ACR) or the acute vascular rejection (AVR) associated with graft outcome. In addition, the blood expression of interferon-stimulated genes (ISGs) might offer a non-invasive way to differentiate AMR from patients with other complication post transplantation (Tx).

Methods

According to the revised Banff criteria 2009/2013, the Borderline changes, T-cell mediated rejection (TCMR) type I and type II/III were defined as low, moderate and high ACR severity, respectively. 270 biopsies were chosen according to the highest ACR severity of each patient, 370 patients experienced no Tx-biopsy were grouped as control.

The three grades of intimal arteritis (v1, v2 and v3) were defined to represent low, moderate and high AVR severity, 148 patients who had at least one AVR episode were enrolled and divided into three types: DSA-C4d- AVR, DSA+C4d-AVR and DSA+C4d+ AVR.

185 patients were recruited, including AMR (n=20), SGF (n=51), UTI (n=17), Borderline (BL) (n=22), TCMR I (n=19), TCMR II/III (n=26) and IFTA (n=30). Total RNA was isolated from whole blood of patients at the time of biopsy. Quantitative real-time PCR and ELISA were performed to measure the expression level of ISGs.

Results

Up to 8-year post Tx, the death-censored graft survival (DCGS) rates of control, Borderline, TCMR I and TCMR II/III groups were 97.6%, 93.3%, 79.6% and 73.6% (log rank test, $p<0.001$), the control group had significantly higher DCGS rate than the three ACR groups (each pair-wise comparison yields $p<0.05$).

The 10-year DCGS rates of DSA-C4d-, DSA+C4d- and DSA+C4d+ AVR groups were 39.5%, 16.7% and 14.3% (overall p=0.06, each pair-wise comparison yields p>0.05). The DCGS rates of v1, v2 and v3- AVR were 43.9%, 11.1% and 0.0% (overall p<0.001, each pairwise comparison to v1-AVR yields p<0.01).

The mRNA expression levels of IFIT1, IFI44L, RSAD2, ETV7, IFIT3, IFI44 were significantly up regulated in blood of AMR patients compared to the SGF group (*P*<0.05).

Conclusions

All types of ACR affect long-term graft survival. The vascular or late ACR predict inferior graft survival. The AVR severity is more closely associated with the long-term graft failure rather than the types. The measurement of ETV7 mRNA expression level might offer a novel and non-invasive approach differentiating AMR from other complications after kidney transplantation.

1 INTRODUCTION

1.1 Renal transplantation

Chronic kidney disease (CKD) is a worldwide public health problem with increasing prevalence and incidence of end stage of renal diseases (ESRD) substantially throughout world during the next several decades [1-4]. Up to date, Kidney transplantation (either from living or deceased donor) is the most effective treatment for most patients with ESRD despite an increased short-term risk of death after transplantation [5-6]. Besides increasing patients' quality of life (QOL) and reducing the health-care budget, the most important factor is that kidney transplantation can improve long-term patient survival compared with patients on dialysis [7-9].

1.2 Renal allograft biopsy

Despite renal graft and patient survival have improved dramatically over the last two decades, the rate of chronic graft loss remains substantial and did not improve over the last decade and a substantial proportion of patients have returned to chronic dialysis after failed kidney transplant in worldwide [8]. The relationship between histological damage and graft outcome is only incomplete unterstood. So far, many transplant centers routinely consider graft biopsy at the onset of renal dysfunction, the core-needle allograft biopsy is not only the "gold standard" to establish the correct diagnosis, but also provides a good opportunity to study the relationship between histological damage and transplant outcome [10].

1.3 Updated Banff classification

The Banff working classification of renal allograft pathology is a guideline to interpret the allograft biopsy by consensus definitions and arbitrary thresholds [11]. It was published in 1997 and is updated every 2 years during the last 2 decades [12-15]. With considerable progress in capturing, standardizing and incorporating the histological, immunohistochemical and serological factors, the revised Banff classification has improved sensitivity in the diagnosis of allograft rejection and tries to relate histology with allograft survival [11]. However, Banff classification describes histological "types" rather than "grade"

of rejection [16], and it remains unclear how the updated classification and scoring relates to outcome and if the timing of rejection plays a role in graft survival.

1.4 Acute cellular Rejection

According to Banff classification, the principle diagnostic lesions of acute cellular rejection (ACR) include interstitial inflammation (i), tubulitis (t) and intimal arteritis (v), which are graded by arbitrary consensus rules [12]. Borderline changes and TCMR are defined as two categories of ACR, and TCMR is consisted of five subtypes [13]. Therefore, ACR encompasses distinct histological features of different scored lesions, and relates to variable initial response to anti-rejection treatment, allograft function and outcome [16-17].

1.5 Acute vascular rejection

The acute vascular rejections (AVR) are commonly considered as a severe form of acute rejection characterized by infiltration of mononuclear cells beneath the endothelium or by the presence of arteritis, and is traditionally categorized into TCMR and AMR based on the presence of donor specific HLA-antibodies (DSA) and C4d staining. In general, the antibody mediated vascular rejection is supposed to relate to the poorer initial responses to antirejection treatment, allograft function and graft outcome compared to the T-cell mediated vascular rejection [12-13, 17]. The new consensus criteria for C4d-negative AMR has been described in the 2013 Banff meeting report [15]; the AVR episodes are further reclassified into T-cell mediated vascular rejection, C4d-negative/ C4d-positive antibody-mediated vascular rejection. However, it remains unclear if the revised AVR types and severity defined by the Banff scores of intimal arteritis also affect graft survival.

