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Aus der Klinik mit Schwerpunkt Nephrologie und Intensivmedizin
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DISSERTATION

Use of interferon-stimulated gene expression level in renal transplant patients' blood as biomarkers for diagnosis of rejection

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List of abbreviations

ABMR	antibody-mediated rejection
APCs	antigen-presenting cells
AR	acute rejection
AUC	area under the curve
BL	borderline
CAN	chronic allograft nephropathy
CKD	chronic kidney disease
CNI	calcineurin inhibitor
CR	chronic rejection
CXCL10	C-X-C motif chemokine 10
DCs	dendritic cells
DGF	delayed graft function
DSAs	donor-specific antibodies
EMT	epithelial-mesenchymal transition
ETS	E26 transformation-specific
ETV7	ETS variant transcription factor 7
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
IFI44	interferon-induced protein 44
IFI44L	interferon-induced protein 44-like
IFIT1	interferon-induced protein with tetratricopeptide repeats 1
IFIT3	interferon-induced protein with tetratricopeptide repeats 3

IFN	interferon
IFTA	interstitial fibrosis/tubular atrophy
IL	interleukin
IP-10	interferon-inducible protein 10
IRI	ischemia-reperfusion injury
ISG	interferon-stimulated gene
ISGF-3	interferon-stimulated growth factor-3
LR	likelihood ratio
MHC	major histocompatibility complex
MICA	major histocompatibility complex class I-related chain A
mPCR	multiplex polymerase chain reaction
MVI	microvascular inflammation
QOL	quality of life
ROC	receiver operating characteristics
ROS	reactive oxygen species
RSAD2	radical S-adenosyl methionine domain containing 2
SGF	stable graft function
TCMR	T cell-mediated rejection
TGF-β	transforming growth factor beta
Th1	type 1 helper T cell
Th2	type 2 helper T cell
Tregs	regulatory T cells
UTI	urinary tract infection

Abstrakt (Deutsch)

Einleitung: Antikörper-vermittelte Abstoßung (ABMR) wurde als Hauptursache für Transplantatverlust erkannt. Eine frühe Diagnose ist entscheidend für eine wirksame Behandlung von Patienten mit ABMR. Der Nachweis der Expression von Interferon-stimulierten Gene (ISG) könnte einen nicht-invasiven Weg zur Differenzierung von ABMR von Patienten mit stabiler Transplantatfunktion (SGF), Harnwegsinfektion (UTI), T-Zell-vermittelter Abstoßung (TCMR) und interstitieller Fibrose / tubuläre Atrophie (IFTA) bieten.

Methoden: Insgesamt wurden 185 adulte Nierentransplantationsempfänger einschließlich ABMR (n=20), SGF (n=51), UTI (n=17), Borderline (BL) (n=22), TCMR Typ I (n =19), TCMR Typ 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 ISG zu messen, die *IFIT1, IFIT3, RSAD2, ETV7, IFI44, IFI44L* enthielten. Die mRNA- und Proteinexpressionsniveaus von ISG in verschiedenen Patientengruppen wurden durch den Mann-Whitney-*U*-Test miteinander verglichen. Eine Receiver Operating Characteristics (ROC) Analyse wurde durchgeführt, um den diagnostischen Wert für die Unterscheidung von ABMR-Patienten von anderen zu bewerten.

Ergebnisse: Die mRNA-Expressionsspiegel von *IFIT1, IFIT3, RSAD2, ETV7* waren im Blut von ABMR-Patienten im Vergleich zur SGF-Gruppe signifikant erhöht ($P<0,05$). Der Proteinexpressionsgrad zeigte keine signifikanten Veränderungen im Vergleich zur SGF-Gruppe ($P>0,05$). *ETV7*-mRNA Expressionsniveau in ABMR war signifikant höher als in den SGF, BL, TCMR Typ II / III-Gruppen und signifikant niedriger in der UTI-Gruppe ($P<0,05$). Der *ETV7*-Fläche unter der ROC-Kurve (AUC), der ABMR von allen anderen Gruppen unterscheidet, ist 0,69 (95% CI: 0,60-0,78, $P=0,006$) mit einer Sensitivität von 0,95 und einer Spezifität von 0,50. Die AUC von ABMR zur Unterscheidung von der SGF- Gruppe ist 0,83 (95% CI: 0,74-0.92, $P<0,001$) mit einer

Sensitivität von 0,95 und einer Spezifität von 0,70.

Schlussfolgerungen: Die Messung der ETV7-mRNA-Expression könnte einen neuen und nicht-invasiven Ansatz darstellen, eine ABMR von anderen Komplikationen nach Nierentransplantation zu differenzieren.

Abstract (English)

Purpose: Antibody-mediated rejection (ABMR) has been recognized as a major cause of graft loss. Early diagnosis is crucial for the effective treatment of patients with ABMR. Detecting the blood expression of interferon-stimulated gene (ISG) might offer a non-invasive way to differentiate patients with ABMR from patients with stable graft function (SGF), urinary tract infection (UTI), T cell-mediated rejection (TCMR) and interstitial fibrosis/tubular atrophy (IFTA).

Methods: A total of 185 adult kidney transplant recipients were recruited, including ABMR (n=20), SGF (n=51), UTI (n=17), Borderline (BL) (n=22), TCMR type I (n=19), TCMR type II/III (n=26) and IFTA (n=30) patients. Total RNA was isolated from the whole blood of patients at the time of biopsy. Quantitative real-time PCR and ELISA were performed to measure the expression levels of ISGs, which included *IFIT1, IFIT3, RSAD2, ETV7, IFI44, IFI44L*. The mRNA and protein expression levels of ISGs in different patient groups were compared with each other by the Mann-Whitney *U* test. Receiver operating characteristics (ROC) analysis was performed to assess the diagnostic value for those markers distinguishing ABMR patients from other patients.

Results: mRNA expression levels of *IFIT1, IFIT3, RSAD2, ETV7* were significantly up-regulated in the blood of ABMR patients compared to the SGF group ($P<0.05$). The protein expression of these genes showed no significant differences compared to those of the SGF group ($P>0.05$). The *ETV7* mRNA expression level in ABMR was significantly higher than in the SGF, BL, TCMR type II/III groups and significantly lower in the UTI group ($P<0.05$). The area under the ROC curve (AUC) for *ETV7* mRNA expression distinguishing ABMR from all other groups is 0.69 (95% CI: 0.60-0.78, $P=0.006$) with a sensitivity of 0.95 and a specificity of 0.50. The AUC for *ETV7* mRNA expression distinguishing the ABMR group from the SGF group is 0.83 (95% CI: 0.74-0.92, $P<0.001$) with a sensitivity of 0.95 and a specificity of 0.70.

Conclusions: The measurement of the *ETV7* mRNA expression level might offer a novel and non-invasive approach for differentiating ABMR from other complications after kidney transplantation.

1 INTRODUCTION

1.1 Kidney transplantation

Chronic kidney disease (CKD) is a worldwide public health problem with increasing incidence and prevalence [1-4]. Patients with CKD may develop the end-stage renal disease (ESRD), resulting in lifelong renal replacement therapy (dialysis or transplantation), which puts a high burden on the patient and the health system [5]. Dialysis alone takes about 2% of health-care budgets with only less than 0.1% of overall patients in Europe [6]. Kidney transplantation (from either a living or a deceased donor) is the most effective treatment for most patients with ESRD despite an increased short-term risk of death after transplantation. 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].

The number of active patients listed waiting for kidney transplantation exceeds the number of kidney transplantations in the Eurotransplant region [10]. A total of 10,476 patients were on kidney waiting lists on 31 December 2016 at Eurotransplant (for comparison: 10,400 patients were on waiting lists at Eurotransplant on 31 December 2015) [11] (Fig. 1). The numbers of both active and inactive adult candidates listed for kidney transplantation decreased in 2016 compared with 2015, which might be due to the death or deteriorating medical condition of candidates in the USA. The total number of kidney transplants has increased, but there are still thousands of candidates waiting for transplantation, reflecting the ongoing organ shortage all over the world [12] (Fig. 2). The imbalance of organ supply and the number of patients waiting for kidney transplantation are challenges for society.

A

Waiting list type	Composition	2012	2013	2014	2015	2016	2015/2016
Kidney	kidney	10151	10757	10689	10400	10476	8.5%
	kidney + heart	25	17	12	14	19	35.7%
	kidney + lung	1	1	1	0	1	--
	kidney + liver	67	57	55	62	57	3.6%
	kidney + liver + pancreas	1	1	1	1	1	0.0%
	kidney + pancreas	280	287	322	320	347	12.3%
Kidney	**Total**	**10525**	**11120**	**11080**	**10797**	**10901**	**8.6 %**

B

Type of transplant	2012	2013	2014	2015	2016	2015/2016
Kidney-only	3139	2951	3086	3171	3046	-3.9 %
Kidney en bloc	40	16	36	37	28	-24.3 %
Kidney + heart	18	8	9	7	3	-57.1 %
Kidney + double lungs	0	0	0	1	1	0.0%
Kidney + split liver	4	4	3	2	2	0.0%
Kidney + whole liver	35	39	38	30	41	36.7 %
Kidney + whole liver + pancreas	1	0	1	0	0	0.0 %
Kidney + pancreas	195	164	175	175	156	-10.9 %
Kidney en bloc + pancreas	0	1	0	0	1	--
Total	**3432**	**3183**	**3348**	**3423**	**3278**	**-4.2 %**

Fig. 1: Waiting lists and transplantation in Eurotransplant.

A. Active Eurotransplant waiting lists at year-end, from 2012 to 2016; B. Kidney transplants (deceased donor) from 2012 to 2016 [11].

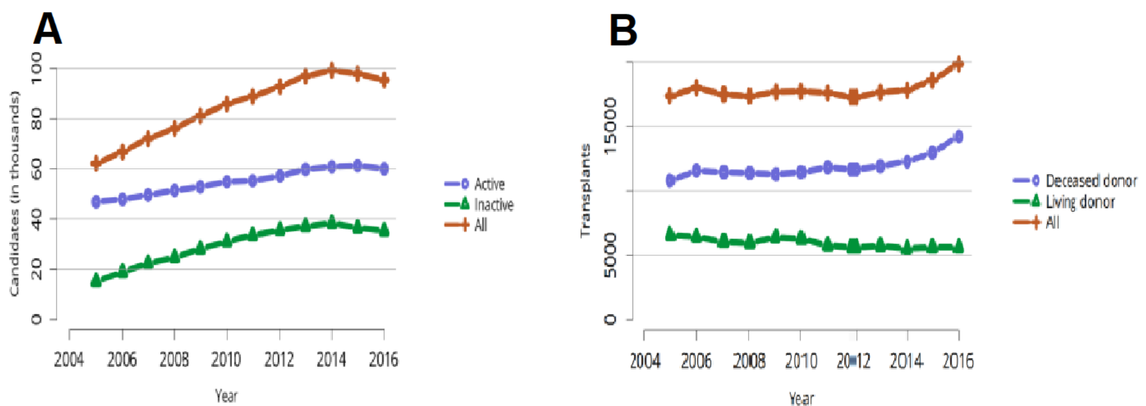


Fig. 2: Candidates and kidney transplants in the USA.

A. New adult candidates added to kidney transplant waiting lists in the USA; B. Total kidney transplants in the USA (All kidney transplant recipients, including adult and pediatric, retransplant, and multi-organ recipients.) [12].

Over the past 20 years, along with the introduction of more effective calcineurin inhibitor (CNI)-based immunosuppressive regimens and advances in tissue typing, organ preservation technology, diagnostics and new medical and surgical therapies, the 1-year renal allograft survival rate has increased to 95% or even higher [13]. In contrast, long-term graft survival is still disappointing due to multiple complications leading to constant graft attrition after transplantation, which has only marginally improved in the last decades [14, 15].

1.2 Renal allograft rejection

After transplanting a graft from a genetically disparate individual, a series of inflammatory and immunological events in both host and graft may occur. Some of the responses come up as a result of trauma related to ischemia-reperfusion injury (IRI), transplantation surgery, etc., whereas others involve the direct effect of immune system incompatibility between recipients and donors, which if not controlled will destroy the graft [16, 17]. This immune response to an allogeneic-transplanted graft is called rejection, which involves both innate and adaptive immune response mechanisms [18].

Renal allograft rejection can be classified in different ways: as hyperacute rejection, accelerated rejection, acute rejection (AR), or chronic rejection (CR) from the time of onset and progression to injury [19]. It can also be classified based on other features such as severity, response to treatment (glucocorticoid resistance or not), the involvement of deterioration of graft function (subclinical rejection or not), and finally, according to the predominant immunologic mechanism: T cell-mediated rejection (TCMR), antibody-mediated rejection (ABMR) or mixed rejection.

Hyperacute rejection occurs immediately after the graft is reperfused. It is caused by pre-existing complement-fixing antibodies to graft antigens and is an irreversible process [20]. Accelerated rejection is associated with pretransplantation humoral and cellular components of the immune response against donor antigens [21]. It occurs within

several days or several weeks and usually does not respond to anti-rejection therapy. Both of these kinds of rejection are rare since cross-matching technique and effective anti-rejection treatment have improved.

AR can occur at any time after transplantation [19]. The occurrence, timing, and the number of AR episodes are also associated with increased risk of graft loss [22]. The clinical manifestation of CR is the progressive reduction of graft function combined with hypertension and proteinuria. Its pathological features are renal interstitial fibrosis, renal tubular atrophy, and vascular changes. The gradual progression of CR ultimately results in transplanted kidney fibrosis and causes graft failure [23].

1.3 Antibody-mediated rejection in kidney transplantation

Antibody-mediated rejection, also termed humoral rejection, accounts for 20-30% of all AR episodes after kidney transplantation and it has been recognized as a major cause of graft loss [24]. Antibodies that cause ABMR include those against donor human leukocyte antigen (HLA) class I, class II, or non-major histocompatibility complex (MHC) antigen on the endothelium. The types of ABMR include a) hyperacute rejection, which occurs immediately after renal revascularization; b) acute ABMR, which occurs within several days or several weeks after transplantation; and c) chronic ABMR, which usually develops more than one year after transplantation.

1.3.1 Hyperacute rejection

As mentioned above, hyperacute rejection occurs due to preformed donor-specific antibodies (DSAs) against HLA expressed on the endothelium of the glomeruli and microvasculature [25]. The classic complement cascade is activated, which eventually results in endothelial necrosis and platelet deposition [26].

1.3.2 Acute ABMR

Compared to 7% incidence of acute ABMR in HLA-matched renal graft recipients, as

many as 50% of HLA-incompatible patients suffer from this complication [27, 28]. Besides anti-HLA antibodies, numerous pathogenic non-HLA anti-endothelial cell antibodies, such as major histocompatibility complex class I-related chain A (MICA) antibody, angiotensin II type 1 receptor antibody, etc., have been identified to play roles in the process of ABMR [29, 30]. Regardless of antibody origin, after binding to its respective target on the graft endothelial, damaged endothelial cells release various injurious molecules such as cytokines, chemokines, as well as the chemoattractants C3a and C5a [31]. As a result of cell adhesion, the classical complement pathway is activated, which causes localized endothelial necrosis and apoptosis [17]. Activation of the complement cascade by DSAs might be the primary mechanism of acute ABMR [32].

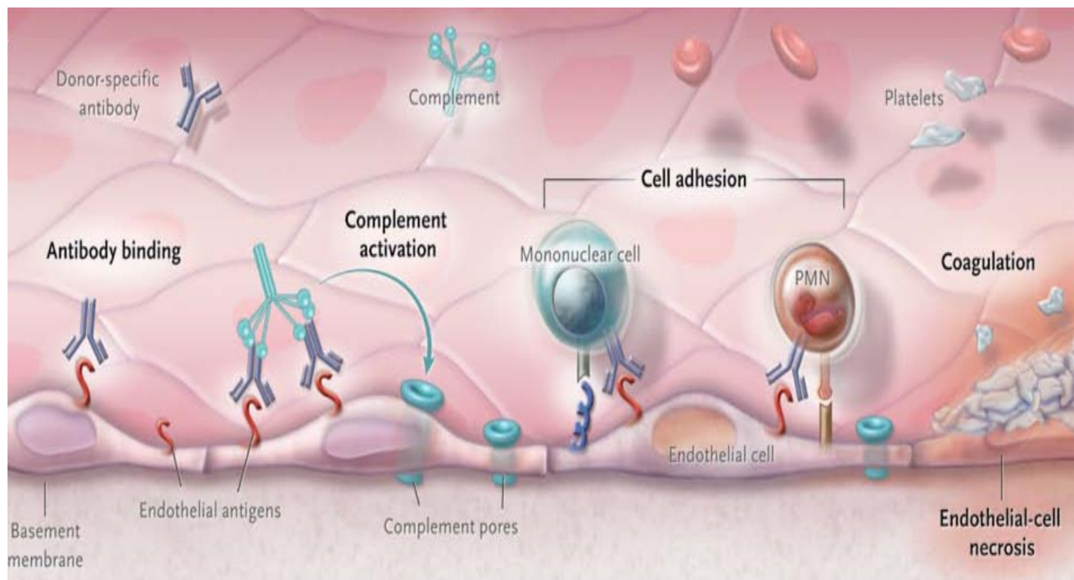


Fig. 3: The process of antibody-mediated rejection in kidney transplantation [17].