1.6 The expression of the Interferon-stimulated genes

Interferon-stimulated genes (ISGs) are described as the genes whose expression are induced or regulated by interferon, which play pivotal roles in immune system defense against infection [18]. However, this classical definition is not comprehensive enough to cover all aspects of ISGs. Recent observations demonstrate that the expression of ISGs also can be the

response to a variety of stimulatory factors like injury, inflammation, stress, and other events [19, 20]. Emerging functional roles of ISGs besides their antiviral effect remain to be elucidated.

In addition to systemic lupus erythematosus and cancer, the involvement of ISGs have been described in solid organ transplantation [21, 22]. Saiura et al found interferon-γ-inducible genes are upregulated in murine cardiac transplantation mode during the late phase of AR [23]. Interferon-γ related genes also significantly changed in AR compared to no rejection patients after lung transplantation [24]. The early activation of ISGs in human liver allografts was related to the risk of acute cellular rejection. However, unlike other organ transplantation, the induction of ISGs after liver transplantation might also due to the recurrence of hepatitis C as an antiviral response [25].

Rascio et al compared peripheral blood molecular signature of 29 chronic AMR patients with eight IFTA and 29 stable transplant recipients as controls. They found genes involved in type I interferon signaling upregulated in chronic AMR [26]. Akalin et al used an oligoarray to analyze three normal renal allograft biopsy samples and seven human TCMR samples, six of which shown up-regulated interferon-stimulated growth factor-3 (ISGF-3), the activator ISGs [27]. The capability of ISGs to distinguish between the different types of rejection in kidney transplantation still needs confirmation.

1.7 Aims and objectives

From Jan.1996 until Oct. 2018, nearly 2400 for-cause kidney graft biopsies have been performed in Charite Campus Mitte. The results of the biopsies have been reviewed and revaluated according to the updated Banff working classification 2009/2013. All the data of the scored lesions and categories of each biopsy have been documented and saved in the transplantation date base (Tbase) which provide the complete and computerized data of histological evaluation for the treatment, clinical study, and research. Therefore, we performed two retrospective studies to address the following issues:

1) whether the severity of ACR is associated with the long term graft loss;

2) whether the types and severity of AVR is relevant to distinct short-term (reversibility of the rejection episode with antirejection therapy) and long-term

clinical outcome (graft survival);

In addition, to find out non-invasive markers for sensitive and specific diagnosis of AMR, we focused on six genes out of the identified gene set and validated IFIT1, RSAD2, and ETV7 in a large patient cohort with different pathologies including AMR, TCMR, and infection. Measuring these three markers alone allows the diagnosis of AMR with high specificity and sensitivity.

2 MATERIALS AND METHODS

2.1 Patient and data collection

We reviewed all adult (≥18 years) patients who received kidney transplant between Jan. 1996 and dec. 2012 at the Kidney Transplant Centre of Charité Campus Mitte.

Regarding to the ACR study, 270 patients who had at least one episode of ACR were chosen as study group; In case one patient experienced multiple episodes of ACR, the TCMR type II/III were preferentially chosen, followed by TCMR type I and Borderline changes. 370 patients who experienced no biopsy post Tx were employed as the control group.

In consideration of AVR study, 148 patients were collected who had at least one for-cause graft biopsy that demonstrated histological features of AVR. In case one patient experienced multiple categories of AVR, the DSA+C4d+ AVR were preferentially chosen, followed by DSA+C4d- AVR and DSA-C4d- AVR. In addition, Banff v1, v2 and v3-lesions were defined as the low, moderate and high AVR severity, respectively. In case there were multiple AVR episodes in one AVR category, v3- AVR was firstly chosen, followed by v2 and v1-AVR.

With respect to the ISGs study, 185 Tx-recipients were recruited, including AMR (n=20), SGF (n=51), UTI (n=17), Borderline (BL) (n=22), TCMR I (n=19), TCMR II/III (n=26) and IFTA (n=30). Of which 117 serum and plasma samples were also collected. This study was approved by the ethical committee of the Charité-Universitätsmedizin Berlin (EA1/091/10). All the patients received and signed written informed consent.

Graft loss was defined as returning to chronic dialysis or death with functioning grafts. Death-censored graft failure was decided as returning to chronic dialysis. All clinical and laboratory data were recorded in our transplant database system (TBase) at each visit.

2.2 Pathologic review of biopsies

An ultrasound-guided graft biopsy was performed when clinically indicated, i.e. elevating the concentration of serum creatinine (Scr). All patients with DGF, defined as needing dialysis within 1-week post Tx [28] underwent protocol biopsy on the 7th day post Tx.

Biopsy specimens were processed with standard techniques in the Department of Pathology, Charite Campus Mitte. Adequate sample involved minimal of seven glomeruli and one artery. Indirect immunofluorescent staining of C4d was performed on paraffin sections (polyclonal anti-C4d antibody, Dianovo, Germany). Biopsies from the pre-C4d era were retrospectively tested for C4d. All light microscopy slides were reviewed by two pathologists (B.R and K. Wu), and diagnosed according to the 2009/2013 revised Banff classification [13]. Each sample was scored on the following: glomerulitis (g), peritubular capillaritis (ptc), transplant glomerulopathy (cg), intimal arteritis (v), interstitial inflammation (i), tubulitis (t), mesangial matrix increase (mm), vascular intimal fibrosis (cv), arteriolar hyaline thickening (ah), interstitial fibrosis (ci) and tubular atrophy (ct). In the ACR study, Borderline changes referred to histological indices t1/i1-2 or i1/t1-2 [29]. TCMR I was defined as v0, t2-3, i2-3, and TCMR II/III defined as v1-3, t0-3, i0-3. Borderline, TCMR I and TCMR II/III group represented low, middle and high ACR severity. The microcirculation inflammation (MI) lesions included g≥1, or ptc≥1, the biopsies were chosen which showed the highest ACR severity of each patient, free of MI-lesions and negative for C4d or DSA. Diagnosis of AMR depends on simultaneous presence of DSA, positive C4d staining and allograft pathology. ‘C4d negative AMR’ was considered if C4d was negative but DSA and morphologic moderate MI-lesions present. In the study of AVR, T-cell mediated vascular rejection was defined as v1-3, C4d 0 and free of DSA; antibody-mediated vascular rejection was diagnosed on simultaneous presence of v1-3, C4d 1-3 and DSA; C4d negative antibody-mediated vascular rejection referred to v1-3, C4d 0 plus

DSA.

2.3 HLA-antibody screening

DSA level was monitored as previously described [30]. All serum samples which were collected once a year or at biopsy were qualitatively screened for HLA antibodies by two ELISA based screening systems (PRA-STAT and LAT) from 1996 to 2006 or the Luminex-based bead assay LABScreen Mixed (One Lambda, Canoga Park, CA, USA) from 2007 on. All tests were performed according to the manufacturer's guidelines [31].

2.4 Immunosuppressive protocol and anti-rejection treatment

The immunosuppression protocol comprised of triple immunosuppression with a calcineurin pathway agent Cyclosporin A (CyA)/Tacrolimus (Tac), and anti-proliferative agent (mycophenolate mofetil (MMF)/ mycophenolic acid (MPA) or m-Tor inhibitor with methylprednisolone. The recommended initial oral daily CyA dose was 300 mg/m2 subsequently maintaining the whole-blood concentrations within the range of 150–250 ng/ml for the first 4 weeks and 100–150 ng/ml from 2-6 months and 60-120 ng/ml thereafter. The recommended initial oral daily Tac dose was 0.3 mg/kg, administered in two divided doses; and to maintain the target whole-blood trough levels within the range of 10–15 ng/ml from day 0 to day 30 and 6–10 ng/ml from day 30 to 90 onwards and 5-8 ng/ml thereafter. Anti-rejection therapy involved two broad steps: 1) the pulse therapy of corticosteroids; 2) grafts with steroid-resistance or positive DSA received therapeutic PPH plus antibody therapy, which included one or more of following reagents: ATG (rabbit antithymocyte globulin); IVIG (intravenous immune globulin); Rituximab (anti-CD 20 globulin); Bortezomib (therapeutic proteasome inhibitor); Eculizumab (anti-Complement protein 5). Complete, partial and non-reversible responses were defined by comparing the 1-month post biopsy Scr with the pre-biopsy concentration [32].

2.5 mRNA and protein expression of ISGs

Fresh blood samples (2.5 ml/sample) were collected into PAXgene blood RNA tubes from patients based on the manufactures instruction at the time of biopsy (PreAnalytiX; Becton Dickinson, Heidelberg, Germany). Candidate genes IFIT1, IFI44L, RSAD2, ETV7, IFIT3, and IFI44, had been previously picked

after Next Generation Sequencing experiments and gene expression analyses with RNA from six AMR, six stable graft function and four TCMR patients [33]. Total RNA was isolated from whole blood using PAXgene blood miRNA kit (PreAnalytix; Qiagen, Hilden, Germany) based on manufacturers' instructions. The concentration of the sample was measured by NanoDrop lite spectrophotometer (prqlab, Erlangen, Germany). The Maxima first strand cDNA synthesis kit (Thermo Scientific) was used for reverse transcription of RNA into cDNA. Quantitative real-time PCR (RT-PCR) was performed with candidate mRNAs IFIT1, IFI44L, RSAD2, ETV7, IFIT3, IFI44, and HPRT1 as housekeeping gene using TaqMan™ Universal Master Mix II, with UNG (Thermo Scientific) which included AmpliTaq Gold® DNA Polymerase, Uracil-N-Glycosylase (UNG), dNTPs with dUTP, passive reference 1, optimized buffer components. The housekeeping gene HPRT1 was used for normalization of each cDNA sample. For quantification of protein expression, serum samples were used to measure ETV7 (ETV7 ELISA Kit by MyBioSource, San Diego, CA, USA) and plasma samples were used to measure RSAD2 (RSAD2 ELISA Kit; Aviva Systems Biology, San Diego, CA, USA) and IFIT1 (Cusabio, Houston, TX, USA) according to the manufacuters instruction in AMR, SGF, and TCMR patients.