Based on the mechanism of ABMR, the Banff 2015 classification scheme was changed to include three necessary diagnostic criteria, which are a) presence of donor-specific antibodies, b) evidence of current/recent antibody interaction with vascular endothelium, and c) histologic evidence of acute tissue injury [33].

1.3.3 Chronic ABMR

With the optimization of immunosuppressive regimens and better tissue typing, the

one-year renal graft survival rate has increased to >90%, whereas long-term survival is still not satisfactory, with a 10-year renal allograft survival rate of around 50% [13]. The development of DSAs to foreign antigens is considered a major cause of late renal allograft dysfunction and graft loss [34]. The histological features of chronic active ABMR, are a) microvascular remodeling, b) moderate microvascular inflammation (MVI), and c) new-onset arterial intimal fibrosis [35]. As a supplement, the revised 2013 Banff classification assumed chronic ABMR was accompanied by significant MVI as chronic active ABMR [36].

1.4 T cell-mediated rejection in kidney transplantation

T cell-mediated rejection is another independent risk factor for graft loss [37]. TCMR can serve as a sensitizing event that triggers the development of *de novo* DSA and subsequent ABMR and thereby affects long-term graft outcome.

Most B cells require help from T cells to initiate the production of alloantibody. A study including 795 cross-match negative patients showed that TCMR could accelerate graft inflammation and fibrosis [38]. A study including 69 C4d-negative biopsies patients with TCMR-arteritis showed an 8-year graft survival rate of 72.7% compared with 93.3% in the matched-control group without arteritis [39]. Also, a common clinical observation is the combination of ABMR and TCMR, called mixed rejection [40].

1.4.1 Pathophysiology of TCMR

For TCMR, the activation of T cells requires multiple signals, which include two prerequisite signals: antigen recognition and costimulation [41]. The most common form of allograft rejection is triggered as soon as donor alloantigens are presented to the T lymphocytes of the recipients by antigen-presenting cells (APCs). Both dendritic cells (DCs) and macrophages can serve as APCs, and even a B cell can present antigen to T cells efficiently by their surface immunoglobulins and MHC class II molecules [42].

The recipient's T lymphocytes respond to donor alloantigen via either the direct pathway (alloantigens displayed by the donor's APCs) or the indirect pathway (alloantigens displayed by the recipient's APCs) [43]. When T cells recognize alloantigens by either of these two pathways, antigen-specific signals are delivered. Coupled with the interaction of costimulatory molecules with their ligands on both the T cells and APCs, naïve T cells are activated [44].

Following activation, depending on the microenvironment and the extra signals T cells received, they will differentiate into different kinds of cells that possess various cytokine signatures and functions [45]. Immune status of the recipients at the time of transplantation, immunosuppressive regimens, degree of HLA mismatch, or antibody load will serve as influence factors that determine the way and result of T cell differentiation [41]. T cells mediate allograft injury directly by contact with tubular epithelial cells and the release of cytokines locally.

1.4.2 T cell subsets

Helper T cells play an important role in the immune system, particularly in the adaptive immune response. By releasing some T cell cytokines, helper T cells regulate the activity of other immune cells. Overall, type 1 helper T (Th1) cells can mediate rejection, while in contrast type 2 helper T (Th2) cells facilitate tolerance. However, evidence also shows that Th2 cells can take part in the allograft rejection process [46].

$CD8^{+}$T cells, also known as cytotoxic lymphocytes, mediate cytotoxicity, while $CD4^{+}$T cells can produce all kinds of cytokines such as interleukin (IL) and interferon (IFN), which may drive both cellular and humoral immune response [47].

Regulatory T cells (Tregs) characterized by $CD4^{+}$ $CD25^{+}$ $FOXP^{3+}$ have shown an important role in maintaining immune homeostasis, limiting allograft rejection response, and even in promoting graft tolerance [48]. After transplantation, Tregs migrate from the blood into grafts and draining lymph nodes and they can have a role in suppressing

effector T cell proliferation by cell contact and suppressive cytokines [49].

1.5 Interstitial fibrosis/tubular atrophy in kidney transplantation

Based on the Banff 97 expert consensus, chronic allograft nephropathy (CAN) was defined as progressively impaired renal graft function with the presence of interstitial fibrosis and tubular atrophy independent of etiology [50]. CAN was replaced by interstitial fibrosis/tubular atrophy (IFTA) as a pathological classification in the Banff score system to prevent CAN being misunderstood as a specific disease entity [51]. IFTA is a non-specific lesion caused by various immune and non-immune injuries to the renal allograft [52].

IFTA occurs in about 40% of renal grafts at 3-6 months after transplantation and this number increases to 65% at 2 years after transplantation [53]. Regardless of whether a specific disease was diagnosed in the allograft, IFTA is an independent risk factor for graft loss, especially in grafts from expanded criteria donors [54, 55].

Understanding the mechanism of progressive IFTA in the renal allograft is crucial for early diagnosis and further development of treatment strategies to prolong allograft survival. In the past several decades, numerous efforts have been made to conduct a series of research studies to explore the pathogenesis of IFTA. Multiple factors such as IL, reactive oxygen species (ROS), hepatocyte growth factor (HGF), transforming growth factor beta (TGF-β), and various mechanisms have been demonstrated to be involved in the progress of IFTA, including IRI, immunosuppressant toxicity, ABMR, epithelial-mesenchymal transition (EMT) [53, 56-60].

1.6 Non-invasive diagnosis of complication in kidney transplantation

After the graft is transplanted to the recipient, a series of complications may occur. Among these complications, IFTA and rejection, regardless of type or timing, significantly reduce graft survival [61,62]. Therefore, early diagnosis and effective treatment are of

great significance. Approximately 10-20% of the renal transplant recipients suffer from one or multiple episodes of AR, which occur mainly in the first year after surgery [63].

The current gold standard for the diagnosis of rejection and IFTA is renal allograft biopsy [64]. However, biopsy is an invasive examination, carrying the potential risk of damaging precious kidney grafts, which limits repetitive and dynamic observations. Patients with coagulation disorders cannot undergo biopsies. Due to operative procedures, only a small portion of r nal allograft tissue can be obtained via biopsy, which presents a challenge for the accurate evaluation of all pathological changes [65].

Therefore, many attempts have been made to find a non-invasive method to diagnose rejection and IFTA at an early stage and to offer a sensitive marker to evaluate the effect of treatment. However, few of these are used for clinical routine due to their limited sensitivity and specificity as well as their relatively low predictive values regarding renal allograft outcomes [66, 67].

1.7 Interferon-stimulated genes

Interferon is a cytokine used for the communication between cells to trigger the immune defenses [68]. IFN can be divided into three classes: Type I, Type II, and Type III based on the type of signal receptor. IFN-α and IFN-β belong to Type I IFN, which is produced mainly by fibroblasts and monocytes. Type II IFN or IFN-γ is produced mostly by CD4+ and CD8+ T cells and NK cells. These IFNs have complex effects on immune cells and induce a range of downstream gene expression [69]. IFN-γ modulates the allogeneic responses in organ transplantation and IFN-γ-induced genes have been reported to be involved in TCMR and ABMR [70-72].

Interferon-induced protein with tetratricopeptide repeats 1 (*IFIT1*), interferon-induced protein with tetratricopeptide repeats 3 (*IFIT3*), radical S-adenosyl methionine domain containing 2 (*RSAD2*), ETS variant transcription factor 7 (*ETV7*), interferon-induced protein 44 (*IFI44*), and interferon-induced protein 44-like (*IFI44L*) these six genes are

members of interferon-stimulated genes (ISGs). ISGs are described as genes whose expression is induced or regulated by interferon and play pivotal roles in immune system defense against infection [73]. However, this classic definition is not comprehensive enough to cover all aspects of ISGs. Recent observations demonstrate that the expression of ISGs can also be a response to a variety of stimulatory factors such as injury, inflammation, stress, and other events [74, 75]. Emerging functional roles of ISGs besides their antiviral effect remain to be elucidated.

ISGs can be subdivided further into type I (stimulated mainly by interferon α, β), type II (interferon γ), and type III (interferon λ). One single ISG can belong to one subtype or several subtypes, which means this gene can be stimulated by one kind or several kinds of interferon [76]. Both *IFIT1* (also known as *ISG56*) and *IFIT3* (also known as *ISG60*) belong to the *ISG56/IFIT1* gene family, which can be induced by type I and type III interferons, especially IFN-α/β [77, 78]. *RSAD2* (also known as viperin) belongs to the S-adenosylmethionine enzyme superfamily and can be induced by type I, II and III IFN in a variety of cell types [79]. *ETV7* or *TEL2*, a poorly studied transcription factor member of ETS family, is known to be induced by IFN-α [80,81]. *IFI44L* gene is a type I ISG and belongs to the *IFI44* family [82].

ISGs play vital roles in both innate and adaptive immune responses through different types of IFN signaling [76]. The involvement of ISGs has also been described in solid organ transplantation, in addition to systemic lupus erythematosus and cancer [83, 84]. Saiura et al. found that interferon-γ-inducible genes are up-regulated in murine cardiac transplantation mode during the late phase of AR [85]. Interferon-γ related genes also significantly changed in AR compared to patients without rejection after lung transplantation [86]. The early activation of ISGs in human liver allografts has been related to the risk of acute cellular rejection. However, unlike other organ transplantation, the induction of ISGs after liver transplantation might also be due to the recurrence of hepatitis C as an antiviral response [87].

In the field of kidney transplantation, Rascio et al. compared the peripheral blood molecular signature of 29 chronic ABMR patients with eight IFTA and 29 stable transplant recipients as controls. They found that several types I ISGs including *IFIT1* and *IFIT3* are up-regulated in chronic ABMR patients [88]. Akalin et al. used an oligoarray to analyze three normal renal allograft biopsy samples and seven human TCMR samples, six of which showed up-regulated interferon-stimulated growth factor-3 (ISGF-3), the activator of ISG [89]. Maluf et al. evaluated gene expression profiles of kidney biopsies from 24 normal kidneys, six normal allografts, and seventeen allografts with IFTA using high-density oligonucleotide microarray. Several genes including *RSAD2, IFI44*, and *IFI44L* were overexpressed in IFTA samples compared with normal allografts and normal kidneys [90]. The capability of ISGs to distinguish between the different types of rejection and complications in kidney transplantation with a larger sample size still needs confirmation.

1.8 Aims and objectives

This study aimed to find non-invasive markers for sensitive and specific diagnosis of ABMR. To achieve this aim, the following objectives were formulated:

1. To measure the mRNA expression levels of candidate ISGs in the whole blood cells in different kidney transplant recipients.

2. To investigate the protein expression levels of ISG markers in the serum or plasma of different patients.

3. To study the feasibility of the mRNA and protein expression levels of ISGs in blood as markers to diagnose ABMR.

4. To validate which marker can be the most effective tool to diagnose ABMR.

5. To provide a potential clue to the mechanism of ABMR.

2 MATERIALS and METHODS

2.1 Study design

The present study is a single-center study comparing the peripheral blood expression levels of six ISGs in different kidney transplant recipients and providing further proof of the feasibility of these markers to diagnose ABMR. The pre-/peritransplant period information was retrieved from the transplantation database (TBase) retrospectively and posttransplant creatinine was measured at the time of biopsy. Samples were measured without knowledge of grouping.

2.2 Patients and sample collection

2.2.1 Eligibility criteria and clinical data collection

The inclusion criteria for this study included adult kidney transplant recipients regardless of donor types, and patients who had given their written consent. Exclusion criteria included pediatric patients, recipients of multiple organ transplantations, patients with a serious infectious disease, patients who had developed dialysis-requiring chronic allograft failure, and patients with any factors that limited their ability to cooperate during the study (e.g., mental disorder or substance abuse). 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.

Clinical data that included the demographics of patients, creatinine at the time of biopsy, the patient’s immunosuppressive regimen, etc. were collected.

2.2.2 Biopsy specimens

Ultrasound-guided graft biopsy was performed when clinically indicated due to rising creatinine or proteinuria or in the case of prolonged delayed graft function (DGF). The histological analysis was performed by two independent pathologists in a blinded fashion.

The pathological changes were graded according to the 2009/2013 Banff classification.

2.2.3 Blood samples collection

Fresh blood samples (2.5 ml/sample) were collected into PAXgene blood RNA tubes from patients at the time of biopsy according to the manufacturer's instructions, and the tubes were kept in the -80°C freezer until the RNA isolation procedure began.

Besides, serum and plasma samples were taken at the time of biopsy. Serum separator tubes were used to collect the patients' peripheral blood, and samples were clotted for two hours at room temperature before being centrifuged for 10 minutes at 3000×g. The serum was removed and stored at -80°C. The plasma was collected using EDTA tubes and the samples were centrifuged for 10 minutes at 3000×g. The supernatant was removed and the samples were stored at -80°C.

2.3 Candidate markers selection

As described before [91], candidate genes were selected through next-generation sequencing, gene set enrichment analysis, and further verified by quantitative real-time PCR (RT-PCR) using whole peripheral blood from six biopsy-proven ABMR, four biopsy-proven TCMR patients and six patients with stable graft function (SGF). *IFIT1, IFIT3, RSAD2, ETV7, IFI44* were the most significantly regulated genes in ABMR compared to SGF and TCMR. *IFI44L* was chosen as one of the most ABMR-SGF discriminating candidates and paralog of the *IFI44* gene. Significant enrichment for IFN-α and IFN-γ signaling which included these markers was also found. The mRNA expression difference between ABMR and SGF patients was statistically significant after validation. Therefore, all these markers were measured in a large cohort of kidney transplant patients with different complications.

2.4 Isolation of blood RNA

Total RNA was isolated from the whole blood using PAXgene blood miRNA kit

(PreAnalytix; Qiagen, Hilden, Germany) based on the manufacturer's instructions. Samples in PAXgene blood RNA tubes were incubated for 2 hours at room temperature and then centrifuged for 10 minutes at 4600×g. The supernatant was removed. 4 ml of RNase-free water was added to the pellet. After thorough mixing, the tube was sealed by a new secondary Hemogard. The tube was centrifuged for 10 minutes at 4600×g. The upper phase was removed by decanting.

The further RNA isolation procedure was carried out with the PAXgene blood RNA kit, which contains PAXgene RNA spin columns, PAXgene shredder spin columns, processing tubes, Buffer BM1 (resuspension buffer), Buffer BM2 (binding buffer), Buffer BM3 (wash buffer concentrate), Buffer BM4 (wash buffer concentrate), Buffer BR5 (elution buffer), proteinase K, and an RNase-free DNase set.

The sample was resuspended in Buffer BM1 and then transferred to a 1.5 ml microcentrifuge tube. 300 μl of Buffer BM2 and 40 μl of proteinase K were added. After being incubated for 10 minutes in a 55°C shaker-incubator at 900 rpm, the sample was pipetted into a PAXgene shredder spin column and then centrifuged for 3 minutes at 14,000 rpm. The entire supernatant and 700 μl of isopropanol were mixed by shaking. The sample was pipetted into the PAXgene RNA spin column and centrifuged for 1 minute at 14,000 rpm.

After the sample was washed by 350 μl of Buffer BM3, the DNase I incubation Mix was pipetted directly onto the PAXgene RNA spin column membrane and incubated on the benchtop (20–30°C) for 15 minutes. After being washed with 350 μl of Buffer BM3 once and with 500 μl of Buffer BM4 twice, the spin column was placed in a new 2 ml processing tube, which was centrifuged at 14,000 rpm for 1 minute. 40 μl of Buffer BR5 was pipetted directly onto the spin column membrane to elute the RNA. And the elution step was repeated.