2.6 Statistical Analysis

All patients were followed up until the end of study or graft failure. All data were assessed for completeness. Continuous variables were expressed as mean± standard deviation. Categorical variables were expressed as N and percentage of total. Student's t-test was used to compare 2 groups of continuous variables and chi-square for categorical data. The survival curves were analyzed by Kaplan-Meier graphs and statistically compared by log-rank test. To test putative risk factors for long-term graft loss, Banff scored lesions were tested in univariate Cox-regression analysis; those of which with significant association ($p<0.05$) were then entered into multivariate analysis. For analysis of different expression level of markers from the different group, KS normality test was performed to test whether the data was normally distributed if not the nonparametric Mann-Whitney *U* test was used to compare the difference of each two patient groups. Logistic regression was used to test

the diagnostic value of significantly changed markers in last step. The receiver operating characteristics (ROC) analysis was performed. Area under the curve (AUC), sensitivity, and specificity were used to specify the performance of markers in discriminating AMR from the comparators (None-AMR patients or SGF patients). All statistics were performed by using SPSS16.0 (SPSS Inc., Chicago, IL). P-value < 0.05 was considered significant.

3 RESULTS

3.1 Study 1: Different types of ACR and the graft survival [34]

270 ACR cases were chosen and classified into three groups: Borderline (n=90, 33.3%), TCMR I (n=108, 40.0%) and TCMR II/III (n=72, 26.7%). At biopsy, the mean Scr concentration of TCMR II/III group was statistically higher than that of TCMR I group (5.8 vs. 4.6 mg/mL, p=0.04). At 1-month post biopsy, the mean Scr value of Borderline group (2.3 mg/mL) was statistically lower than that of TCMRI group (2.8 mg/mL, p=0.03) or TCMR II/III group (3.6 mg/mL, p=0.007). The Borderline group showed the highest proportion of complete reversibility, in contrast, TCMR II/III group presented with the highest fraction of partial/non reversibility, although significantly more patients of TCMR II/III group received therapeutic PPH and antibody therapy.

Among the three ACR groups, the mean scores of Banff lesions ah and mm showed similar grades. The mean t-lesion score of TCMR I group was significantly higher than that of borderline or TCMR II/III group; the mean i-lesion score of TCMR I or TCMR II/III group was significantly higher than that of borderline group; the mean ci/ct score of TCMR I or TCMR II/III group was statistically higher compared with Borderline group.

Kaplan-Meier graft-survival analysis showed the control group had the best graft survival, and the patient survival was similar among four groups. The 8-year DCGS rates of control and Borderline groups were significantly higher than tha of TCMR I group or TCMR II/III group (each pair-wise comparison yields p<0.001), and the DCGS rate of control group was statistically higher than that of Borderline group (p=0.03). No statistical difference of DCGS rate

was found between TCMR I and TCMR II/III groups (p=0.20). Defined by the Banff scores of t and v-lesions, we divided TCMR I into Ia and Ib types, and TCMR II into IIa and IIb types. The patients with TCMR Ia showed significantly better graft and patient survival than those with TCMR Ib or TCMR IIb/III; the patients with TCMR Ib and IIa had the same graft and patient survival; the lowest graft and patient survival was found in TCMR IIb/III group. Compared with late overall ACR, early overall ACR showed significantly better graft survival but same patient survival. The GS and DCGS rates of early and late Borderline group were similar; the graft survival was significantly higher in early TCMR I or II/III group, comparing with late TCMR I or type II/III group. Either in the three early or late ACR groups, the graft survival of TCMR II/III group was significantly lower than that of borderline or TCMR I group, but there was no significant difference between Borderline and TCMR I groups.

3.2 Study 2: Three kinds of AVR and the graft survival [35]

A total of 148 patients with AVR episodes were chosen and categorized into three AVR groups: DSA-C4d- AVR (n=85, 57.4%), DSA+C4d- AVR (n=37, 25.0%) and DSA+C4d+ AVR (n=26, 17.6%). Of which, 64.7% DSA-C4d- AVR, 41.7% DSA+C4d- AVR and 37.0% DSA+C4d+ AVR showed v1-lesion (overall $p<0.05$, each pair comparison to DSA-C4d- AVR, yields $p<0.05$); in contrast, 25.9% DSA+C4d+ AVR and 5.9% DSA-C4d- AVR had v3-lesion (p=0.003).

There was no significant difference in recipient and transplant characteristics among the three AVR groups. The median biopsy timing of DSA+C4d+ AVR was statistically longer than that of DSA-C4d- AVR (400 vs. 10 days post Tx, p=0.01). The variation of Scr concentration (pre-, at and post AVR) were comparable among the three AVR groups. Significantly more patients of DSA+C4d- AVR and DSA+C4d+ AVR received therapeutic PPH and antibodies therapy compared to DSA-C4d- AVR (each pair-wise comparison to DSA-C4d- AVR group yields $p<0.05$). However, no significant differences of the responses to the antirejection therapy were found among the three AVR groups. In aspect of the AVR severity, significantly more patients with v2-AVR and v3-AVR received therapeutic PPH and antibody therapy compared to patients with v1-AVR (each pair-wise comparison to v1-AVR group yields $p<0.05$). The grafts with v1-AVR showed a significantly higher proportion of

complete reversibility than that of v3-AVR (56.2% vs. 23.5%, p=0.02); the v2- and v3-AVR presented with a significantly higher fraction of non-reversibility than that of v1-AVR (each pair-wise comparison to v1-AVR yields p<0.05), but similar response was found between v2-AVR and v3-AVR.

The DSA+C4d+ AVR group had statistically higher grade (score≥2) of g, cg, ptc and mm-lesions in comparison to the DSA-C4d- AVR group (each pair-wise comparison yields p<0.01); the high grade of g and ptc-lesions occurred statistically more often in the DSA+C4d+ AVR group compared to the DSA+C4d- AVR group (each pair-wise comparison, yields p<0.05). A significantly higher proportion of v2 and v3-lesions was found in the DSA+C4d- AVR and DSA+C4d+ AVR groups compared to the DSA-C4d- AVR group (each pair-wise comparison to DSA-C4d- AVR group, yields p<0.05).

The 1-, 5- and 10-year DCGS rates were comparable among the three AVR categories (overall p=0.06, each pair-wise comparison between two AVR groups yields p>0.05). However, the grafts with v2 and v3-AVR showed significantly lower DCGS rates than that of v1-AVR at 1-, 5- and 10-years post Tx (each pair-wise comparison to AVR with v1-lesion, yields p<0.01); moreover, the DCGS rate of v3-AVR was statistically lower than v2-AVR at 5-years post Tx (p=0.03). Within the DSA-C4d- AVR group, the 10-year DCGS rates of grafts with v1, v2 and v3-lesions were 51.9%, 11.0% and 0.0%, respectively (each pair-wise comparison yields p<0.05, Table 5). Within DSA+C4d- AVR group, the 10-year DCGS rate of v3-group was significantly lower than that of v1 or v2-group; within the DSA+C4d+ AVR group, the 10-year DCGS rate of v3-group was significantly lower than that of v1-group. The grafts with the same v-score showed the similar 10-year DCGS rate in spite of the ACR types. By multivariate analysis, the presence of ptc-lesions and donor specific HLA-antibodies class I appeared to be independent factors inversely related to graft loss.

3.3 Study 3: The expression of ISGs und its value for AMBR [33]

A total of 111 genes were able to discriminate AMR from controls and from TCMR. The sum of 85 genes were differentially expressed between control patients and TCMR, whereas 14 of these differentiated controls from AMR and

from TCMR. Nine specific genes distinguished TCMR from AMR and controls. Six candidates were selected according to their ability to distinguish AMR from TCMR or SGF or both. Furthermore, the candidates were picked due to their expression and the variability in their expression within the single patients cohorts. IFIT1, RSAD2, IFIT3, IFI44, and ETV7 were picked from the group of genes that distinguished AMR from SGF and additionally from TCMR, whereas IFI44L was chosen as AMR- SGF discriminating candidate and paralog of the candidate IFI44 gene.

A significant expression difference was observed between AMR and SGF patients for all candidate genes after performing a nonparametric Mann- Whitney U Test. IFIT1, IFI44, and RSAD2 additionally displayed a significantly lower expression in TCMR compared to AMR, whereas expression levels for IFIT3 varied between SGF and TCMR.

The candidate expression in the patients with AMR, SGF, UTI, BL, TCMR, and IFTA was analyzed with a nonparametric Kruskal- Wallis test. Significant distinctions were observed for IFIT1 ($P < 0.001$), IFIT3 ($P < 0.001$), IFI44 ($P < 0.05$), IFI44L ($P < 0.01$), ETV7 ($P < 0.0001$), and RSAD2 ($P < 0.0001$). Hence, a two- stage step- up test of Benjamini, Krieger, and Yekutieli between any two groups was performed. The expression of IFIT3, IFI44, and IFI44L in AMR patients was significantly upregulated compared to SGF patients, but not when comparing it to the other patient groups. The sequencing and validation results for IFIT1 could be confirmed, and the expression in AMR patients differed significantly from patients with SGF and TCMR. Additionally, IFIT1 expression in SGF was lower when comparing it to UTI and IFTA ETV7 and RSAD2 were significantly upregulated in AMR patients when comparing their expression to SGF, BL, and TCMR.

The ROC analysis of gene expression in patients with AMR vs patients with SGF demonstrated high specificities and high sensitivities for IFIT1 ($AUC = 0.761$; $P \leq 0.0006$), ETV7 ($AUC = 0.84$; $P \leq 0.0001$), and RSAD2 ($AUC = 0.761$; $P \leq 0.0006$; Figure 6A- C). After cross- validation, AUCs of 0.64, 0.68, and 0.70 were observed for IFIT1, ETV7, and RSAD2.

4 DISCUSSION

4.1 Study1: The severity of ACR and the clinical graft outcome

We retrospectively studied 270 biopsies, which showed the highest ACR severity of each patient after the AMR was excluded. Firstly, our data proved that any type of ACR, regardless of severity, was associated with deterioration of graft function overtime; even patients of Borderline group, in terms of the low ACR severity, could not sustain the same DCGS rate as the patients of control group. The significant deterioration of graft survival might relate to partial ACR, which responded incompletely to anti-rejection treatment. The incompletely reversible ACR could lead to more for-cause biopsies, increase the risk for subsequent late rejection, both of which result in persistent and progressive parenchymal damage and accelerate graft failure [36, 37].

Secondly, based on the Banff scored lesions, the higher ACR severity predicted the poorer anti-treatment response and graft survival. The patients of Borderline group showed significantly higher graft survival than those of TCMR type I or TCMR type II/III group. However, there was no significant difference of graft survival between TCMR type I and TCMR type II/III. Our data was in accordance with a recent study of Lefaucheur et al [38], who reported that compared with acute cellular interstitial rejection; the risk of graft loss was 9.07 times higher in AMR with v-lesion, 2.93 times higher in AMR without v-lesion while there was no significant increasing in acute cellular vascular rejection. Overall, in ACR patients, the v and t-lesions showed significant association with long-term graft failure and v-lesion was proven as an independent predictor of subsequent graft loss regardless of the timing of biopsy. Among three types of interstitial rejection, patients with t3-lesion (TCMR Ib) had a demonstrable worse graft outcome than those with t1/t2-lesions (Borderline or TCMR Ia).