After being incubated for 5 minutes at 65°C in the shaker–incubator without shaking, the

eluate was chilled immediately on ice. The concentration of the sample was measured using a NanoDrop lite spectrophotometer.

2.5 Reverse transcription

The Maxima first strand cDNA synthesis kit (Thermo Scientific), which contains 10× dsDNase buffer, dsDNase, 5× reaction mix, Maxima enzyme mix and RNase-free water was used for reverse transcription of RNA into cDNA.

1µg of RNA was diluted in a total volume of 50µl of nuclease-free water. 3µl of RNA, 1µl of 10x dsDNase buffer, 1µl of dsDNase, and 5µl of RNAse-free water were mixed gently. The reagents were incubated in a thermal block for 2 minutes at 37°C to eliminate contaminating genomic DNA. After being chilled on ice, 4µl of 5× reaction mix, 2 µl of Maxima enzyme mix, and 4µl of RNase-free water were added. This mix was incubated in thermal blocks for 10 minutes at 25°C, followed by 15 minutes at 95°C. The reaction was terminated by heating at 85°C for 5 minutes. The reverse transcriptase minus negative control, which included all components except the Maxima enzyme mix, was used to verify the result. The cDNA samples were stored at -20°C until further use.

2.6 Quantitative real-time PCR

RT-PCR was performed with candidate genes *IFIT1, IFIT3, RSAD2, ETV7, IFI44, IFI44L* 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, ROX passive reference, and optimized buffer components.

For each reaction, a mixture of 5 µl of TaqMan™ Universal Master Mix II, with UNG, 0.5 µl of Primer TaqMan mRNA Assay, and 3.5 µl of nuclease-free water were transferred to the wells of a 96-Well Reaction Plate with Barcode (Thermo Scientific) before adding 1µl of cDNA sample (1:2 diluted). The final volume of each reaction mixture was 10µl and

reactions were carried out in duplicate. Negative controls, which replaced cDNA with the same volume nuclease-free water, were included in each run. The reaction was run on a QuantStudio 3 Real-Time PCR System (Thermo Scientific) based on the program below (Table 1). The results were analyzed using the QuantStudio™ Design & Analysis Software v1.4.1 (Thermo Scientific). Relative quantification of gene expression was calculated using the $2^{-\Delta Ct}$ method. The housekeeping gene *HPRT1* was used to normalize each cDNA sample.

Table 1 Quantitative real-time PCR program

Step	Temperature	Duration	Cycles
UNG incubation	50°C	2 minutes	1
Enzyme activation	95°C	10 minutes	1
Denature	95°C	15 seconds	40
Anneal/Extend	60°C	1 minute	40

2.7 ELISA

Serum was used to measure ETV7 and plasma was used to analyze the concentration of IFIT1 and RSAD2. All reagents were brought to room temperature before use for 30 minutes. Serial dilution was made for standards according to the instructions. 100μl of standards and samples were added per well in duplicates, and the sample diluent served as a blank. The plate was covered with a plate lid and incubated at 37°C for 2 hours. The liquid in the wells was discarded, and 100 μL of the detector antibody was added to each well. The plate was covered with a plate lid and incubated at 37°C for 1 hour. Each well was aspirated and washed three times by filling each well with 200-300μl of wash buffer.100μl of HRP-conjugate reagent was added to each well and the plate was covered with a plate lid. The plate was washed 4-5 times after being incubated at 37°C

for 1 hour. 90μl of TMB Substrate (for RSAD2 and IFIT1) or 50μl of chromogen solution A and 50μl of chromogen solution B (for ETV7) were added to each well. The plate was incubated in the dark for 15 minutes at 37°C. 50μl of stop solution was added to each well, then the optical density (OD) of each well was determined within 5 minutes using the DTX 880 Multimode Detector (Beckman Coulter) at 450 nm.

The mean duplicate readings for each standard and sample were used to make standard curves after subtracting the average OD of the blank. The concentration of ETV7, RSAD2, and IFIT1 were interpolated by GraphPad Prism 8.3.0.

2.8 Statistical analysis

Continuous variables with normal distribution were summarized as mean±standard deviation; other continuous variables were expressed as medians with interquartile ranges. For categorical variables, the N and percentages in each category were shown. To analyze the different expression levels of markers from the different groups, a Kolmogorov-Smirnov normality test was performed to test whether the data were normally distributed; if not, the nonparametric one−way analysis of variance (ANOVA) on ranks (Kruskal−Wallis test) was conducted. When significant differences between the groups were observed, the post hoc nonparametric Mann-Whitney *U* test was used to compare the difference between every two patient groups. The two-stage step-up method of Benjamini, Krieger and Yekutieli correction was applied to control the false discovery rate in multiple comparisons. The univariate logistic regression was performed in each single candidate marker to test the diagnostic value. The receiver operating characteristics (ROC) analysis was performed to present diagnostic utility. The area under the curve (AUC), sensitivity, and specificity were determined to specify the performance of markers in discriminating ABMR from the comparators (None ABMR patients or SGF patients). The best cut-off point for each marker was defined at the maximal Youden index. The multivariate logistic regression including all candidate

markers was used to test the diagnostic value of the combination of the genes. A *P* value<0.5 was considered statistically significant.

2.9 Materials

2.9.1 Reagents and Kits

Nuclease-Free Water	Ambion
Isopropanol	J.T. Baker
Ethanol	J.T. Baker
PAXgene Blood RNA Kit	PreAnalytiX
Maxima First Strand cDNA Synthesis Kit	Thermo Scientific
TaqMan™ Universal Master Mix II, with UNG	Thermo Scientific
TaqMan™ Gene Expression Assays	Thermo Scientific
Human IFIT1 ELISA Kit	Cusabio Biotech
RSAD2 ELISA Kit (Human)	AVIVA Systems Biology
Human Transcription Factor ETV7 ELISA Kit	MyBioSource

2.9.2 Laboratory equipment and devices

Centrifuges:

Heraeus Multifuge 3 S-R	Thermo Scientific
Refrigerated Microcentrifuge, Model 5417R	Eppendorf
Mini Plate Centrifuge NG040	Nippon Genetics

Thermal cyclers:

Personal Cycler	Biometra
QuantStudio 3 Real-Time PCR System	Thermo Scientific

Pipettes:

Pipette, 2.5 µl/10 µl/100ml/200ml/1ml/5ml	Eppendorf
Pipette tips, 10 µl/100 µl/200 µl/1ml/5ml	Sarstedt
NeoPipette Controller D-6017	neoLab Migge GmbH
Serological Pipette, 5ml	Falcon
E1-ClipTip Electronic Pipette, 2-125µl	Thermo Scientific
Multipette plus	Eppendorf

Mixers:

Vortex Mixer	Scientific Industries
Thermomixer Compact	Eppendorf

Tubes, plates, and other consumables:

PAXgene Blood RNA Tubes (2.5 ml)	Becton Dickinson
Safe-lock tubes 0.5ml/1.5ml	Eppendorf
PCR Tubes 0.2ml	Fisher Scientific
96-Well Reaction Plate with Barcode, 0.1 mL	Thermo Scientific
PCR film	Eppendorf

Other equipment:

Holten Horizontal Laminar Airflow Clean Bench	Thermo Scientific
Scotsman AF80 Ice Flaker	Scotsman
NanoDrop Lite Spectrophotometer	Thermo Scientific
PCR Workstation	VWR Peqlab
XT Cooling Core	Corning

CoolRack XT PCR96	Corning
Ultralow-temperature freezer	Panasonic
Heraeus B6 incubator	Thermo Scientific
DTX 880 Multimode Detector	Beckman Coulter

2.9.3 Software

QuantStudio™ Design & Analysis Software v1.4.1	Thermo Scientific
GraphPad Prism 8.3.0	GraphPad Software
Office software word/excel/access	Microsoft
SPSS for Windows, Version 20	IBM
Transplantation date bank, "T-Base 3.0"	Charité
Multimode Analysis Software	Beckman Coulter

3 RESULTS

3.1 Patient population

One hundred and twenty-two indicated biopsy patients who met the inclusion criteria were collected consecutively from both outpatients and inpatients in the Department of Nephrology, Campus Mitte, Charité-Universitätsmedizin Berlin from 2010 to 2017. Fifty-five patients with SGF and 18 patients with urinary tract infection (UTI) were selected randomly at the same time. A total of 195 patients were selected, of whom 10 patients were excluded due to insufficient data or inadequate sample material. The flow chart (Fig. 4) shows the process of patient selection and subgroup distribution.

A total of 185 adult kidney transplant recipients were analyzed, in which 20 patients were diagnosed as Banff-2 ABMR (Banff2-ABM), 22 patients with borderline rejection (Banff3-BL), 19 patients with TCMR Banff4-I, 26 patients with TCMR Banff4-II/III, 30 patients with Banff5-IFTA, 51 patients with SGF, as well as 17 patients with UTI. The distribution of demographic data and other patient characteristics is shown in Table 2.

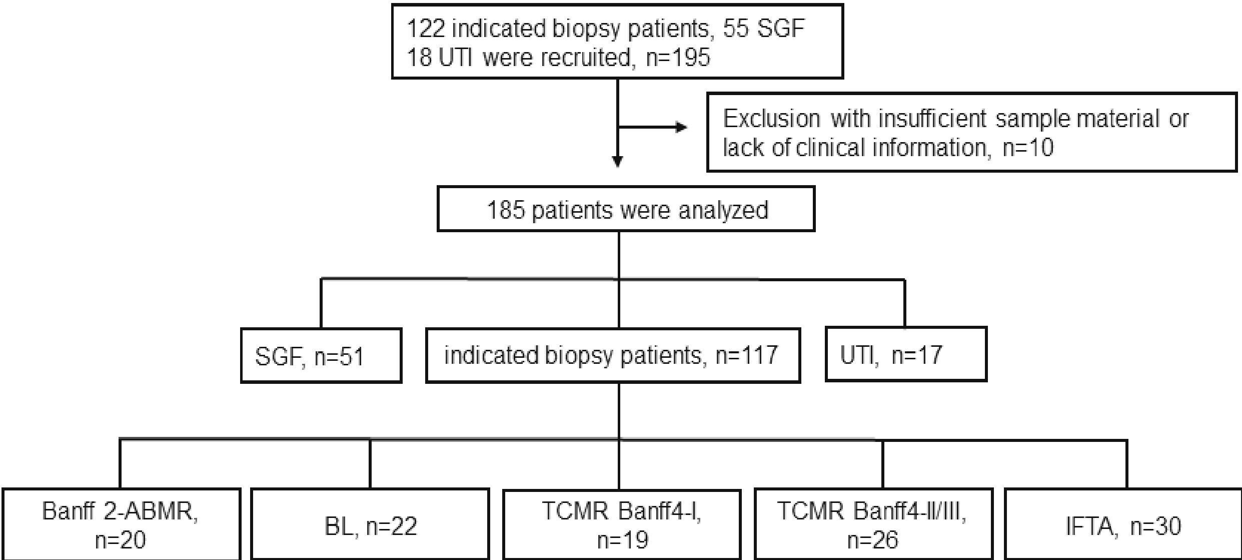


Fig. 4: Flowchart of patient enrollment and classification.

Table 2 Patients' demographics and clinical characteristics*

	Banff2-ABM (n=20)	Control (n=51)	UTI (n=17)	Banff3-BL (n=22)	Banff4-I (n=19)	Banff4-II/III (n=26)	IFTA (n=30)
Age	46.25±12.36	51.96±15.16	51.41±16.73	45.32±16.27	49.11±14.10	51.62±15.10	56.73±14.89
Gender (m/f)	17/3	33/18	2/15	13/9	15/4	23/3	17/13
Creatinine (mg/dL)	3.03 (2.06,4.56)	1.50 (1.16,1.69)	1.98 (1.30,2.93)	2.96 (2.07,3.66)	3.26 (2.58,5.19)	6.86 (3.59,9.30)	2.97 (2.46,4.62)
Previous kidney Tx (0/1/2)	17/3/0	50/1/0	10/6/1	21/1/0	18/1/0	20/3/2/1ns	24/3/1/2ns
Month post Tx	85.62 (34.37, 112.60)	0.40 (0.33, 0.68)	62.15 (37.66, 101.10)	12.65 (1.38, 48.27)	4.40 (1.02, 20.75)	0.30 (0.23,4.80)	47.40 (7.67, 115.20)
Donor living/non living (l/nl)	10/10	23/28	6/9/2ns	13/9	7/12	2/22/2ns	3/23/4ns
Donor related/non related (r/nr)	6/2/2ns	12/10/1ns	3/2/1ns	7/1/5ns	4/1/2ns	1/1	1/2
Cold ischemia time (h)	4.60 (2.52, 13.12)	5.83 (2.38, 9.40)	11.25 (3.00,14.39)	3.62 (2.26, 11.02)	7.65 (2.81, 11.54)	11.23 (7.08, 14.38)	7.88 (6.02, 14.00)
Etiologic of ESRD							
Glomerulonephritis	7	22	4	8	9	5	8
Polycystic disease	0	12	3	3	2	2	5
Diabetic nephropathy	1	0	1	0	0	4	3
Htn/nephrosclerosis	1	4	1	0	0	0	2
Pyelonephritis	2	0	1	0	0	1	1
Reflux nephropathy	3	0	1	0	1	1	0
Interstitial Nephritis	0	3	0	1	1	2	1
Other causes	6	10	5	10	6	9	7
ns	0	0	1	0	0	2	3
Steroid-free regimen (n/y)	13/7	51/0	8/9	13/9	18/1	25/1	22/8
DSA (+/-)	15/5	-	-	-	-	-	-

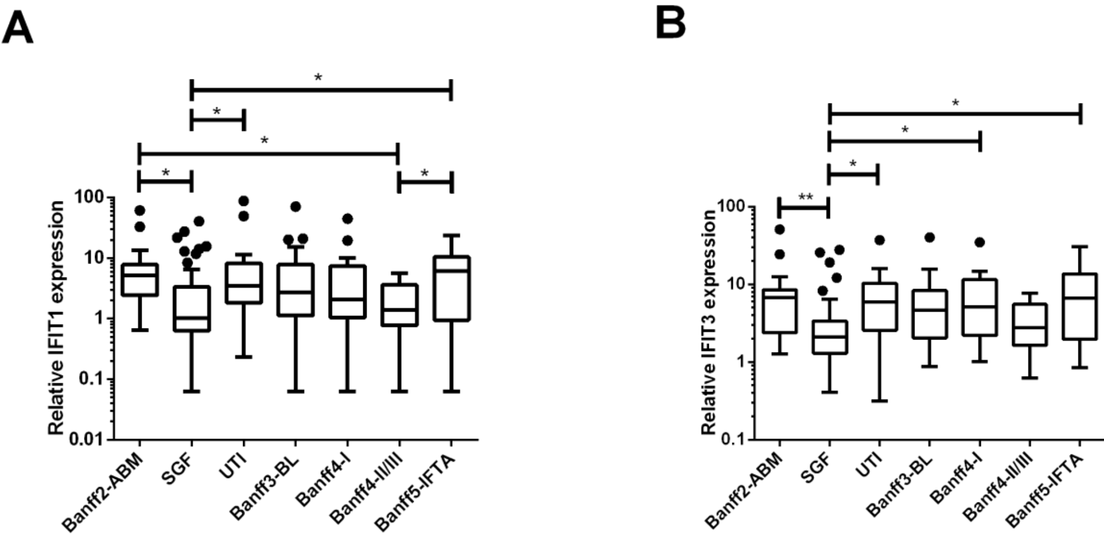
*Values are presented as mean±SD or median (IQR); Tx: transplantation, ns: not specified.

3.2 Interferon-stimulated gene mRNA expression in whole blood cells after kidney transplantation

All candidate ISGs, which included *IFIT1, IFIT3, RSAD2, ETV7, IFI44,* and *IFI44L* mRNA expression in whole blood cells, showed significant differences with varied medians between the groups after being compared with a nonparametric one-way ANOVA (Kruskal-Wallis test), $P<0.05$. A nonparametric Mann-Whitney *U* test was performed between every two groups (21 comparisons for every single marker) and a two-stage

step-up method of Benjamini, Krieger and Yekutieli was used subsequently to control the false discovery rate. By comparing the difference within the groups excluding SGF patients using Kruskal-Wallis test, *IFIT1* (*P*=0.033), *RSAD2* (*P*=0.002), and *ETV7* (*P*<0.001) showed significant different medians, while *IFIT3* (*P*=0.167), *IFI44* (*P*=0.781), and *IFI44L* (*P*=0.540) showed no significant differences.