Late ACR was supposed to be more difficult to reverse and had a higher risk of subsequent graft loss than early ACR, considering of its higher scores of chronic scarring (ci/ct, mm), vascular diseases (ah, cv) and active immune inflammation (i, t). Histologically, interstitial fibrosis and tubular atrophy (ci/ct) were common in late allografts indicating the cumulative burden of injury and diseases such as chronic allograft nephropathy (CAN), which was defined as

progressive allograft dysfunction occurring at least three months post Tx and is the cause of 44% of graft loss after the first year post Tx [39]. However, ci/ct-lesion was not a disease itself, but a feature of all progressive kidney diseases. In addition, the chronic vascular damage (ah, cv) undoubtedly contributed to late graft loss, which also resulted from CNI use, as biopsy samples from both native and transplanted kidney exposed to CNI demonstrate that arteriolar hyalinosis (ah) eventually develops into an obliterative vasculopathy, and finally leads to tubulointerstitial damage and striped fibrosis [40]. Moreover, except for the infiltration of vessels by mononuclear cells, the histological characteristics of vascular rejection, including endothelial-cell apoptosis and the synthesis of matrix proteins and collagens by intimal myofibroblasts, enhanced the development of arteriosclerosis. This probably explains why the grade of cv in TCMR II/III group was significantly higher than that of early TCMR II/III group, whereas no prominent difference was found between early and late Borderline as well as TCMR I group.

4.2 Study 2: The severity of AVR and the clinical graft outcome

The DSA-C4d- AVR group had a relatively higher DCGS rate at 1, 5 and 10 years post Tx than that of DSA+C4d- AVR group or DSA+C4d+ AVR group, whereas no significant difference was found among the three AVR groups or even from each pair-wise comparison. The three AVR groups presented with similar responses to antirejection treatment. Our data were in accordance with a recent study of Lefaucheur *et al*, who reported that the risk of graft loss in AMR with v-lesions was not significantly increased in acute cellular vascular rejection. On the other hand, the three AVR types were not mutually exclusive and could coexist; the late onset or severe form of TCMR was often in conjunction with unrecognized AMR [41]. For minimizing the influence of humoral components in the T-cell-mediated AVR group, any biopsies showing C4d and/or DSA positivity were excluded. However, it was impossible to completely rule out the mixture of acute or chronic active AMR because the laboratory evidence of C1q-fixing DSA or non-HLA antibodies were not routinely detected [42], or histological evidence from multilayering of the peritubular capillary basement membrane, could only be obtained through

electron microscopy [43], a procedure not widely performed.

Haas *et al* demonstrated that all AVR cases with fibrinoid necrosis lost grafts within a year [44]. The AVR severity, classified using the Banff v-lesion scores, associated with the well-known poor graft outcome, the higher v-lesion scores indicated the poorer graft outcomes, even though the v2 and v3-AVR were more often treated with the antibody-directed therapy, the fraction of non-reversibility was clearly higher compared to v1-AVR. Conversely, AVR with the same v-lesion score presented with similar responses to antirejection treatment regardless of the positivity of DSA or C4d staining. The irreversible AVR leads to more clinically indicated biopsies and increases the risk for subsequent late rejection, both of which result in persistent and progressive renal parenchymal damage and accelerate graft failure [45, 46]. So far, the ideal treatment of the different severities of vascular rejection remains to be determined, but a more aggressive attempt to treat and prevent the high severity of AVR would be an effective strategy and a real impetus for improving kidney survival [47].

The preexisting or de novo DSA was proved to compromise renal allograft survival [48, 49]. The v-lesions occured in AMR, mixed AMR/TCMR and TCMR, albeit the v-lesions in the presence of DSA frequently coexist with glomerulitis and/or peritubular capillaritis because DSA directly induced endothelial cell injury and activated both complement dependent and complement independent pathways [50,51]. The criteria for diagnosis of C4d-negative AMR required moderate MI-lesions (g+ptc≥2) or elevated expression of gene transcripts indicative of endothelial injury in the biopsy tissue [15], which was supported by our data because the DSA+C4d- AVR with MI-lesions showed inferior graft outcomes than those lacking MI-lesions.

The MI-lesions were considered a humoral component of AMR and has been previously proven to be a negative prognostic feature in late biopsies independent of C4d staining [52, 53]. However, MI-lesions existed not only in AMR but also in TCMR, the latter in absence with DSA has good graft prognosis [54]. In our study, the AVR episodes presented with MI-lesions had significantly poorer prognosis than the AVR free of MI-lesions; whereas the inferior impact of MI-lesions on allograft outcome was independent of the

extent of MI-lesions since the graft outcome was similar when the comparison was performed between AVR with low and high grade of MI-lesion.