All markers were significantly elevated in Banff2-ABM patients compared with patients with SGF except for *IFI44* and *IFI44L* (Fig. 5A-F). The significant differences between the Banff4-I and the SGF groups were observed in the *IFIT3, RSAD2* and *ETV7* mRNA expression levels (Fig. 5B-D). No significant differences were found between the Banff4-II/III and the SGF groups in all markers (Fig. 5A-F). All markers were significantly elevated in IFTA patients compared with patients with SGF except for *IFI44* (Fig. 5A-F). Patients with UTI also had significantly higher marker expression than those with SGF except for *IFI44* and *IFI44L* (Fig. 5A-F). Differences between Banff3-BL and SGF were not significant in all markers (Fig. 5A-F).



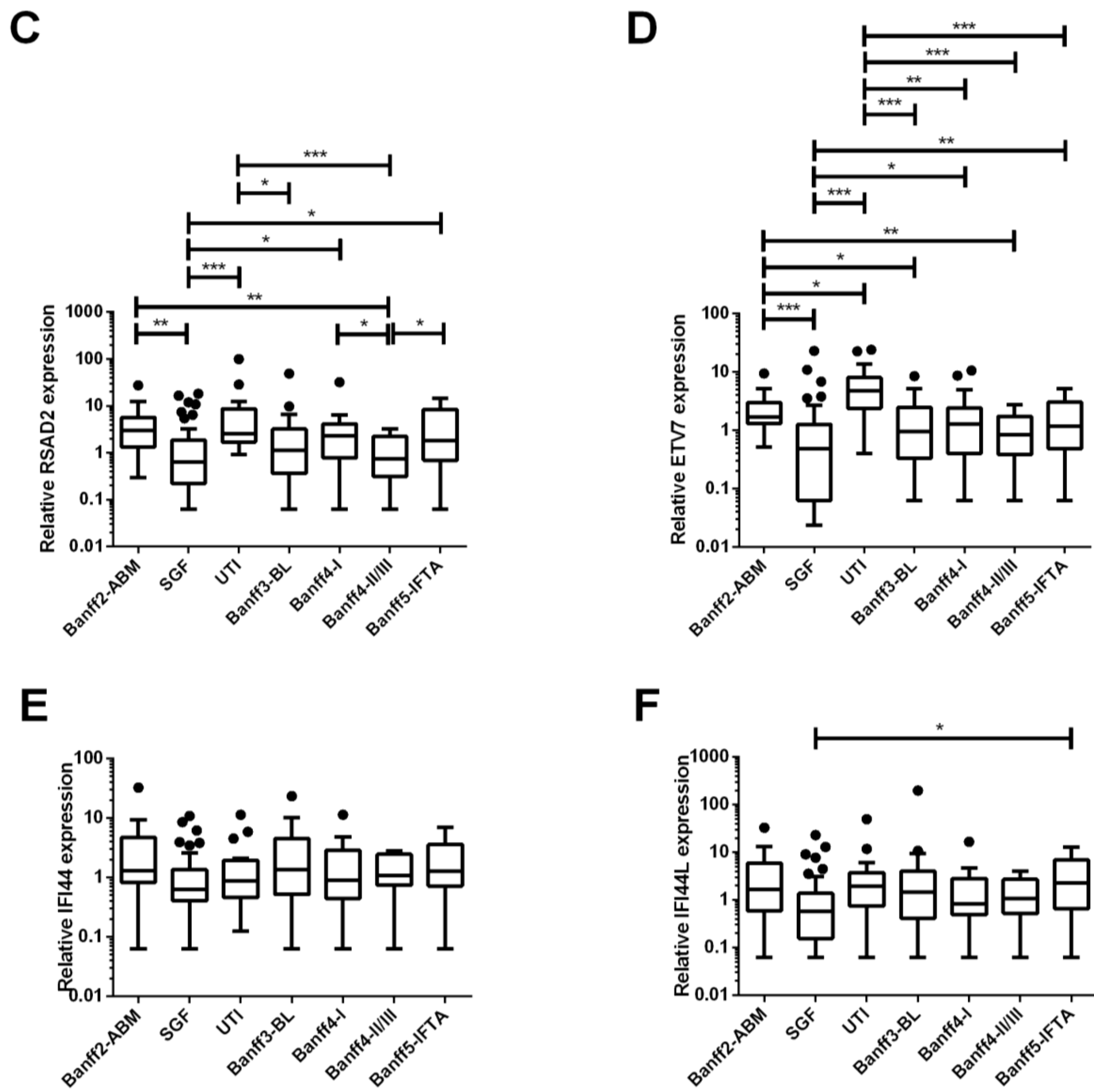


Fig. 5: mRNA expression of ISGs in blood cells after kidney transplantation.

The mRNA expression levels of *IFIT1* (A), *IFIT3* (B), *RSAD2* (C), *ETV7* (D), *IFI44* (E), *IFI44L* (F) were presented by Box-Whisker-Plots and the outliers were specified by the Tukey method. The boxes indicate the median and the lower and upper quartiles. The maximum and minimum values excluding outliers are shown as whiskers above and below the boxes. After performing the nonparametric one-way ANOVA (Kruskal-Wallis test), a nonparametric Mann-Whitney *U* test was performed between every two groups (21 comparisons for every single marker) and a two-stage step-up method of Benjamini, Krieger and Yekutieli was used subsequently to correct *P* values. (**P*<0.05; ***P*<0.01; ****P*<0.001).

Moreover, the *IFIT1* mRNA expression level was significantly lower in the Banff4-II/III group than that in the Banff2-ABM and IFTA groups (Fig. 5A). Similarly, *RSAD2* also

showed relatively lower expression in the Banff4-II/III patients compared with the Banff2-ABM, UTI, Banff4-I, and IFTA patients. The Banff3-BL patients showed lower *RSAD2* expression than the UTI patients (Fig. 5C). *ETV7* expression in the blood cells was at the highest level in the UTI patients compared with all the other 6 groups. Patients in the Banff2-ABM group expressed a higher *ETV7* level than those in the Banff3-BL and Banff4-II/III groups (Fig. 5D).

3.3 Diagnostic value of interferon-stimulated gene mRNA expression levels in whole blood cells for ABMR

ROC curves were used to assess the utility of ISG mRNA expression levels in whole blood cells for differentiating the ABMR patients from all the other patient groups and from the patients with SGF. When compared with all the other patient groups, the ABMR ROC test of IFIT1, RSAD2, ETV7 showed AUCs of over 0.65 (0.656, 0.668, and 0.689 respectively) (Fig. 6).

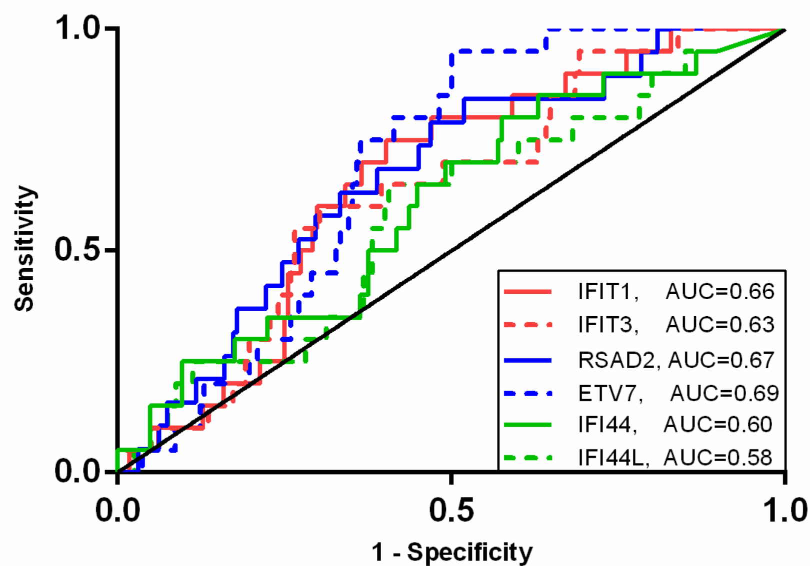


Fig. 6: ROC curves for single ISG mRNA expression levels in blood cells to discriminate ABMR from all other patients.

AUCs are shown in figures and the corresponding *P* values are *IFIT1* (*P*=0.023), *IFIT3* (*P*=0.052), *RSAD2* (*P*=0.017), *ETV7* (*P*=0.006), *IFI44* (*P*=0.129), *IFI44L* (*P*=0.242).

Both univariate and multivariate logistic regression were used to test the diagnostic value of these markers, whereas none of these single or combined markers entered the logistic regression equation (Table 3).

Table 3 Logistic regression analysis of ISG mRNA expression levels in relation to the occurrence of ABMR in all patients

Parameter	Univariate analysis			Multivariate analysis		
	B	P-value	OR (95% CI)	B	P-value	OR (95% CI)
IFIT1	0.008	0.571	1.008 (0.980, 1.037)	-0.065	0.286	0.937 (0.831, 1.056)
IFIT3	0.033	0.167	1.033 (0.986, 1.082)	0.022	0.770	1.022 (0.884, 1.181)
RSAD2	0.009	0.641	1.010 (0.970, 1.051)	0.036	0.615	1.037 (0.901, 1.193)
ETV7	0.023	0.697	1.023 (0.911, 1.149)	0.01	0.903	1.01 (0.857, 1.191)
IFI44	0.077	0.080	1.080 (0.991, 1.177)	0.262	0.070	1.299 (0.979, 1.724)
IFI44L	0.000	0.992	1.000 (0.973,1.028)	-0.046	0.576	0.955 (0.813, 1.122)

Univariate and multivariate logistic regression were performed. The multivariate logistic regression including all candidate markers (*IFIT1, IFIT3, RSAD2, ETV7, IFI44,* and *IFI44L*) was used to test the diagnostic value of the combination of the genes. OR: odds ratio; CI: confidence interval.

The diagnostic properties of all the markers of mRNA expression levels for the diagnosis of ABMR in all other patient groups are presented in Table 4. The *IFIT1, RSAD2, ETV7* mRNA expression levels showed relatively high specificity and high sensitivity, with AUCs of over 0.65 compared with all the other groups' patients.

Table 4 Diagnostic performance of single markers of mRNA expression levels for ABMR from all patients

Parameter	cutoff	Youden Index	AUC (95% CI)	Sensitivity* (95% CI)	Specificity* (95% CI)	LR+* (95% CI)	LR-* (95% CI)
IFIT1	>3.18	0.35	0.66 (0.55-0.76)	0.75 (0.51-0.91)	0.60 (0.52-0.67)	1.86 (1.4-2.6)	0.42 (0.2-0.9)
IFIT3	>6.05	0.30	0.63 (0.52-0.75)	0.60 (0.36-0.81)	0.70 (0.62-0.77)	1.98 (1.3-3.0)	0.57 (0.3-1.0)
RSAD2	>1.03	0.32	0.67 (0.55-0.78)	0.84 (0.60-0.97)	0.48 (0.40-0.56)	1.62 (1.3-2.1)	0.33 (0.1-0.9)
ETV7	>0.84	0.45	0.69 (0.60-0.78)	0.95 (0.75-1.00)	0.50 (0.42-0.58)	1.90 (1.6-2.3)	0.10 (0.01-0.7)
IFI44	>0.82	0.22	0.60 (0.48-0.73)	0.80 (0.56-0.94)	0.42 (0.35-0.50)	1.39 (1.1-1.8)	0.47 (0.2-1.2)
IFI44L	>1.47	0.24	0.58 (0.45-0.71)	0.65 (0.41-0.85)	0.59 (0.51-0.67)	1.60 (1.1-2.3)	0.59 (0.3-1.1)

ROC analysis of data from all patients to determine optimal thresholds for the diagnosis of ABMR and to estimate the diagnostic value of each mRNA expression level. CI: confidence interval; LR+: positive likelihood ratio; LR-: negative likelihood ratio; * value at the optimal Youden index.

Whereas the ROC analysis of mRNA expression in patients with ABMR versus patients with SGF demonstrated relatively high specificity and sensitivity for *ETV7*, with an AUC of over 0.8, *IFIT1, IFIT3, RSAD2, IFI44, IFI44L* also showed AUCs of over 0.7 (0.748, 0.766, 0.761, 0.708 and 0.701 respectively) (Fig. 7).

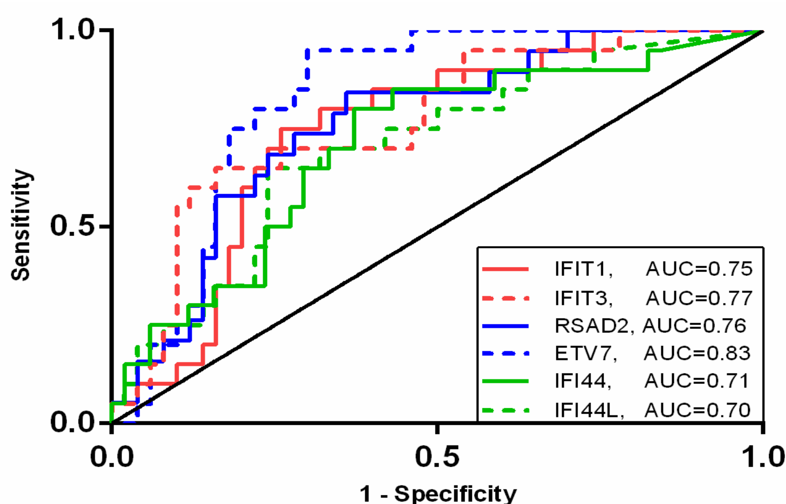


Fig. 7: ROC curves for single ISG mRNA expression levels in blood cells to discriminate ABMR from SGF patients.

AUCs are shown in figures and the corresponding *P* values are *IFIT1* (*P*=0.001), *IFIT3* (*P*=0.001), *RSAD2* (*P*=0.001), *ETV7* (*P*<0.001), *IFI44* (*P*=0.007), *IFI44L* (*P*=0.009).

One of the possible ways to increase the diagnostic accuracy was to combine different markers by the logistic regression approach. However, no markers entered the multivariate logistic regression equation (*P*>0.05), while *IFIT3* and *RSAD2* showed the significant *P* value, which might due to the limited sample size (Table 5).

Table 5 Logistic regression analysis of ISG mRNA expression levels in relation to the occurrence of ABMR in SGF patients

Parameter	Univariate analysis			Multivariate analysis		
	B	P-value	OR (95% CI)	B	P-value	OR (95% CI)
IFIT1	0.045	0.101	1.046 (0.991,1.103)	-0.063	0.395	0.939 (0.813, 1.085)
IFIT3	0.082	0.049	1.085 (1.000, 1.177)	0.054	0.57	1.055 (0.877, 1.270)
RSAD2	0.112	0.048	1.118 (1.001, 1.249)	0.040	0.844	1.041 (0.697, 1.555)
ETV7	0.086	0.281	1.089 (0.933, 1.272)	0.089	0.577	1.093 (0.799, 1.496)
IFI44	0.180	0.082	1.197 (0.977, 1.466)	0.406	0.236	1.501 (0.767, 2.940)
IFI44L	0.092	0.088	1.096 (0.960,1.218)	-0.174	0.401	0.840 (0.560, 1.261)

Univariate and multivariate logistic regression were performed. The multivariate logistic regression including all candidate markers (*IFIT1, IFIT3, RSAD2, ETV7, IFI44,* and *IFI44L*) was used to test the diagnostic value of the combination of the genes. OR: odds ratio; CI: confidence interval.

The diagnostic properties of all the markers of mRNAs expression levels for the diagnosis of ABMR in all patients with SGF are presented in Table 6. The *ETV7* mRNA expression level showed relatively high specificity and high sensitivity with an AUC of over 0.80 when compared with other markers.

Table 6 Diagnostic performance of single markers of mRNA expression levels for ABMR from SGF patients

Parameter	cutoff	Youden Index	AUC (95% CI)	Sensitivity* (95% CI)	Specificity* (95% CI)	LR+* (95% CI)	LR-* (95% CI)
IFIT1	>2.84	0.49	0.75 (0.63-0.87)	0.75 (0.51-0.91)	0.74 (0.60-0.85)	2.88 (1.7-4.9)	0.34 (0.2-0.7)
IFI44L	>1.27	0.41	0.70 (0.57-0.83)	0.65 (0.41-0.85)	0.76 (0.62-0.87)	2.71 (1.5-4.9)	0.46 (0.2-0.9)
RSAD2	>1.00	0.48	0.76 (0.64-0.88)	0.84 (0.60-0.97)	0.64 (0.49-0.77)	2.34 (1.5-3.6)	0.25 (0.09-0.7)
ETV7	>0.84	0.65	0.83 (0.74-0.92)	0.95 (0.75-1.00)	0.70 (0.55-0.82)	3.17 (2.0-4.9)	0.07 (0.01-0.5)
IFIT3	>4.70	0.49	0.77 (0.65-0.89)	0.65 (0.41-0.85)	0.84 (0.71-0.93)	4.06 (2.0-8.3)	0.42 (0.2-0.8)
IFI44	>0.81	0.43	0.71 (0.58-0.84)	0.80 (0.56-0.94)	0.63 (0.48-0.76)	2.15 (1.4-3.3)	0.32 (0.1-0.8)

ROC analysis of data from patients with SGF and ABMR to determine the optimal thresholds for classification of blood samples and to estimate the diagnostic value of each marker of mRNA expression levels. CI: confidence interval; LR+: positive likelihood ratio; LR-: negative likelihood ratio; * value at the optimal Youden index.

3.4 Interferon-stimulated gene protein expression levels in serum and plasma after kidney transplantation

The protein expression levels of IFIT1, RSAD2 in the plasma, and ETV7 in the serum were further measured by ELISA because of the significant various expression levels of mRNA in whole blood cells. The nonparametric one-way ANOVA (Kruskal-Wallis test) found that IFIT1 and ETV7 originated from the different distribution ($P<0.05$), while the P value of RSAD2 was 0.108. A nonparametric Mann-Whitney U test was performed between every two groups (21 comparisons for every single marker), and a two-stage step-up method of Benjamini, Krieger and Yekutieli was subsequently used to control the false discovery rate. By comparing the difference within the groups excluding SGF patients using Kruskal-Wallis test, IFIT1 ($P=0.011$) and ETV7 ($P<0.001$) showed significant different medians, while RSAD2 ($P=0.059$) showed no significant differences.

The plasma IFIT1 protein expression level in Banff5-IFTA group was significantly higher than that of all the other groups except the UTI and Banff4-I groups (Fig. 8A). No significant differences in RSAD2 protein expression level were found between different patient groups (Fig. 8B). Banff5-IFTA patients showed significantly the lowest ETV7 serum expression level compared with all the other patients except for Banff2-ABM group (Fig. 8C).

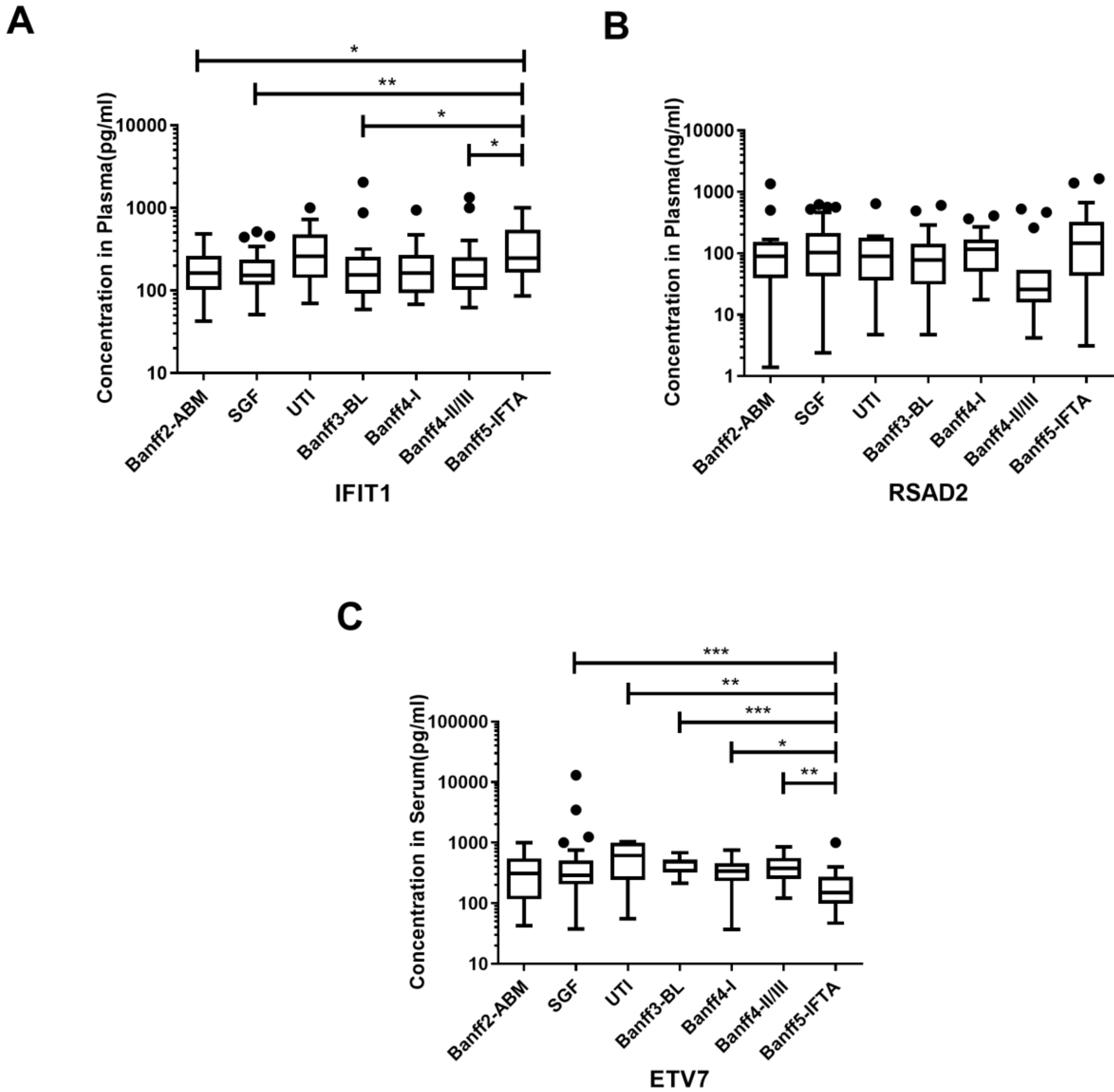


Fig. 8: Protein expression of ISGs in plasma (IFIT1, RSAD2) and serum (ETV7) after kidney transplantation.

The protein expression levels of IFIT1 (A), RSAD2 (B), ETV7 (C) are presented by Box-Whisker-Plots and the outliers are specified by the Tukey method. The boxes indicate the median and the lower and upper quartiles. The maximum and minimum values excluding outliers are shown as whiskers above and below the boxes. After performing the nonparametric one-way ANOVA (Kruskal-Wallis test), a nonparametric Mann-Whitney *U* test was performed between every two groups (21 comparisons for every single marker) and a two-stage step-up method of Benjamini, Krieger and Yekutieli was used subsequently to correct *P* values. (**P*<0.05; ***P*<0.01; ****P*<0.001).

3.5 Diagnostic value of interferon-stimulated gene protein expression levels in serum and plasma for ABMR

ROC curves were used to assess the utility of ISG protein expression levels in serum and plasma for differentiating the ABMR patients from all the other patient groups and from the patients with SGF. When compared with all the other group patients, the markers in plasma and serum did not show any diagnosis value, with AUCs lower than 0.60 and *P* values of over 0.05 (Fig.9). When compared with SGF patients, the markers in plasma and serum also did not show any diagnosis value, with AUCs lower than 0.60 and *P* values of over 0.05 (Fig.10).

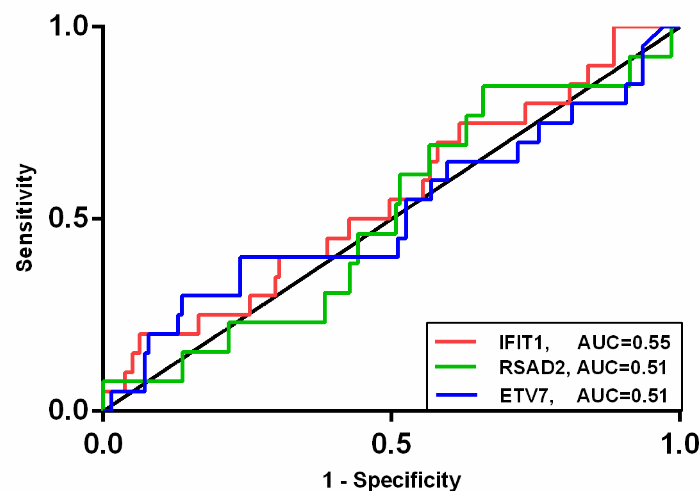


Fig. 9: ROC curves for single IFIT1, RSAD2, ETV7 protein expression levels in plasma (IFIT1, RSAD2) and serum (ETV7) to discriminate ABMR from all other patients.

AUCs are shown in figures, and the corresponding *P* values are IFIT1 (*P*=0.450), RSAD2 (*P*=0.916), ETV7 (*P*=0.842).

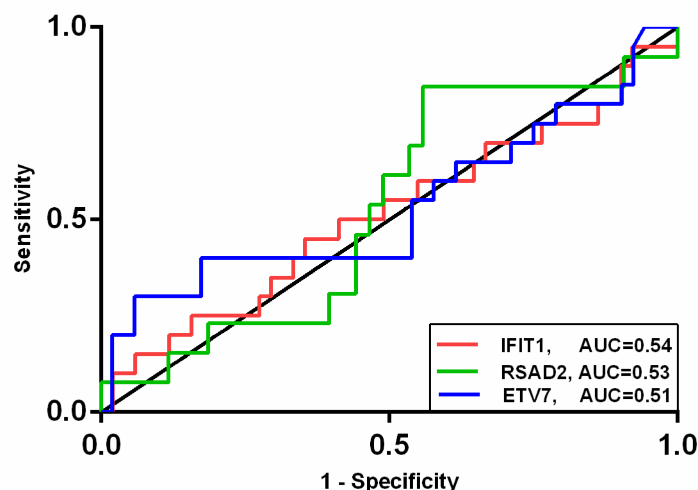


Fig. 10: ROC curves for single IFIT1, RSAD2, ETV7 protein expression levels in plasma (IFIT1, RSAD2) and serum (ETV7) to discriminate ABMR from SGF patients.

AUCs are shown in figures and the corresponding *P* values are IFIT1 (*P*=0.868), RSAD2 (*P*=0.734), ETV7 (*P*=0.637).

The diagnostic properties of all the markers of protein expression levels for the diagnosis of ABMR from all other patient groups are presented in Table7A and from SGF patients in Table7B.

Table 7 Diagnostic performance of single markers of protein expression levels for ABMR

Parameter	cutoff	Youden Index	AUC (95% CI)	Sensitivity* (95% CI)	Specificity* (95% CI)	LR+* (95% CI)	LR-* (95% CI)
IFIT1[A]	≤70.53	0.14	0.55 (0.42-0.69)	0.20 (0.57-0.44)	0.94 (0.89-0.97)	3.12 (1.1-9.0)	0.85 (0.7-1.1)
RSAD2[A]	≤166.53	0.19	0.51 (0.35-0.66)	0.85 (0.55-0.98)	0.34 (0.26-0.43)	1.28 (1.0-1.7)	0.45 (0.1-1.7)
ETV7[A]	≤118.20	0.16	0.51 (0.36-0.66)	0.30 (0.12-0.54)	0.86 (0.80-0.92)	2.19 (1.0-4.8)	0.81 (0.6-1.1)
IFIT1[B]	>96.58	0.11	0.51 (0.35-0.67)	0.75 (0.51-0.91)	0.14 (0.06-0.26)	0.87 (0.7-1.1)	1.82 (0.7-5.1)
RSAD2[B]	≤166.53	0.29	0.53 (0.36-0.71)	0.85 (0.55-0.98)	0.44 (0.29-0.60)	1.52 (1.1-2.2)	0.35 (0.09-1.3)
ETV7[B]	≤118.20	0.24	0.54 (0.37-0.70)	0.30 (0.12-0.54)	0.94 (0.84-0.99)	5.20 (1.4-18.8)	0.74 (0.6-1.0)

ROC analysis of data from all patients to determine optimal thresholds for the diagnosis of

ABMR and to estimate the diagnostic value of each protein expression level (A). ROC analysis of data from patients with SGF and ABMR was performed to determine optimal thresholds for the classification of blood samples and to estimate the diagnostic value of each marker of the protein expression level (B). CI: confidence interval; LR+: positive likelihood ratio; LR-: negative likelihood ratio; * value at the optimal Youden index.

3.6 Serum creatinine levels and diagnostic value for ABMR after kidney transplantation

The serum creatinine was measured routinely at the same time. The nonparametric one-way ANOVA (Kruskal-Wallis test) was performed and significant difference was found within groups ($P<0.001$). A nonparametric Mann-Whitney *U* test was performed between every two groups (totally 21 comparisons), and a two-stage step-up method of Benjamini, Krieger and Yekutieli was subsequently used to control the false discovery rate. By comparing the difference within the groups excluding SGF patients using Kruskal-Wallis test, significant difference was also found ($P<0.001$).

The serum creatinine is significantly elevated in all groups compared with the SGF group (Fig. 11A). When compared with all the other patient groups, the serum creatinine did not show any diagnosis value, with the AUC lower than 0.6 and a *P* value of over 0.05 (Fig. 11B). When compared with SGF patients, the serum creatinine showed AUC of over 0.85 with *P* values of < 0.0001(Fig. 11 B, C).

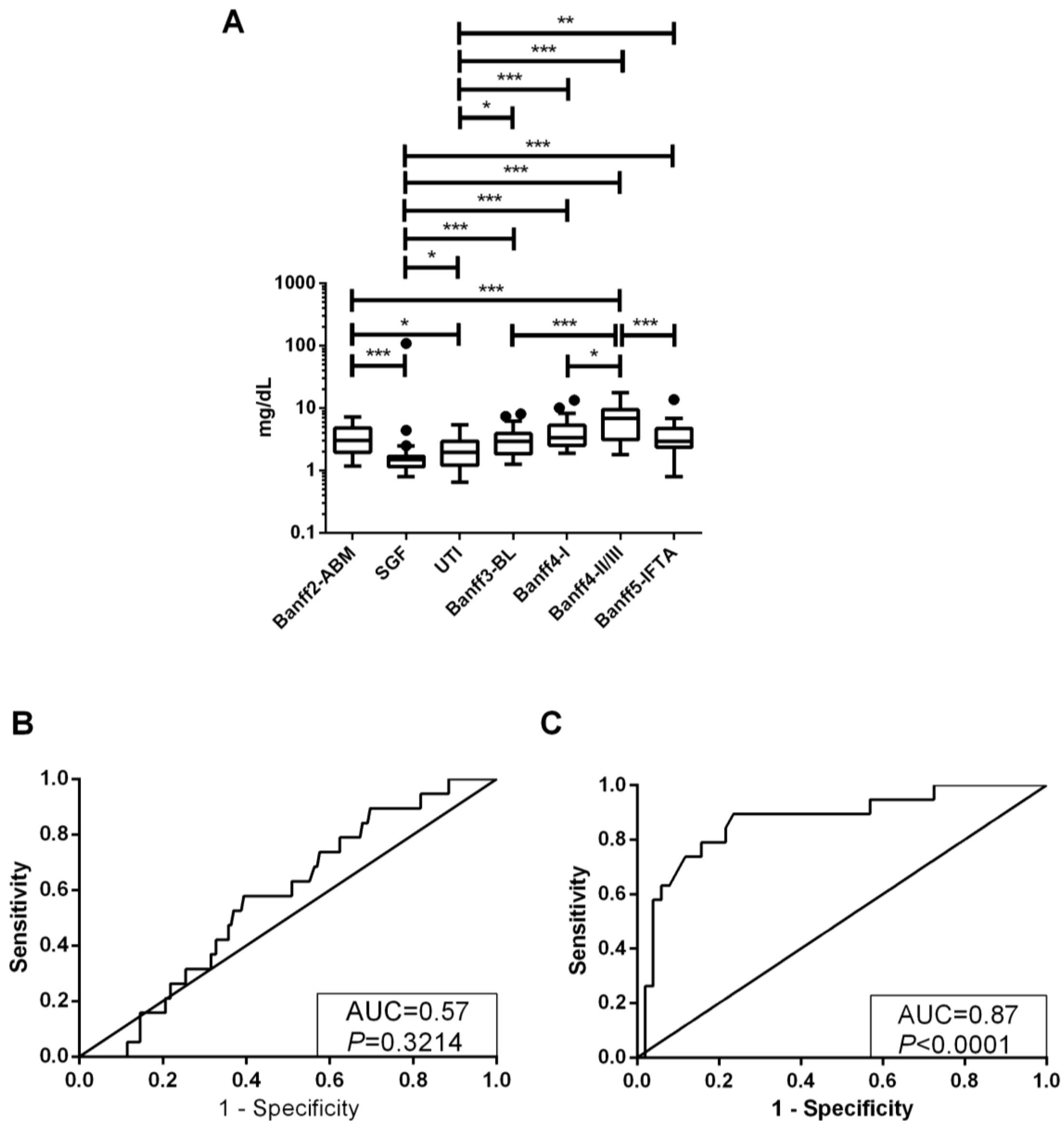


Fig. 11: Serum creatinine and ROC curves.