4.3 Study 3: The expression of ISGs as biomarkers for AMR diagnosis

Next-generation sequencing revealed several genes which were regulated in blood cells of patients with AMR, TCMR and SGF. Particularly, the IFN type I signaling was relevant in the crosstalk between adaptive and innate immunity [55] originating from the modulation through TLR agonists [56]. The TLR expression on leukocytes was increased in Tx patients with developing allograft dysfunction [57]. Recently, IFN type I co-acts with Th17 was reported to associate with AMR [58, 59]. Our study confirmed the finding that IFIGs are upregulated in AMR episodes after Tx since the majority of the 25 most significantly regulated genes between AMR and TCMR or SGF was involved in the IFN type I signaling pathway. Notably, we were additionally able to include a far greater number of patients in the RT-PCR study. We included control groups like TCMR and infection, which were crucial for the selection of candidates and subsequently for the conclusion that specific gene signatures might be AMR specific. Nevertheless, we would highly suggest to include even more control groups with matched demographics and immunosuppressive therapy in further validation studies, including patients with viral infections and patients with combined TCMR and AMR.

Interferon-inducible genes might exhibit immunomodulatory properties that could be involved in the molecular post TX and AMR mechanisms. IFIT1 and IFIT3 are highly conserved with multiple repeats of tetratricopeptide repeat helix-turn-helix motifs. IFIT1 might be a negative feedback regulator of IFNs [60]. RSAD2 belongs to the S-adenosylmethionine (SAM) enzyme superfamily and is localized in the ER, the golgi apparatus, and the mitochondria. The precise functions and substrates of this IFIG are unknown, but it displays high antiviral activity against a wide range of DNA and RNA pathogens, including hepatitis C virus [61, 62]. Apart from the role in inhibiting viral proliferation, it has been hypothesized that RSAD2 might be involved in Th2 response regulation [63]. ETV7 belongs to the ETS family of transcription factors, which involved in a wide variety of processes including cellular differentiation, proliferation, and apoptosis [64]. These three candidate markers showed a

significant upregulation of gene expression in blood cells of AMR patients compared to TCMR and SGF patients. The findings on mRNA level could not be translated into the protein level in serum/plasma, suggesting that the cell types which upregulate the expression of these three intercellular factors upon AMR do not release IFIT1, RSAD2, and ETV7.

4.4 Limitations and further perspectives

As many clinical studies may encounter, heterogeneity of the patient may lead to the decrease of accuracy. Thus, a larger group cohort, multi-center recruitment, application of random selection and blind method can help prevent the bias of future research.

For patient measuring, it is better to choose control patients matching in age or time after Tx so that we can distinguish the influence of these factors. Due to patients having not experienced protocol biopsies, the control group we used was diagnosed based on the clinical finding, which may have included patients with subclinical rejection.

To minimize any possible effect of AMR, we excluded any biopsies showing positivity of C4d or DSA or MI-lesions. Nevertheless we cannot completely rule out the mixture of ACR with chronic active AMR because the multilayering of peritubular capillary basement membrane can only be obtained through the electron microscopy, while this procedure is not widely performed, in addition, C4d negative AMR while C1q-fixing DSA or non-HLA antibodies has not been routinely checked.

The prognostic value of the ISGs markers for the survival of graft or the patients should be analyzed in order to provide more information for clinicians to decide the timely and appropriate treatment. Most AMR patients enrolled in this study encountered AMR more than one year after kidney transplantation and most of the patients in AMR group encountered chronic AMR or chronic active AMR. Since the goal of this study is to find a better way to diagnose AMR, more detailed subgroup with a larger population should be considered in the future study.

4. 5 Conclusion remarks

Allograft rejection (AR) can occur at any time after transplantation. The occurrence, timing, and number of AR episodes are also associated with increased risk of graft loss. The gold standard for diagnosis is the needle biopsy, although the Banff classification has evolved based on continuous new research, but more areas need to be determined for further clarification.

Results from our studies indicate that the types and severity of ACR und AVR defined by Banff classification are associated overtime with renal allograft dysfunction; ACR with v-lesions and late occurring ACR predict poorer long-term graft prognosis. The severity of AVR is robustly associated with over time renal allograft dysfunction despite of the negative impacts of anti-HLA antibodies and microcirculation injuries. For AVR with mild intimal arteritis, the aggressive immunosuppressive therapeutic interventions might improve the reversibility of rejection and long-term clinical outcomes. The elevated expression levels of these ISGs genes gives us a clue: related genes could widen the candidate markers circle so that more potential markers could be identified to yield a better diagnosis of AMR. For another, searching for the origin of these genes from specific cell subsets and tracking the molecular pathway about these genes might offer a better understanding of the mechanism during AMR.

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Eidesstattliche Versicherung

„Ich, Kaiyin Wu, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: ［Association of the Banff-categories of acute rejections and the Expression of interferon-stimulated genes with the clinical outcomes after kidney transplantation Assoziierung der Banff-Rejektionskategorien und der Expression von Interferon-stimulierten Genen mit dem klinisch-Outcome nach Nierentransplantation］selbstständig und ohne nicht offengelegte Hilfe Dritter verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel genutzt habe.

Alle Stellen, die wörtlich oder dem Sinne nach auf Publikationen oder Vorträgen anderer Autoren beruhen, sind als solche in korrekter Zitierung kenntlich gemacht. Die Abschnitte zu Methodik (insbesondere praktische Arbeiten, Laborbestimmungen, statistische Aufarbeitung) und Resultaten (insbesondere Abbildungen, Graphiken und Tabellen werden von mir verantwortet.