The serum creatinine levels are presented by Box-Whisker-Plots and the outliers are specified by the Tukey method. The boxes indicate the median and the lower and upper quartiles. The maximum and minimum values excluding outliers are shown as whiskers above and below the boxes. After performing the nonparametric one-way ANOVA (Kruskal-Wallis test), a nonparametric Mann-Whitney *U* test was performed between every two groups (21 comparisons for every single marker) and a two-stage step-up method of Benjamini, Krieger and Yekutieli was used subsequently to correct *P* values. (*$P<0.05$; **$P<0.01$; ***$P<0.001$) (A). ROC curves for serum creatinine on the diagnostic value of ABMR versus all other groups (B) and versus stable graft function (C).

Univariate logistic regression was used, whereas serum creatinine did not enter the

logistic regression equation (*P*=0.730 vs All, *P*=0.912 vs SGF). The diagnostic properties of serum creatinine for the diagnosis of ABMR in all other patient groups and in the SGF group are presented in Table 8.

Table 8 Diagnostic performance of serum creatinine levels for ABMR

Parameter	cutoff	Youden Index	AUC (95% CI)	Sensitivity* (95% CI)	Specificity* (95% CI)	LR+* (95% CI)	LR-* (95% CI)
Cr [A]	>1.69	0.20	0.57 (0.50-0.64)	0.89 (0.67-0.99)	0.30 (0.23-0.38)	1.28 (1.10-1.50)	0.35 (0.09-1.30)
Cr [B]	>1.69	0.66	0.87 (0.77-0.94)	0.89 (0.67-0.99)	0.76 (0.63-0.87)	3.80 (2.30-6.40)	0.14 (0.04-0.50)

ROC analysis of data from all patients to determine optimal thresholds and to estimate the diagnostic value of serum creatinine for the diagnosis of all ABMR (A) and from patients with SGF (B). CI: confidence interval; LR+: positive likelihood ratio; LR-: negative likelihood ratio; * value at the optimal Youden index.

4 DISCUSSION

Kidney transplantation is widely recognized as the most effective treatment for ESRD. Over the past 20 years, along with the application of CNI-based immunosuppressive regimens and advances in tissue typing and organ preservation technology, the 1-year renal allograft survival rate has increased to 90% or higher [12]. However, acute and chronic rejections, IFTA, and other causes are responsible for suboptimal long-term graft survival [13]. Among all the causes of graft loss, ABMR is considered a major cause of long-term renal graft loss [14]. Therefore, early diagnosis and the development of better treatment options are of great significance.

The clinical symptoms of ABMR are increasing serum creatinine, elevated blood pressure, decreased urine output, and proteinuria. Current strategies for monitoring ABMR have limitations, as severe damage may precede an increase in creatinine, and the development of proteinuria already reflects a later stage of injury. The gold standard for the diagnosis of ABMR is renal allograft biopsy. However, it is difficult to detect ABMR at an early stage. Moreover, biopsy is an invasive procedure carrying the potential risk of damaging precious kidney grafts, which limits repetitive and dynamic observations. Therefore, many attempts have been made to find non-invasive markers for the diagnosis of ABMR in an early stage. Such a sensitive and specific marker might offer the opportunity for early therapeutic intervention. Detecting a cheap and reliable marker for distinguishing potential ABMR would also be valuable, especially for longitudinal observations (e.g. for treatment effects). However, there is still a lack of good markers with sufficient sensitivity and specificity [66].

In this study, *IFIT1, IFIT3, RSAD2, ETV7* showed significantly different expression in ABMR patients. The selection of these markers was based on previous sequencing results [91]. To compensate for the limitations of sequencing and to enable the application of these markers for clinical use, this study was organized into large patient

cohorts that were thoroughly matched against a control group in order to identify the diagnostic value of these markers and thus find a more effective clinical application.

4.1 Levels of interferon-stimulated gene expression

The interferon response protects cells from pathogen invasions by stimulating the expression of ISGs. ISGs show immunomodulating effects and are reported to take part in the regulation of autoimmune disease, cancer, and hepatitis [92-95]. Interferon-inducible protein 10 (IP-10), also known as C-X-C motif chemokine 10 (CXCL10), is considered an ISG. It has been reported to promote the generation and migration of effector T cells [96, 97]. IP-10 has also been found to be a diagnostic marker for acute cellular rejection in kidney allografts [98]. As shown in section 3.2, our study found that from mRNA levels, candidate ISGs, which included *IFIT1, IFIT3, RSAD2, ETV7*, were higher expressed in the ABMR group compared to the SGF group. While none of the markers in serum or plasma showed significant differences between the ABMR group and the SGF group, this could be due to different gene expression profiles between blood cells and body fluids. Similar to our previous work [99], this result suggests that blood cells are a better marker resource for diagnosis than serum or plasma.

Like the ABMR patients, the IFTA patients also showed elevated mRNA levels of ISGs. Additionally, the protein level of IFIT1 in plasma and ETV7 in serum were also significantly changed in the IFTA group compared with the SGF group.

Interferon is used as an immunomodulator to treat viral infection in clinical settings. It plays a crucial role in physiological and pathological immune responses by regulating innate and adaptive immune responses [100]. IFN-γ is known to be important in modulating the allogeneic responses in transplantation. Transcript changes involving IFN-γ are also found in TCMR and ABMR patients [70,71]. Similarly, activation of immune responses by IFN-α increases the risk of allograft rejection too [101, 102]. After

administration of interferon, ISGs will express, which is also a sign of activated immune response. This could be the reason why ISGs are highly expressed during ABMR.

Halloran et al. showed that ABMR manifested as transcript changes that reflected IFN-γ effects, and the molecular feature of TCMR was dominated by IFN-γ-induced genes [71,72]. Rascio et al. also found up-regulated genes involved in type I interferon signaling in chronic ABMR and IFTA patients which included *IFIT1* and *IFIT3*. Their results are similar to our founding, but they did not analyze these markers' expression levels in a large patient cohort, and so were unable to determine the diagnostic value of these markers [88]. They thought the expression of these markers might relate to B cell differentiation, DC maturation, and polarization of $CD4^+$ T cells. Although the exact mechanism is unknown, this provides us with a further research direction in the pathophysiology of ABMR.

mRNA expression levels of ISGs were upregulated in patients with UTI compared to the SGF group. Similar results were also found in previous works on non-transplantation patients [103, 104].

The TCMR patients showed no significant differences in expression levels compared to the SGF groups in *IFIT1, IFI44, IFI44L*, while, *IFIT3, RSAD2, ETV7* up-regulated mRNA levels in the Banff4-I TCMR patients compared with the SGF group. *IFIT1, RSAD2, ETV7* mRNA expression levels in the Banff4-II/III TCMR group were lower than in the ABMR group, which may be because no significant difference was found between the Banff4-II/III TCMR and the SGF patients.

Many biopsies with inflammation changes that are not sufficient to be TCMR are labeled as BL. De Freitas et al. compared the molecular phenotype of the BL biopsies in TCMR and SGF patients and found that BL rejection as defined by histopathology is found to be nonrejection by molecular phenotyping [105]. Their work may help to explain why markers in the Banff3 BL group did not show any significant differences between patients

with SGF.

Among these uniquely expressed markers, *ETV7* showed the highest discrepancy between groups. While this marker is relatively new and has not yet been studied in larger cohorts, *ETV7* is a member of the E26 transformation-specific (ETS) family of the transcriptional regulators, and it is an IFN-stimulated gene. *ETV7* plays a key role in blood cell development and differentiation as well as tumor genesis [80]. *ETV7* was highly expressed in the UTI patients compared to all other patients at mRNA levels. The ABMR patients also expressed *ETV7* mRNA differently from all patients with the exception of the Banff4-I TCMR and IFTA patients. Apart from the Banff3 BL and Banff4-II/III TCMR groups, the *ETV7* mRNA expression levels in all other groups was higher than in the SGF group.

RSAD2 was also highly expressed in Banff2-ABMR, UTI, Banff4-I TCMR, and IFTA patients compared to SGF patients. *RSAD2* is a specific marker necessary for DCs maturation, mature DCs then triggers allogeneic immune responses [106, 107].

ISG56/IFIT1 Gene Family markers *IFIT1* and *IFIT3* showed similar mRNA expression differences. Both markers showed elevated expression in Banff2-ABMR, UTI, and IFTA patients compared to the SGF group. However, both *IFI44* and *IFI44L* showed no significant difference within each group except *IFI44L* elevated in IFTA patients compared to the patients with SGF.

Cumulatively, these differences create a foundation for the diagnosis of ABMR from all other complications.

4.2 Diagnostic performance of interferon-stimulated genes

Acute renal graft dysfunction is a common complication after kidney transplantation. Despite its limitations, serum creatinine remains the routine test to monitor the graft function. When ABMR patients show elevated serum creatinine, graft damage already

exists. Also, elevated serum creatinine cannot distinguish between various types of graft changes requiring different treatments. The diagnostic uncertainty of existing markers triggers the decision for an invasive allograft biopsy, which carries the potential risk of damaging precious kidney grafts. Thus, there is an urgent need to develop a noninvasive way to diagnose ABMR.

We have successfully validated several markers in blood for the noninvasive diagnosis of ABMR. The diagnostic accuracy of ISG mRNA expression levels for the diagnosis of ABMR was remarkable compared to that of serum creatinine (Table 4, 6, 8), while the ISG protein expression levels were not helpful (Table 7).

There was a relatively fair performance characteristic of *ETV7* in discriminating ABMR patients from non-ABMR patients (AUC=0.69 with 0.95 sensitivity and 0.50 specificity cutoff>0.84) and from patients with SGF (AUC=0.83 with 0.95 sensitivity and 0.70 specificity cutoff>0.84). Notably, *ETV7* possesses more value for diagnosing ABMR in all patients as compared with serum creatinine (AUC=0.57 with 0.89 sensitivity and 0.30 specificity cutoff>1.69). This can also be seen from the obvious trend that the *ETV7* expression level in ABMR patients is distinct from that in all the other groups.

The ROC analysis of markers distinguishing ABMR from patients with SGF is able to diagnose ABMR early without allograft function impairment. Patients with SGF might also encounter potential ABMR or be in the early stage of ABMR, which shows no significant elevated serum creatinine. All six markers showed good performance, with AUCs over 0.70.

Regretfully, none of the single variable or multi-variables entered the logistic regression equation to predict the onset of ABMR in all other patients or in the SGF group, which is also the case with serum creatinine. A possible explanation for this could be the small sample size and the heterogeneity of patients. However, multi-parameter measurements can still increase the sensitivity by reaching any one or even all of the parameter cutoffs

(parallel test) and elevate the specificity by reaching all the cutoffs of the parameters (serial test).

4.3 Clinical implications

The final goal of biomarker research is to find noninvasive markers to diagnose ABMR directly instead of using graft biopsy. Due to the complexity of ABMR, this goal is still far away. However, with the help of some specific markers, unnecessary biopsies may be avoided, and this can make the diagnosis of ABMR more cost-effective and precise. The use of circulating RNA or protein as biomarkers offers several advantages: the sample can be collected easily and the quantification of RNA or protein is economical and reproducible, and can even be ward-based in the future [108].

Currently, the clinical routine of ABMR diagnosis is based on elevated creatinine and the presence of DSAs. Whereas there are still so many reasons that may explain the increase of creatinine, DSAs are also only a risk factor for ABMR and many patients are bearing DSAs without ABMR. The stratification of patients based on the levels of biomarkers will enable clinicians to use an invasive tool with greater precision for diagnosing high-risk patients. In this case, the implementation of a marker with a low LR- might prevent a large number of patients from undergoing a pointless biopsy. Based on our results, a single use of the *ETV7* mRNA expression level conveys a LR- 0.10, and the high LR+1.90 could increase the probability of ABMR before a biopsy is performed.

A series of markers was analyzed and showed relatively fair efficacy for the diagnosis of ABMR in all patients or patients with SGF in our study. The sensitivity and specificity for the diagnosis of ABMR will improve with the measurement of these markers. An increase of sensitivity will have to be compromised with a decrease of specificity. Thus, it is of great importance to adjust the cutoff value in order to achieve a better diagnosis of ABMR. For patients with SGF, a missed diagnosis of potential or subclinical ABMR should be avoided. Therefore, a more sensitive diagnosis is necessary. For patients with

elevated serum creatinine, an indicated biopsy could be performed, which allows less misdiagnosis, and in such cases the cutoff value should be adjusted to achieve higher specificity.

The perfect diagnostic marker tests should also have a favorable cost-benefit ratio, be prepared expediently, and be reproducible so that they can be used as clinical routine. In this study, the most positive result is on the basis of mRNA expression level measurement, which can be tested by RT-PCR. This technique is an extremely sensitive, rapid, easily operative method, which, hopefully, will become a valuable tool for detecting the expression levels of specific markers for the diagnosis of ABMR. A point-of-care multiplex polymerase chain reaction (mPCR) system has also been developed, and it could simplify all the steps of the traditional system into a multiplexed, automated, and closed system [109]. This reliable system may someday be applicable for monitoring the changes of markers in the blood of ABMR patients. Since this technique can be practiced routinely in clinics, the most important task will be to determine the most effective marker or marker combination scheme.

4.4 Limitations and future study

As may be encountered in many clinical studies, patient heterogeneity can lead to a decrease in accuracy. Thus, a larger group cohort, multi-center recruitment, and the application of random selection and the blind method could help prevent bias in future research. More frequent marker monitoring at different time points after transplantation is also needed to determine the dynamic trend of the expression level of markers. Moreover, a single cohort analysis of markers is prone to the inaccurate diagnostic effect of biomarkers by chance. Thus, including a replication test from an independent patient cohort would be important in order to further validate the diagnostic utility of these markers.

The elevated expression levels of these genes give us a clue: related genes could be

targeted to increase the list of candidate markers so that more potential markers could be identified to achieve a better diagnosis of ABMR. Furthermore, searching for the origin of these genes from specific cell subsets and tracking the molecular pathway of these genes might offer a better understanding of the mechanism of ABMR.

For patient measuring, it is better to choose control patients matched in age or time after transplantation so that we can better determine the influence of these factors. Because the patients had not undergone protocol biopsies, the control group we used was diagnosed based on the clinical findings, which may have included patients with subclinical rejection. As may be encountered in a clinic, the recurrence of primary diseases and viral infections such as cytomegalovirus or BK virus can also impair the graft function, which needs to be further qualified. However, due to the limited patient numbers, we were unable to compare more diseases.

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

4.5 Concluding remarks

Despite the advances in immunosuppressive therapy, which have greatly improved the short-term graft survival, ABMR is still a threat to long-term graft survival. Timely diagnosis of ABMR and adjustments of immunosuppressant drugs can salvage the precious graft. A major limitation of the current management is that serum creatinine is not a sensitive and specific marker for ABMR. Therefore, the discovery of a new ABMR marker is essential to distinguish patients at risk for ABMR from other patients. Based on

the clinical needs, we included in this study the most common complications after kidney transplantation.

After measurement and comparison of the mRNA and protein levels of the ISG markers, the mRNA levels of *IFIT1, RSAD2, ETV7* showed significant diagnostic value for distinguishing patients at risk for ABMR from other patients. The combination of these three markers will increase the overall sensitivity and specificity by serial test and parallel test. This procedure could make it possible to diagnose ABMR accurately and in this way greatly benefit kidney transplant patients.

5 SUMMARY

ABMR is considered to be an elemental factor affecting the long-term survival of renal allograft. The gold standard for diagnosing ABMR is the needle biopsy, which is far away from being widely practiced as clinical routine due to its invasiveness. The graft function test, like serum creatinine measurement, is neither sensitive nor specific enough for the diagnosis of ABMR. Moreover, when the serum creatinine is elevated, the precious graft has been damaged inevitably and the best chance for treatment might have been missed. Hence, there is an urgent need for discovering effective and repeatable markers for the diagnosis of ABMR to facilitate an earlier intervention.

ISGs are the effectors of interferon actions and play crucial roles in innate immune defense against pathogens. More and more evidence has revealed that ISG might be induced during various autoimmune diseases such as systemic lupus erythematosus or systemic sclerosis. Our previous sequencing results also showed a high expression level of ISGs in ABMR patients. All these findings suggest that the elevation of ISG levels in the blood could be an indicator of ABMR.

The present study provides ISG expression information on renal transplantation recipients from a large patient cohort at both the RNA and the protein levels based on RNA sequencing results. This work also validates the effects of these genes for the diagnosis of ABMR and thus provides a foundation for future research. Of all the candidate ISGs, *ETV7* shows the highest sensitivity and specificity, even though the combination of these candidate markers using logistic regression failed.

In conclusion, the serum ISG RNA is an effective marker for the detection of ABMR. However, further prospective multicenter and longitudinal studies are warranted to determine the utility of these markers for future clinical practice.

Reference

1. Hallan SI, Coresh J, Astor BC, Åsberg A, Powe NR, Romundstad S, Hallan HA, Lydersen S, Holmen J. International comparison of the relationship of chronic kidney disease prevalence and ESRD risk. Journal of the American society of nephrology. 2006 Aug 1;17(8):2275-84.
2. Zhang L, Wang F, Wang L, Wang W, Liu B, Liu J, Chen M, He Q, Liao Y, Yu X, Chen N. Prevalence of chronic kidney disease in China: a cross-sectional survey. The lancet. 2012 Mar 3;379(9818):815-22.
3. Coresh J, Selvin E, Stevens LA, Manzi J, Kusek JW, Eggers P, Van Lente F, Levey AS. Prevalence of chronic kidney disease in the United States. Journal of the American medical association. 2007 Nov 7;298(17):2038-47.
4. Levey AS, De Jong PE, Coresh J, Nahas ME, Astor BC, Matsushita K, Gansevoort RT, Kasiske BL, Eckardt KU. The definition, classification, and prognosis of chronic kidney disease: a KDIGO Controversies Conference report. Kidney international. 2011 Jul 1;80(1):17-28.
5. Grassmann A, Gioberge S, Moeller S, Brown G. ESRD patients in 2004: global overview of patient numbers, treatment modalities and associated trends. Nephrology dialysis transplantation. 2005 Oct 4;20(12):2587-93.
6. El Nahas AM, Bello AK. Chronic kidney disease: the global challenge. The lancet. 2005 Jan 22;365(9456):331-40.
7. Matas AJ, Halbert RJ, Barr ML, Helderman JH, Hricik DE, Pirsch JD, Schenkel FA, Siegal BR, Liu H, Ferguson RM. Life satisfaction and adverse effects in renal transplant recipients: a longitudinal analysis. Clinical transplantation. 2002 Apr;16(2):113-21.
8. De Vecchi AF, Dratwa M, Wiedemann ME. Healthcare systems and end-stage renal disease (ESRD) therapies-an international review: costs and reimbursement/funding of ESRD therapies. Nephrology dialysis transplantation. 1999 Dec 1;14(suppl_6):31-41.
9. Wolfe RA, Ashby VB, Milford EL, Ojo AO, Ettenger RE, Agodoa LY, Held PJ, Port FK. Comparison of mortality in all patients on dialysis, patients on dialysis awaiting transplantation, and recipients of a first cadaveric transplant. New England journal of medicine. 1999 Dec 2;341(23):1725-30.
10. Breidenbach T. Organ donation and transplantation in Germany. In: Jox R., Assadi G, Marckmann G, eds. Organ transplantation in times of donor shortage. Cham, Germany: Springer, 2016: 115-26.

11. Eurotransplant Annual Report 2016 (Accessed 2017, at https://www.eurotransplant.org/cms/mediaobject.php?file=Eurotransplant+JV+PDF.pdf)

12. Hart A, Smith JM, Skeans MA, Gustafson SK, Stewart DE, Cherikh WS, Wainright JL, Kucheryavaya A, Woodbury M, Snyder JJ, Kasiske BL. OPTN/SRTR 2015 annual data report: kidney. American journal of transplantation. 2017 Jan; 17:21-116.

13. Lamb KE, Lodhi S, Meier-Kriesche HU. Long-term renal allograft survival in the United States: A critical reappraisal. American journal of transplantation. 2011 Mar;11(3):450-62.

14. 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):1146-54.

15. Koo EH, Jang HR, Lee JE, Park JB, Kim SJ, Kim DJ, Kim YG, Oh HY, Huh W. The impact of early and late acute rejection on graft survival in renal transplantation. Kidney research and clinical practice. 2015 Sep 1;34(3):160-4.

16. Ball S, Dallman MJ. Immunology of graft rejection. In: Morris PJ, Knechtle SJ, eds. Kidney transplantation-principles and practice. 7th ed. Edinburgh, UK: Elsevier Health Sciences, 2013: 10-38.

17. Nankivell BJ, Alexander SI. Rejection of the kidney allograft. New England journal of medicine. 2010 Oct 7;363(15):1451-62.

18. Andrade CF, Waddell TK, Keshavjee S, Liu M. Innate immunity and organ transplantation: the potential role of toll-like receptors. American journal of transplantation. 2005 May;5(5):969-75.

19. Gritsch HA, Blumberg JM. Renal transplantation. In Wein AJ, Kavoussi LR, Partin AW, Peters CA, eds. Campbell-Walsh Urology. 11th ed. Philadelphia, USA: Elsevier Health Sciences, 2016:1069-88.

20. Kissmeyer-Nielsen F, Olsen S, Petersen VP, Fjeldborg O. Hyperacute rejection of kidney allografts, associated with pre-existing humoral antibodies against donor cells. The lancet. 1966 Sep 24;288(7465):662-5.

21. Paul LC, Claas FH, van Es LA, Kalff MW, Graeff JD. Accelerated rejection of a renal allograft associated with pretransplantation antibodies directed against donor antigens on endothelium and monocytes. New England journal of medicine. 1979 May 31;300(22):1258-60.

22. Wu O, Levy AR, Briggs A, Lewis G, Jardine A. Acute rejection and chronic nephropathy: a systematic review of the literature. Transplantation. 2009 May 15;87(9):1330-9.

23. Chapman JR, O'Connell PJ, Nankivell BJ. Chronic renal allograft dysfunction. Journal of the American society of nephrology. 2005 Oct 1;16(10):3015-26.
24. Sureshkumar KK, Hussain SM, Carpenter BJ, Sandroni SE, Marcus RJ. Antibody-mediated rejection following renal transplantation. Expert opinion on pharmacotherapy. 2007 May 1;8(7):913-21.
25. Williams GM, Hume DM, Hudson Jr RP, Morris PJ, Kano K, Milgrom F. Hyperacute renal-homograft rejection in man. New England journal of medicine. 1968 Sep 19;279(12):611-8.
26. Colvin RB. Antibody-mediated renal allograft rejection: diagnosis and pathogenesis. Journal of the American society of nephrology. 2007 Apr 1;18(4):1046-56.
27. Puttarajappa C, Shapiro R, Tan HP. Antibody-mediated rejection in kidney transplantation: a review. Journal of transplantation. 2012 Apr 10; 193724:1-9.
28. Orandi BJ, Chow EH, Hsu A, Gupta N, Van Arendonk KJ, Garonzik-Wang JM, Montgomery JR, Wickliffe C, Lonze BE, Bagnasco SM, Alachkar N. Quantifying renal allograft loss following early antibody-mediated rejection. American journal of transplantation. 2015 Feb;15(2):489-98.
29. Terasaki PI, Ozawa M, Castro R. Four-year follow-up of a prospective trial of HLA and MICA antibodies on kidney graft survival. American journal of transplantation. 2007 Feb;7(2):408-15.
30. Deltombe C, Gillaizeau F, Anglicheau D, Morelon E, Trébern-Launay K, Le Borgne F, Rimbert M, Guérif P, Malard-Castagnet S, Foucher Y, Giral M. Is pre-transplant sensitization against angiotensin II type 1 receptor still a risk factor of graft and patient outcome in kidney transplantation in the anti-HLA Luminex era? A retrospective study. Transplant international. 2017 Nov;30(11):1150-60.
31. Davis S, Cooper JE. Acute antibody-mediated rejection in kidney transplant recipients. Transplantation reviews. 2017 Jan 1;31(1):47-54.
32. Stegall MD, Chedid MF, Cornell LD. The role of complement in antibody-mediated rejection in kidney transplantation. Nature reviews nephrology. 2012 Nov;8(11):670-8.
33. Loupy A, Haas M, Solez K, Racusen L, Glotz D, Seron D, Nankivell BJ, Colvin RB, Afrouzian M, Akalin E, Alachkar N. The Banff 2015 kidney meeting report: current challenges in rejection classification and prospects for adopting molecular pathology. American journal of transplantation. 2017 Jan;17(1):28-41.
34. Gaston RS, Cecka JM, Kasiske BL, Fieberg AM, Leduc R, Cosio FC, Gourishankar S, Grande J, Halloran P, Hunsicker L, Mannon R. Evidence for antibody-mediated injury as

a major determinant of late kidney allograft failure. Transplantation. 2010 Jul 15;90(1):68-74.
35. Hara S. Current pathological perspectives on chronic rejection in renal allografts. Clinical and experimental nephrology. 2017 Dec 1;21(6):943-51.
36. Haas M, Sis B, Racusen LC, Solez K, Glotz D, Colvin RB, Castro MC, David DS, David-Neto E, Bagnasco SM, Cendales LC. Banff 2013 meeting report: inclusion of c4d-negative antibody-mediated rejection and antibody-associated arterial lesions. American journal of transplantation. 2014 Feb;14(2):272-83.
37. Randhawa P. T-cell-mediated rejection of the kidney in the era of donor-specific antibodies: diagnostic challenges and clinical significance. Current opinion in organ transplantation. 2015 Jun 1;20(3):325-32.
38. Gago M, Cornell LD, Kremers WK, Stegall MD, Cosio FG. Kidney allograft inflammation and fibrosis, causes and consequences. American journal of transplantation. 2012 May;12(5):1199-207.
39. Wu KY, Budde K, Schmidt D, Neumayer HH, Rudolph B. Acute cellular rejection with isolated v-lesions is not associated with more favorable outcomes than vascular rejection with more tubulointerstitial inflammations. Clinical transplantation. 2014 Apr;28(4):410-8.
40. Everly MJ, Everly JJ, Susskind B, Brailey P, Arend LJ, Alloway RR, Roy-Chaudhury P, Govil A, Mogilishetty G, Rike AH, Cardi M. Bortezomib provides effective therapy for antibody-and cell-mediated acute rejection. Transplantation. 2008 Dec 27;86(12): 1754-61.
41. Wood KJ, Zaitsu M, Goto R. Cell mediated rejection. In: Zachary AA, Leffell MS, eds. Transplantation immunology: methods and protocols. 2nd ed. Totowa, NJ: Humana Press, 2013:71-83.
42. Kreisel D, Krupnick AS, Balsara KR, Riha M, Gelman AE, Popma SH, Szeto WY, Turka LA, Rosengard BR. Mouse vascular endothelium activates CD8+ T lymphocytes in a B7-dependent fashion. The journal of immunology. 2002 Dec 1;169(11):6154-61.
43. Vella JP, Vos L, Carpenter CB, Sayegh MH. Role of indirect allorecognition in experimental late acute rejection. Transplantation. 1997 Dec 27;64(12):1823-8.
44. Kinnear G, Jones ND, Wood KJ. Costimulation blockade: current perspectives and implications for therapy. Transplantation. 2013 Feb 27;95(4):5276.
45. Burrell BE, Bishop DK. Th17 cells and transplant acceptance. Transplantation. 2010 Nov 15;90(9):945.

46. Loverre A, Divella C, Castellano G, Tataranni T, Zaza G, Rossini M, Ditonno P, Battaglia M, Palazzo S, Gigante M, Ranieri E. T helper 1, 2 and 17 cell subsets in renal transplant patients with delayed graft function. Transplant international. 2011 Mar;24(3):233-42.
47. Csencsits KL, Bishop DK. Contrasting alloreactive CD4+ and CD8+ T cells: there's more to it than MHC restriction. American journal of transplantation. 2003 Feb;3(2):107-15.
48. Hu M, Wang YM, Wang Y, Zhang GY, Zheng G, Yi S, O' Connell PJ, Harris DC, Alexander SI. Regulatory T cells in kidney disease and transplantation. Kidney international. 2016 Sep 1;90(3):502-14.
49. Zhang N, Schröppel B, Lal G, Jakubzick C, Mao X, Chen D, Yin N, Jessberger R, Ochando JC, Ding Y, Bromberg JS. Regulatory T cells sequentially migrate from inflamed tissues to draining lymph nodes to suppress the alloimmune response. Immunity. 2009 Mar 20;30(3):458-69.
50. Racusen LC, Solez K, Colvin RB, Bonsib SM, Castro MC, Cavallo T, Croker BP, Demetris AJ, Drachenberg CB, Fogo AB, Furness P. The Banff 97 working classification of renal allograft pathology. Kidney international. 1999 Feb 1;55(2):713-23.
51. Solez K, Colvin RB, Racusen LC, Sis B, Halloran PF, Birk PE, Campbell PM, Cascalho M, Collins AB, Demetris AJ, Drachenberg CB. Banff'05 meeting report: differential diagnosis of chronic allograft injury and elimination of chronic allograft nephropathy (‘CAN’). American journal of transplantation. 2007 Mar;7(3):518-26.
52. Sellares J, De Freitas DG, Mengel M, Reeve J, Einecke G, Sis B, Hidalgo LG, Famulski K, Matas A, Halloran PF. Understanding the causes of kidney transplant failure: the dominant role of antibody-mediated rejection and nonadherence. American journal of transplantation. 2012 Feb;12(2):388-99.
53. Boor P, Floege J. Renal allograft fibrosis: biology and therapeutic targets. American journal of transplantation. 2015 Apr;15(4):863-86.
54. Naesens M, Kuypers DR, De Vusser K, Vanrenterghem Y, Evenepoel P, Claes K, Bammens B, Meijers B, Lerut E. Chronic histological damage in early indication biopsies is an independent risk factor for late renal allograft failure. American journal of transplantation. 2013 Jan;13(1):86-99.
55. Azancot MA, Moreso F, Salcedo M, Cantarell C, Perello M, Torres IB, Montero A, Trilla E, Sellarés J, Morote J, Seron D. The reproducibility and predictive value on outcome of renal biopsies from expanded criteria donors. Kidney international. 2014 May 1;85(5):1161-8.