Meine Anteile an etwaigen Publikationen zu dieser Dissertation entsprechen denen, die in der untenstehenden gemeinsamen Erklärung mit dem/der Betreuer/in, angegeben sind. Für sämtliche im Rahmen der Dissertation entstandenen Publikationen wurden die Richtlinien des ICMJE (International Committee of Medical Journal Editors; www.icmje.og) zur Autorenschaft eingehalten. Ich erkläre ferner, dass mir die Satzung der Charité – Universitätsmedizin Berlin zur Sicherung Guter Wissenschaftlicher Praxis bekannt ist und ich mich zur Einhaltung dieser Satzung verpflichte.

Die Bedeutung dieser eidesstattlichen Versicherung und die strafrechtlichen Folgen einer unwahren eidesstattlichen Versicherung (§156,161 des Strafgesetzbuches) sind mir bekannt und bewusst.“

Datum Unterschrift

Anteilserklärung an den erfolgten Publikationen

Kaiyin Wu hatte folgenden Anteil an den folgenden Publikationen:

1. **Wu K,** Budde K, Lu H, Schmidt D, Liefeldt L, Glander P, Neumayer HH, Rudolph B. The severity of acute cellular rejection defined by Banff classification is associated with kidney allograft outcomes.Transplantation. 2014 Jun 15; 97(11):1146-1154.

Contribution in detail:

Tested and designed the protocol; reviewed all biopsies; analyzed the data and prepared all figures und tables; drafted the manuscript text and edited the publication during the review process.

2. **Wu K,** Budde K, Schmidt D, Neumayer HH, Rudolph B. The Relationship of the Severity and Category of Acute Rejection With Intimal Arteritis Defined in Banff Classification to Clinical Outcomes. Transplantation. 2015 Aug; 99 (8):105-114.

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3. Matz M, Heinrich F, Zhang Q, Lorkowski C, Seelow E, **Wu K,** Lachmann N, Addo RK, Durek P, Mashreghi MF, Budde K. The regulation of interferon type I pathway-related genes RSAD2 and ETV7 specifically indicates antibody-mediated rejection after kidney transplantation. Clinical transplantation. 2018 Dec; 32(12):e13429.

Contribution in detail:

Wu K: histological diagnose of all the allograft biopsies, analyzed the data of Figure 1 and 6.

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Unterschrift, Datum und Stempel des betreuenden Hochschullehrers/der betreuenden Hochschullehrerin

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Unterschrift des Doktoranden/der Doktorandin

Auszug aus der Journal Summary List (ISI Web of KnowledgeSM)

Publikation 1 (Seite 36-44) und Publikation 2 (Seite 45-54): Im Fachbereich „Transplantation“ ist das Journal „Transplantation“ an siebter Stelle (von 25 der nach Impact Factor sortierten Journals; Rangfolge siehe unten) gelistet. Das Journal verfügt über einen Impact Factor von 3,960 und einem Eigenfaktor von 0,03096.

Publikation 3 (Seite 55-63): Im Fachbereich „Transplantation“ ist das Journal „Clinical Transplantation“ an achtzehnter Stelle (von 25 der nach Impact Factor sortierten Journals; Rangfolge siehe unten) gelistet. Das Journal verfügt über einen Impact Factor von 1,518 und einem Eigenfaktor von 0,0079.

Journal Data Filtered By: **Selected JCR Year: 2017** Selected Editions: SCIE,SSCI
Selected Categories: **“TRANSPLANTATION”** Selected Category Scheme: WoS
Gesamtanzahl: 25 Journale

Rank	Full Journal Title	Total Cites	Journal Impact Factor	Eigenfactor Score
1	JOURNAL OF HEART AND LUNG TRANSPLANTATION	11,129	7.955	0.028970
2	AMERICAN JOURNAL OF TRANSPLANTATION	23,460	6.493	0.051290
3	XENOTRANSPLANTATION	1,479	4.717	0.002550
4	NEPHROLOGY DIALYSIS TRANSPLANTATION	25,654	4.600	0.038260
5	BONE MARROW TRANSPLANTATION	12,506	4.497	0.020810
6	BIOLOGY OF BLOOD AND MARROW TRANSPLANTATION	10,583	4.484	0.026940
7	TRANSPLANTATION	24,731	3.960	0.030960
8	LIVER TRANSPLANTATION	9,930	3.752	0.013900
9	STEM CELLS AND DEVELOPMENT	7,589	3.315	0.016440
10	TRANSPLANT INTERNATIONAL	4,709	3.196	0.009890
11	CELL TRANSPLANTATION	5,255	2.885	0.009420
12	Current Opinion in Organ Transplantation	1,859	2.869	0.005640
13	Transplantation Reviews	705	2.364	0.002030
14	ARTIFICIAL ORGANS	3,768	2.111	0.004060
15	Transplant Infectious Disease	2,361	1.869	0.005910
16	ASAIO JOURNAL	2,893	1.842	0.005500
17	TRANSPLANT IMMUNOLOGY	1,307	1.655	0.002170
18	CLINICAL TRANSPLANTATION	4,205	1.518	0.007900

1. The severity of acute cellular rejection defined by Banff classification is associated with kidney allograft outcomes.

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2. The Relationship of the Severity and Category of Acute Rejection With Intimal Arteritis Defined in Banff Classification to Clinical Outcomes.

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3. The regulation of interferon type I pathway-related genes RSAD2 and ETV7 specifically indicates antibody-mediated rejection after kidney transplantation. Clinical transplantation.

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Lebenslauf

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Publikationsliste

Als Erstautor der Publikationen

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