56. Liptak P, Ivanyi B. Primer: Histopathology of calcineurin-inhibitor toxicity in renal allografts. Nature reviews nephrology. 2006 Jul;2(7):398-404.
57. Cosio FG, Gloor JM, Sethi S, Stegall MD. Transplant glomerulopathy. American journal of transplantation. 2008 Mar;8(3):492-6.
58. Rastaldi MP, Ferrario F, Giardino L, Dell'Antonio G, Grillo C, Grillo P, Strutz F, Müller GA, Colasanti G, D'Amico G. Epithelial-mesenchymal transition of tubular epithelial cells in human renal biopsies. Kidney international. 2002 Jul 1;62(1):137-46.
59. Li X, Zhuang S. Recent advances in renal interstitial fibrosis and tubular atrophy after kidney transplantation. Fibrogenesis & tissue repair. 2014 Dec;7(1):15.
60. Timsit MO, Yuan X, Floerchinger B, Ge X, Tullius SG. Consequences of transplant quality on chronic allograft nephropathy. Kidney international. 2010 Dec 1;78:S54-8.
61. Einecke G, Sis B, Reeve J, Mengel M, Campbell PM, Hidalgo LG, Kaplan B, Halloran PF. Antibody-mediated microcirculation injury is the major cause of late kidney transplant failure. American journal of transplantation. 2009 Nov;9(11):2520-31.
62. Nankivell BJ, Shingde M, Keung KL, Fung CL, Borrows RJ, O'Connell PJ, Chapman JR. The causes, significance and consequences of inflammatory fibrosis in kidney transplantation: the Banff i-IFTA lesion. American journal of transplantation, 2018 Feb, 18(2): 364-76.
63. DeVos JM, Gaber AO, Teeter LD, Graviss EA, Patel SJ, Land GA, Moore LW, Knight RJ. Intermediate-term graft loss after renal transplantation is associated with both donor-specific antibody and acute rejection. Transplantation. 2014 Mar 15;97(5):534-40.
64. De Vlaminck I, Valantine HA, Snyder TM, Strehl C, Cohen G, Luikart H, Neff NF, Okamoto J, Bernstein D, Weisshaar D, Quake SR. Circulating cell-free DNA enables noninvasive diagnosis of heart transplant rejection. Science translational medicine. 2014 Jun 18;6(241):241ra77.
65. Erpicum P, Hanssen O, Weekers L, Lovinfosse P, Meunier P, Tshibanda L, Krzesinski JM, Hustinx R, Jouret F. Non-invasive approaches in the diagnosis of acute rejection in kidney transplant recipients, part II: omics analyses of urine and blood samples. Clinical kidney journal. 2016 Sep 6;10(1):106-15.
66. Gwinner W. Renal transplant rejection markers. World journal of urology. 2007 Oct 1;25(5):445-55.
67. Anglicheau D, Naesens M, Essig M, Gwinner W, Marquet P. Establishing biomarkers in transplant medicine: a critical review of current approaches. Transplantation. 2016 Oct 1;100(10):2024-38.

68. Parkin J, Cohen B. An overview of the immune system. The lancet. 2001 Jun 2;357(9270): 1777-89.
69. Borden E C, Sen G C, Uze G, Silverman RH, Ransohoff M, Foster GR, Stark GR. Interferons at age 50: past, current and future impact on biomedicine. Nature reviews Drug discovery. 2007 Dec; 6(12): 975.
70. Hidalgo LG, Halloran PF. Role of IFN-gamma in allograft rejection. Critical reviews in immunology. 2002, 22(4): 317-49.
71. Halloran PF, Famulski K, Reeve J. The molecular phenotypes of rejection in kidney transplant biopsies. Current opinion in organ transplantation. 2015 Jun; 20(3): 359-67.
72. Halloran PF, Reeve JP, Pereira AB, Hidalgo LG, Famulski KS. Antibody-mediated rejection, T cell-mediated rejection, and the injury-repair response: new insights from the Genome Canada studies of kidney transplant biopsies. Kidney international. 2014Feb; 85(2): 258-64.
73. Chow KT, Gale M. SnapShot: interferon signaling. Cell. 2015 Dec 17;163(7):1808.
74. Green R, Ireton RC, Gale M. Interferon-stimulated genes: new platforms and computational approaches. Mammalian genome. 2018 Aug 1;29(7-8):593-602.
75. John SP, Sun J, Carlson RJ, Cao B, Bradfield CJ, Song J, Smelkinson M, Fraser ID. IFIT1 exerts opposing regulatory effects on the inflammatory and interferon gene programs in LPS-activated human macrophages. Cell reports. 2018 Oct 2;25(1):95-106.
76. Schneider WM, Chevillotte MD, Rice CM. Interferon-stimulated genes: a complex web of host defenses. Annual review of immunology. 2014 Feb 6;32(1):513-45.
77. Fensterl V, Sen GC. The ISG56/IFIT1 gene family. Journal of interferon & cytokine research 2011 Jan 1; 31(1):71-8.
78. Zhou X, Michal JJ, Zhang L, Ding B, Lunney JK, Liu B, Jiang Z. Interferon induced IFIT family genes in host antiviral defense. International journal of biological sciences. 2013 Feb 11; 9(2):200-8.
79. Seo JY, Yaneva R, Cresswell P: Viperin: a multifunctional, interferon-inducible protein that regulates virus replication. Cell host & microbe. 2011 Dec 15; 10(6):534-39.
80. Rasighaemi P, Ward AC. ETV6 and ETV7: Siblings in hematopoiesis and its disruption in disease. Critical reviews in oncology/hematology. 2017 Jun 3; 116:106-15.
81. Irudayam JI, Contreras D, Spurka L, Subramanian A, Allen J, Ren S, Kanagavel V, Nguyen Q, Ramaiah A, Ramamoorthy K, French SW, Klein AS, Funari V, Arumugaswami V. Characterization of type I interferon pathway during hepatic differentiation of human pluripotent stem cells and hepatitis C virus infection. Stem cell research. 2015 Aug 15; 15(2):354-64.

82. Huang WC, Tung SL, Chen YL, Chen PM, Chu PY: IFI44L is a novel tumor suppressor in human hepatocellular carcinoma affecting cancer stemness, metastasis, and drug resistance via regulating met/Src signaling pathway. BMC Cancer. 2018 May 30; 18(1):609.
83. Smith S, Fernando T, Wu PW, Seo J, Gabhann JN, Piskareva O, McCarthy E, Howard D, O'Connell P, Conway R, Gallagher P. MicroRNA-302d targets IRF9 to regulate the IFN-induced gene expression in SLE. Journal of autoimmunity. 2017 May 1; 79:105-11.
84. Post AE, Smid M, Nagelkerke A, Martens JW, Bussink J, Sweep FC, Span PN. Interferon-stimulated genes are involved in cross-resistance to radiotherapy in tamoxifen-resistant breast cancer. Clinical cancer research. 2018 Apr 16;24 (14):3397-408.
85. Saiura A, Mataki C, Murakami T, Umetani M, Wada Y, Kohro T, Aburatani H, Harihara Y, Hamakubo T, Yamaguchi T, Hasegawa G. A comparison of gene expression in murine cardiac allografts and isografts by means DNA microarray analysis. Transplantation. 2001 Jul 27;72(2):320-9.
86. Gimino VJ, Lande JD, Berryman TR, King RA, Hertz MI. Gene expression profiling of bronchoalveolar lavage cells in acute lung rejection. American journal of respiratory and critical care medicine. 2003 Nov 15;168(10):1237-42.
87. Minisini R, Giarda P, Grossi G, Bitetto D, Toniutto P, Falleti E, Avellini C, Occhino G, Fabris C, Pirisi M. Early activation of interferon-stimulated genes in human liver allografts: relationship with acute rejection and histological outcome. Journal of gastroenterology. 2011 Nov 1;46(11):1307-15.
88. Rascio F, Pontrelli P, Accetturo M, Oranger A, Gigante M, Castellano G, Gigante M, Zito A, Zaza G, Lupo A, Ranieri E. A type I interferon signature characterizes chronic antibody-mediated rejection in kidney transplantation. The journal of pathology. 2015 Sep;237(1):72-84.
89. Akalin E, Hendrix RC, Polavarapu RG, Pearson TC, Neylan JF, Larsen CP, Lakkis FG. Gene expression analysis in human renal allograft biopsy samples using high-density oligoarray technology. Transplantation. 2001 Sep 15;72(5):948-53.
90. Maluf DG, Mas VR, Archer KJ, Yanek K, Gibney EM, King AL, Cotterell A, Fisher RA, Posner MP: Molecular pathways involved in loss of kidney graft function with tubular atrophy and interstitial fibrosis. Molecular medicine 2008 May 01, 14(5-6):276-85.
91. 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.

92. Oliveras-Ferraros C, Vazquez-Martin A, Queralt B, Adrados M, Ortiz R, Cufí S, Hernández-Yagüe X, Guardeño R, Báez L, Martin-Castillo B, Pérez-Martínez MC. Interferon/STAT1 and neuregulin signaling pathways are exploratory biomarkers of cetuximab (Erbitux®) efficacy in KRAS wild-type squamous carcinomas: a pathway-based analysis of whole human-genome microarray data from cetuximab-adapted tumor cell-line models. International journal of oncology. 2011 Dec 1;39(6):1455-79.

93. Muskardin TL, Niewold TB. Type I interferon in rheumatic diseases. Nature reviews rheumatology. 2018 Apr;14(4):214-28.

94. Robinson MW, Swann R, Sigruener A, Barclay ST, Mills PR, McLauchlan J, Patel AH. Elevated interferon-stimulated gene transcription in peripheral blood mononuclear cells occurs in patients infected with genotype 1 but not genotype 3 hepatitis C virus. Journal of viral hepatitis. 2015 Apr;22(4):384-90.

95. Rice GI, Forte GM, Szynkiewicz M, Chase DS, Aeby A, Abdel-Hamid MS, Ackroyd S, Allcock R, Bailey KM, Balottin U, Barnerias C. Assessment of interferon-related biomarkers in Aicardi-Goutieres syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study. The lancet neurology. 2013 Dec 1;12(12):1159-69.

96. Dufour JH, Dziejman M, Liu MT, Leung JH, Lane TE, Luster AD. IFN-γ-inducible protein 10 (IP-10; CXCL10)-deficient mice reveal a role for IP-10 in effector T cell generation and trafficking. The Journal of Immunology. 2002 Apr 1;168(7):3195-204.

97. Taub DD, Longo DL, Murphy WJ. Human interferon-inducible protein-10 induces mononuclear cell infiltration in mice and promotes the migration of human T lymphocytes into the peripheral tissues and human peripheral blood lymphocytes-SCID mice. Blood. 1996 Feb 15;87(4):1423-31.

98. Suthanthiran M, Schwartz JE, Ding R, Abecassis M, Dadhania D, Samstein B, Knechtle SJ, Friedewald J, Becker YT, Sharma VK, Williams NM. Urinary-cell mRNA profile and acute cellular rejection in kidney allografts. New England journal of medicine. 2013 Jul 4;369(1):20-31.

99. Matz M, Lorkowski C, Fabritius K, Durek P, Wu K, Rudolph B, Neumayer HH, Mashreghi MF, Budde K. Free microRNA levels in plasma distinguish T-cell mediated rejection from stable graft function after kidney transplantation. Transplant immunology. 2016 Nov 1; 39:52-9.

100. González-Navajas JM, Lee J, David M, Raz E. Immunomodulatory functions of type I interferons. Nature reviews immunology. 2012 Feb;12(2):125.
101. Magnone M, Holley JL, Shapiro R, Scantlebury V, McCauley J, Jordan M, Vivas C, Starzl T, Johnson JP. Interferon-α-induced acute renal allograft rejection. Transplantation. 1995 Apr 15;59(7):1068-70.
102. Wéclawiack H, Kamar N, Mehrenberger M, Guilbeau-Frugier C, Modesto A, Izopet J, Ribes D, Sallusto F, Rostaing L. Alpha-interferon therapy for chronic hepatitis C may induce acute allograft rejection in kidney transplant patients with failed allografts. Nephrology dialysis transplantation. 2007 Oct 3;23(3):1043-7.
103. Madan I, Than NG, Romero R, Chaemsaithong P, Miranda J, Tarca AL, Bhatti G, Draghici S, Yeo L, Mazor M, Hassan SS. The peripheral whole-blood transcriptome of acute pyelonephritis in human pregnancya. Journal of perinatal medicine. 2014 Jan 1;42(1):31-53.
104. Lutay N, Ambite I, Hernandez JG, Rydström G, Ragnarsdóttir B, Puthia M, Nadeem A, Zhang J, Storm P, Dobrindt U, Wullt B. Bacterial control of host gene expression through RNA polymerase II. The journal of clinical investigation. 2013 Jun 3;123(6): 2366-79.
105. De Freitas DG, Sellares J, Mengel M, Chang J, Hidalgo LG, Famulski KS, Sis B, Einecke G, Halloran PF. The nature of biopsies with "borderline rejection" and prospects for eliminating this category. American journal of transplantation. 2012 Jan;12(1):191-201.
106. Jang JS, Lee JH, Jung NC, Choi SY, Park SY, Yoo JY, Song JY, Seo HG, Lee HS, Lim DS. Rsad2 is necessary for mouse dendritic cell maturation via the IRF7-mediated signaling pathway. Cell death & disease, 2018 Aug 01, 9(8): 823.
107. Reich B, Viehmann S F, Kurts C. Plasmacytoid dendritic cells: important players in human kidney allograft rejection. Kidney international, 2018 Feb; 93(2): 301-3.
108. Petralia S, Conoci S. PCR technologies for point of care testing: progress and perspectives. ACS sensors. 2017 Jul 18;2(7):876-91.
109. Craw P, Balachandran W. Isothermal nucleic acid amplification technologies for point-of-care diagnostics: a critical review. Lab on a chip. 2012;12(14):2469-86.

Eidesstattliche Versicherung

„Ich, Qiang Zhang, versichere an Eides statt durch meine eigenhändige Unterschrift, dass ich die vorgelegte Dissertation mit dem Thema: Use of interferon-stimulated genes expression level in renal transplant patients' blood as biomarkers for diagnosis of rejection 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 etwaigen erfolgten Publikationen

Qiang Zhang hatte folgenden Anteil an der Publikation:

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.

Beitrag im Einzelnen:

Qiang Zhang war als Co-Autor des Manuskripts zusammen mit Dr.rer.medic. Mareen Matz und Prof. Dr. med. Klemens Budde an der gemeinsamen Erarbeitung des Studiendesigns sowie an der Erhebung, Verarbeitung, und Auswertung der Daten beteiligt. Ebenso hat er wesentlich an der Erstellung und Korrektur des Manuskriptes beigetragen.

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

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Unterschrift des Doktoranden/der Doktorandin

Curriculum Vitae

My curriculum vitae does not appear in the electronic version of my paper for reasons of data protection.

List of publications

1. **Zhang Q,** Yu Z, Zeng S, Liang L, Xu Y, Zhang Z, Tang H, Jiao W, Xue W, Wang W, Zhang X, Jiang T, Hu X. Use of intravoxel incoherent motion imaging to monitor a rat kidney chronic allograft damage model. BMC nephrology, 2019 Oct 10, 20(1): 1-10.
2. Zeng S, Liang L, **Zhang Q**, Xu Y, Tang H, Zhang Z, Zhang X, Jiang T, Hu X. Using functional magnetic resonance imaging to evaluate an acute allograft rejection model in rats. Magnetic resonance imaging. 2019 May 1;58:24-31.
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.
4. Matz M, Heinrich F, Lorkowski C, Wu K, Klotsche J, **Zhang Q**, Lachmann N, Durek P, Budde K, Mashreghi MF. MicroRNA regulation in blood cells of renal transplanted patients with interstitial fibrosis/tubular atrophy and antibody-mediated rejection. Plos one. 2018 Aug 13;13(8): e0201925.
5. Zhang Z, Liu L, Tang H, Jiao W, Zeng S, Xu Y, **Zhang Q**, Sun Z, Mukherjee A, Zhang X, Hu X. Immunosuppressive effect of the gut microbiome altered by high-dose tacrolimus in mice. American journal of transplantation. 2018 Jan 9.
6. Xu Y, Zeng S, **Zhang Q**, Zhang Z, Hu X. Stem Cell Mobilizers: Novel Therapeutics for Acute Kidney Injury. Current protein and peptide science. 2017 Dec 1;18(12):1195-9.
7. Xue W, Zhang Z, Zeng S, Xu Y, **Zhang Q**, Wang W, Zhang Y, Zhang X, Hu X. Expression and Clinical Significance of Tissue Inhibitor of Metalloproteinases-1 (TIMP-1) and a Disintegrin and Metalloproteinase with Thrombospondin Type 1 Motif 1 (ADAMTS1) in Post-Kidney-Transplant Bladder Tumors. Annals of transplantation. 2017 Oct;22:622-30.
8. Xu Y, **Zhang Q**, Xue W, Zeng S, Zhang Z, Zhang X, Hu X. CXC Chemokine Receptor 4 (CXCR4) Antagonist, a Novel Pathway to Prevent Chronic Allograft Nephropathy. Annals of transplantation. 2016 Nov;21:728-34.
9. **Zhang Q**, Yu Z, Xu Y, Zeng S, Zhang Z, Xue W, Wang W, Zhang X, Hu X. Use of contrast-enhanced ultrasonography to evaluate chronic allograft nephropathy in rats and correlations between time-intensity curve parameters and allograft fibrosis. Ultrasound in medicine & biology. 2016 Jul 1;42(7):1574-83.
10. Jinesh GG, Taoka R, **Zhang Q**, Gorantla S, Kamat AM. Novel PKC-ζ to p47 phox interaction is necessary for transformation from blebbishields. Scientific reports. 2016 Apr 4; 6:23965.

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